
University of Southampton

Institute of Developmental Sciences

Faculty of Natural and Environmental Sciences

**The Effect of the Level and Timing of Folic Acid
Supplementation during the Life Course on the Regulation of
Breast Cancer Determining Genes, Mammary Morphology and
Implications for Genome Stability and Tissue Differentiation**

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Thesis for the degree of Doctor of Philosophy

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The Effect of the Level and Timing of Folic Acid Supplementation during the Life Course on the Regulation of Breast Cancer Determining Genes, Mammary Morphology and Implications for Genome Stability and Tissue Differentiation

Mark Andrew Burton

Folic acid (FA) intake has increased over the past decade; however, there are concerns that high levels of FA, particularly during early life, may increase breast cancer risk. Here a murine model has been used to directly test FA intake during specific periods of the life-course on the regulation of key cancer determining genes, *Oct-4* and *Brca1*, polycomb regulator genes *Ezh2*, *Bmi1* and *Suz12*, mammary gland morphology and protein expression.

Female C57BL/6 mice were fed a modified AIN93M semi-purified diet containing either 1 mg/kg (1x basal daily recommendation (BDR): 5 mg/kg (5 x BDR), 20 mg/kg (20 x BDR) or a FA deficient diet containing 0.2 mg/kg (0.2 x BDR) for a 4 week period during either the juvenile-pubertal (JP) period or adulthood (A). After this period mice were fed an AIN93M maintenance diet for 4 or 12 weeks. The 2nd and 3rd thoracic mammary glands were collected and mRNA expression of *Brca1*, *Oct-4*, *Ezh2*, *Bmi1* and *Suz12* measured by quantitative real time PCR (qRT-PCR). Levels of *Ezh2* protein and its target H3K27 (tri-methyl) were quantified by Western Blot. Mammary gland epithelial morphology was assessed using mammary whole mounts. Epithelial tree density, branch length, branch junctions and number of terminal end buds (TEBs) were calculated. Genome wide differential gene expression was investigated using RNA-seq in juvenile or adult mice fed 5 mg/kg FA 4 weeks post supplementation. Mammary *Oct-4*, *Brca1*, basal/myoepithelial marker α -smooth muscle actin (α -SMA), luminal marker keratin 8 (K8), cell proliferation marker Ki67 and driver of luminal differentiation Gata-3 proteins were investigated with immunohistochemistry using 3,3'-diaminobenzidine and immunofluorescence.

A persistent increase in *Brca1*, *Oct-4*, *Ezh2*, *Bmi1* and *Suz12* gene expression after FA supplementation at 5 mg/kg was observed during A. In contrast, FA supplementation at 5 mg/kg during JP led to a decrease in *Oct-4* gene expression. FA supplementation at 5 mg/kg increased levels of *Ezh2* protein and levels of H3K27 (tri-methyl) during A. Mammary morphology was largely unchanged; however, epithelial density and number of TEBs showed a persistent increase in JP supplemented mice. *Brca1*, and *Oct-4* proteins were found to be localised in mammary epithelial ducts. Numbers of basal/myoepithelial cells expressing α -SMA were unchanged, however, numbers of cells expressing cell proliferation marker Ki67 increased. RNA-seq analysis identified differential expression in genes associated with extracellular matrix, enriched gene sets included epithelial mesenchymal transition (EMT) and pathways associated with matrix metalloproteinases (MMPs), extracellular matrix components and cancer during A. Interestingly, juvenile mice showed distinct gene sets with minimal overlap with those during A.

JP and adult FA supplementation induce tissue specific alterations in expression of key cancer genes that persist beyond the period of supplementation. The effects of FA are both dose and timing specific and imply that the ability of FA to modify cancer risk extends beyond recent diet and may, in part, reflect intakes during different periods of mammary plasticity. These findings suggest high folic acid intake in mice may increase breast cancer risk especially when consumed during adulthood.

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Academic Thesis: Declaration of Authorship

I, Mark Andrew Burton

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The Effect of the Level and Timing of Folic Acid Supplementation During the Life Course on the Epigenetic Regulation of Cancer Determining Genes and Implications for Genome Stability and Tissue Differentiation

- I confirm that:

 1. This work was done wholly or mainly while in candidature for a research degree at this University;
 2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
 3. Where I have consulted the published work of others, this is always clearly attributed;
 4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
 5. I have acknowledged all main sources of help;

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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
 7. Either none of this work has been published before submission, or parts of this work have been published as: [please list references below]:

Signed:

Date:

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Definitions and Abbreviations

ABL	Average Epithelial Tree Branch Length
ACT	Actin-Like Protein
α-SMA	Alpha-Smooth Muscle Actin
ADH	Atypical Ductal Hyperplasia
APH	Aphidicolin
ASPA	Animals (Scientific Procedures) Act
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia
B2M	Beta-2microglobulin
BARD1	BRCA1 Associated Ring Domain 1
BASC	BRCA1 Associated Genome Surveillance Complex
BDR	Basal Dietary Requirement
BER	Base Excision Repair
BRCA1	Breast Cancer Associated Gene 1
BRCT	BRCA1 C-terminal
BRF	Biomedical Research Facility
BRMS1	Breast Cancer Metastasis Suppressor 1
BSA	Bovine Serum Albumin
BSCs	Breast Cancer Stem Cells
CAK	CDK Activating Kinase
CDK	Cyclin Dependent Kinase
cDNA	Complimentary DNA
CIS	Carcinoma in Situ
CSCLCs	Cancer Stem Cell-Like Cells
CSCs	Cancer Stem Cells
CSCs	Cancer Stem Cells
Ct	Cycle Threshold
CYC	Cyclophilin
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DCIS	Ductal Carcinoma in Situ
DFE	Dietary Folate Equivalents

DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DNMT	DNAmethyltransferase1
DOHaD	Developmental Origins of Health and Disease
DRIs	Dietary Reference Intakes
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ER1	Estrogen Receptor 1
ES	Enrichment Scores
ETD	Epithelial Tree Density
EtOH	Ethanol
EZH2	Enhancer of Zeste Homolog 2
FA	Folic Acid
FACS	Fluorescence Activated Cell Sorting
FEA	Flat Epithelial Atypia
FGCP	Folypoly-γ-glutamate carboxypeptidase
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
GM	Geometric Mean
GO	Gene Ontology
GRN	Gene Regulatory Network
GSEA	Gene Set Enrichment Analysis
H3K27	H3 Lysine 27 Trimethylation
HATs	Histone Acetylation
HATs	Histone Acetyltransferase
HDACs	Histone Deacetylases
HER-2	Human Epidermal Growth Factor Receptor 2
hESCs	Human Embryonic Stem Cell
HKMT	Histone Lysine Methyltransferases
HMTs	Histone Methylation

HRR	Homology Directed Repair
HRT	Hormone Replacement Therapy
HU	Hydroxyurea
ICM	Inner Cell Mass
IDC	Infiltrating Ductal Carcinoma
ILC	Infiltrating Lobular Carcinoma
IOM	Institute of Medicine
IP	Immuno-Positive
IPA	Ingenuity Pathway Analysis
iPSCs	Induced Pluripotent Stem Cells
IR	Immuno-reactive
IR	Ionising Radiation
LCIS	Lobular Carcinoma in Situ
lncRNAs	Long Non-coding RNAs
LSCs	Luminal Stem Cells
MAD	Median Absolute Deviation
MaSCs	Mammary Stem Cells
MATA2A	Methionine Adenosyltransferase 2A
MDP	Methionine-Dependence Phenotype
miRNA	Micro RNA
M-MLV	Maloney Murine Leukaemia Virus
MMR	Mismatch Repair
MMTV	Mammary Tumour Virus
mRNA	Messenger RNA
MRU	Mammary Repopulation unit
MTHFR	Methylenetetrahydrofolate Reductase
MTTR	Methyltetrahydrofolate-homocysteine
NBF	Neutral Buffered Formalin
ncRNA	Non-Coding RNAs
NEJ	Number of Epithelial junctions
NER	Nucleotide Excision Repair
NHEJ	Non Homologous End joining
NIAID	Nation Institute of Allergy and Infectious Diseases

NLS	Nuclear Localisation Signal
NSCs	Neural Stem Cells
NTD	Neural Tube Defects
OCT-4	Octamer Binding Transcription Factor 4
PABA	Para-aminobenzoic Acids
PCA	Principle Component Analysis
PCNA	Proliferating Cell Nuclear Antigen
PgB	Polycomb Gene Bodies
PLCO	Prostate, Lung, Colorectal and Ovarian
PLSC	Pluripotent-Like Stem Cell
PND	Post-Natal Day
PRC2	Polycomb Repressive Complex 2
PTMs	Posttranslational Modifications
PVDF	Polyvinylidene Difluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RISC	RNA Induced Silencing Complex
RN	Random Nonomers
RNA	Ribonucleic Acid
ROI	Region of Interest
RPL	Ribosomal Protein
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SCD	Serine Cluster Domain
SEM	Standard Error of the Mean
sncRNA	Small Non-Coding RNAs
SSCs	Somatic Stem Cells
SWI/SNF	Switch/Sucrose Non Fermentable
TAE	Tris-Acetate EDTA
TE	Tris EDTA
TEB	Terminal End Bud
THF	Tetrahydrofolate
TRI2	Time Resolved Imaging 2
TSG	Tumour Suppressor Genes

TSGs	Tumour Suppressor Genes
UBC	Ubiquitin C
UDH	Usual Ductal Hyperplasia
UILs	Upper Intake Levels
UoS	University of Southampton
WT	Wild Type

Chapter 1: Literature Review

1.1 Breast Cancer

1.1.1 An Overview of Breast Cancer

Breast cancer is the leading form of invasive cancer in females, accounting for an estimated 25.2% of all cases and the cause of 1.7 million (11.7%) new cancer cases worldwide in 2012 (WHO, 2013, Stewart and Wild) (Figure 1.1). Globally, breast cancer is the most common cause of cancer death among women (522,000 in 2012), with 6.3 million females diagnosed in the past five years (Society, 2015a). Since 2008, breast cancer incidence has increased by more than 20% and rates of mortality by 14% (Society, 2015a). An estimated 246,660 new cases of invasive breast cancer are expected to be diagnosed in women and 2600 in men within the US during 2016, with an estimated 40,890 fatalities. Furthermore, it is predicted that 61,000 new cases of carcinoma in situ (CIS) (the earliest form of non-invasive breast cancer) will occur during 2016, of which approximately 83% will constitute ductal carcinoma in situ (DCIS) and 12% lobular carcinoma in situ (LCIS) (Society, 2015a, Society, 2015b). Invasive breast cancer incidence increases with age from 1.9% (1 in 53) between birth to 49 years of age, up to 6.7% (1 in 15) in women aged 70 or above (US) (Siegel *et al.*, 2015). Similarly, non-invasive breast cancer (DCIS and LCIS) incidence increases with age, peaking between age 70-79 (Society, 2015a).

Breast cancer is increasingly prevalent in developed countries and is the leading form of cancer in developing world regions (Key 2001). Despite an increase in breast cancer incidence, mortality rates in the US have decreased since 1989, attributed to a combination of enhanced levels of awareness, early detection and diagnosis, reductions in the use of menopausal hormone therapy (previously known as hormone replacement therapy, HRT) and novel targeted therapeutic treatments (Society, 2015a). Regardless of this decline, breast cancer death rates still ranked second out of ten leading cancer types within the US in 2015 (Siegel *et al.*, 2015). With increased life expectancies in western countries and the frequency of breast cancer highest during older age, breast cancer incidence is forecast to increase as high as 50% in the US by the year 2030 (Rosenberg, 2015). Furthermore, increasingly high levels of breast cancer in less developed countries that lack access to early diagnosis and modern therapies currently contribute to a high global frequency and mortality rate, providing a major contribution to the global burden of disease (WHO, 2013). This highlights the requirement for enhanced intervention that includes novel research, early diagnosis, global access to treatment and a long term strategy to cope with breast cancer in an increasingly aging population.

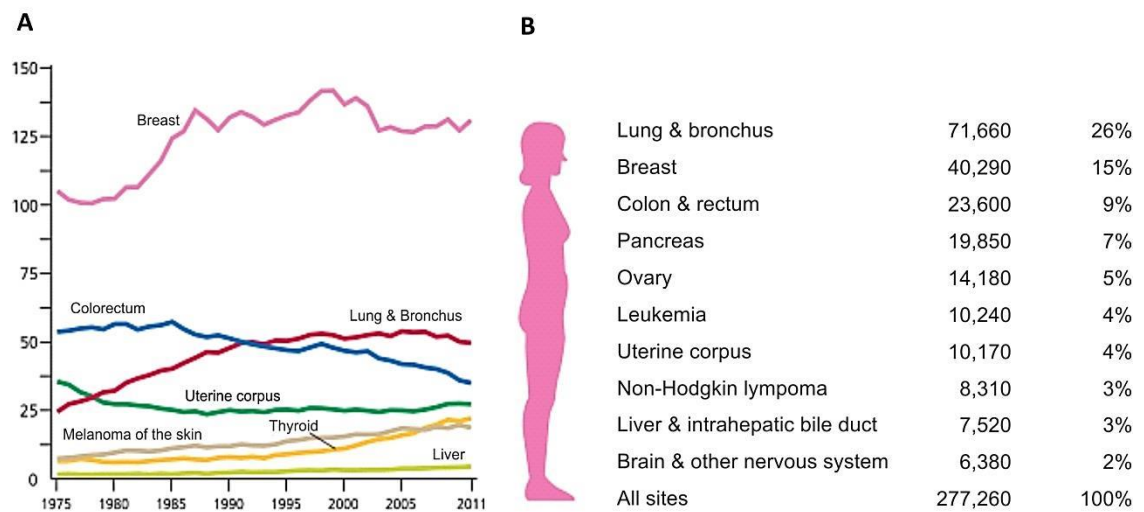


Figure 1.1. Trends in Incidence and Death Rates for Selected Cancers in Females within the United States between 1975 and 2011.

(A) Breast cancer (pink) incidence rate (per 100,000 of the population) (figure is age adjusted to the 2000 US standard population). (B) Breast cancer death rate (15%) compared to 9 leading cancer types in the US in 2015. Modified from (Siegel *et al.*, 2015).

1.1.2 Mammary Gland Function and Development

1.1.2.1 Embryonic Development of the Mouse Mammary Gland

Mammary glands develop primarily postnatally and function in the synthesis and secretion of milk to nourish the offspring (Inman *et al.*, 2015). In mice, mammary gland development begins shortly after mid gestation typically between embryonic (E) 10.5 and 18.5 (Hens and Wysolmerski, 2005). Bilateral epidermal ridges (or milk lines) running between the fore and hind limbs develop on E10.5 from which five pairs of placodes form (E11.5) and invaginate, each forming an embryonic rudimental epithelial bud (anlage) (Hens and Wysolmerski, 2005). Buds remain quiescent until E15.5-E16.5 after which they penetrate into the mesenchyme and extend into a collection of preadipocytes, eventually forming the mammary fat pad (Hens and Wysolmerski, 2005, Robinson, 2007).

1.1.2.2 Postnatal Development of the Mouse Mammary Gland

After birth the anlage remains relatively quiescent, growing allometrically until puberty where, in females, hormonal cues trigger extensive mammary branching morphogenesis and the ductal epithelium of the anlage invades the mammary fat pad (Inman *et al.*, 2015, Lyons, 1958) (Figure 1.2 A, G). Terminal end buds (TEBs), highly proliferative structures formed from cap cells surrounding epithelial cells are located at the leading ends of mammary ducts and extend the epithelial tree into the adipose stroma, followed by primary ducts formed from TEB bifurcation and secondary side branches which develop laterally filling the mammary fat pad (Williams and Daniel, 1983a) (Figure 1.2 B, G). Lobular alveolar structures develop at the end of tertiary branches as the gland reaches maturity (Sternlicht, 2006). The fully developed arboreal in adult virgin mice is composed of a bilayered ductal structure made up from contractile basal/myoepithelial cells which surround apically orientated luminal epithelial cells forming the ductal lumen (Hennighausen and Robinson, 2005, Inman *et al.*, 2015) (Figure 1.2 C, H). Mammary gland morphogenesis can therefore be broken down into three stages of development; embryonic, adolescent and adult, each of which is differentially regulated (Sternlicht, 2006).

Throughout the life course the mammary gland undergoes dramatic changes in both structure and function. Changes in hormone levels induced by the estrous/menstrual cycle induce cyclic expansions in mammary structure (Inman *et al.*, 2015). During pregnancy, dramatic remodelling of the mammary gland occurs, with proliferation of epithelial cells generating alveolar buds that differentiate into distinct alveoli and form lobules that secrete milk during lactation (Macias and Hinck, 2012) (Figure 1.2, D, I). This is followed by a second period of major remodelling that occurs postnatally as the mammary gland ceases to lactate (Lund *et al.*, 1996) (Figure 1.2, E, J). Post lactation involution is characterised by remodelling of the extra cellular matrix (ECM) that includes proteolytic degradation of the basement membrane and apoptosis of secretory epithelial cells undergoing programmed cell death (Lund *et al.*, 1996). The adult mammary gland is composed of a myriad of cell types which include epithelial, adipose, vascular, lymphatic and immune cells that form the architecture of the mature gland and are considered critical for regulation at different stages of mammary development (Inman *et al.*, 2015).

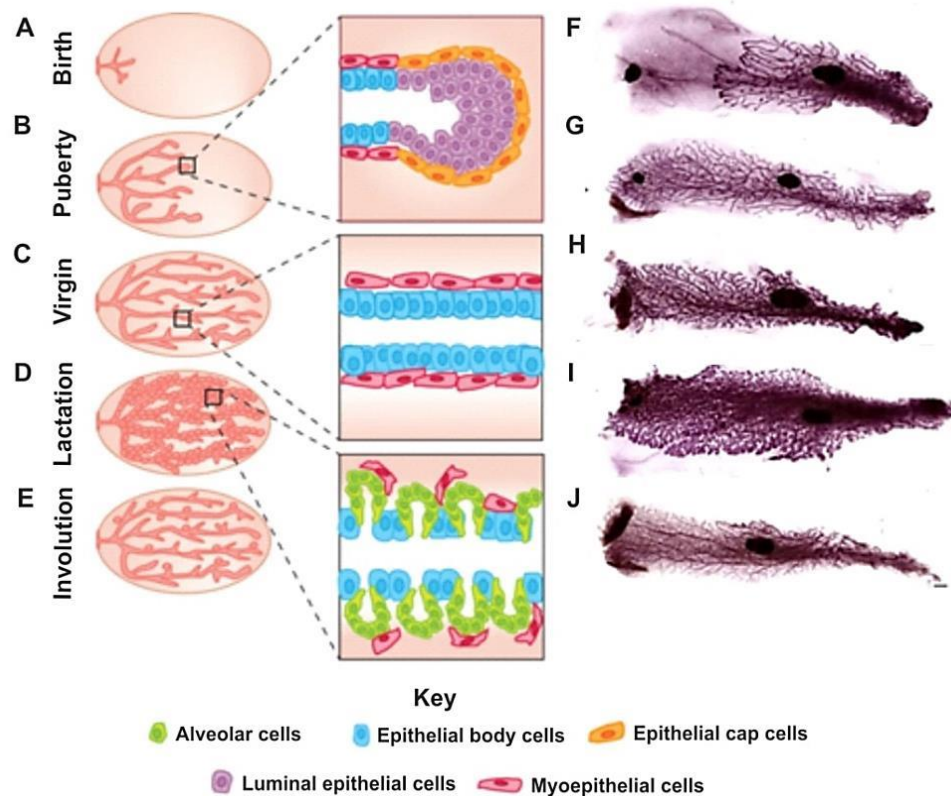


Figure 1.2. Mammary Gland Development from Birth to Adulthood in Mice

(A) At birth the mammary anlage is located in the mammary fat pad and remains quiescent until puberty. (B) During puberty terminal end buds (TEBs) formed from cap cells surrounding epithelial cells located at the ends of ducts extend the anlage into the mammary fat pad forming an epithelial arboreal. (C) In virgin adult mice the epithelial arboreal extends and fills the fat pad via the development of lateral side branches. Mammary ducts are formed from basal/myoepithelial cells surrounding luminal epithelial cells. (D) During pregnancy the expansion of alveolar cells that mature into acini cells that produce milk during lactation. (E) Post pregnancy the mammary gland undergoes a second period of major remodelling returning to that observed prior to pregnancy. (F, G, H, I, J) Carmine stained whole mount mouse mammary glands representative of A, B, C, D, and E respectively. Adapted from (Chuong *et al.*, Crow, 2005, Inman *et al.*, 2015, Olijnyk, 2011).

1.1.2.3 Mammary Epithelial Cells

Mammary epithelial cells are composed of two morphologically distinct populations: basal/myoepithelial cells and luminal cells (Inman *et al.*, 2015). Basal/myoepithelial cells form the outer structure of ducts residing on the basement membrane and express high molecular weight cytokeratin (K) 5, K14, as well as alpha smooth muscle actin (α -SMA) (Smalley *et al.*, 2008, Lazard *et al.*, 1993). Basal/myoepithelial cells demonstrate contractile properties mediated by α -SMA and function primarily to eject milk from the mammary gland (Lazard *et al.*, 1993). Conversely, luminal epithelial cells line the inside of ducts, produce milk from alveoli and express high molecular weight markers K8, K18 and transcription factor GATA-3 a regulator of luminal epithelial cell differentiation (Smalley *et al.*, 2008).

1.1.2.3.1 Mammary Epithelial Stem Cells

Previous research has identified additional populations of epithelial cells within the mammary gland in the form of putative stem and progenitor cells types (Shackleton *et al.*, 2006, Guest *et al.*, 2001, Stingl *et al.*, 2006). Research by Shackleton *et al.*, (2006) has identified a subpopulation of mouse mammary cells (Lin⁻CD29^{hi}CD24⁺) highly enriched for mammary stem cells (MaSCs) which are able to reconstitute multiple breast lineages, termed mammary repopulation units (MRUs) (Shackleton *et al.*, 2006). These cells were isolated using cell surface markers and transplantation. A single mammary cell, labelled with a LacZ transgene and transplanted into a cleared mammary fat pad was shown to be sufficient to reconstitute a complete mammary gland *in vivo*, contribute to both myoepithelial and luminal cell lineages and generate functional lobuloalveolar units during pregnancy (Shackleton *et al.*, 2006). Furthermore, these cells were shown to demonstrate self-renewing capacity by serial transplantation of clonal outgrowth (Shackleton *et al.*, 2006). Research by Stingl *et al.*, (2006) concurs, demonstrating the purification of a rare phenotypically distinct subset of adult mouse mammary cells that possess the capacity to individually regenerate a mammary gland *in vivo* within a six week period and demonstrate a CD49f⁺CD24⁺Sca-1^{low} immunophenotype (Stingl *et al.*, 2006). These MaSCs are rapidly cycling in the adult and can functionally generate mammary epithelial progenitor cells *in vivo* (Stingl *et al.*, 2006).

1.1.2.3.2 Juvenile/Pre-Pubertal Mammary Development

Lineage tracing of cell populations in transgenic animals has shown mammary development during puberty occurs predominantly through several different populations of stem and progenitor cells (Rios *et al.*, 2014). These cells include bipotent stem cells that possess the ability to generate both basal/myoepithelial and luminal cells that form mammary ducts (Rios *et al.*, 2014). Within the pre-pubertal gland, cells labelled for K5, K14 or leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) gave rise to clonal patches of cells from both myoepithelial and luminal lineages. ELF5⁺ cells give rise to alveolar and luminal cells suggesting Elf5⁺ cells contain a luminal progenitor pool (Rios *et al.*, 2014). EZH2, a driver of stem cell differentiation, is required to maintain the luminal cell pool through the regulation of progenitor differentiation and is critical for normal ductal elongation and mammary outgrowth during the pubertal period of mammary development (Michalak *et al.*, 2013). Research by Van Keymeulen *et al.*, (2011) has identified long-lived unipotent stem cells in both luminal and myoepithelial lineages in postnatal mouse mammary glands that can clonally expand during morphogenesis and adult life (Van Keymeulen *et al.*, 2011).

1.1.2.3.3 Adult Mammary Development

Within the adult virgin, murine mammary gland lineage tracing studies have identified epithelial cell turnover and remodelling (Rios *et al.*, 2014). Labelled Elf5⁺ luminal cells derived from the same progenitor have been shown to be present after 8 weeks with reduced numbers of clonal patches observed after 20 weeks, suggesting luminal progenitors decrease with time (Rios *et al.*, 2014). Interestingly, research by Rios *et al.*, (2014) shows that when performing lineage tracing in basal cells using K5⁺, patches of basal and luminal cells were observed, possibly from asymmetric division. (Rios *et al.*, 2014). It appears that over time, clonal patches containing only basal/myoepithelial cells change to contain mainly luminal clonal cells and that luminal progenitor cells derived from bipotent progenitor cells give rise to luminal cells that function to maintain mammary ducts. (Rios *et al.*, 2014).

Over the past decade a growing body of evidence has implicated epithelial mammary stem cells (MaSCs) in the aetiology of breast cancer (Al-Hajj *et al.*, 2003b). Cancer stem cell (CSC) theory is based on the observations that tumour cells demonstrate the ability to self-renew and give rise to differentiated cell lineages of the organ from which they originate (multipotent) (Tan *et al.*,

2006). Isolation and purification of cells from breast cancer tumours show positive expression of cell surface markers which are strongly associated with stem cells and their differentiation (Liu *et al.*, 2011a). Research by Al-Hajj *et al.*, (2003), used fluorescence-activated cell sorting (FACS) to purify isolated cells from human breast carcinoma, based on two tumour cell markers, CD44 and CD24. CD44⁺ CD24⁻ fraction demonstrated tumour initiating activity when implanted into non-obese, immune deficient mice (Al-Hajj *et al.*, 2003b).

1.1.3 Pathophysiology of Breast Cancer

Breast cancer is classified as either invasive or non-invasive. Non-invasive breast cancer exists in two forms; the first, ductal carcinoma *in situ* (DCIS) is a clonal proliferation of cells located in the basement membrane of mammary ducts. The second, lobular carcinoma in situ (LCIS), is located within the mammary lobules (Pinder *et al.*, 2010). Invasive breast cancer is classified into two common histologic types; infiltrating ductal carcinoma (IDC) or infiltrating lobular carcinoma (ILC) (Carcoforo *et al.*, 2012).

DCIS and LCIS, are defined as a proliferation of malignant-appearing cells within ducts and terminal lobular units, respectively, which do not breach the ductal basement membrane (Oliveira *et al.*, 2010). Both DCIS and LCIS are located 'in situ', thus exhibiting cancer cells that have not spread to surrounding tissues. Despite the localised pathophysiology of DCIS and LCIS, both these forms of breast cancer are considered precursors of invasive breast cancer (Oliveira *et al.*, 2010). IDC, a proliferation of the ductal epithelium, originates within the milk duct, where it breaches the duct wall and infiltrates the surrounding fatty tissue of the breast (Carcoforo *et al.*, 2012). From here IDC can metastasise to other parts of the body, commonly the lung, bone and liver via the blood stream and lymph system (Carcoforo *et al.*, 2012). Whilst ILC exhibits similar properties to IDC, it is pathologically distinct, originating within the milk producing glands (lobules) and diverging from IDC, in terms of sites of distant metastasis, more often involving the peritoneal surface, retroperitoneum and gastrointestinal tract (Ferlicot *et al.*, 2004, Winston *et al.*, 2000).

Pathophysiological characterisation of breast cancer has led to the identification of specific lesions defined as precursors of DCIS which are considered high risk factors for subsequent breast cancer development (Schnitt, 2003). These lesions have recently been categorised as flat epithelial atypia (FEA) or more complex proliferative lesions classed as atypical ductal

hyperplasia (ADH). For the ductal subtype, two models have been proposed, the first is a linear multistep process beginning with FEA (considered an obligate precursor to ADH), changing to ADH, which progresses into DCIS and terminates in invasive ductal carcinoma (IDC) (Wellings *et al.*, 1975, Lerwill, 2008). The second ductal model proposes usual ductal hyperplasia (UDH) as an intermediate stage of progression prior to FEA (Page *et al.*, 1985). The lobular subtype of breast cancer proposes ALH and LCIS as precursor stages that lead to ILC (Bombonati and Sgroi, 2011).

Gene expression profiling has resulted in the classification of breast cancer into five distinct subtypes (intrinsic system): luminal A and B, basal-like (triple negative), HER-2 positive and normal breast-like (Bombonati and Sgroi, 2011, Perou *et al.*, 2000). These are based on hormone receptor expression, the genes that they regulate and the molecular and genetic characteristics of the disease (Perou *et al.*, 2000). Luminal A and B, considered the most common breast cancer subtypes, contain estrogen receptors (ER) which are strongly implicated in cellular proliferation (Bombonati and Sgroi, 2011, Perou *et al.*, 2000). Basal-like (triple negative) breast cancer exhibits cells that contain no ER or progesterone hormone receptors and are more common in *BRCA1* mutation carriers (Bombonati and Sgroi, 2011). *HER-2* positive breast cancer contains human epidermal growth factor receptor 2 (HER-2), a proto-oncogene implicated in cellular growth (Perou *et al.*, 2000). Normal breast-like subtype is a heterogeneous group that includes tumours with high stromal content, high lymphocyte infiltration and normal epithelial cell contamination of tumours, with low malignant cell content (Perou *et al.*, 2000).

1.1.4 Genetic and Molecular Aspects of Breast Cancer

1.1.4.1 Breast Cancer Genetics

Breast cancer is considered a heterogeneous age-related malignancy, subdivided into two types; early onset and late onset. A hospital based control study of 1185 women identified correlations between age at childbirth and breast cancer risk, with null parity and late age at first childbirth associated with a higher breast cancer risk (Helmrich *et al.*, 1983). Breast cancer demonstrates age-specific incidence, with 80% of all breast cancers arising in women over the age of 50. The 10-year probability of developing invasive breast cancer increases from less than 1.5% at age 40, to approximately 3% at age 50 and over 4% by age 70 (DevCan, 2006). Breast cancer

development at an early age is strongly correlated with increased familial risk, especially when combined with genetic susceptibility in the form of a Breast Cancer 1 Early Onset (*BRCA1*) gene mutation (Antoniou *et al.*, 2003). A research study in women diagnosed with breast cancer before age 30 identified *BRCA1*, Breast Cancer 2 Early Onset 2 (*BRCA2*) and *TP53* mutations in approximately half who demonstrated family histories of breast cancer and in less than 10% with non-familial breast cancer (Feinberg and Vogelstein, 1983).

Breast cancer is characterised by a series of complex genetic and epigenetic events that culminate in the malignant transformation of normal epithelial cells (Buchholz *et al.* 2002). Uncontrollable malignant growth and cell division within breast tissue is caused by aberrant changes that affect key cellular pathways involved in growth and development (Osborne *et al.*, 2004). Both genetic and non-genetic alterations occur in host cells as well as cells that interact with the tumour microenvironment including immune, vascular and stromal cells (Osborne *et al.*, 2004). Genetic mutations which cause changes to specific nucleotide base pairs within a gene are causally implicated (Buchholz *et al.*, 1999). Genetic mutations can result in either gain or loss of gene function (Buchholz *et al.*, 1999).

Breast cancer is thought to result, in part, from the dysregulation of two main types of gene; proto-oncogenes and tumour suppressor genes (Buchholz *et al.*, 1999). Changes at the level of DNA result in genome instability which occurs in a hostile tumour microenvironment (Beaver *et al.*, 2014). A single genetic change is seldom sufficient to instigate malignant tumour development (Bailey, 2010). Instead, cancer development is considered a multistep process that involves sequential alterations in numerous proto-oncogenes, tumour suppressor genes, microRNA genes and transcription factors (Bailey, 2010). Activation of proto-oncogenes combined with inactivation of tumour suppressor genes, results in high rates of dysregulated cellular proliferation and a loss of the cell's genomic integrity (Bailey, 2010). Therefore, changes in the regulation of genes implicated in breast cancer risk, which lead to changes in gene expression and subsequent protein synthesis, can have profound effects on disease aetiology.

Maladaptive function of a number of genes and cellular pathways involved directly in DNA repair, cell growth and proliferation have been causally implicated in breast cancer aetiology. (Hanahan and Weinberg, 2000, Vogelstein and Kinzler, 2004). Familial breast cancers (20-25%) are most strongly correlated to autosomal dominant germline mutations in the tumour suppressor genes *BRCA1* and *BRCA2*; these high penetrance cancer susceptibility genes are located on chromosome 13 and 17 respectively (Rice *et al.*, 2000, Bissonauth *et al.*, 2008, Olivotto *et al.*,

2001). Both *BRCA1* and *BRCA2* encode for proteins that play pivotal roles in DNA repair, damage, surveillance, cellular regulation and mammary stem cell differentiation (Bissonauth et al., 2008, Smalley et al., 2008). Germline mutations in female carriers of *BRCA1* and *BRCA2* lead to maladaptive function of encoded tumour suppressor proteins, resulting in decreased DNA repair, increased genomic instability and a 60-80% risk of developing breast cancer throughout the life-course (Malone *et al.*, 1998, Rice *et al.*, 2000).

Despite increased risk conferred to individuals with a family history of breast cancer (5-10%), the disease is more commonly attributed to sporadic forms of development (Palmero *et al.*, 2007). Sporadic breast cancer is a complex multifactorial disease, stochastic in nature, not dependent on germline mutations and can occur de novo throughout the life-course (Suter and Marcum, 2007). The aetiology of sporadic breast cancer is poorly understood, however, has been linked with a myriad of factors that include genetics, ageing and lifestyle, as well as reproductive and environmental components (Esteller, 2008, Ferlicot *et al.*, 2004). Current research suggests that sporadic forms of breast cancer arise from an accumulation of acquired mutations in somatic genes, typically mutational activation of oncogenes combined with non-mutational inactivation of tumour suppressor genes (Kenemans *et al.*, 2008). Candidate polymorphic genes linked to sporadic breast cancer include those encoding for enzymes within pathways involved in carcinogen and estrogen metabolism, cell cycle, cell growth, and DNA repair (Mitrinen and Hirvonen, 2003b, Kenemans *et al.*, 2008).

Epidemiological studies have shown that increased or prolonged exposure to reproductive hormones is considered a major risk factor in sporadic breast carcinogenesis (Mitrinen and Hirvonen, 2003a). Endogenous and exogenous estrogens enhance cell proliferation by increasing the number of cell divisions and subsequently the possibility of mutations occurring in mammary tissue (Mitrinen and Hirvonen, 2003a). Furthermore, complex interplay between expression of low penetrance genes and environmental factors are thought to play a key role in sporadic breast cancer aetiology (Kotnis *et al.*, 2005). More recently, sporadic forms of breast cancer have been associated with alterations in the epigenetic regulation of genes. These are stable, heritable molecular alterations of cellular phenotype that do not involve direct changes to the DNA sequence (Rice *et al.*, 2000, Lahtz and Pfeifer, 2011, Jovanovic *et al.*, 2010). Epigenetic modifications associated with breast cancer have been causally linked to changes in hormonal receptors, as well as a number of susceptibility genes that include tumour suppressors, regulators of cell cycle, apoptosis, DNA repair, cellular homeostasis, cell invasion and cell

adhesion (Jovanovic *et al.*, 2010). Such changes are thought to be as a result of genomic adaptation to exposure from environmental factors throughout development and the life-course, such as nutritional status, geographical location and lifestyle, modifying the epigenetic landscape and contributing to the breast cancer phenotype (Bishop and Ferguson, 2015, Teegarden *et al.*, 2012). Notable examples of such changes include the major breast cancer susceptibility gene *BRCA1*, strongly associated with familial breast cancer which has been shown to be aberrantly methylated within the promoter region in breast cancer specimens; and also hypomethylation and overexpression of pluripotency gene octamer-binding transcription factor 4 (*OCT-4*) which has been shown to increase breast cancer risk (Linn *et al.*, 2010, Kumar *et al.*, 2012, Rice *et al.*, 2000).

1.1.4.2 Proto-oncogenes

Proto-oncogenes encode for proteins that are directly involved in the stimulation of cellular division, inhibition of cellular differentiation and cellular apoptosis (Todd and Wong, 1999) (Table 1.1). Dysregulated proto-oncogenes exhibit the potential to become oncogenic, with activation of oncogenic genes considered a prerequisite in cancer development (Osborne *et al.*, 2004). Oncogenes cause a gain of function through several direct mechanisms including gene amplification, point mutations, chromosomal translocation and epigenetic changes, as well as indirectly via modulation of signalling pathways (Osborne *et al.* 2004). Gene amplification causes increased levels of protein synthesis resulting in enhanced function, as seen in *HER-2*, a critical member of the epithelial growth factor receptor (EGFR/ERBB) present in approximately 20% of primary breast cancers (Osborne *et al.*, 2004).

The oncogene *RAS* is a small GTPase guanosine nucleotide binding protein that is critically involved in cellular signalling and encodes for proteins that play an important role in cell growth and differentiation (Downward, 2003) (Table 1.1). Point mutations in *RAS* have been implicated in lung, pancreatic, colon and less commonly breast cancers (Downward, 2003). Activating point mutations in *RAS* codons result in enhanced downstream signalling implicated in cell cycle activation (Downward, 2003). Wild type (WT) *RAS* expression correlates with increased survival of node free breast cancer patients possibly via induced apoptosis of cancer cells (Downward, 2003). Furthermore, cooperative function of oncogenes with mechanisms of epigenetic regulation can play a role in cancer signalling pathways (Baylin and Ohm, 2006).

Table 1.1. Proto-oncogenes, Normal Function and Oncogenic Roles in Cancer

Adapted from (Kopnin, 2000, Bos, 1989).

Proto-oncogenes	Normal function	Cancer
ERBB1 (EGF-R)	Receptor tyrosine kinase	Glioblastoma and other neurogenic tumours
ERBB2 (HER-2)	Receptor tyrosine kinase	Breast Cancer
RAS		Variety of tumour types including; adenocarcinomas of the pancreas, colon, lung, thyroid tumours, myeloid leukaemia.
PRAD1/Cyclin D1	Regulated cell cycle	Breast cancer and salivary gland cancer
C-MYC	Transcription factor, regulates cell cycle, telomerase activity	Burkitt's lymphoma, many forms of neoplasms
ABL	Regulates cell cycle apoptosis	All chronic myeloid leukaemia's, some acute Lymphoblast leukaemia's
CTNNB1 (B-Catenin)	a) transcription factor b) participates in formation of adhesion contacts via binding of cadherin	Hereditary adenomatous polyposis of large intestine; various forms of sporadic tumours
MDM2	Inactivates tumour suppressors p53 pRb.	Osteosarcomas and soft tissue sarcomas

1.1.4.3 Tumour Suppressor Genes

Tumour suppressor genes (TSGs) encode proteins that function to inhibit the formation of tumours via inhibition of cellular differentiation, promotion of apoptosis, modulation of the cell cycle and maintenance of genome fidelity (Osborne *et al.*, 2004, Bensam *et al.*, 2014). These genes negatively regulate functions that affect invasive and metastatic potential, such as cell growth, cell adhesion and protease activity (Osborne *et al.*, 2004) (Table 1.2). TSGs are the primary source of germline mutations that occur in breast cancer; however, somatic acquired mutations can also be present within these genes (Osborne *et al.*, 2004). TSGs are generally recessive, requiring both gene alleles to encode abnormal mutations. This classical "Two Hit" hypothesis can be defined by chromosomal loss of one allele and mutation in the other resulting in loss of function (Knudson 1971).

Currently, four major mechanisms are directly affected by TSG loss of function (Sun and Yang, 2010). Suppression of cellular division is a primary function of most TSGs. The most researched TSG that adopts this mechanism is P53, a protein that is encoded in humans by the *TP53* gene and is altered in breast cancer (Casey *et al.*, 1991) (Table 1.2). The main role of p53 is in the regulation of DNA repair and is thought to suppress cell division by arresting cell growth at the G1/S stage in response to DNA damage and functioning in the activation of DNA repair (Hartwell, 1992). P53 has also been shown to be required to modulate BRCA1 expression and BRCA1 has been shown to regulate p53-dependent gene expression, suggesting a dual role within DNA repair (Arizti *et al.*, 2000, Ouchi *et al.*, 1998). Mutations in *P53* occur in up to 30% of breast cancers and in as many as half of all cancers in humans (Hollstein *et al.*, 1991). Another TSG, p16INK4a, a cyclin dependent kinase (CDK) 4 inhibitor, has been implicated in breast cancer risk (Hui *et al.*, 2000). The P16INK4a gene is frequently inactivated by de novo hypermethylation and switched off in primary breast cancers (Hui *et al.*, 2000). Multiple TSGs function in the induction of apoptosis or programmed cell death. An example of this is *PTEN*, a gene that encodes the phosphatase and tensin analogue protein involved in regulation of the cell cycle and is implicated in breast tumourgenesis and tumour progression (Zhang *et al.*, 2013) (Table 1.2).

TSGs play an important role in the inhibition of cancer metastasis, a major cause of cancer death (Yoshida *et al.*, 2000). Breast cancer metastasis suppressor 1 (*BRMS1*) has been shown to decrease metastasis development in breast malignancies (Seraj *et al.*, 2000). Loss of function of *BRMS1* is associated with worse disease-free survival in subsets of breast cancer patients (Hicks *et al.*, 2006) (Table 1.2). TSGs play a critical role in the repair of damaged DNA (Sun and Yang, 2010). *BRCA1*, *BRCA2* and *P53* function independently or cooperatively to maintain genomic stability through DNA repair mechanisms (Hartman and Ford, 2003). Mutations in these TSGs are strongly associated with increased breast cancer risk (Sherr, 2004).

Table 1.2. Tumour Suppressor Genes, their Normal Function and their Implication in Cancer

Adapted from (Stilgenbauer *et al.*, 1997, Osborne *et al.*, 2004, Hershko, 2010, Pedersen and Brix, 2010, Meijers-Heijboer *et al.*, 2002, Bell *et al.*, 1999, Goldgar *et al.*, 2011).

Tumour Suppressor	Normal function	Cancer
<i>p53</i>	DNA repair, induces cell-cycle arrest, cell-cycle checkpoint activation	Bladder, breast, colorectal, oesophageal, liver, lung, prostate and ovarian carcinomas; brain tumours, sarcomas, lymphomas and leukaemia's.
<i>P27</i>	Inhibit cyclin-dependent protein kinases; arrest cell cycle G1 phase	Colorectal, breast, lung, prostate, ovarian
<i>P16 INK4a</i>	Inhibits cyclin-dependent protein kinases CDK4 and CDK6	Pancreatic adenocarcinoma, breast, oesophageal, gastric
<i>BRCA-1</i>	Regulates DNA transcription; acts to repair damaged DNA, regulation of mammary cell differentiation	Inherited breast cancers, ovarian cancers
<i>BRCA-2</i>	Repairs damaged DNA	Inherited breast cancers, ovarian
<i>CHK2</i>	Cell cycle checkpoint kinase, activates p53 after DNA damage	Breast, prostate, stomach, ovary, endometrium, kidney, prostate.
<i>ATM</i>	Checkpoint kinase, activates CHK2	Breast, T-prolymphocytic leukaemia.
<i>PTEN</i>	Phosphatase, negative regulator of Akt kinase	Cowden syndrome, increased risk of breast and thyroid cancer
<i>Rb</i>	Retinoblastoma gene, repressor of cell cycle and protein translation	Retinoblastoma, sarcomas; bladder, breast, oesophageal, prostate and lung carcinomas.

1.2 BRCA1

1.2.1 BRCA1 Structure

The *BRCA1* gene was identified in 1990 via linkage analysis in large cohorts of families that exhibited hereditary breast cancer and was first cloned in 1994 (Hall *et al.*, 1990, Miki *et al.*, 1994). *BRCA1*, located at 17q21 consists of 24 exons and spans 81KB encoding a 1863 amino acid protein (Bensam *et al.*, 2014, Miki *et al.*, 1994). The BRCA1 protein contains multiple domains; a zinc RING finger domain in the 5' amino terminal region, a serine domain (SCD), two BRCA1 C-terminal (BRCT) domains, a nuclear localisation signal (NLS) and nuclear export signal motifs (NES) (Paterson, 1998a, Henderson, 2005, Miki *et al.*, 1994, Koonin *et al.*, 1996) (Figure 1.3).

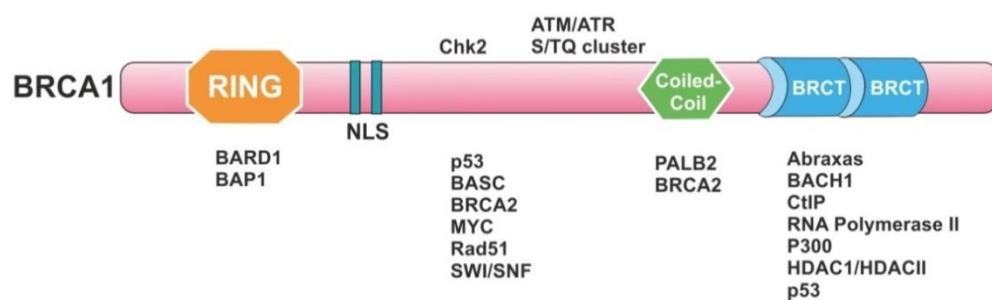


Figure 1.3. Structure of BRCA1 RING: Really Interesting New Gene domain.

NLS: nuclear localization signal; SCD: serine cluster domain; BRCT domains: BRCA1 C-terminal domains. Transcription factors and binding proteins are listed below the domains they interact with: Adapted from (Wang, 2012).

1.2.2 BRCA1 Function

BRCA1 is expressed in multiple tissues including the breast, ovaries, testes and thymus where it functions as a tumour suppressor gene (Marquis *et al.*, 1995b, Hu, 2009, Miki *et al.*, 1994). BRCA1 protein performs multiple cellular roles based on its multi-domain structure. The BRCA1 RING domain mediates protein ubiquitination through E3 ubiquitin ligase activity (Huen *et al.*, 2010). BRCA1 RING domain is further implicated in the heteromeric formation of a dimer with the

protein BARD1 that functions in DNA damage signalling and repair (Huen *et al.*, 2010). The BRCA1 BRCT domain functions in the binding of phosphorylated proteins that are critical in the signalling of DNA damage (Manke *et al.*, 2003, Yu *et al.*, 2003, Meza *et al.*, 1999) (Figure 1.3). *BRCA1* functions as a caretaker gene and its protein product serves to maintain genome integrity through multiple key cellular functions that include DNA repair and recombination, transcriptional regulation, as well as a putative role in the regulation of cell cycle checkpoints (Venkitaraman, 2002, Paterson, 1998b, Kinzler and Vogelstein, 1997, Deng, 2001).

1.2.3 BRCA1 in DNA Repair

DNA damage is a function of the life cycle of a cell and occurs endogenously through dysregulation of cellular processes such as DNA replication, meiotic recombination, or exogenously through environmental factors that include ionizing radiation, air pollution, UV and chemical exposure (Deng and Wang, 2003). Mutations in genes implicated in DNA repair are associated with developmental abnormalities that include cellular proliferation defects, genetic instability and tumourgenesis; key factors in cancer cell development (Deng and Wang, 2003). DNA repair occurs through five major pathways; base excision repair (BER), mismatch repair (MMR), homology-directed repair (HRR), non-homologous end joining (NHEJ) and nucleotide excision repair (NER) (Wood *et al.*, 2001).

Chromosomal double stranded breaks (DSB) are a form of DNA damage that can result in mutagenesis, cell lethality and carcinogenesis (Jeggo, 1998b). Defects in DSB repair are strongly linked to mutations in *BRCA1* and *BRCA2* (Scully and Livingston, 2000a). Murine embryo fibroblasts carrying a targeted *Brca1* deletion on exon 11 exhibit spontaneous chromosomal breakage and defective G2-M checkpoint-mediated growth arrest (Xu *et al.*, 1999b). Embryonic tissues that lack wild type (wt) *Brca1* and *Brca2* demonstrate hypersensitivity to ionizing radiation (IR) and chemical exposure, which is thought to be consistent with defective DSB repair (Sharan *et al.*, 1997, Deng and Scott, 2000, Gowen *et al.*, 1998).

DSBs are repaired by two main pathways; homologous recombination (HRR) and non-homologous end joining (NHEJ) (Jeggo, 1998a) (Figure 1.4). HRR utilises a homologous sequence from an identical sister chromatid to repair damaged DNA sequences with high accuracy (Bernstein *et al.*, 2002). Conversely, NHEJ involves the addition and deletion of nucleotides from the ends of a DNA sequence, followed by ligation which restores covalent continuity with less

accuracy (Bernstein *et al.*, 2002). BRCA1 play a functional role in HRR, NHEJ and nucleotide excision repair (NER) in response to DNA damage (Pierce *et al.*, 2001, Moynahan *et al.*, 1999, Moynahan *et al.*, 2001, Venkitaraman, 2002). BRCA1 functions as a component of the BRCA1-associated genome surveillance complex (BASC), a large multi-subunit protein complex that forms in response to DNA damage (Wang *et al.*, 2000). BASC contains multiple genes including tumour suppressors, signal transducers and DNA damage sensors which are thought to act synergistically as a sensor for DNA repair (Wang *et al.*, 2000).

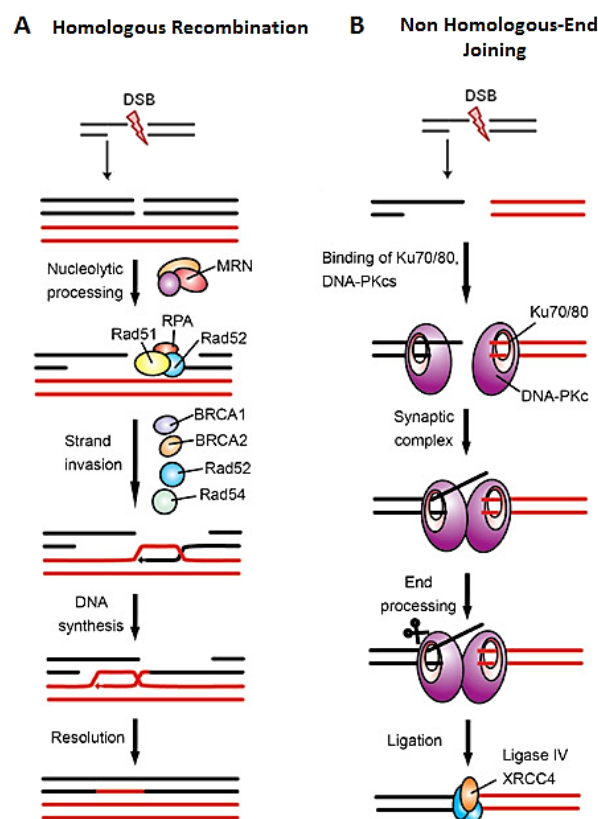


Figure 1.4. Homologous Recombination (HRR) and Non-Homologous End-Joining (NHEJ)

(A) HRR utilises a homologous sequence from an identical sister chromatid to repair damaged DNA sequences with high accuracy. Firstly the ends of DNA are processed by MRN (Mre11/Rad50/Nbs1) complex creating single-strand overhangs. Secondly Rad51, Rad52 and RPA associate with DNA overhangs forming a joint molecule between strands which is repaired by template guided DNA synthesis. (B) NHEJ forms a synaptic complex between the ends of damaged DNA consisting of two DNA PKcs and two Ku70/80 molecules. DNA is subsequently repaired by IV/XRCC4 ligase. Adapted from (Weterings and Chen, 2008).

DSBs result in activation of the mammalian checkpoint kinases ATM (mutated in ataxia telangiectasia) and ATR (ataxia telangiectasia) (Tibbetts *et al.*, 2000, Abraham, 2001, Caestecker and Van de Walle, 2013). ATM co-localises with, and phosphorylates BRCA1, both in vivo and in vitro in response to DNA damaging radiation (Cortez *et al.*, 1999). ATR phosphorylates BRCA1 in response to UV light or DNA replication inhibitors hydroxyurea (HU) and aphidicolin (APH) (Tibbetts *et al.*, 2000). Importantly, mutated BRCA1, missing two phosphorylation sites, fails to rescue radiation hypersensitivity in a BRCA1 deficient cell line, implicating BRCA1 as a critical component in the response to DSBs (Cortez *et al.*, 1999).

BRCA1 co-localises with mammalian homologue of E-coli RecA protein (RAD51) which functions in meiotic and mitotic recombination and DSB repair (Tashiro *et al.*, 1996). Co-localisation of BRCA1 and RAD51 occurs through a BRCA1 interaction domain, at sites of DNA replication that occur in response to DNA damage where they function in HRR of DSBs (Scully *et al.*, 1997b). BRCA1 also co-localizes with RAD50 within discrete nuclear foci forming a BRCA1, RAD50, Mre11 and p95/Nbs1 (MRN) complex in response to DNA damaging irradiation (Zhong *et al.*, 1999). MRN has been shown to play a critical role in homologous recombination, NHEJ and meiotic recombination (Lamarche *et al.*, 2010). These discrete foci are dramatically reduced in BRCA1-deficient HCC/1937 BC cells, however, can be restored via wild-type BRCA1 transfection (Zhong *et al.*, 1999). Activated ATM phosphorylates CtIP and ATR, a global regulator of the DNA damage response (Wang *et al.*, 2000, Cortez *et al.*, 1999). In addition to the function of ATR and ATM, PALB2 proteins play an important role in enrolling BRCA1 into DNA damage foci (Caestecker and Van de Walle, 2013). Abraxas/RAP80 act to regulate HR and PALB2 recruits RAD51 and BRCA2 proteins to regulate exchange of genetic material between homologous and damaged DNA (Caestecker and Van de Walle, 2013). BRCA1 forms a highly stable heterodimer with BRCA1 associated ring domain 1 protein (BARD1), through interactions within the BRCA1 N-terminal RING domain (Wu *et al.*, 1996). BARD1 shares homology with BRCA1 through a conserved N-terminal RING domain and two carboxyl terminal BRCT domains (Wu *et al.*, 1996). BRCA1/BARD1 complex exhibits ubiquitin ligase E3 activity, which is induced in response to endogenous (errors in DNA replication) or exogenous (genotoxic agents) genomic insults (Baer and Ludwig, 2002). In response to DNA damage BRCA1/ BARD1 E3 ligase functions may be activated by damage inducible kinases ATM, ATR or CDS1 (Baer and Ludwig, 2002). BRCA1/BARD1 may migrate to areas of DNA damage where induction of its ubiquitin E3 ligase activity may modulate cell cycle control via the inhibition of RNA processing (Baer and Ludwig, 2002).

BRCA1/BARD interaction has been shown to be disrupted by amino acid substitutions within the RING domain (Hashizume *et al.*, 2001). These tumorigenic germ line mutations are correlated with breast, ovarian and uterine cancers (Hashizume *et al.*, 2001). Breast cancer derived RING mutations in BRCA1 disrupt BRCA1/BARD ubiquitin ligase activity. Furthermore, BRCA1^{C61G} (BC-derived BARD1-interaction deficient mutant) mice have been shown not to exhibit ubiquitin ligase activity in vitro (Hashizume *et al.*, 2001). This implicates BRCA1/BARD1 ubiquitin ligase activity as a critical component in BRCA1 mediated DNA damage signalling and tumour suppressor function (Figure 1.5).

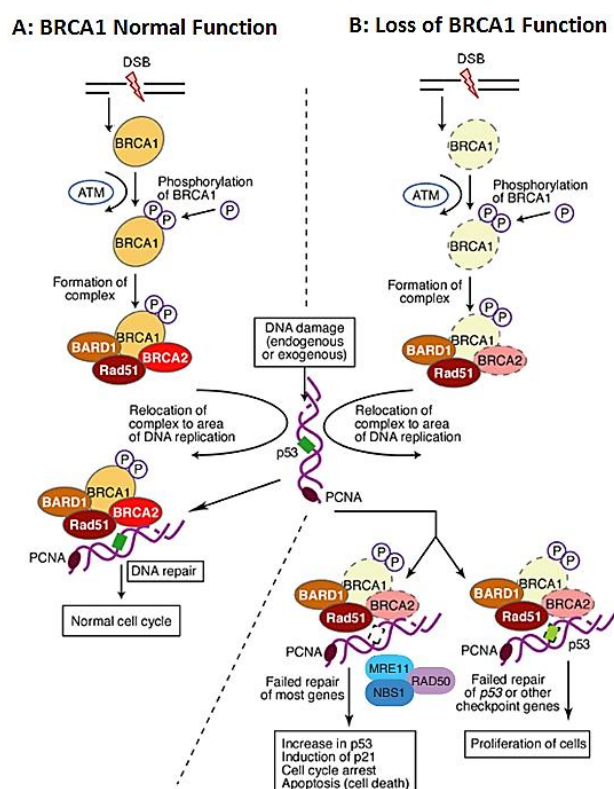


Figure 1.5. Function of WT BRCA1 and loss of function of mutant BRCA1 in DNA repair.

(A) In response to DNA damage (double stranded break) BRCA1 is phosphorylated by ATM. BRCA1 forms a macromolecular complex consisting of BRCA1, BRCA2, RAD51 and BARD1. This complex relocates to regions of the chromosome undergoing DNA replication marked by proliferating cell nuclear antigen (PCNA). Here BRCA1 recruits MRE11, NBS1 and RAD50 (MRN complex) and functions as a component of DNA repair machinery. (B) Loss of BRCA1 function results in the inability to repair DNA damage, failed repair of genes that control cell cycle, apoptosis (P53), or cell checkpoint which results in aberrant cellular proliferation. Adapted and modified from (Arnold and Goggins, 2001, Yoshida and Miki, 2004).

1.2.4 BRCA1 in Transcriptional Regulation

Mechanisms for BRCA1's role in transcriptional regulation have been linked to both RNA pol II and chromatin remodelling complexes (Anderson *et al.*, 1998). RNA Pol-II functions as a catalyst for DNA transcription and synthesis of ribonucleic acids (RNA) (Hahn, 2004). BRCA1 has been shown to co-purify with Pol-II holoenzyme complex via interactions with RNA helicase A, implicating BRCA1 as a component of essential transcriptional machinery (Anderson *et al.*, 1998). Cancer associated point mutations in BRCA1 have been shown to disrupt BRCA1-pol-II interactions (Scully *et al.*, 1997a). In addition, BRCA1 modulates the phosphorylation status of RNA pol-II C-terminal domain, which negatively regulates phosphorylation via CDK-activating kinase (CAK), a cyclin dependent kinase activator, suggesting a modulatory role for BRCA1 in cell cycle control (Moisan *et al.*, 2004). BRCA1/BARD1 may target RNA pol II and RNA processing machinery for degradation at sites of DNA damage, clearing the site and allowing access for DNA repair machinery (Kleiman *et al.*, 2005).

BRCA1 C-terminus (amino acids) 1528-1865) fused to a GAL4 DNA-binding domain has been shown to transactivate a GAL4 reporter which is prevented in disease associated mutations that truncate the BRCA1 C-terminus (Chapman and Verma, 1996). This suggests the interaction of BRCA1 with specific target genes that may control DSB repair and cell cycle checkpoint control, of which a number of transcription factors including p53, c-Myc, p21 and GADD45 have been identified (Welsh *et al.*, 2000).

1.2.5 BRCA1 and Chromatin Remodelling

BRCA1 has been shown to recruit histone deacetylases HDAC1 and HDAC2, components of SWI/SNF complex proteins as well as histone acetylase Rb associated proteins, which are critical for chromatin remodelling (Yarden and Brody, 1999, Bochar *et al.*, Pao *et al.*, 2000). Current mechanisms for BRCA1's roles in HDAC regulated transcriptional regulation are not clear, however, they may involve BRCA1 mediated chromatin activation or condensation (Bochar *et al.*, 2000, Pao *et al.*, 2000). BRCA1 may displace HDAC from specific gene promoter regions resulting in transcriptional activation. Conversely BRCA1 may function in the recruitment of HDAC complex's to specific promoters, removing acetyl groups from histones and preventing transcription factor binding, regulating specific gene expression (Bochar *et al.*, 2000, Pao *et al.*,

2000). Mutations in BRCA1/BRCT domain reduce transcriptional activity as well and disrupt binding of BRCA1 to the HDAC complex (Anderson *et al.*, 1998).

BRCA1 demonstrates a stable association with a Switch/Sucrose Non-Fermentable (SWI/SNF) ATP-dependant chromatin remodelling complex, through direct interaction with BRG1 SWI/SNF subunit (Bochar *et al.*, 2000). SWI/SNF functions to alter the tightly packaged DNA nucleosome structure, exposing otherwise protected regions of chromatin, which allows access to DNA binding proteins such as TFs (Tang *et al.*, 2010). Modulation of chromatin structure by BRCA1/SWI/SNF therefore provides a mechanism for BRCA1 in transcriptional gene regulation (Bochar *et al.*, 2000)

More recently, BRCA1 has been linked with chromatin remodelling polycomb repressive complex 2 (PRC2) (Wang and Huang, 2013). Research by Wang *et al.*, (2013) has identified BRCA1 binds directly to enhancer of zeste homolog 2 (EZH2), the catalytic subunit of PRC2, in both human breast cancer and mouse ESCs (Wang and Huang, 2013) (Figure 1.6). EZH2 functions as a methyltransferase, directly responsible for histone H3 lysine 27 trimethylation (H3K27me3) and epigenetic gene silencing through chromatin modification (Wang and Huang, 2013). .

1.2.6 BRCA1, Mammary Gland Differentiation and Mammary Stem Cells

BRCA1 is implicated in the control of mammary gland differentiation (Foulkes, 2004, Lim *et al.*, 2009). Both BRCA1 and BRCA2 function in the regulation of proliferation during embryogenesis and have been shown to be upregulated in breast epithelial cells undergoing proliferation during puberty, pregnancy and lactation (Welsh *et al.*, 2000, Rajan *et al.*, 1997). Research by Kubista *et al.*, (2002) has identified that down regulation of endogenous *BRCA1* expression, as well as ectopic overexpression of *BRCA1* affects regulation of mammary epithelial cell differentiation (Kubista *et al.*, 2002). Research by Liu *et al.*, (2007) has shown that mammary epithelial cells induced to differentiate by attachment to collagen coated dishes demonstrate a 4-fold increase in BRCA1 mRNA and protein levels (Liu *et al.*, 2008a). Furthermore, Furuta *et al.*, (2005) have shown *BRCA1* knockdown abrogates the formation of acini in 3D culture in MCF10A mammary epithelial cells, suggesting BRCA1 plays an important role in the regulation of mammary epithelial cell development (Furuta *et al.*, 2005).

A recent study by Buckley *et al.*, (2013) suggests a mechanism for regulation of mammary development through the Notch pathway (Buckley *et al.*, 2013). BRCA1 activates the Notch pathway in tumorigenic breast cancer cells via Δ NP63 dependent regulation of JAG1 gene (Buckley *et al.*, 2013). siRNA knockdown of components involved in Notch signalling results in loss of markers associated with basal and luminal differentiation, however, increases expression of genes associated with proliferation and markers of basal like breast cancer. This suggests a role for BRCA1, via transcriptional activation of the Notch pathway, in the regulation of mammary cell fate (Buckley *et al.*, 2013).

Current research has linked BRCA1 to a role in stem cell regulation (Liu *et al.*, 2008b, Buckley *et al.*, 2013). Research has identified adult stem cell populations within the mammary gland which are critical components of normal mammary cell self-renewal and differentiation during different stages of mammary development (Ezeh *et al.*, 2005a). These pluripotent-like stem cells (PLSCs) are present in large numbers within the lactating mammary glands and have also been identified in smaller populations to exhibit in vitro and in vivo pluripotency features in normal resting mammary tissue from non-pregnant, non-lactating women (Hassiotou *et al.*, 2012, Roy *et al.*, 2013). Mammary PLSCs form a heterogeneous population of cells that drive mammary remodelling via hierarchal differentiation and include early stage stem cells that exhibit pluripotent features, luminal and basal progenitor cells and myoepithelial cells (Hassiotou *et al.*, 2013b, Seymour *et al.*, 2015). Research by Liu *et al.*, (2008) has shown *BRCA1* expression is required for ER- α -negative stem/progenitor cell differentiation to ER- α -positive luminal cells (Liu *et al.*, 2008b). BRCA1 may function to regulate luminal differentiation by controlling transcriptional activation of ER- α (Hosey *et al.*, 2007). Research by Bai *et al.*, (2014) has shown that germline or epithelium-specific mutation of BRCA1 in p18-deficient mice activate epithelial-to-mesenchymal transition (EMT) and induces dedifferentiation of luminal stem cells (LSCs) (Bai *et al.*, 2014).

Interactions between BRCA1 and polycomb regulator EZH2 have been linked with ESC regulation (Wang and Huang, 2013). Transient knockdown of *Brca1* or genetic deletion of a single allele of *Brca1* in mouse ESC cell lines has been shown to promote genome-wide Ezh2 re-targeting and an increase in H3K27me3 at PRC2 target loci (Wang and Huang, 2013). Furthermore, increased EZH2 activity in response to decreased expression of BRCA1 results in inhibition of ESC differentiation and an aggressive breast cancer phenotype that includes increased cell migration and invasion (Wang and Huang, 2013). Collectively, these findings suggest that BRCA1 functions

as a negative modulator of EZH2 (Wang and Huang, 2013). Research by Wang et al., (2013) has further identified links between HOTAIR lncRNA, EZH2 and BRCA1. Overexpression of BRCA1 results in subsequent binding to EZH2 in a region that has been shown to be bound by HOTAIR, thus blocking HOTAIR-mediated PRC2 targeting on chromatin (Figure 1.6). Conversely, BRCA1 loss enhances EZH2 and HOTAIR interaction, levels of PRC2 occupancy and increases methylation of H3K27 in breast cancer cells (Wang and Huang, 2013). Wang et al., (2013) therefore suggests that reduced levels of BRCA1 expression, possibly due to promoter methylation, increases activity of EZH2, which in turn functions to prevent committed cell lineage differentiation within SCs or reprogramming of differentiated luminal cells into basal-like, aggressive neoplastic cells (Wang and Huang, 2013).

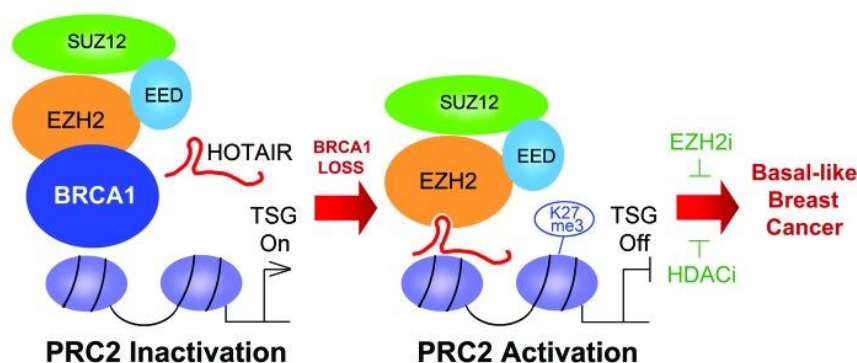


Figure 1.6 The Effect of BRCA1 Loss on PRC2 function and Cancer Development

BRCA1 binds to EZH2 which inhibits HOTAIR long non-coding RNA (lncRNA)/EZH2 interactions on chromatin, resulting in transcriptional activation of TSGs. BRCA1 loss results in HOTAIR binding to EZH2 and targeting of PRC2 to chromatin, where it functions via methylation of H3K27 to silence TSGs Adapted from Wang *et al.*, (2013).

1.2.7 BRCA1 and Breast Cancer

Germline mutations in *BRCA1* are implicated in a large population of families with inherited breast and ovarian cancers (80-90%), as well as approximately 50% of hereditary breast cancers (Hu, 2009). Mutations in *BRCA1* lead to protein truncation and to a lesser extent missense mutations which cause amino acid substitutions (Couch and Weber, 1996). Missense mutations

are generally located in clusters in the BRCA1 5' amino terminal RING domain, or the BRCT domain located at the 3' C-terminus (Paterson, 1998b) (Figure 1.3). Interestingly, somatic mutations in the *BRCA1* coding region have not been found in sporadic breast cancer and non-coding mutations are seldom implicated (Futreal *et al.*, 1994, Papa *et al.*, 1998).

Research suggests that aberrant function of BRCA1 in the regulation of DNA repair and cell cycle checkpoints are critical factors in the loss of genome stability that contribute to breast cancer development (Scully and Livingston, 2000a, Jasin, 2002, Pierce *et al.*, 2001). Studies using cell culture models to explore how *BRCA1* functions within tumour growth show *BRCA1* overexpression is lethal (Wilson *et al.*, 1997). The breast cancer cell line HCC1937 that lacks *wtBRCA1* demonstrates hypersensitivity to IR and delayed DSB repair which is reversed by stable expression of *wtBRCA1* (Scully and Livingston, 2000b). Similarly, *BRCA1* and *BRCA2* null embryos die during gestation, demonstrating proliferative defects and induction of the p53-dependent cell cycle inhibitor p21, suggesting the activation of a DNA damage dependent checkpoint (Hakem *et al.*, 1996, Scully and Livingston, 2000a). Mice with mutated BRCA1, specifically in mammary epithelium, develop late onset breast cancer, with the introduction of a p53 null allele significantly enhancing mammary gland tumour formation (Deng and Scott, 2000). p53-mediated checkpoint functions may therefore play a pivotal role in the control of proliferation in *BRCA1*-deficient breast ductal cells (Deng and Scott, 2000).

BRCA1 is associated with increased cancer risk in both a gender and tissue-specific manner (Hu, 2009). Antisense inhibition of *BRCA1* causes increased rates of tumour growth that demonstrate specificity to breast and ovarian cells (Wilson *et al.*, 1997). Interestingly, DNA repair and regulation of cell cycle checkpoints, both regulated by BRCA1, are fundamental components that are universal amongst cell types. One caveat that is currently unclear pertains to how aberrations in BRCA1 affect these processes mainly within female breast tissues and the molecular mechanisms responsible for this gender and tissue specific carcinogenesis (Welch *et al.*). Current research has implicated BRCA1 with novel roles in modulation of gene expression within breast cancer (Wang and Huang, 2013). One such role proposes that clinical, molecular and pathological features of breast cancer in BRCA1 mutation carriers are linked to BRCA1 function in the regulation of stem cells and adult progenitor cell populations (Foulkes, 2004).

Previous research by Lim *et al.*, (2009) identified an aberrant expanded luminal progenitor population of cells within the breast in *BRCA1* mutation carriers (Lim *et al.*, 2009). Furthermore, a conditional mutation of *BRCA1* results in blunted ductal morphogenesis and tumour formation

in mammary epithelial cells (Xu *et al.*, 1999a). More recently, direct interactions between BRCA1 and TWIST promoter show that BRCA1 suppresses TWIST and inhibits epithelial to mesenchymal transition (EMT) and LSC differentiation. Furthermore, interactions between BRCA1 and polycomb proteins have shown that increased BRCA1 gene expression (which binds directly to EZH2) results in increased level of EZH2 activity which in turn inhibits ES cell differentiation and subsequent development of a breast cancer phenotype (Wang and Huang, 2013).

1.3 Mammary Cancer Stem Cells

Over the past decade a growing body of evidence has alluded to a role for stem cells in the formation of carcinogenesis (Beltran *et al.*, 2011, Tan *et al.*, 2006, Petersen and Polyak, 2010). Cancer stem cell (CSC) theory is based on the observations that tumour cells demonstrate the ability to self-renew and give rise to differentiated cell lineages of the organ from which they originate (multipotent) (Tan *et al.*, 2006). Research by Al-Hajj *et al.*, (2003) has implicated epithelial mammary stem cells (MaSCs) in the aetiology of breast cancer (Al-Hajj *et al.*, 2003b). Fluorescence-activated cell sorting (FACS) was used to purify isolated cells from human breast carcinoma, based on two tumour cell markers, CD44 and CD24 (Al-Hajj *et al.*, 2003a). CD44⁺ CD24⁻ fraction demonstrated tumour initiating activity when implanted into non-obese, immune deficient mice (Al-Hajj *et al.*, 2003a). Furthermore, isolation and purification of cells from breast cancer tumours show positive expression of cell surface markers which are strongly associated with stem cells and their differentiation (Liu *et al.*, 2011a). Thus, within cancerous tissue, a small population of immortal tumour initiating cells with the capability of maintaining pluripotency, self-renewal and differentiation into other cancers may function to sustain cancer development.

Despite research linking stem cell function with breast cancer aetiology, the relationship between genes that function in stem cell differentiation and the clinic-pathological characteristics of breast cancer are far from clear (Liu *et al.*, 2011b). Pluripotency factors have been implicated as playing a functional role in pathways associated with breast cancer development (Redell and Twardy, 2006) (Zerbini, 2001). Pluripotency factors bind to sequence specific DNA targets (cognate recognition sequences or response elements) to regulate transcription levels of genes associated with stem cell self-renewal and differentiation (Warnmark *et al.*, 2003, Brent and Ptashne, 1984). Pluripotency transcription factors Octamer Binding Transcription Factor 4 (OCT-4), Sex Determining Region Y Box 2 (SOX-2) and Nanog

Homobox (NANOG) are considered the master regulators of pluripotency and function primarily within human embryonic stem cells (hESC's) as repressors of differentiation and in the maintenance of cellular pluripotency and self-renewal during development (Liu *et al.*, 2013, Boyer *et al.*, 2005).

CSC characteristics are observed in adult mammary stem cells which can divide and produce progenitor cells that demonstrate unlimited division capacity, providing parallels between adult somatic cellular regeneration and CSCs in tumorigenesis (Tan *et al.*, 2006). Breast cancer stem cells (BCSC) are strongly implicated in the formation of solid tumours, tumour recurrence and therapy resistance within breast cancer (Liu *et al.*, 2011b). These BCSCs demonstrate aberrant regulation of gene expression and signalling pathways (Chen *et al.*, 2013). *OCT-4*, *SOX-2* and *NANOG* are expressed in embryonic stem cells (ESC) and cancer stem cell-like cells (CSCs) (Liu *et al.*, 2013). Furthermore, research by Ezh et al., (2005) has identified *OCT-4*, *SOX-2* and *NANOG* expression in breast carcinoma (Ezh et al., 2005b). This suggests a specific role for pluripotency factors during the various stages of mammary gland development and remodelling both during and post pregnancy in the development and regulation of breast cancer stem cells. However, currently it is unclear exactly what may cause aberrant pluripotency factor expression that leads to development of CSCs and subsequent effects on breast cancer development and reoccurrence.

1.4 OCT-4

1.4.1 OCT-4 Structure

OCT-4 (also known as OCT-3) is a homeodomain transcription factor of the POU (pit-oct-unc) family, encoded from the *POU5F1* gene (Takeda *et al.*, 1992). OCT-4 exists in two isoforms within humans, OCT-4A and OCT-4B, which contain 360 and 265 amino acids, respectively (Takeda *et al.*, 1992). The structure of OCT-4 consists of a proline rich N-terminal domain and a proline, serine and threonine-rich C-terminal domain, as well as a POU domain made up of two sub domains: a 60 amino-acid carboxyl-terminal homeodomain (POUh) and a 75 amino acid terminal POU specific (POUs) region (Schöler, 1991, Jin *et al.*, 2002) (Figure 1.7).

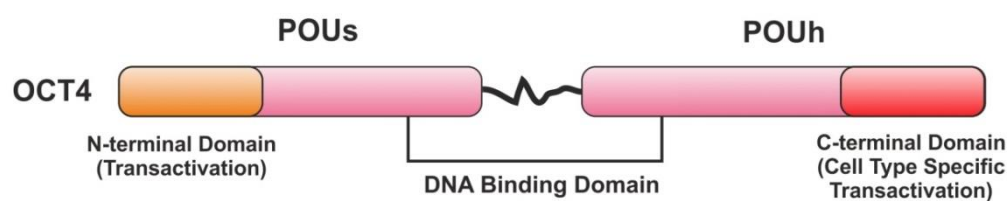


Figure 1.7 Structure of OCT-4

N-terminal domain functions in transactivation and C-terminal domain functions in cell type specific transactivation. The POU domain is made up from POU and POUh domains which are connected by a variable linker of 15 to 56 amino-acids. POU and POUh specifically bind DNA through a helix turn helix structure. Adapted from (Jin *et al.*, 2002).

Both N- and C-terminal domains have been implicated to play a role in transactivation (Schöler, 1991, Brehm *et al.*, 1997). Experiments fusing N- or C-terminal regions to a GAL4 DNA binding domain demonstrate that both function as transactivation domains in multiple cell lines (Takeda *et al.*, 1992). Furthermore, replacement of OCT-4 POU DNA binding domain with a GAL4 DNA binding domain does not affect transactivation function (Brehm *et al.*, 1997). OCT-4 POU domain is critically involved in DNA binding through DNA contact with both POUh and POU subdomains

(Jin *et al.*, 2002) (Figure 1.7). POUh and POUi domains are linked via a flexible linker that is variable in length, allowing each domain to bind independently to sequence specific targets on cognate DNA sequences (Herr and Cleary, 1995) (Figure 1.7). This formation allows multiple DNA binding conformations and interactions with numerous cofactors (Herr and Cleary, 1995). OCT-4 activates the transcription of specific genes that contain cis acting elements via binding to an octameric sequence motif of an AGTCAAAT consensus sequence located within target gene enhancer or promoter regions (Schöler, 1991, Pesce and Schöler, 2001).

1.4.2 Role of *OCT-4* in Pluripotency and Embryonic Development

OCT-4 is expressed in the unfertilized oocyte, ESCs and germ cells (Schöler *et al.*, 1989). Within these specialised cells *OCT-4* plays critical roles in the regulation of embryonic development as well as cell maintenance and cellular differentiation (Nichols *et al.*, 1998a). *OCT-4* is initially expressed in the embryo within blastomeres where it functions to maintain cellular pluripotency and suppress cellular differentiation (Thomson *et al.*, 1998). During embryonic development *OCT-4* expression becomes downregulated in the trophoblast and is upregulated in the inner cells of the blastocyst, (ICM) (Pesce and Schöler, 2001). Loss of *OCT-4* at the blastocyst stage results in differentiation of the ICM into trophectoderm, suggesting *OCT-4* functions to suppress differentiation of ESC in the ICM (Pesce and Schöler, 2001). Research using in vivo mutagenesis concurs with this theory, showing *OCT-4* expression is only detected in undifferentiated stem cells within the ICM and the epiblast (Pesce and Schöler, 2001). Furthermore, *OCT-4* targeted disruption in mice results in the development of embryos that lack a pluripotent ICM (Nichols *et al.*, 1998b). Interestingly, despite the essential role of *OCT-4* in maintaining cellular pluripotency, research suggests that it is not required for initiating totipotency/pluripotency in embryos (Wu *et al.*, 2013, Frum *et al.*, 2013). *OCT-4* plays a pivotal role in the self-renewal of undifferentiated embryonic stem cells (ESC) and is a key regulator of cellular differentiation (Turner *et al.*, 2007, Zaehres *et al.*, 2005).

1.4.3 **OCT-4 Expression in Embryonic and Adult Stem Cells**

ESCs, derived from the ICM, can divide continuously as undifferentiated cells (self-renewal) as well as maintain the ability to differentiate into any cell within the human body (pluripotency) (Reya *et al.*, 2001). Regulation of ESC differentiation is achieved via complex signalling pathways that express and activate the core regulatory TSFs OCT-4, SOX-2 and NANOG (Wang *et al.*, 2012b). OCT-4 functions synergistically with SOX-2 in the transcriptional regulation of target genes and the activation of its own expression through a positive auto regulatory loop in ESCs (Okumura-Nakanishi *et al.*, 2005, Ambrosetti *et al.*, 1997). This provides a possible mechanism by which OCT-4 maintains precise expression levels required to maintain ESC pluripotency (Okumura-Nakanishi *et al.*, 2005). OCT-4–SOX-2 complex is thought to drive NANOG transcription in mammals, regulating maintenance of this expression in ESCs (Rodda *et al.*, 2005).

Pioneering research by Takahashi and Yamanaka in 2006, reprogrammed mouse embryonic or adult fibroblasts to a pluripotent stem cell state through the forced expression of OCT-3/4, SOX-2, C-MYC, and KLF4 in these cells (Takahashi *et al.*). These reprogrammed pluripotent cells were termed ‘induced pluripotent stem cells’ (iPSCs). iPS cells were subsequently generated from adult human fibroblasts in 2007, using the same four TSFs (Takahashi *et al.*). iPS cells demonstrate characteristics that are highly similar to ESC (Takahashi *et al.*). The combinations of transcription factors required to initially generate iPS cells can vary, however, OCT-4 is considered essential (Kim *et al.*, 2009a). Current research suggests that OCT-4 alone is sufficient to induce pluripotency in neural stem cells (NSCs) (Kim *et al.*, 2009a).

Somatic stem cells (SSCs) are multipotent, meaning they can self-renew and divide into all the distinct cell types of the organ from which they originate (Barker *et al.*, 2010). SSCs function in the maintenance and supply of differentiated cells required to maintain organ function and repair of damaged tissue (Barker *et al.*, 2010). *Oct-4* expression has been demonstrated in pluripotent adult human stem cells within multiple tissues (Tai *et al.*, 2005, Trosko, 2006). Human breast epithelial, liver and pancreatic stem cells demonstrate diminished expression of OCT-4 when stimulated to differentiate (Tai *et al.*, 2005).

This suggests a role for OCT-4 in the maintenance of pluripotency in SSCs, which is consistent with that seen in embryonic stem cells. Interestingly, two studies by Lengner *et al.*, (2007) and Zangrossi *et al.*, (2007) have challenged this hypothesis. Lengner *et al.*, (2007) showed no OCT-

4 expression in murine adult stem cells (Lengner *et al.*, 2007). Furthermore, Lazarri (2007) identified low levels of *OCT-4* expression in adult stem cells which was mirrored in their stem cell-absent controls (Zangrossi *et al.*, 2007). More recently, *OCT-4* has been identified in the epithelium and stroma of human normal (non-pregnant/non-lactating) mammary glands (0.9%) co-localised with *NANOG* and shown to increase (11.4%) during lactation (Hassiotou *et al.*, 2013b).

1.4.4 *OCT-4* and Breast Cancer

Isolation and purification of cells from breast cancer tumours show positive expression of cell surface markers which are strongly associated with stem cells and their differentiation (Liu *et al.*, 2011a). *OCT-4* has been shown to be positively expressed in adult tumour cells that demonstrate stem cell characteristics (Liu *et al.*, 2011a). Transduction of *Oct-4* into normal breast preparations produces cells capable of tumour initiation and colonization capabilities, which cause differentiated carcinoma in nude mice (Beltran *et al.*, 2011). Research by Liu *et al.*, (2011) using human cell culture models and immunohistochemistry, has shown *OCT-4* is differentially expressed between CD44+/CD24- tumour cells and non-CD44+/CD24- tumour cells (Liu *et al.*, 2011a). *Oct-4* protein was more highly expressed in cancerous tissues than adjacent tumour cells and was related to histological type, lymph node status and molecular type of breast cancer (Liu *et al.*, 2011a). Furthermore, cases with highly expressed *OCT-4* protein attained significantly poorer postoperative disease-specific survival compared to those with low expression (Liu *et al.*, 2011a).

Research by Kim *et al.*, (2011) has determined whether *OCT-4* gene expression is associated with the maintenance and expansion of CSCs by the introduction of an *OCT-4* reporting vector into 4T1 mouse breast cancer cells and sorting populations of *OCT-4* high and low cells (Kim and Nam, 2011). *OCT-4* (high) 4T1 cells demonstrate increased tumorigenic potential in vivo, an increased ability to form tumorspheres and high levels of stem cell markers that include *ALDH1*, *CD34*, *Sca-1* in comparison to *OCT4*(low) 4T1 cells (Kim and Nam, 2011).

More recently, research by Wang *et al.*, (2014) has implicated *Oct-4* and *Nanog* in the regulation of epithelial-mesenchymal transition (EMT) and metastasis of breast cancer (Wang *et al.*, 2014). Levels of *Oct-4* and *Nanog* expression were associated with the pathology of tumours and poor

prognosis in breast cancer patients ((Wang *et al.*, 2014). Oct-4 and Nanog knockdown demonstrated lower expression and inhibited spontaneous changes in the expression of EMT-related genes (Wang *et al.*, 2014). Conversely, Oct-4 and Nanog over-expression was shown to enhance spontaneous changes in EMT-related gene expression in CSCs (Wang *et al.*, 2014). OCT-4, therefore, functions in the positive regulation of EMT and may act as a biomarker for initiation, progression and differentiation of cells towards a breast cancer phenotype (Wang *et al.*, 2014).

1.5 Risk Factors for Breast Cancer

1.5.1 Non Modifiable Risk Factors

The primary non-modifiable risk factor for breast cancer is attributed to a positive family history of the disease (Anderson, 1992). As high as 10% of breast cancers in western countries are associated with a heritable genetic predisposition (Bombonati and Sgroi, 2011). The most common influential inherited mutations in genes, include *BRCA1* and *BRCA2* with *P53*, *ATM*, *pTEN* and *CHEK2* further correlated with increased breast cancer risk (Society, 2013). Autosomal dominant inherited mutations in *BRCA1* and *BRCA2* result in a single copy of the altered gene in each cell sufficient to increase breast cancer risk (Anderson, 1992). Sporadic breast cancer, in which two somatic mutations occur on two homologous chromosomes, is generally considered to reflect tumour suppressor gene inactivation or loss and dysregulated cellular proliferation (Anderson, 1992).

Exposure to the hormone estrogen has been implicated as an important non-modifiable risk factor for breast cancer (Perou *et al.*, 2000). Prolonged exposure to endogenous estrogens due to early menarche or late menopause are considered high risk factors (Lacey *et al.*, 2009). Research in rodents has identified hydroxylated metabolites of 17- β estradiol induce DCIS and adenocarcinoma mammary tumours (Turan *et al.*, 2004). Elevated blood and urine levels of endogenous estrogen in postmenopausal women are associated with increased breast cancer risk (Bombonati and Sgroi, 2011).

1.5.2 Modifiable Risk Factors

A number of epidemiological studies support the role of environmental factors in a wide range of human cancers (Boyd *et al.*, 1998). Research suggests that 30-40% of all cancers can be prevented by lifestyle and dietary factors (Boyd *et al.*, 1998). Modifiable risk factors associated with lifestyle and breast cancer risk include diet and vitamin intake, body weight, number of pregnancies, cigarette smoking, levels of physical activity, alcohol intake, oral contraceptives, exogenous estrogens, ionizing radiation and chemical exposure (Kenemans *et al.*, 2008, van der Rhee *et al.*, 2007, Broeks *et al.*, 2007, Krishnadasan *et al.*, 2007, Bissonauth *et al.*, 2008, Suter and Marcum, 2007, Ferlicot *et al.*, 2004). Dietary and nutritional factors including the

consumption of single food groups such as fruits, meat and fish, selected micro and macro elements and single macronutrients such as vitamins, antioxidants and essential fatty acids have been linked to the modulation of breast cancer risk (Braga et al., 1997, Bissonauth et al., 2008). Of these, levels of alcohol intake and consumption of dietary fats which is linked to body weight have previously been shown to increase breast cancer risk (Byers, 1994). Research using cohort studies have shown that the risk of alcohol related breast cancer in women who have never smoked increases with as little as one alcoholic drink per day (Cao *et al.*, 2015). Furthermore, clear associations have been established between increased dietary fat intake, obesity and breast cancer risk (van den Brandt *et al.*, 2000).

More recently, levels of micronutrient intake and breast cancer risk have been linked (Roswall *et al.*, 2010, Cui and Rohan, 2006). A number of studies, although not all, have shown that folate status (either as dietary folate intake or levels of blood folate) is inversely related to the risk of developing colorectal, ovarian, prostate or breast cancers (Navarro Silvera et al., 2006, Shannon et al., 2009). However, current epidemiologic research has suggested that high levels of folate intake, largely from dietary folic acid and high plasma folate concentrations, may increase breast cancer risk (Stolzenberg-Solomon et al., 2006b). Both animal and human studies investigating dietary folic acid intake have recently shown associations between high levels of folic acid intake and increased mammary tumour development (Deghan Manshadi et al., 2014, Stolzenberg-Solomon et al., 2006b). Furthermore, both the timing and dose of folic acid intake are considered important factors in the development and progression of breast cancer (Kim, 2006b).

1.6 Folate

1.6.1 An Overview

Folate is used to denote naturally occurring water soluble moieties of vitamin B9 and is formed from a pteridine ring, para-aminobenzoic acid (PABA) and variable numbers of glutamate residues (Hoffbrand and Weir, 2001) (Figure 1.8). Folate cannot be synthesised *de novo* in mammals; thus folate is obtained through the diet from a variety of foods that include green leafy vegetables, fruits, nuts and legumes (Tucker *et al.*, 1996). Derivatives of folate can be obtained via dietary supplementation or food fortification with the synthetic forms of folate, referred to as folic acid.

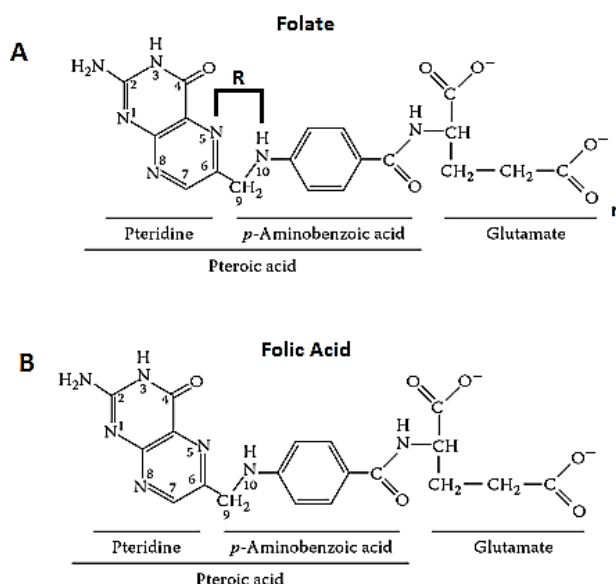


Figure 1.8. Structure of Folate and Folic Acid

(A) Folate is formed from a pteridine ring, para-aminobenzoic acid (PABA) and up to 9 glutamate residues joined by gamma peptide linkages. Additional single carbon units, including methyl (CH₃), formyl-CHO, methylene (CH₂), and methenyl (CH₄), can be attached to the N5 or N10 nitrogen atoms. (B) Folic acid is a synthetic form of folate formed from a pteridine ring, para aminobenzoic acid (PABA) residue and a single glutamate residue. Adapted from (Hoffbrand and Weir, 2001).

Folic acid (pteroyl glutamic acid) (FA), a synthetic form of folate in which the pteridine (2-amino-4-hydroxypteridine) ring remains unreduced, differs from naturally occurring folate in three ways; the addition of three glutamate residues (polyglutamates), reduction to di or tetra hydroforms and additional carbon units attached to N5 or N10 nitrogen atoms (Hoffbrand and Weir, 2001) (Figure 1.8).

FA structure is resistant to chemical oxidation and highly stable when compared to naturally occurring folate (Figure 1.8). This stability is reflected in the bioavailability of folic acid which is much greater than that of its naturally occurring counterpart. (Wang and Fenech, 2003b). Using isotopes to measure changes in serum folate levels, the bioavailability of folate derived from food was shown to be 78%, equating to 85% of the bioavailability of folic acid (Winkels *et al.*, 2007). Folic acid demonstrates superior stability and higher bioavailability when compared to naturally occurring folate found in foods (Winkels *et al.*, 2007).

1.6.2 Biochemical Functions of Folate and Folic Acid

Folate is an essential nutrient and biological co-factor which acts as a one-carbon source in DNA repair, synthesis and methylation reactions (Weinstein *et al.*, 2003, Crider *et al.*, 2012, Bailey and Gregory, 1999). Folate is essential in the development of pyrimidines and purines, critical nucleotides required in DNA synthesis and replication (Crider *et al.*, 2012). Furthermore, folate is directly involved in the regulation of DNA CpG methylation, via the primary methyl donor S-adenosyl methionine (SAM) (Friedel *et al.*, 1989).

Dietary folate exists in polyglutamate form and requires hydrolysis to monoglutamate form by the enzyme folypoly- γ -glutamate carboxypeptidase (FGCP) in the apical brush border of the intestine (Chandler *et al.*, 1991, Blom and Smulders, 2011). Folic acid's structure is monoglutamate (Figure 1.9), thus it is quickly absorbed through the intestinal mucosa without the requirement of a hydrolysis step (Bailey, 2010). Absorbed monoglutamate forms of folate and folic acid are further reduced to dihydrofolate (DHF) and subsequently tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR) (Bailey, 2010). THF is methylated to 5,10-methylene THF and reduced by the enzyme methylenetetrahydrofolate reductase (MTHFR) to form 5,10-methyltetrahydrofolate (5-methyl-THF), the primary biochemically active circulating form of folate in plasma and mammalian cells (Bailey, 2010).

5-methyl-THF functions as a methyl donor which is exclusively donated to homocysteine, forming methionine and tetrahydrofolate, catalysed by the vitamin B12 dependent methionine synthase (Wang and Fenech, 2003a). Methionine is a precursor of S-Adenosyl methionine (SAM), a co-substrate involved in the transfer of methyl groups to DNA, RNA and proteins (Ghandour *et al.*, 2002, Jacob *et al.*, 1998).

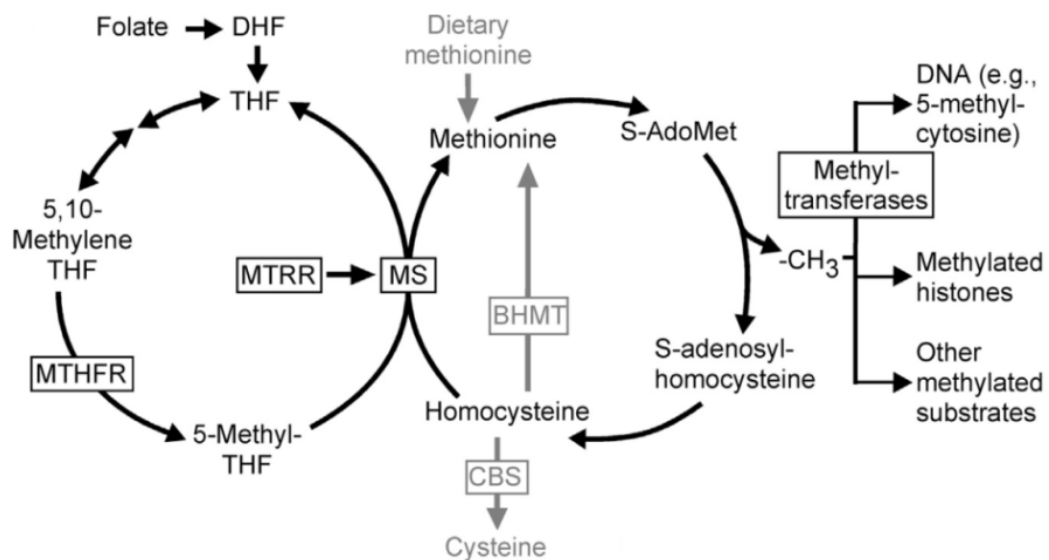


Figure 1.9. Folate Metabolism

Folate is metabolised to DHF and THF by DHFR. THF is converted to 5, 10-methylene THF by SHMT which is converted to 5-methyl THF (Folate) by MTHFR. 5, 10-methylene THF is used to convert dUMP to dTMP which is used in DNA synthesis. 5-methyl THF is used in metabolism of homocysteine to methionine. Methionine is converted to SAM which acts as a methyl donor. Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; dTMP, thymidylate; dUMP, deoxyuridine monophosphate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase (Adapted from (Padmanabhan *et al.*, 2013)).

1.6.3 Folic Acid Intake and Dietary Recommendations

Naturally occurring folate found in foods exists in polyglutamate form compared to folic acid which exists in fully oxidised monoglutamate form (Medicine, 1998). Folate derived from diet requires hydrolysis to monoglutamate form prior to absorption, thus is absorbed more slowly than folic acid (Chandler *et al.*, 1991, Bailey, 2010). To account for higher bioavailability of folic acid compared to folate derived from diet, the Institute of Medicine (IOM) developed intake recommendations based on dietary reference intakes (DRIs) measured as dietary folate equivalents (DFE) (Medicine, 1998). One microgram (μg) DFE is equivalent to 1 μg of folate from food. For folic acid, 1 μg DFE is equal to 0.6 μg of folic acid from fortified foods or dietary supplements consumed with foods and 0.5 μg of folic acid derived from dietary supplements that are taken on an empty stomach (Medicine, 1998).

The recommended dietary allowance of folate varies based on age and conventional status (Table 1.3). Current RDA for infants (birth to 12 months) is 65 μg of DFE, established from an average intake that is equivalent to the mean folate intake in healthy breastfed infants in the US (Medicine, 1998). Folate RDA increases with age up to 400 μg of DFE in adult males and females, and 600 μg of DFE in pregnant and lactating females (Table 1.3).

Table 1.3. Recommended Dietary Allowances (RDAs) for Folate (Medicine, 1998)

Age	Male	Female	Pregnant	Lactating
Birth to 6 months	65 μg DFE*	65 μg DFE*		
7–12 months*	80 μg DFE*	80 μg DFE*		
1–3 years	150 μg DFE	150 μg DFE		
4–8 years	200 μg DFE	200 μg DFE		
9–13 years	300 μg DFE	300 μg DFE		
14–18 years	400 μg DFE	400 μg DFE	600 μg DFE	500 μg DFE
19+ years	400 μg DFE	400 μg DFE	600 μg DFE	600 μg DFE

* *Adequate Intake (AI)*

Table 1.4. Tolerable Upper Intake Levels (UILs) for Folate (Ball, 1998).

Age	Male	Female	Pregnant	Lactating
Birth to 6 months	Not possible to establish	Not possible to establish		
7–12 months	Not possible to establish	Not possible to establish		
1–3 years	300 µg	300 µg		
4–8 years	400 µg	400 µg		
9–13 years	600 µg	600 µg		
14–18 years	800 µg	800 µg	800 µg	800 µg
19+ years	1000 µg	1000 µg	1000 µg	1000 µg

Fortification of foods with folic acid in many countries combined with supplements containing folic acid has raised concerns with regards to upper safety limits of folic acid intake and possible effects on masking vitamin B12 deficiency and accelerating pre-neoplastic cancer development (Brouwer and Verhoef, 2007, Deghan Manshadi *et al.*, 2014). The IOM has established guidelines for tolerable upper intake levels (UILs) of folic acid through food and supplementation (Table 1.4). Currently it is not possible to establish UILs for infants (Birth to 12 months). Folic acid UILs increase with age up to a maximum of 1000 µg in adult males and adult pregnant and lactating females (Table 1.4).

1.6.4 Folic Acid Fortification and Dietary Supplementation

1.6.4.1 Pregnancy and Neural Tube Defects

Over the past half century a plethora of research has highlighted the protective effects of dietary folate intake against neural tube defects (NTD), specifically spina bifida (Hibbard, 1964). Current opinion suggests that women who intend to become pregnant should ingest folic acid prior to, and throughout the duration of pregnancy to protect against NTD (Pitkin, 2007b). Guidelines suggest healthy adult females with no previous history of NTD consume between 400–600 µg of folic acid per day during pregnancy (Medicine, 1998) and females at high risk due to previous NTD as high as 4 mg per day (Control, 1991). Initially, recommendations for increasing folate did not improve NTD, which led to mandatory folic acid fortification in several countries including the US. Following mandatory fortification of staple foods with folic acid, a significant increase in

folate indexes and reduction in NTD frequency (25-30%) was observed (Mills and Signore, 2004, Society, 2015b).

1.6.4.2 Dietary Fortification and Supplementation

Over the past decade folic acid intake has increased, primarily due to mandatory fortification of staple foods as well as increased general and periconceptional folic acid supplementation (Wilson *et al.*, 2007, Bailey *et al.*, 2010c). Since 1998 the US Food and Drug Administration (FDA) have stipulated a requirement for folic acid addition to a wide range of food sources that include bread and cereals (U.S. Food and Drug Administration, 1996). The increase in intake of folic acid was projected to be 100 µg/day, however, mean folic acid intakes in the US were increased to 190 µg/day (Whittaker *et al.*, 2001, Choumenkovitch *et al.*, 2002).

Along with food fortification an increase in the availability and use of folic acid supplements has further contributed towards increased levels of folic acid intake. Folic acid is available either alone or as part of multivitamin complexes in a variety of different doses, often 400 µg. Research suggests that approximately 28% of children (1-13 years) and 35% of adults currently take folic acid containing supplements (Bailey *et al.*, 2010a, Bailey *et al.*, 2010d). Sources of dietary folic acid, including fortification of staple foods and supplementation within the US, have resulted in large populations of individuals consuming excessively high ($\geq 1000\mu\text{g}$) amounts of folic acid daily, near or above the upper safety limit (Bailey *et al.*, 2010c). A current report by NHANES has suggested that 2.6 million Americans between the ages of 51-70 currently exceed the tolerable upper limit of 1000 µg of folic acid per day (Bailey *et al.*, 2010b). This population-based increase in exposure to folic acid has raised major concerns with regards to its effects on immediate and long term health (Qin *et al.*, 2013).

Many European states have demonstrated resistance towards the introduction of folic acid fortification, due to associations between increased levels of folic acid and potential health risks that include a decrease in levels of natural killer cell cytotoxicity, increased risk of insulin resistance in pregnant women and associated obesity in their children as well as potential interactions between folic acid and medications (Smith *et al.*, 2008, Butterworth and Tamura, 1989). Recently, mandatory fortification of flour with folic acid is under debate by health ministers within the UK (Walker, 2016). Current UK recommendations have identified populations of individuals who might be at greater risk of cancer (individuals over the age of 50

and those with a history of cancer) and advised these individuals to limit folic acid supplementation, not exceeding 200 µg per day, due to the role folic acid might play in contributing to cancer risk in susceptible individuals. With evidence from the US folic acid fortification program demonstrating higher than expected increases in folic acid intake post fortification, it would appear that regulating folic acid intake in populations with known cancer risk or individuals who are at risk but not aware of their current folic acid status may prove complex.

As well as mandatory fortification of folic acid in some countries such as the US, levels of folic acid intake through supplementation is currently unknown. Epidemiologic studies show that vitamins and supplements are commonly used with higher frequency among newly diagnosed cancer patients as well as cancer survivors when compared to the general population (Giovannucci and Chan, 2010, Velicer and Ulrich, 2008, Holmes *et al.*, 2010). 54-72% of women with early-stage breast cancer have been shown to consume multivitamins pre and post cancer diagnoses respectively (Kwan *et al.*, 2011). Interestingly, Caucasian female breast cancer patients and survivors demonstrate the highest supplemental use of folic acid (Velicer and Ulrich, 2008). Furthermore, 17% of women with a high risk of hereditary breast and ovarian cancer (who underwent BRCA1/2 susceptibility genetic testing) reported using multivitamins and 51% using one dietary supplement (including multivitamins) (Alamian *et al.*, 2006). It is clear that mandatory fortification of staple foods increases levels of folic acid consumption, but when combined with high levels of folic acid intake through supplementation, such as levels found in some vitamin and dietary supplements, individuals could easily be regularly consuming excessively high levels of folic acid without being aware. This is particularly concerning in individuals with increased disease risk such as those who currently have or have had breast cancer.

1.6.5 Folic Acid and Breast cancer

Folate status has been inversely linked to breast cancer risk (Thorand *et al.*, 1998, Potischman *et al.*, 1999, Shrubsole *et al.*, 2001, Rohan *et al.*, 2000, Graham *et al.*, 1991, Zhang *et al.*, 2003, Levi *et al.*, 2001). However, many previous cohort and case control studies investigating the relationship between dietary folate intake and breast cancer risk are inconsistent and demonstrate a significant or equivocal inverse relationship that does not demonstrate statistical

significance (Thorand *et al.*, 1998, Potischman *et al.*, 1999, Zhu *et al.*, 2003, Cho *et al.*, 2003, Feigelson *et al.*, 2003). Research by Ulrich (2007) suggests a nonlinear relationship between folate status and breast cancer risk (Ulrich, 2007). Ulrich identifies epidemiologic studies that suggest higher folate status may reduce breast cancer risk in postmenopausal women with very low folate intakes; however, a lack of evidence suggests women with adequate folate intakes derive any additional benefit from increased folate intake. In fact research suggests that high folate intakes, attributable to supplemental use in the form of folic acid, may in fact increase breast cancer risk. Current research by Chen *et al.*, (2014) using a meta-analysis from prospective studies and case controlled studies has demonstrated a non-linear relationship between dietary folate intake and breast cancer risk (Chen *et al.*, 2014). Daily dietary folate intake in women between 153 μg and 400 μg demonstrates a significantly reduced risk of breast cancer when compared to those consuming less than 153 μg ; however, not for those consuming greater than 400 μg (Chen *et al.*, 2014). This suggests a U-shaped relationship exists between folate intake and breast cancer risk, with both low and high folate intake potentially linked to adverse breast cancer risk (Chen *et al.*, 2014). Research suggests that folate in the form of folic acid may play a dual role in cancer development by providing protection in individuals with low folate status; however, it may promote breast carcinogenesis at high doses (Ulrich, 2007).

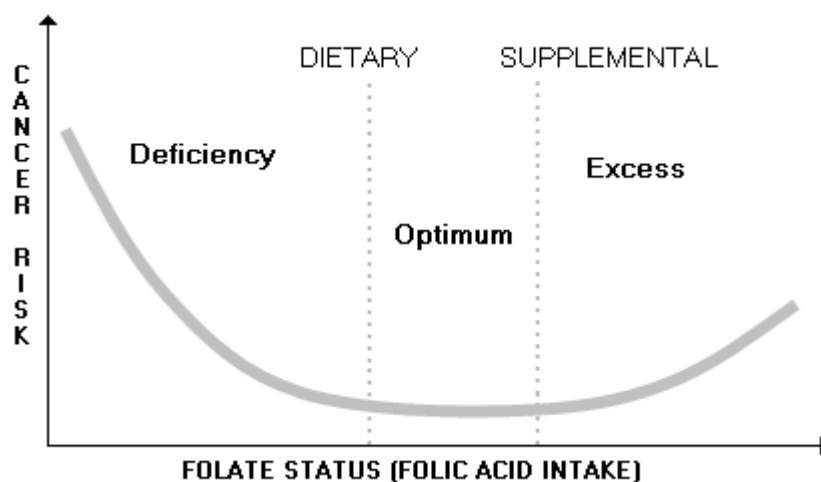


Figure 1.10. Hypothesised Relationship between Folate Status and Breast Cancer Risk.

Dietary folate deficiency below, and excess folate above the optimum daily allowance through folic acid intake increase breast cancer risk. Adapted from Ulrich, (2007).

Research has identified significant interactions between individuals who consume alcohol, a well-established risk factor for breast cancer and inhibitor of folate metabolism, and folate intake in modifying breast cancer risk (Zhang *et al.*, 1999, Sellers *et al.*, 2001, Baglietto *et al.*, 2005, Tjonneland *et al.*, 2006). Interestingly, increased breast cancer risk in individuals with low folate and high alcohol intake has been shown to be limited to estrogen receptor negative breast cancer (Sellers *et al.*, 2002, Zhang *et al.*, 2005a). Research investigating the association between dietary folate, alcohol consumption and postmenopausal breast cancer from the prostate, lung, colorectal and ovarian (PLCO) study, identified a 20 percent increase in breast cancer risk in women who consume ≥ 400 $\mu\text{g}/\text{d}$ of supplemented folic acid, compared with no supplemental intake (Stolzenberg-Solomon *et al.*, 2006a). Furthermore, folate intake from foods was not significantly related to breast cancer risk; however, total folate intake, mainly from folic acid supplementation, increased breast cancer risk by 32% (Stolzenberg-Solomon *et al.*, 2006a). Therefore, high folate, generally attributed to folic acid supplementation, may increase breast cancer risk in post-menopausal women (Stolzenberg-Solomon *et al.*, 2006a).

More recently, high folic acid intake (and high plasma folate concentration) in animal models have been linked with an increased risk of breast cancer development and promoting the development of pre-existing neoplasms. Research by Manshadi *et al.*, (2014) has shown that folic acid supplementation promotes the progression of established mammary tumours in female Sprague-Dawley rats (Deghan Manshadi *et al.*, 2014). Rats placed on a control diet (2 mg/kg folic acid) and given a single intragastric dose of 7,12-dimethylbenza[a]anthracene at puberty (7 weeks of age) to induce single mammary tumour formation were supplemented (once tumour size was between 0.7–0.9 cm) with 2, 5, 8 or 10 mg/kg of folic acid. Folic acid supplementation significantly promoted the progression of sentinel mammary tumours with the most significant and consistent effects observed in the 5 mg/kg (2.5 x BDR) supplemented group. Interestingly, no dose response effects were observed, suggesting that the tumour promoting effects of folic acid are concentration specific within mammary tumours (Deghan Manshadi *et al.*, 2014). The effects of folic acid appear to be dependent on dose (amount of folic acid supplementation), timing of exposure, as well as the stage of cell transformation at the time of folic acid supplementation, suggesting folic acid possesses dual modulatory effects on cancer development (Kim, 2006a).

Mechanisms associated with tissue folate status and cancer risk implicate imbalances in nucleotide synthesis, disruption of DNA repair, and changes in DNA or RNA methylation through

epigenetic mechanisms (Choi and Mason, 2000). De novo synthesis of purines and the pyrimidine thymidylate, critical components of DNA synthesis, are dependent on folate derived one carbon groups. Thus, inadequate levels of folate cause folate coenzymes involved in biological methylation and nucleotide synthesis to compete, compromising folate availability for nucleotide synthesis (Choi and Mason, 2000). Adequate folate availability promotes the synthesis of nucleotide precursors, a critical component of DNA synthesis, integrity, replication, repair and genome stability (Padmanabhan *et al.*, 2013). Abundant folate may facilitate hyperproliferation of rapidly dividing cells, a common feature of both precancerous and cancerous neoplasms, via the provision of nucleotide precursors. Unbalanced levels of dNTPs may contribute to increased uracil misincorporation and subsequent compromised DNA integrity (Choi and Mason, 2000). Uracil misincorporation increases the requirement for DNA repair which is also compromised by the reduced pool of deoxyribonucleotides (Choi and Mason, 2000). Furthermore, modulation in levels of folate derived one carbon groups due to changes in folic acid intake may affect methylation reactions, critical components of gene regulation. One mechanism by which folate has been suggested to influence cancer risk is through the altered epigenetic regulation of genes.

1.7 Epigenetics

1.7.1 An Overview of Epigenetics

Epigenetic processes are functional changes that occur to the genome and induce stable changes in gene expression without direct changes to the primary DNA coding sequence (Jirtle and Skinner, 2007). These processes include methylation at the 5' position of cytosine bases (CpG dinucleotides) within DNA, structural changes to chromatin and post-translational covalent modifications including acetylation, methylation, phosphorylation and ubiquitination of histone proteins (Bird, 2002, Sharma *et al.*, 2010). Epigenetic processes play key roles in tissue specific regulation of transcription and gene expression (Sharma *et al.*, 2010). Epigenetic modulation of transcription can occur in very short time frames (minutes), or can persist throughout the life course (Barrès *et al.*, Gluckman *et al.*, 2007). Changes in the epigenetic regulation of genes strongly influences phenotype and are implicated in a range of human diseases including many cancers (Bloushtain-Qimron *et al.*, 2008, Widschwendter and Jones, 2002).

1.7.2 DNA Methylation

CpG promoter methylation (a form of alkylation) is the most common form of DNA modification in eukaryotic genomes and is essential for proper embryonic development (Cooper and Krawczak, 1989, Li *et al.*, 1992). CpG methylation occurs via the covalent addition of a CH₃ methyl group to carbon 5 of cytosine situated next to a guanine, forming 5-methylcytosine in CpG dinucleotides pairs (Goldberg *et al.*, 2007) (Figure 1.11). CH₃ is donated to a cytosine via the methionine sulphur atom present in the co-substrate S-adenosyl methionine (SAM). This reaction is catalysed by the actions of DNA methyltransferases (DNMT'S), a family of enzymes that utilise SAM as a methyl donor (Moore *et al.*, 2013).

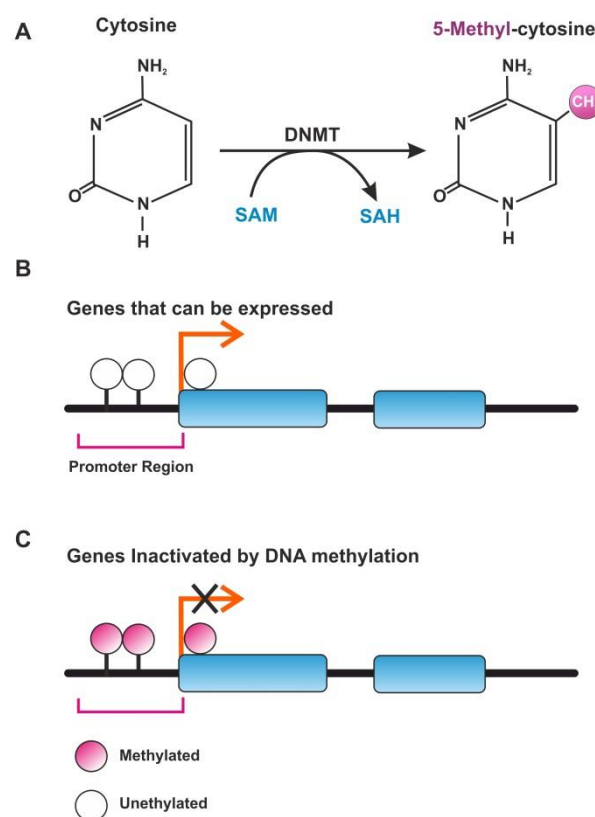


Figure 1.11. DNA Methylation and Transcriptional Silencing

(A) The addition of a methyl (CH_3) group to the 5 position in cytosine forms 5'methyl-cytosine. CH_3 is donated by SAM and catalysed by DNA methyltransferase (DNMT). **(B)** Methylation of CpG's within gene promoter region inactivates genes and silences transcription. Abbreviations: SAM, S-Adenosyl-methionine; SAH, S-Adenosyl-homocysteine. Adapted from (Zakhari, 2013, Institute, 2010).

A high percentage (~50-90%) of CpG residues are methylated within the mammalian genome (Bird, 1986). CpG location is not random, with approximately 1% of vertebrate genomes accounting for CpG rich regions (15% of all CpG dinucleotide), often clustered at the 5' end of genes in areas termed CpG islands (Cooper, 1983, Bird, 1986). CpG islands are generally considered unmethylated regions (Cross and Bird, 1995). Methylation of CpGs within islands is strongly associated with transcriptional gene repression (Keshet *et al.*, 1985). Epigenetic transcriptional regulation is achieved via 5-methylcytosine preventing binding of basal transcription machinery or transcription factors to CpGs in gene promoter regions, as well as transcriptional repressors that recognise methylated CpGs modifying chromatin structure and

silencing genes (Kass *et al.*, 1997). High levels of methylation (hypermethylation) are associated with gene silencing and low levels (hypomethylation) associated with increased levels of transcriptional activation (Berger, 2007).

1.7.3 Histone Modifications

Histones are positively charged proteins that associate with, and package, DNA into nucleosomes within the cell nucleus (Ramakrishnan, 1997). Histone proteins exist in five major families: H1/H5 H2A, H2B, H3 and H4 (Ramakrishnan, 1997). Two copies of the major histones H2A, H2B, H3 and H4 form an octomer, which DNA (145-147 bp) coils around forming a nucleosome core (Luger *et al.*, 1997b, McGhee and Felsenfeld, 1980). Histones contain a globular C-terminal domain and highly basic N-terminal amino tail (Luger *et al.*, 1997a). The N-terminal tail protrudes from the nucleosome and functions as a site for histone modifications as well as making contact with adjacent nucleosomes (Luger *et al.*, 1997b, Kuo and Allis, 1998).

Histone proteins undergo post-translational modifications (PTMs) via acetylation, methylation, phosphorylation, deamination, ADP ribosylation, ubiquitination and sumoylation (Bannister and Kouzarides, 2011). The most characterised PTMs include histone acetylation and methylation (Bártová *et al.*, 2008). Histone acetylation of lysine residues located within histone tails is regulated by two sets of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Bannister and Kouzarides, 2011). HATs catalyse the transfer of an acetyl group (from acetyl co-A) to the NH_3^+ on lysine side chains located mainly in histone N-terminal tails, but also to a lesser extent within the globular histone core (Tjeertes *et al.*, 2009, Bannister and Kouzarides, 2011). Acetylation removes lysine's positive charge which decreases interactions between lysine residues and negatively charged phosphate groups present within DNA (Bannister and Kouzarides, 2011). This changes the chromatin structure from highly condensed (heterochromatin) to more relaxed (euchromatin), increasing chromatin accessibility and allowing transcription factors access to bind to DNA response elements, increasing levels of gene transcription (Gorisch *et al.*, 2005). Conversely, HDACs function to remove acetyl groups from lysine residues restoring positive charge, strengthening interactions with DNA and repressing transcriptional activation (Kuo and Allis, 1998).

1.7.3.1 Histone Methylation

Histones can be methylated on lysine and arginine side chain residues (Murray, 1964). Methylation of histones is achieved via histone lysine methyltransferases (HKMT), or arginine methyltransferases (Rme1), enzymes that catalyse methyl group transfer from SAM to E-amino groups in lysine, or SAM to guanidino group in arginine (Bannister and Kouzarides, 2011). Methylation of lysine residues occurs in histones H3 and H4. Within these histones, six lysine residues have been shown to be either mono, di, or tri methylated (Martin and Zhang, 2005). Histone methylation functions to activate or repress transcription.

1.7.4 Non Coding RNAs

Non coding RNAs (ncRNA) are RNA molecules that are not translated into proteins and include microRNAs, small noncoding RNAs (snoRNA's) and long noncoding RNAs (lncRNA's) (Mattick and Makunin, 2006). Many ncRNAs have been shown to be overexpressed in disease, including breast tumours (Mattick and Makunin, 2006).

1.7.4.1 Micro RNA Genes

MicroRNA (miRNA) genes encode a single 21-23 nucleotide non-coding RNA strand that functions in the post transcriptional regulation of gene expression (Bartel, 2004). MicroRNA nucleotides anneal to complementary sequences present within messenger RNA (mRNA) and function to block translation of proteins, or degrade targeted mRNA (Bartel, 2004) (Figure 1.12). Research has identified the location of numerous microRNA genes in regions of the chromosome that undergo deletions, amplifications and rearrangements in cancer cells (Calin *et al.*, 2004). Furthermore, recent studies have identified miRNAs that are dysregulated in human breast cancer, including mir-125b, mir-145, mir-21, and mir-155 (Iorio *et al.*, 2005, Volinia *et al.*, 2006). More recently, long non-coding RNAs (lncRNAs) have been implicated in transcriptional and posttranscriptional regulation, organelle biogenesis and subcellular trafficking with dysregulation of lncRNAs linked with breast cancer development (Guffanti *et al.*, 2009, Redvers, 2013).

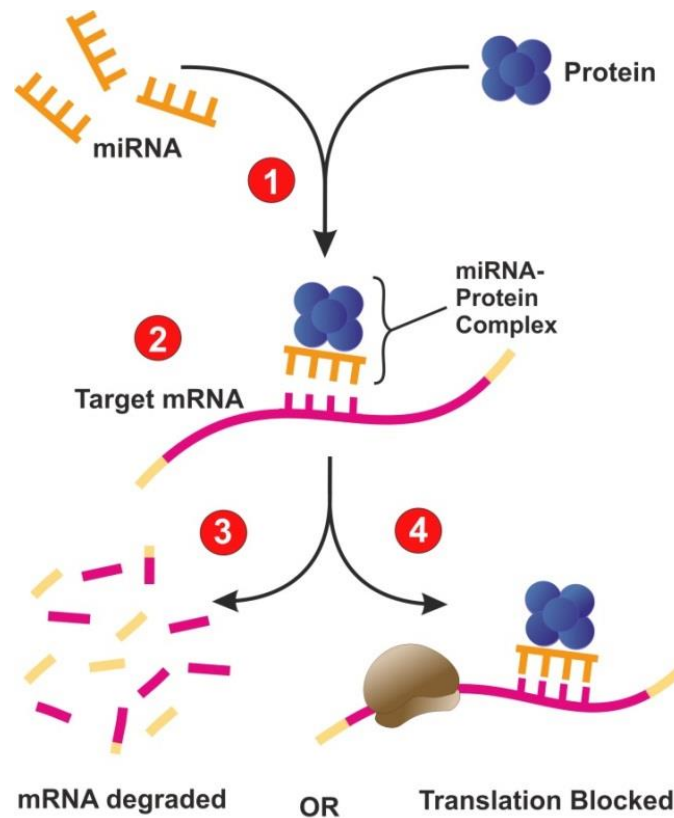


Figure 1.12. Simplified miRNA Post-Transcriptional Regulation

(1) Mature miRNA forms a RNA induced silencing complex (RISC) with a number of proteins including Dicer. **(2)** miRNA protein complex binds to the complementary target mRNA sequence with high specificity. **(3)** Complete complementation between miRNA and mRNA target sequence results in mRNA degradation. **(4)** Incomplete complementation results in the blocking of translation. Adapted from (Education, 2009).

1.7.4.2 Long Non Coding RNAs

LncRNAs are transcribed RNA non-protein coding transcripts with a length greater than 200 nucleotides (Kung et al., 2013). Current research has implicated lncRNAs in disease development and progression (Gupta et al., 2010). Research by Gupta et al., (2010) has identified lncRNAs in the HOX loci that become systematically dysregulated during the progression of breast cancer (Gupta et al., 2010). One such lncRNA, HOTAIR, is highly overexpressed in mammary tumours and metastasis and levels of HOTAIR expression can be used as a predictor of breast cancer metastasis and poor survival (Gupta et al., 2010). Mechanisms associated with these changes

have been linked to specific chromatin remodelling activities, specifically, HOTAIR has been shown to alter H3K27 methylation through regulation of the polycomb PRC2 complex, the initiation of chromatin re-modelling and repressing inhibitors of breast cancer progression such as HOXD10 (Gupta *et al.*, 2010, Rinn *et al.*, 2007).

1.7.5 Epigenetics and Breast Cancer

1.7.5.1 CpG Methylation and Breast Cancer

It is well established that global DNA hypomethylation and regional hypermethylation of genes occur in breast cancer (Baylin *et al.*, 2001a, Narayan *et al.*, 1998). Whole genome analysis has identified methylation signatures of breast cancer genes that may be used as markers of cancer stage progression. (Huang *et al.*, 1999, Huang *et al.*, 1997, Chen *et al.*, 2003). The levels and patterns of DNA methylation provide key markers within cancer cells (Rodriguez-Paredes and Esteller, 2011, Delpu *et al.*, 2013). Cancerous tumours often exhibit global DNA hypomethylation when compared to normal tissues, mainly attributed to low methylation of repetitive DNA sequences, and de-methylation of introns and coding regions (Feinberg and Vogelstein, 1983). Global hypomethylation is often accompanied by regional hypermethylation within gene promoter regions, silencing specific genes (Jones and Baylin, 2002). Aberrant expression of DNMT'S, the protein machinery that catalyses DNA methylation, is further implicated in cancer development (Baylin *et al.*, 2001b, Issa *et al.*, 1993, Ehrlich, 2002). This epigenetic signature reflects a change in methylation status from a normal to diseased state, which results in direct changes to levels of transcriptional gene expression (Szyf, 2012). Inactivation of tumour suppressor genes (TSGs), which control cellular proliferation via hypermethylation of CpG islands located in their promoters, is characteristic of the origins of many cancers including breast cancer (Feinberg and Tycko, 2004). Hypomethylation of genes that regulate cancer growth and metastasis are present in many cancers (Narayan *et al.*, 1998). Epigenetic silencing of genes involved in cell proliferation can act as a step in the Knudson 'two-hit' model of cancer development (Sharma *et al.*, 2010).

1.7.5.2 **BRCA1 CpG Methylation and Breast Cancer**

BRCA1 is associated with hypermethylation in sporadic breast cancer (Niwa *et al.*, 2000). A study by Rice *et al.*, (2000) detected aberrant cytosine methylation in the *BRCA1* promoter region, in three out of 21 (14%) breast cancer specimens (Rice *et al.*, 2000). Mancini *et al.*, (1998) identified CpG methylation adjacent to the *BRCA1* transcriptional start site in a series of randomly sampled breast carcinomas (Mancini *et al.*, 1998). Research by Niwa *et al.*, (2007), analysed 32 sporadic breast cancers of which 10 (31%) demonstrated DNA methylation within the *BRCA1* promoter region, and 18 (56%) were positive for *BRCA1* immunoreactivity (Niwa *et al.*, 2000). Furthermore, a significant inverse relationship was observed between *BRCA1* methylation and expression in sporadic breast cancers (Niwa *et al.*, 2000).

Research by Shukla *et al.*, (2010) has shown that impaired function of *BRCA1* effects the regulation of DNA methyltransferase 1 (*DNMT1*) gene, which results in global DNA hypomethylation, an open chromatin configuration and loss of genomic imprinting (Shukla *et al.*, 2010). Furthermore, proto-oncogenes *C-Myc*, *Ha-Ras* and *c-Fos* gene expression was increased (Shukla *et al.*, 2010).

1.7.5.3 **OCT-4 CpG Methylation and Breast Cancer**

Despite links between *OCT-4* expression levels and breast cancer, methylation of the *OCT-4* gene has not been well studied. Research by Cantz *et al.*, (2007) identified high levels of *OCT-4* promoter methylation and negative *OCT-4* expression in the breast cancer cell line MCF7 (Cantz *et al.*, 2008). A study by Wang *et al.*, (2011) identified hypermethylation in the distal, proximal enhancer and exon 1 of the *OCT-4* A isoform in MCF7 cells, which was also not expressed (Wang *et al.*, 2011). Further research is required to fully understand methylation patterns in *OCT-4* within breast cancer.

1.7.5.4 **Histone Modifications and Breast Cancer**

Histone modifications have been implicated in playing a key regulatory role in breast cancer aetiology (Elsheikh *et al.*, 2009). Research by Elsheikh *et al.*, (2009) examined 880 human breast cancer specimens identifying low levels of H4K16ac and high levels of H3K18ac and H4K20me3

in the majority of cases (Elsheikh *et al.*, 2009). Furthermore, low levels of H3K9ac, H4R3me2 and H4K16ac have been associated with large tumour size (Elsheikh *et al.*, 2009). Changes in levels of enzymes involved in histone acetylation (HAT's), histone deacetylation (HDACs) and histone methylation (HMTs) are critical components of nucleosome remodelling complexes that regulate chromatin structure and levels of gene expression regulation in breast cancer (Park *et al.*, 2011, Dumitrescu, 2012). Several HATs have been implicated in breast cancer, including mutations in CBP and P300, increased expression of Hbol 1 and decreased expression of hMOF (Iyer *et al.*, 2004, Hyman *et al.*, 2002). Histone deacetylases (HDACs) have also been shown to play a role, with HDAC 1, 6 and 8 shown to regulate the expression of matrix metalloproteinases (MMPs), important mediators of the extra cellular matrix that become dysregulated in breast cancer (Park *et al.*, 2011).

1.7.5.5 Polycomb Complexes and Breast Cancer

Polycomb gene expression has been shown to be overexpressed or implicated in several cancers including breast, prostate, endometrial, and melanoma (Bachmann *et al.*, 2006). Polycomb repressive proteins form two main complexes, namely polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 complexes containing BMI1, MEL 18 or NSPC1 are involved in gene silencing, either independently or synergistically with PRC2 (Margueron and Reinberg, 2011). PRC2 functions in gene silencing and recruits PRC1 to promoters of common target genes (Tolhuis *et al.*, 2006). EZH2 forms a component of the PRC2 complex, along with embryonic ectoderm development (EED), retinoblastoma (Rb)-associated proteins 46/48 (RbAp46/48) and suppressor of zeste 12 (EED) (Kim and Roberts, 2016). EZH2 functions as the catalytic subunit of PRC2 with critical roles in gene repression through H3K27 methylation and as a scaffold for DNA methyltransferases, forming a link between histone methylation and promoter DNA methylation (Vire *et al.*, 2006).

1.7.5.5.1 EZH2

EZH2 is a tumour suppressor gene that encodes the EZH2 protein and functions as a catalyst in the addition of methyl groups to specific residues on histone proteins that regulate transcriptional repression (Kim and Roberts, 2016). EZH2 overexpression has been identified in

several specific cancer types that include breast, bladder, melanoma and prostate (Holm et al., 2012). Increased levels of *EZH2* expression has been linked to the expansion of breast tumour initiating cells and elevated protein levels of EZH2 have been shown to indicate a pre-cancerous state in morphologically normal breast tissue, which increases as the cancer develops (Ding et al., 2006a). EZH2 binds to unmethylated histone gene promoter regions present in stem cells, marking specific genes for hypermethylation and acts in histone assisted silencing of P16INK4a in colon cancer cells (Bachman *et al.*, 2003). Within breast progenitor cells, epigenetic programming can become dysregulated, with aberrant signalling involved in cellular differentiation linked with upregulation of EZH2 and associated polycomb repressor proteins that include SUZ12, BMI1, which is part of PRC1, G9a and SUV39H, which silences tumour suppressor genes (Dimri et al., 2002, Kleer et al., 2003). Polycomb regulated gene silencing leads to abnormal proliferation of undifferentiated or semi-differentiated cells (Dimri et al., 2002, Kleer et al., 2003). Additional DNA methylation of these genes can result in permanent transcriptional inactivation, leading to persistent changes in gene expression, deregulated cellular differentiation and cancer phenotype (Kleer et al., 2003, Dumitrescu, 2012).

High EZH2 expression is significantly associated with triple-negative breast cancer (Hussein et al., 2012). Research by Hussein et al., (2012), using tissue microarrays and *in vivo* EZH2 gene silencing, has shown that out of 261 cases of invasive breast cancer, high levels of EZH2 expression was detected in 87 (33%) cases, and was strongly associated with a triple-negative breast cancer phenotype and poorer overall survival (Hussein et al., 2012). Furthermore, EZH2 gene silencing was shown to result in a significant reduction in tumour growth in the orthotopic MB-231 breast carcinoma mouse model (Hussein et al., 2012).

Mechanisms associated with increased levels of EZH2 in breast cancers are linked with EZH2 mediated trimethylation of repressive chromatin mark H3K27 (Yoo and Hennighausen, 2012). Increased EZH2 expression has been associated with trimethylation of H3K27 in the promoter regions of *RAD51*, *CDKN1C*, *FOXC1*, *RUNX3* and E-Cadherin with decreased levels of gene expression (Yoo and Hennighausen, 2012). Furthermore, elevated concentrations of EZH2 in ER-negative breast cancer cells have been shown to block the phosphorylation of BRCA1 (Ser1423). This promotes an increase in Cdc25C, which functions as a critical regulator of G2-M checkpoint control and induces Cdc2/cyclinB1 activity, resulting in increased cell proliferation (Gonzalez et al., 2009). Ezh2 also activates Akt-1 (Ser473) via phosphorylation which suppresses nuclear localisation of BRCA1 protein in the breast cancer cell line CAL51 (Gonzalez et al., 2011).

Current research has associated EZH2 function with mammary cell differentiation. A study by Wu et al., (2015), using in vitro transplantation of mammary cancer stem cells transduced with EZH2 or JMD3 (which opposes the action of EZH2) short hairpin RNAs (shRNAs) has shown that EZH2 promotes mammary stem and luminal progenitor cell expansion and metastasis (Wu and Crowe, 2015). Research suggests that the luminal progenitor cell population demonstrates tumorigenic properties in murine models of mammary cancer and is also considered the progenitor population of human basal breast cancer (Wu and Crowe, 2015). Thus, EZH2 plays a critical role in mammary cell differentiation through regulation of luminal progenitor cells and mammary cancer risk.

1.7.6 Environment, Epigenetics and Breast Cancer

The Developmental Origins of Health and Disease concept (DoHAD) suggests that maternal exposure to both environment and diet during early embryonic development influences the phenotype of offspring later in life (Barker and Osmond, 1986, Barker et al., 1989, Hanson and Gluckman, 2014). Furthermore, early life environmental exposures, including nutritional status, are important determinants of disease risk and susceptibility that occur during adulthood (Bird, 2002, Roth, 2012, Szyf *et al.*, 2008). Over the past 25 years many experimental and epidemiological studies have implicated epigenetic programming as the underlying mechanism behind persistent alternations in disease risk, including many cancers (Lillycrop et al., 2007, Lillycrop et al., 2008, Godfrey et al., 2007). Population based studies show that breast cancer incidence varies dramatically around the world (Nelson, 2006, Bray *et al.*, 2004). Japanese women demonstrate a much lower incidence of breast cancer when compared to American women; however, breast cancer incidence increases in Japanese women who emigrate to live in the US (Nelson, 2006). This suggests that modifiable environmental factors rather than pre-genetically determined factors associated with Western culture contribute towards a breast cancer phenotype (Nelson, 2006).

The epigenome is influenced by many factors that include environment, parenting and nutritional status (nutriepigenomics) (Bird, 2002, Roth, 2012, Szyf *et al.*, 2008). Growing evidence in animal studies has shown that maternal diet during pregnancy causes stable changes in the epigenome through altered epigenetic regulation of specific genes (Lillycrop *et al.*, 2005, Burdge *et al.*, 2007). Nutritional status of a mother correlates to disease risk of her progeny in

adult life (Ravelli *et al.*, 1998). Furthermore, nutritional imbalances that occur at critical periods of development have been linked with non-communicable diseases that include cardiovascular disease, obesity and breast cancer (Gallou-Kabani *et al.*, 2007). Therefore, environmental factors such as early life nutrition, a period of increased susceptibility, can induce persistent changes in gene and protein expression that can increase disease risk during adulthood (Ravelli *et al.*, 1998).

More recently, folate status through folic acid intake has been implicated as a risk factor for breast cancer, possibly through epigenetic regulatory mechanisms (Deghan Manshadi *et al.*, 2014, Padmanabhan *et al.*, 2013). However, it is unclear if a relationship exists between early life exposure to folic acid and breast cancer development later in life, as well as the mechanism behind such changes (Wilson *et al.*, 2007).

1.7.7 Folic Acid and the Epigenome

1.7.7.1 Folic Acid and DNA methylation

Dietary folic acid, metabolised to 5-methylTHF during intestinal absorption and first-pass in the liver, functions in the maintenance of methyl group flux required to remethylate homocysteine to methionine (Crider *et al.*, 2012). Methionine acts as a critical substrate for SAM which functions as a methyl donor in the biological methylation of DNA, RNA, histones, proteins, phospholipids and other small molecules (Crider *et al.*, 2012). SAM has many regulatory roles that include maintenance of genome stability and regulation of gene expression through methylation reactions (Miranda and Jones, 2007).

1.7.7.2 Low Folate Status, Folic Acid Supplementation and DNA Methylation

Folate depletion has been shown to diminish the methyl pool which is critical for the generation and maintenance of SAM (Miller *et al.*, 1994). Folate depletion decreases SAM and increases S-adenosylhomocysteine (SAH) which functions as an inhibitor of methyltransferases, critical regulators of methylation reactions (Kim *et al.*, 2009b).

In vitro studies in untransformed cell lines have demonstrated folic acid induced changes in both global and gene specific methylation vary between cell types (Branda and Blickensderfer, 1993). Folate deficiency has been shown to alter global methylation in untransformed Chinese hamster

ovarian cell line CHO-K1 and NIH/3TE fibroblast cells (Branda and Blickensderfer, 1993). A study investigating folate deprivation on HTC116 colorectal cancer cells has shown that when cultured in folate deplete medium, HTC116 cells display enhanced CpG methylation located in the promoter of Sonic Hedgehog (SHH) gene, combined with increased levels of SHH gene expression (Wang *et al.*, 2012a).

In vivo animal studies have shown that diets deficient in methyl donors including folic acid, induce DNA hypomethylation both globally and in the proto-oncogenes c-myc, c-fos and c-Ha-ras in rats (Christman *et al.*, 1993). Interestingly, severe folate deficiency in rats has been shown to increase global DNA methylation by 56%, however, DNA hypomethylation (40%) within mutation associated exons 6 and 7 but not 8 of the p53 tumour suppressor gene was induced (Kim *et al.*, 1997). Folic acid deficiency, therefore, induces both site and gene specific methylation patterns that do not necessarily reflect the direction of changes in global methylation (Kim *et al.*, 1997).

Insufficient levels of folate during cellular division has been shown to reduce the production of thymidine, a critical base required for DNA, resulting in uracil incorporation into DNA strands (Miller *et al.*, 1994). Furthermore, low levels of folic acid results in micronuclei formation which is indicative of chromosomal breakage. Despite associations between low levels of folic acid and impairment of DNA methylation, research suggests that low folate can also lead to hypermethylation which has been implicated in regulation of gene expression (Herman and Baylin 2003, Jaenisch and Bird).

1.7.7.3 High Folate Status, Folic Acid Supplementation and DNA Methylation

In vitro studies have shown high levels of folic acid supplementation (2.9-9.3 μ M) induce gene specific CpG island hypomethylation in FHC colon epithelial cells and WI-38 fibroblasts (Charles *et al.*, 2012). Furthermore, a supramaximal concentration of 20 μ M folic acid supplemented within culture medium has been shown to increase CpG promoter methylation of tumour suppressor genes Estrogen receptor 1 (ESR1), p16INK4a and p15INK4b in Caco2 human epithelial colorectal cancer cells (Berner *et al.*, 2010).

Despite a plethora of research on folate deficiency, few studies have investigated the effects of high or supramaximal levels of folic acid supplementation and methylation in animal models. A

study by Kotsopoulos *et al.*, (2008) supplementing weanling rats (3 weeks old) with 8 mg/kg (4 x BDR) of folic acid for 5 weeks showed no difference in levels of hepatic genomic DNA methylation (Kotsopoulos *et al.*, 2008). Research by Achon *et al.*, (2007) supplementing 3 week old rats with a supramaximal (40 mg/kg) concentration of folic acid (28 days) showed a reduction in homocysteine and creatinine concentrations but no changes in hepatic DNA methylation or the ratio of SAM/SAH (Achon *et al.*, 2007). Conversely, a study by Choi *et al.*, (2005) supplementing 1 year old rats with 18 µM/kg folic acid showed an increase in hepatic DNA methylation when compared to a folate deficient group, but in a similar study in colon, no changes in the levels of methylation were shown, suggesting changes in levels of DNA methylation in response to folic acid supplementation are both concentration and tissue specific (Choi *et al.*, 2005, Choi *et al.*, 2003).

Several studies in humans have demonstrated changes in levels of DNA methylation in response to folic acid supplementation. A study by Quinlevan *et al.*, (2008) supplementing women with either 100, 400, or 4000 µg of folic acid daily for a period of 6 months demonstrated decreased levels of genomic DNA methylation (13%) which further decreased (23%) after a 3 month period of folic acid withdrawal (Quinievan *et al.*, 2008). A study by Christensen *et al.*, (2010) investigating 1,413 autosomal CpG loci associated with 773 cancer-related genes in primary breast tumours from 162 women, identified tumour grade, size, triple negative status and estrogen and progesterone receptor status are significantly associated with the altered methylation of CpG loci (Christensen *et al.*, 2010).

1.7.8 Folic acid, DNA methylation and Gene Expression

Changes in levels of DNA methylation in response to folic acid supplementation have been identified in a large number of genes implicated in cancer risk. Research by Pietruszewska *et al.*, (2013) testing the effects of folic acid supplementation on the DNA promoter methylation and gene expression of tumour suppressor genes RARbeta2, APC and PTEN in MDA-MB-231 and MCF-7 breast cancer cell lines has demonstrated a dose dependent down regulation of tumour repressor genes with increasing concentrations of folic acid that may be linked to gene promoter methylation (Lubecka-Pietruszewska *et al.*, 2013).

A study by Crott *et al.*, (2008) in which rats were fed a folate deplete diet resulted in the upregulation of immune-related genes and downregulation of adhesion molecule genes in the

colon when compared to rats fed 8 mg/kg of folic acid (Crott *et al.*, 2008). A total of 136 genes were altered in young rats and 62 in old rats, demonstrating an age dependent difference in both numbers and types of differentially regulated genes (Crott *et al.*, 2008).

Several studies have linked folic acid to MTHFR gene regulation (Sohn *et al.*, 2009). High levels of FA supplementation have been shown to induce pseudo-MTHFR deficiency, altered lipid metabolism and liver injury in mice (Christensen *et al.*, 2015). Research investigating the MTHFR gene C677T polymorphism, associated with altered one-carbon transfer reactions via modulation of intracellular cofactors, in MDA-MB-435 breast cancer cells has shown decreased levels of genomic DNA methylation and decreased uracil incorporation when folate levels (from supplemental folic acid) are either adequate or high (Sohn *et al.*, 2009). This suggests that modulating levels of intracellular co-factors (through the MTHFR gene c677t polymorphism) directly affects genomic DNA methylation in a breast cancer cell line (Sohn *et al.*, 2009). Furthermore, the same study has shown that these changes are cell specific, suggesting a way in which changes in levels of folate through folic acid supplementation may induce tissue specific methylation (Sohn *et al.*, 2009).

1.7.9 Folic Acid, the Life-course and Breast Cancer Risk

1.7.9.1 Folic Acid and its Effect on the Epigenome

Dietary folic acid intake during early life has been shown to affect the phenotype of the offspring. Research by Waterland *et al.*, (2003) fed Agouti mice (that harbour a transposable element in the agouti gene and under normal conditions give birth to obese yellow offspring) methyl donors including folic acid, choline or B12 and choline (Waterland and Jirtle, 2003). Mice fed methyl donor nutrition give birth to thin brown pups and demonstrate increased CpG methylation in the agouti gene altering the offspring phenotype and implicating DNA methylation as a mechanism by which dietary methyl donors may contribute to altered disease risk (Waterland and Jirtle, 2003).

Our research group has previously shown that folic acid supplementation during the juvenile-pubertal period can modify the epigenotype in rats (Burdge *et al.*, 2009). Furthermore, dietary protein restriction in pregnant rats results in epigenetic modification of hepatic genes that can be prevented by folic acid supplementation (Lillycrop *et al.*, 2007). More recently, research by

Ly et al., (2011), analysing maternal and post weaning folic acid intake on mammary tumour risk in the offspring of mice, has shown that high intrauterine and post weaning folic acid intake accelerates the rate and multiplicity of mammary adenocarcinoma in the offspring. Furthermore, the tumour promoting effects caused by folic acid intake may be regulated by changes in DNA methylation and levels of DNMT activity (Ly et al., 2011). A further study by Sie et al., (2013) suggests that both maternal and post weaning intake of folic acid (5 mg/kg) modulates global as well as gene-specific DNA methylation in the livers of rat offspring (Sie et al., 2013). Collectively, these findings suggest that supplementation with folic acid modifies tumour risk through changes in epigenetic programming during early life. This suggests that the epigenome is more susceptible to change during early life due to increased levels of developmental plasticity and less so during adulthood.

Preliminary data from our research group (unpublished data) has shown that folic acid supplementation at 5 mg/kg over a period of 28 days in rats results in changes in *Brca1* and *Oct-4* gene expression in adipose and muscle tissue which lasts for a minimum of 28 days post supplementation (Figure 1.13). Furthermore, folic acid supplementation in adipose tissue induces a significant increase in *Brca1* mRNA expression and decrease in *Oct-4* mRNA expression. Conversely, folic acid supplementation was shown to decrease *Brca1* mRNA expression and increase *Oct-4* mRNA expression in muscle tissue (Figure 1.13). Changes in *Brca1* and *Oct-4* mRNA expression were accompanied by changes in the methylation status of a number of CpG's within *Brca1* and *Oct-4* promoters (Figure 1.14) (unpublished data). Folic acid supplementation (5 mg/kg) increased the methylation of CpGs – 15, +14, +33 and +47 in adipose tissue and CpG +33 in muscle tissue.

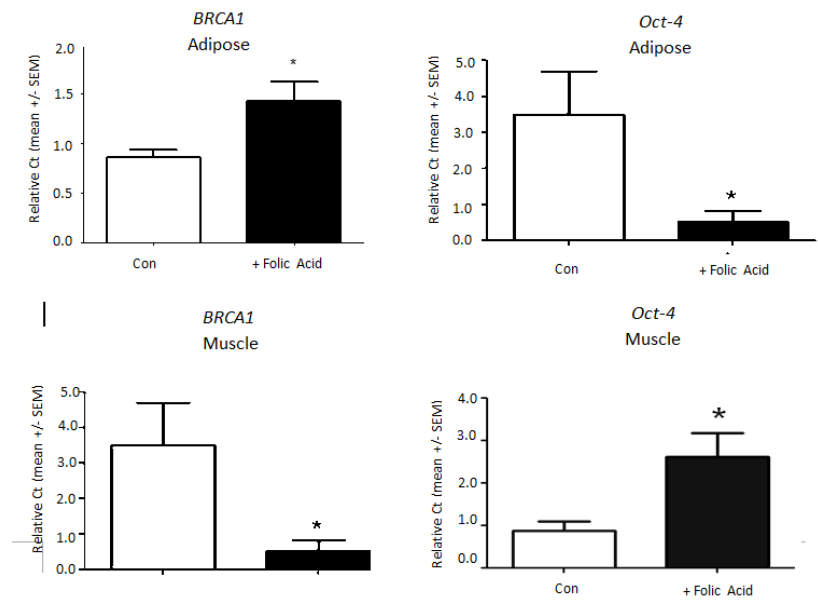


Figure 1.13. Effect of Folic Acid Supplementation during the Juvenile Period on *Brca1* and *Oct-4* Gene Expression in Muscle and Adipose Tissue.

Brca1 methylation is significantly increased adipose and decreased in muscle tissue. *Oct-4* methylation is significantly decreased in adipose and increased in muscle tissue (Relative Ct mean $\pm$ SEM).

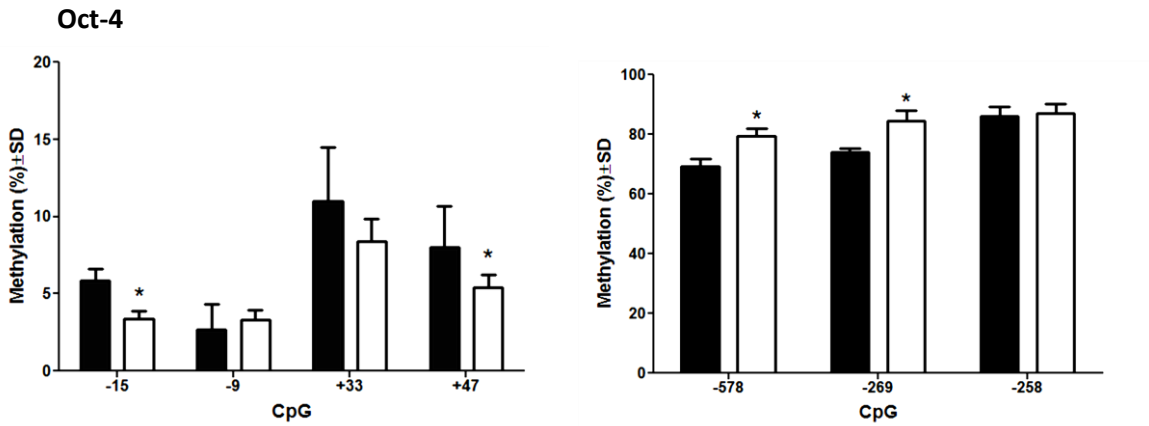


Figure 1.14. Effect of Folic Acid Supplementation during the Juvenile Period on *Brca1* and *Oct-4* Methylation in Adipose Tissue.

Brca1 methylation was significantly decreased at CpG -15 and +47. *Oct-4* methylation was significantly increased in CpG's -578 and -269 (Methylation (%) $\pm$ SD).

Our preliminary data suggests that changes in folate status directly affect methylation and gene expression of *Brca1*, critically involved in DNA surveillance and repair and *Oct-4*, a major regulator of cellular pluripotency and differentiation, both key cellular regulatory functions implicated in cancer development. Findings from preliminary data, coupled with current research implicating *Brca1* and *Oct-4* genes as major regulators of breast cancer risk, suggest an important role for folate status on the epigenetic regulation of these genes.

1.7.9.2 Folic Acid and Adult Life

Research by Jacob *et al.*, (1998) in humans has identified global reductions in DNA methylation in older women in response to folate depletion (Jacob *et al.*, 1998). Interestingly, this effect was not observed in young female adults, suggesting age dependent changes in DNA methylation in response to changes in folate status (Shelnutt *et al.*, 2004).

Currently, increasing uncertainty exists with regards to levels of dietary folate and folic acid supplementation and how this affects cancer risk (Wilson *et al.*, 2007, Bailey *et al.*, 2010c). It is clear that folate and folic acid intake regulates key molecular processes that play roles in breast cancer development. However, it is unclear exactly how folic acid supplementation affects the molecular, genetic and epigenetic mechanisms that are thought to be critically involved in the development of breast cancer and to what extent both concentration of folic acid and timing of supplementation may contribute to breast cancer phenotype (Boyd *et al.*, 1998).

1.8 Hypothesis and Aims

Over the past decade, folic acid intake in many countries has dramatically increased due to mandatory food fortification and the advice for periconceptional folic acid supplementation in women of child bearing age. Increased levels of dietary folic acid intake mean that it is likely that individuals at all stages of the life course will be exposed to high levels of folic acid. However, the relative plasticity of the epigenome at different phases of the life course is largely unknown. Therefore, the aim of this project is to use an animal model to directly assess the effect of different levels of folic acid intake during different periods of the life course on persistent changes in *Brca1* and *Oct-4* expression in the mammary gland and the downstream processes that they control.

1.8.1 Hypothesis

Variations in the concentration of folic acid intake during the juvenile period or adulthood induce dose and timing persistent changes in stem cell regulator genes, morphology, and protein markers associated with DNA repair, mammary cell differentiation, and cell lineage in the mammary glands of C57BL/6 virgin female mice.

1.8.2 Chapter by Chapter Aims and Thesis Outline

1.8.2.1 Chapter 3: The Effect of Folic Acid Supplementation during the Juvenile Period or Adulthood on Weight, Energy Intake, Folate, Hormone Concentrations and Expression of Stem Cell Differentiation Genes and Proteins in the Female C57BL/6 Mammary Gland

To establish the long term effects of 0.2 mg/kg, 1 mg/kg, 5 mg/kg and 20 mg/kg of dietary folic acid for 4 weeks during the juvenile period or adulthood in virgin C57BL/6 female mice on weight, energy intake, plasma folate and hormones estradiol-17 β or progesterone. Furthermore, to quantify gene expression levels of *Brca1*, *Oct-4* and polycomb genes *Ezh2*, *Bmi1* and *Suz12* and to follow this up with the quantification of *Ezh2* and its target H3K27 (tri-methyl) protein levels in response to high folic acid supplementation.

1.8.2.2 Chapter 4: The Effects of Folic Acid Supplementation during the Juvenile Period or Adulthood on Mammary Morphology in Female C57BL/6 Mice

To investigate changes in mammary gland morphology including epithelial density, number of epithelial branches, number of epithelial junctions and number of TEBs in response to different concentrations of folic acid 4 or 12 weeks after supplementation during the juvenile or adult period.

1.8.2.3 Chapter 5: The Effects of Folic Acid Supplementation during Adulthood on Protein Expression of Epithelial and Cell Differentiation Markers in the Female C57BL/6 Mouse Mammary Gland

To investigate mammary stem cell protein markers *Brca1*, *Oct-4* and *Gata3*; basal/myoepithelial marker α smooth muscle actin (α -SMA), luminal cell marker Keratin 8 and proliferation marker Ki-67, 4 weeks after high folic acid intake during the adult period.

1.8.2.4 Chapter 6: The Effect of Folic Acid Supplementation during the Juvenile Period or Adulthood on the Mammary Gland Transcriptome of Female C57BL/6 Mice. A Whole Transcriptome Shotgun Sequencing Approach.

To follow up candidate gene expression analysis with investigation of the whole transcriptome (RNA-seq) 4 weeks after high (5 mg/kg) folic acid supplementation during the juvenile period or adulthood.

1.8.2.5 Chapter 7: The Effects of Folic Acid Deficiency during the Juvenile Period or Adulthood on Mammary Stem Cell Differentiation Genes and Mammary Gland Morphology in the Female C57BL/6 Mouse Mammary Gland

To establish the long term effects of a folic acid deficient diet of 0.2 mg/kg for 4 weeks during the juvenile period or adulthood on gene expression levels of *Brca1*, *Oct-4* and polycomb genes *Ezh2*, *Bmi1* and *Suz12* within the mammary glands of C57BL/6 mice. To follow this up by investigating changes in mammary morphology in response to folic acid deficiency.

Chapter 2: Methods

Table 2.1. Reagent/Chemical/Material for Animal Procedures from Folic Acid Treated C57BL/6 Mice

Reagent/Chemical/Material	Supplier
Ethanol 100%	Fisher, Loughborough, UK
Microscope Slides	Leica, Solms, Germany
Diethyl Ether	Fisher, Loughborough, UK
Nitrogen Gas	BOC, UK
Progesterone ELISA kit	ENZO Lifesciences, SWI
17- β Estradiol High Sensitivity ELISA kit	ENZO Lifesciences, SWI
CO ₂ Gas	BOC, UK

2.1 Animal Procedures

2.1.1 Animal Model

C57BL/6 mice were selected for folic acid dietary intervention studies as this strain exhibit low incidence of endogenous levels of mouse mammary tumour virus (MMTV) sequences that are transcriptionally active within their genome and are considered refractory to many tumours (Ihle *et al.*, 1976). MMTV is associated with decreased genome stability and increased rates of spontaneous mammary tumours (Ponnaiya *et al.*, 1997). C57BL/6 mice provide a model for research into the effects of folic acid supplementation on long term gene regulation and mammary gland morphology in normal untransformed mammary epithelial cells. With 97.5% concordance between murine and human genomes, substantial conservation of genes and pathways identified between human and mouse epithelial signatures, this experimental model provides direct translatable insight into the effects of folic acid supplementation in humans (Lim *et al.*, 2010).

2.1.2 Selection of Folic Acid Diets

Modified diets comprising varying concentrations of folic acid are considered a standard method of controlling dietary folate intake in rodents and have been used in a myriad of animal studies (Kotsopoulos *et al.*, 2003, Lillycrop *et al.*, 2005, Kotsopoulos *et al.*, 2005). A Rat and Mouse No.1 (RM1) maintenance diet (SDS, UK) (Table 2.1) and five modified AIN93M semi-purified commercially prepared diets were designed (Test Diet, USA) (Table 2.2). Diets were formulated to represent a range of folic acid concentrations relative to the recommended basal dietary requirement (BDR) that would mirror the global variation in levels of folic acid intake currently found in humans through consumption of folic acid fortified foods and supplementation.

Power calculations were performed for the main outcomes of the study based on data collected from a pilot study investigating the effects of different levels of folic acid supplementation on *Brca1* and *Oct-4* gene expression in rats. Calculation of the experimental means of control and test data along with the standard deviation showed that ≥ 7 animals in each dietary group was sufficient to provide statistical power of at least 80% with a $P < 0.05$.

Test diets were formulated to contain low (0.2 mg/kg), basal (1 mg/kg or 2.1 mg/kg), high (5 mg/kg) or supra-maximal (20 mg/kg) levels of folic acid. Concentrations of folic acid between 1 mg/kg (control diet) and 2.1 mg/kg (maintenance diet) are generally considered within the range of the basal dietary requirement (BDR) for rodents and selected to represent the recommended daily requirement (~200-400 µg/day of dietary folate equivalents) in humans (Huang *et al.*, 2014). Folic acid at a concentration of 5 mg/kg diet (5 x BDR) was selected to represent increased levels of folic acid intake frequently observed in humans which is approximately 5 times the recommended daily requirement at ~1000 µg/day derived from mandatory folic acid fortification of staple foods in North America combined with supplementation through multivitamins and food supplements, and the recommended daily intake advised to women planning a pregnancy (Kim, 2008, Deghan Manshadi *et al.*, 2014, Wilson *et al.*, 2003). Folic acid at a concentration of 20 mg/kg (20 mg/kg) was selected to represent a supra-maximal concentration of 20 x BDR which approximates the likely total intake in humans of ~3000 – 4000 µg/day. This concentration was included to examine toxicity at maximum levels and represents an enriched folic acid fortified diet combined with high levels of supplementation recommended during pregnancy to women with a history of neural tube defects. A concentration of 0.2 mg/kg FA (0.2 x BDR) was selected to represent folate deficiency (Lin, 1991). All diets were stored at -20°C in the BRF in line with recommended storage specifications. Diets were provided *ad lib* at approximately ~100-200 g per cage and were replenished as diet depleted to ensure food was constantly available and diet remained fresh. To aid the discussion of results, the effect of folic acid supplementation is investigated in chapters 3 to 6 and the effects of folic acid deficiency in chapter 7.

Table 2.2 Nutritional Profile of RM1 Modified Folic Acid Diet Supplemented to C57BL/6 Mice Prior to Folic Acid Dietary Intervention.

Nutritional Profile	RM1 Maintenance Diet (mg/kg)
Folic Acid	0.79
Vitamin B-12	0.00749
Choline (Vitamin B4/7)	1080.14
Starch	449700
Sugar	40500
Powdered Cellulose	43200
Nutritional Profile	%
Fat	2.71
Protein	14.38
Carbohydrate	83.76
Total Energy (kcal/g)	3.5179

* Vitamin Mix: Vitamin A, 8554.27 IU/kg; Vitamin D-3, 621.70 IU/kg; Vitamin E, 84.1 IU/kg; Vitamin K, 10.17 mg/kg; Thiamine Hydrochloride, 8.58 mg/kg; Riboflavin, 4.33 mg/kg; Pantothenic Acid, 20.17 mg/kg; Pyridoxine, 4.81 mg/kg; Biotin, 277.13 µg/kg; Choline, 1080.14 mg/kg; Ascorbic Acid, 2.59 mg/kg; β-Carotene, 0.16 mg/kg; Retinol, 2566.38 µg/kg; Cholecalciferol, 15.54 µg/kg; α-Tocopherol, 76.45 mg/kg; Nicotinic Acid, 61.32 mg/kg; Inositol, 2369.59 mg/kg.

** Minerals: Calcium, 0.73%; Phosphorus, 0.52%; Potassium, 0.67%; Magnesium, 0.23%; Sodium, 0.25%; Chloride, 0.38%; Fluorine, 10.49 mg/kg; Iron, 159.30 mg/kg; Zinc, 35.75 mg/kg; Manganese, 72.44 mg/kg; Copper, 11.5 mg/kg; Cobalt, 634.10 µg/kg; Iodine, 1202.69 µg/kg; Selenium, 298.99 mg/kg; Fluorine, 10.49 mg/kg.

Table 2.3 Nutritional Profile of AIN-93M Modified Folic Acid Diets Supplemented to C57BL/6 Mice during Juvenile/Pre-Pubertal Development or Adulthood.

Nutritional Profile	AIN-93M Low FA (mg/kg)	AIN-93M Basal FA (mg/kg)	AIN-93M High FA (mg/kg)	AIN-93M Maximal FA (mg/kg)	AIN-93M Maint (mg/kg)
Folic Acid	0.20000	1.00000	5.10000	20.1000	2.10000
Vitamin B-12	0.027000	0.02700	0.02700	0.02700	0.02800
Choline Chloride	0.175000	0.175000	0.175000	0.175000	125000
Corn Starch	465192	465192	465192	465192	465692
Maltodextrin	155000	155000	155000	155000	155000
Casein	140000	140000	140000	140000	140000
Sucrose	100000	100000	100000	100000	100000
Powdered Cellulose	500000	500000	500000	500000	500000
Soybean Oil	400000	400000	400000	400000	400000
AIN 93M Mineral Mix **	350000	350000	350000	350000	350000
AIN 93M Vitamin Mix*	100000	100000	100000	100000	100000
Choline Bitartrate	0.02500	0.02500	0.02500	0.02500	0.02500
L-Cysteine	0.01800	0.01800	0.01800	0.01800	0.01800
t-Butylhydroquinone	0.00008	0.00008	0.00008	0.00008	0.00008
Orange Dye	0.00500	0.00000	0.00000	0.00000	0.00000
Red Dye	0.00000	0.00500	0.00000	0.00500	0.00000
Yellow Dye	0.00000	0.00000	0.00500	0.00000	0.00000
Blue Dye	0.00000	0.00000	0.00000	0.00500	0.00500
Nutritional Profile	%	%	%	%	%
Fat	4.1	4.1	4.1	4.1	4.1
Protein	13.0	13.0	13.0	13.0	13.0
Carbohydrate	76.5	73	72.1	73.0	73.0
Total Energy (kcal/g)	3.77	3.81	3.77	3.77	3.81

* Vitamin Mix: Vitamin A, 4 IU/kg; Vitamin D-3, 1 IU/kg; Vitamin E, 78.8 IU/kg; Vitamin K, 0.75 mg/kg; Thiamine Hydrochloride, 6.0 mg/kg; Riboflavin, 6.5 mg/kg; Niacin, 30 mg/kg; Pantothenic Acid, 16 mg/kg; Pyridoxine, 5.8 mg/kg; Biotin, 0.2 mg/kg; Choline, 1080.14 mg/kg; Ascorbic Acid, 2.59 mg/kg.

** Minerals: Calcium, 0.50-0.51%; Phosphorus, 0.31%; Potassium, 0.36%; Magnesium, 0.05%; Sodium, 0.13%; Chloride, 0.20%; Fluorine, 1.0 mg/kg; Iron, 39 mg/kg; Zinc, 35 mg/kg; Manganese, 11 mg/kg; Copper, 6.0 mg/kg; Cobalt, 0.0 mg/kg; Iodine, 0.21, mg/kg; Chromium, 1.0 mg/kg; Molybdenum, 0.14 mg/kg; Selenium, 0.22 mg/kg.

2.1.3 Experimental Design

All animal procedures were carried out within the University of Southampton (UOS) Biomedical Research Facility (BRF) in strict accordance with the Home Office Animals (Scientific Procedures) Act (ASPA) 1986.

2.1.3.1 Juvenile Mice

Female (virgin) C57BL/6 mice (PND 28) were bred onsite from animals housed in the BRF (UOS). The juvenile folic acid dietary intervention study design utilised animals that were previously under breeding conditions, minimising the requirement for external breeding, animal transport and acclimatisation prior to dietary intervention and adhering to the principles of reduction and refinement outlined in the 3R's of humane animal research (ASPA, 1983).

Age matched, randomised (non-sibling) juvenile mice (PND 21) were split into three groups, comprising two groups of 40 animals housed in cages of 10 (test) and a single group of 20 animals housed in cages of 5 (supplementary control). All mice were weaned directly onto a RM1 maintenance diet (SDS, UK) (Table 2.1) from post-natal day (PND) 21 to PND 28. At PND 28 mice were placed on a modified AIN93M semi-purified diet (Test Diet, USA), containing either 0.2 mg/kg (0.2 x Basal Dietary Recommendation, BDR), 1 mg/kg (1 x BDR), 5 mg/kg (5x BDR), 20 mg/kg (20 x BDR) for a period of 28 days. After 28 days (PND 56) mice were transferred onto a maintenance diet containing 2.1 mg/kg folic acid for either 28 or 84 days respectively (Figure 2.1). All diets for experimental parameters were provided ad-libitum.

2.1.3.2 Adult C57BL/6 Mice

Female (virgin) C57BL/6 mice (60 at post-natal day (PND) 56-63 and 40 at PND 42-49 randomised (non-sibling) adults were split into three groups comprising two groups of 40 animals housed in cages of 10 (test) and a single group of 20 animals housed in cages of 5 (supplementary control). All mice were fed an RM1 diet (SDS, UK) (Table 2.1) prior to dietary intervention.

Groups were further subdivided, with half of the mice in each test cage (n = 5) allocated either 4 week or 12 week post folic acid supplementation with the maintenance diet to minimise variability between groups. At PND 74, mice were placed on a modified AIN93M semi-purified diet (Test Diet, USA), containing either 0.2 mg/kg (0.2 x BDR), 1 mg/kg (1 x BDR), 5 mg/kg (5 x

BDR) or 20 mg/kg (20 x BDR) for a period of 28 days. After 28 days (PND 102) mice were transferred onto a maintenance diet (Test Diet, USA) containing 2.1 mg/kg folic acid for a further 28 or 84 days respectively (Figure 2.1).

A

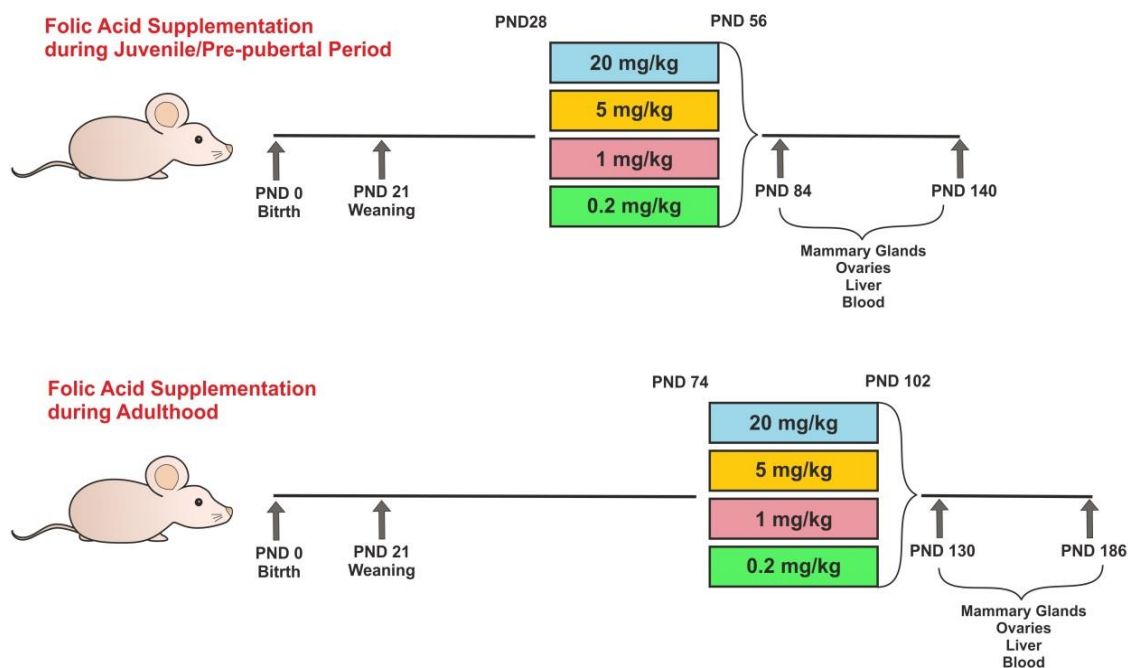


Figure 2.1 Experimental Design of Folic Acid Supplementation and Tissue Collection in C57BL/6 mice.

(A) Juvenile female C57BL/6 mice at PND 28 were randomised and supplemented for 28 days on a semi-purified AIN93M diet (Test Diet™) containing either low (0.2 mg/kg), baseline (1 mg/kg), high (5 mg/kg) or supramaximal (20 mg/kg) folic acid. After 28 days (PND 56) mice were transferred to an AIN93M maintenance diet for 28 days (PND 84) or 84 (PND 140) days and tissue was collected. (B) Adult female C57BL/6 mice at PND 74 were randomised and supplemented for 28 days at the same folic acid concentrations as the juveniles (see A). After 28 days (PND 102) adult mice were transferred to an AIN93M maintenance diet for 28 days (PND 130) or 84 days (PND 186) and tissue was collected.

2.1.4 Animal Housing

Mice were housed in groups of 5 (supplementary control) or 10 (test) in plastic bottomed, grated metal topped MB1 or MB3 cages (NKP Cages, UK). MB1 cages (overall dimensions 45 x 28 x 13 cm, internal dimensions 13 cm high and 960cm² floor area) housed 10 animals and MB3 cages (overall dimensions 48 x 15 x 13 cm 4, internal dimensions 13 cm high and 490 cm² floor area) housed 5 animals. Cages contained wood chip bedding, shredded paper for nesting and mice were provided with environmental enrichment that included crimson Perspex nesting houses and compressed cotton wool pellets. Cages and bedding were changed bi-weekly and all components were autoclaved sterile. Cages were housed in a temperature (20.5 ± 2°C) and humidity (50-55%) controlled diet room within the BRF, with a 12 hour light/dark cycle (lights on at 7 am, lights off at 7 pm).

2.1.5 Animal Weight and Energy Intake

Individual mouse weights (g) and energy intakes (kJ per 10 g of mouse) were measured throughout the duration of the folic acid dietary intervention study. All mice were assigned a code and ear punched to enable identification. Mice were weighed at the start of each dietary intervention, after 10 days (to adjust for staggered supplementation of animals) and at 7 day intervals throughout the duration of the study. Animals were individually removed from cages, cleaned of residual bedding, weighed and replaced. Weights were averaged (mean) between experimental groups.

Energy intakes of individual mice were calculated once a week. After animals were weighed, food was removed from each cage, weighed and replaced. Mice were allowed to feed ad-libitum for a period of 24 hours prior to food being re-weighed. Total food weight (g) after 24 hours was subtracted from initial food weight and divided by the number of animals in the cage. Energy intake (kJ per 10 g of mouse) was calculated using the following equation:

$$\text{Energy Intake (kJ per 10 g of mouse)} = \frac{(\text{food intake (g in 24 hours)} * (\text{Calorie Content of diet}))}{\text{Mouse Weight (g)} * 4.2} \times 10$$

Energy intakes were averaged (mean) between experimental groups and baseline corrected.

2.1.6 Animal Dissections

2.1.6.1 Blood Collection

All mice were fasted for 6 hours (7.30 am to 13.00 pm) prior to tissue collection. Female juvenile (PND 84 or 140) or adult (PND 130 OR 186) C57BL/6 mice were individually sacrificed using a gradual rising CO₂ concentration, followed by cervical dislocation (Animal Scientific Procedures Act 1986, Schedule 1). Blood was immediately collected via cardiac puncture (multisampling needle, 22 g x 1.5 inch, Sarstedt, Germany) into a blood collection tube (1.2 ml EDTA coated S-Monovette, Sarstedt, Germany), transferred to a 1.5 ml Eppendorf and centrifuged at 1800 rpm for 10 minutes. Plasma was extracted using a 200 µl pipette and snap frozen in liquid nitrogen. Blood was extracted, centrifuged and stored at -80°C within 15 minutes of cervical dislocation to preserve folate and homocysteine metabolites.

2.1.6.2 Measurement of Plasma Folate

Blood plasma extracted directly after the 4 week period of folic acid supplementation during the juvenile period or adulthood was analysed for 5-meTHF by Bevitel (Bergen, Norway) using the *Lactobacillus Casei* microbiological assay (Horne and Patterson, 1988).

2.1.6.3 Measurement of Plasma 17β-Estradiol and Progesterone

Due to the large number of animals (200) used within the study, time requirements for individual animal dissections and preparation of mammary whole mounts, it was not feasible to identify the phase of the estrus cycle in each mouse and cull animals in one specific stage of the cycle. As such, results reflect the effects of folic acid supplementation across all four phases of the estrus cycle. However, to confirm that differences in estrus cycle between groups did not influence the outcome of results, blood was extracted 4 weeks after the period of folic acid supplementation during both the juvenile period and adulthood and was analysed for 17β-estradiol and progesterone using the 17β-estradiol or progesterone enzyme linked immunosorbent assay (ELISA) kits (Enzo Life Sciences, SWI). Primary data was then checked against hormone concentrations to confirm that the effects of folic acid supplementation or deficiency were independent of estrogen and progesterone levels.

2.1.6.3.1 17 β -Estradiol Extraction

Plasma (50 μ l) samples were aliquoted into glass vials and diethyl ether (Fisher, UK) was added at a 5:1 (v/v). Samples were vortexed for 2 minutes and phases were allowed to separate for 2 minutes prior to transferring the organic phase into glass test tubes containing 1ml of dH₂O and allowed to separate for 2 minutes. The organic phase was transferred to new glass test tubes and dried under nitrogen gas for 5 minutes. Samples were resuspended in Assay Buffer (Enzo) and stored at -20.

2.1.6.3.2 Progesterone Extraction

Plasma (25 μ l) from each sample was aliquoted into glass vials and diethyl ether (Fisher, UK) was added at a 5:1 (v/v) ratio. Samples were shaken for ~1 minute and phases were allowed to separate for 2 minutes prior to transferring the organic phase into glass test tubes. The addition of diethyl ether was repeated a further 2 times and the organic phases were added to each tube and then dried under nitrogen gas for 5 minutes. Sample dilutions were optimised prior to sample analysis using plasma extracted from pregnant and non-pregnant adult female mice to ensure that concentrations of progesterone fitted into the progesterone standard curve limits of the ELISA assay.

2.1.6.3.3 17 β -Estradiol and Progesterone ELISA

17 β -Estradiol or Progesterone standards were formulated from a concentrated stock using serial dilutions. 100 μ l of standards, samples and controls were pipetted into respective wells on a 17 β -Estradiol or progesterone Elisa plate (Enzo Lifesciences, SWI). Samples were mixed with 17 β -Estradiol or progesterone conjugate and antibody and incubated for 120 minutes on a shaker (500 rpm) at room temperature. Wells were washed 3 times in wash buffer and the plates were tapped dry. pNPP substrate was added to each well and the plate was incubated for 45 minutes at room temperature with no shaking. After 45 minutes stop solution was added to each well and the absorbance at 405 nm was immediately measured on an ELX800 Universal Micro Plate Reader (Biotek Instruments INC, USA). Absorbance readings were calculated by first subtracting the blank wells (BI) from all the samples, followed by subtracting the non-specific binding wells (NSB) from all samples. The net OD values from the samples were then divided by

the maximum binding (B_0) values and multiplied by 100. The percent bound (B/B_0) versus the concentration of 17- β estradiol or progesterone from the standards was plotted in graphical format (Figure 2.2). The concentration of 17- β estradiol or progesterone (pg/ml) for the unknown samples was calculated by interpolation. To correlate progesterone values with those within current literature, interpolated progesterone concentrations (pg/25 μ l) were converted into ng/ml. Test samples from freshly dissected mice were run during optimisation and compared directly with values obtained from samples that had been stored at -80 to ensure sample quality had not diminished. Test samples were compared to gene expression data, morphology data and RNA-seq data using linear regression to ascertain if there was any relationship.

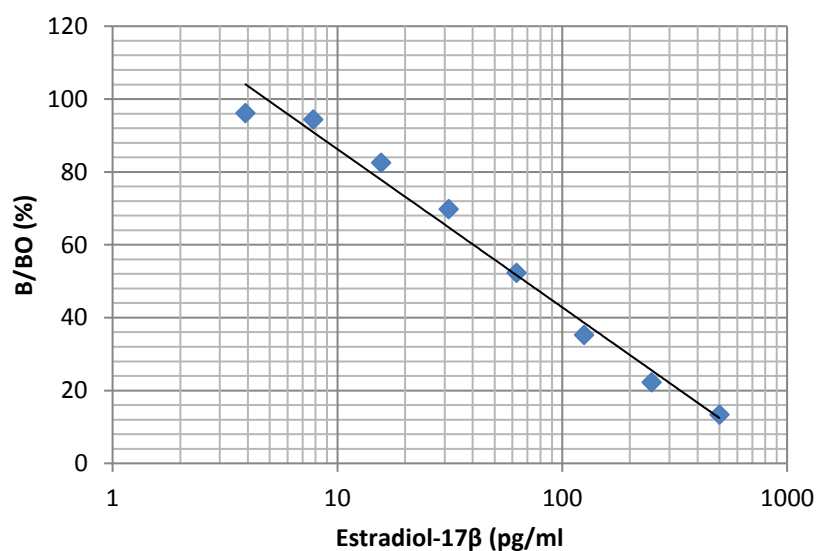


Figure 2.2 Estradiol-17 β Standard Curve

Estradiol-17 β standards were serial diluted and run alongside test samples. Absorbance was measured at 405 nm and used to plot the percent bound (B/B_0) versus the concentration of Estradiol-17 β for each standard. The concentrations of Estradiol-17 β for each plasma sample were calculated using interpolation in Excel (Microsoft 2010).

2.1.6.4 Tissue Collection

Mice were laid supine and pinned to a cork board exposing the thoracic and abdominal regions (Figure 2.3). Laboratory grade ethanol (EtOH, 99.9%, Fisher, UK) was applied to the skin and fur across the thorax and a midline incision was made from the lower abdomen to the upper thorax. An inverted Y incision was made from the midline towards the hind legs on both sides and the skin was peeled back and pinned, exposing the inguinal abdominal, thoracic and cervical mammary glands (Figure 2.3 B). The left inguinal glands were excised and submerged in 10% neutral buffered formalin (NBF) for 24 hours prior to paraffin embedding and immunohistochemical analysis (Figure 2.3 C). Left thoracic mammary glands were excised, whole mounted onto glass slides (Figure 2.3 E, F) and placed in Carnoy's Fixative (75% EtOH, 25% laboratory grade acetic acid). Remaining mammary glands (left and right cervical and right abdominal, thoracic and inguinal), liver, ovaries and brain were excised, snap frozen in liquid nitrogen and stored at -80°C. Mice were culled individually and dissections carried out within 15 minutes of cervical dislocation to minimise effects of tissue degradation post mortem.

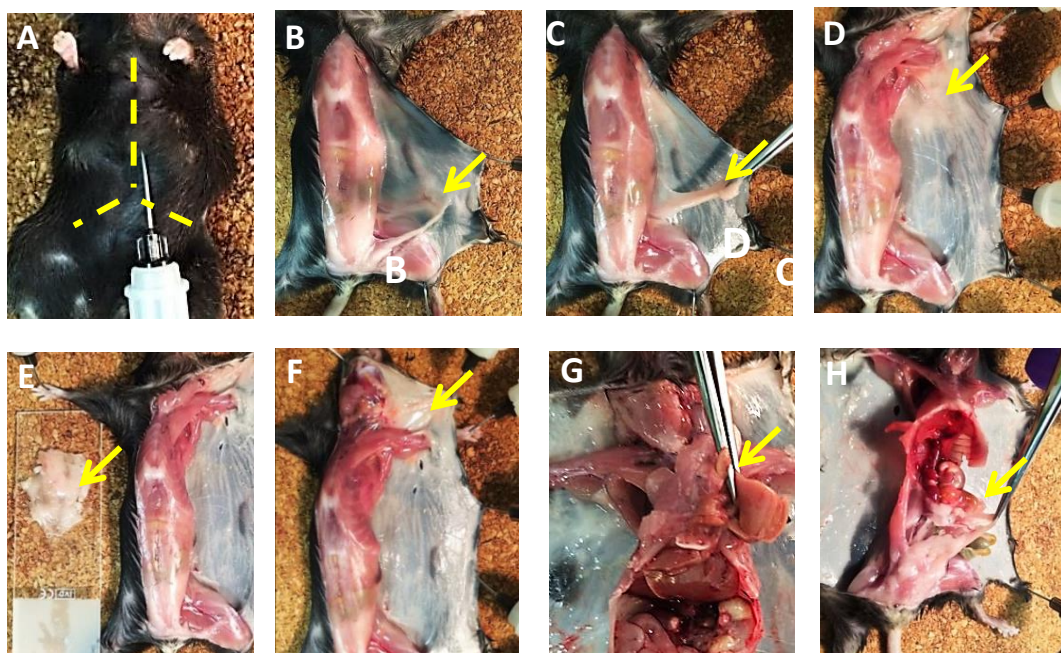


Figure 2.3 C57BL/6 Mouse Dissections.

(A) Location of midline incision and cardiac puncture site. (B) Location of left inguinal and left abdominal mammary glands. (C) Removal of left inguinal and abdominal mammary glands. (D) Location of left thoracic mammary gland. (E) Removal and transfer of left thoracic mammary glands to slide for whole mount. (F) Left cervical mammary gland. (G) Removal of liver. (H) Removal of ovaries.

Table 2.4. Reagent/Chemical/Material for Preparation of Mammary Gland Whole Mounts from Folic Acid Treated C57BL/6 Mice.

Reagent/Chemical/Material	Supplier
Aluminium Potassium Sulfate	Sigma Aldrich, Dorset, UK
Carmin Stain	BDH Stains, Cambridge, UK
Ethanol 100%	Fisher, Loughborough, UK
Glacial Acetic Acid	Fisher, Loughborough, UK
Neutral buffered Formalin (10%) (NBF)	Leica, Solms, Germany
Pertex	Leica, Solms, Germany
Microscope Slides	Leica, Solms, Germany
Coverslips	Leica, Solms, Germany
Clearine Solution	Leica, Solms, Germany
Paraffin	Leica, Solms, Germany

2.2 Mammary Gland Whole Mounting and Slide Processing.

Left inguinal and abdominal mammary gland fat pads were excised together, intact and whole mounted on to glass slides (Figure 2.3 D and E). Glands were positioned onto individual slides corresponding to the original size, dimension and orientation as observed *in situ* (Figure 2.3 E). Mounted glands were left to air dry for 10 minutes and fixed in Carnoy's solution containing 75% EtOH and 25% Glacial Acetic Acid, Fisher, UK) for 3-5 days.

Fixed glands were washed in 70% EtOH for 60 minutes followed by distilled water for 30 minutes at room temperature to remove Carnoy's fixative and rehydrate tissues. Carmine alum stain was prepared by adding 1g of carmine red (BDH Stains, UK) and 2.5g of aluminium sulfate (Fisher, UK) to 500 ml of distilled H₂O. Staining solution was boiled for 30 minutes (100°C), adjusted to a final volume of 500ml and stored at room temperature protected from light (de Assis et al., 2010).

Fixed glands were stained in Carmine alum for 2-4 days or until the lymph nodes had stained through. Slides were washed in 70% EtOH to remove residual stain and dehydrated through a graded series of EtOH (70%, 90%, 100% and 100%) for 60 minutes at each concentration. Dehydrated slides were placed in Clearine (Leica, UK) for one hour (repeated three times) to delipidate the mammary fat pad and increase tissue transparency. Slides were cover slipped using Pertex (Leica, Germany) and allowed to air dry for 2 days prior to imaging.

2.2.1 Mammary Whole Mount Analysis and Quantification

Imaging of whole mount mammary tissue sections was performed using a DotSlide 2.1 Virtual Slide System (Olympus, UK). Each slide was scanned (x 2.5 magnification) and overview images were collected and used for morphological analysis. Quantification of epithelial tree density (ETD), number of epithelial junctions (NEJ), average epithelial tree branch length (ABL) and the number of terminal end buds (TEBS) were calculated from overview images using Image Processing and Analysis in Java (Image J, Maryland, USA).

2.2.2 Quantification of Epithelial Tree Density

Whole mount scanned images (VSi) were scaled using OlyVia Image Viewer Software Version 3.7 (Olympus, UK). Image measurement parameters were adjusted to scale bars (2 mm) using Image J. Each mammary whole mount image was divided into two halves (medial and distal) and a region of interest (ROI) was selected and converted to an 8 BIT (greyscale) file (Figure 2.4 A, B, C). The ROI was then threshold adjusted, a mask was created and the area of the mask (representative of the total epithelial tree area) was measured (Figure 2.4 D, E). Epithelial density (area of epithelial tree) was calculated for each mammary region using three independent ROI measurements (three from the medial and three from the distal sections) taken randomly from each whole mount. The ROI (area) used was standardised between measurements to ensure accurate reproducibility of epithelial area calculations.

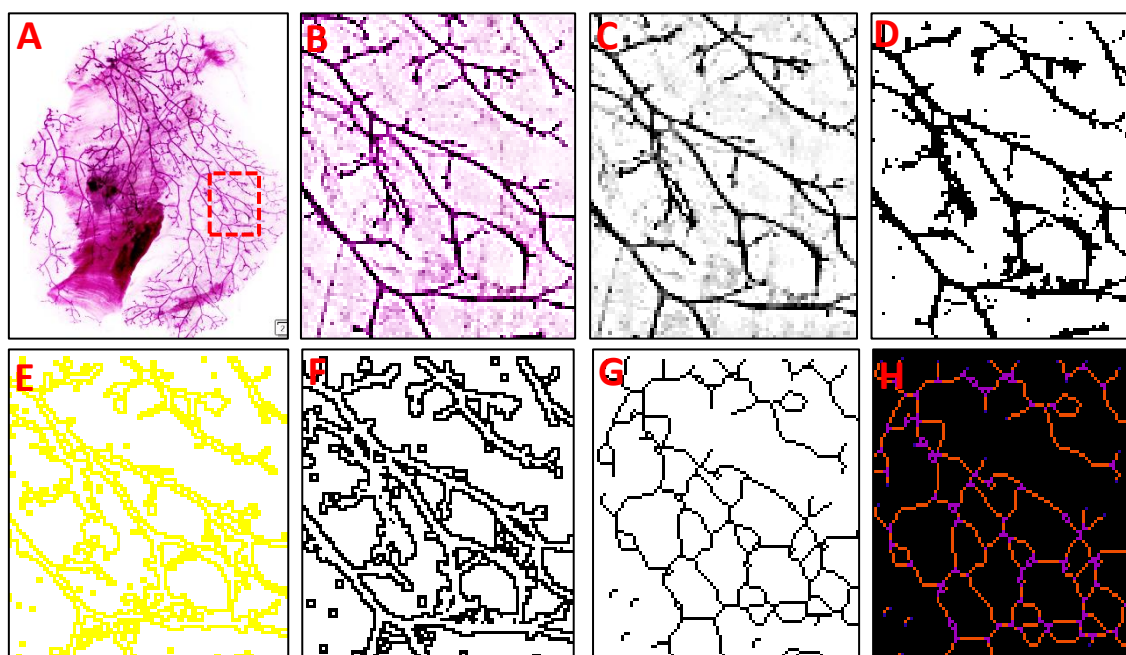


Figure 2.4. Quantification of Mammary Gland Epithelial Tree Density, Junctions and Branches
 (A) Carmine stained mammary gland whole mount (left 2nd and third thoracic). (B) ROI selected from image A (highlighted in yellow). (C) ROI converted to 8 BIT. (D) ROI threshold adjusted (E) ROI converted into a mask to measure epithelial area/density. (F) ROI outlined in binary format. (G) ROI skeletonised. (H) Calculation of epithelial branch number (red), junctions (purple) and average branch length.

2.2.3 Epithelial Tree Junctions, Branches and Branch Length

Whole mount scanned images, (scaled and adjusted) were used to generate an ROI which was selected and thresholded (Figure 2.4 D). Thresholded images were inverted (image changed from black to white) and the epithelial tree outlined, dilated and skeletonized using binary function settings (Image J) (Figure 2.4 G). Skeletonised images were analysed using Analyse Skeleton software plug in (Image J). The number of epithelial branches, junctions and average branch length were calculated for 6 separate ROI's as outlined in section 2.2.2 (Figure 2.4 H).

2.2.4 Epithelial Tree Terminal End Buds

Whole mount scanned images (scaled and adjusted) were used to generate an ROI. The number of terminal end buds was calculated manually by counting each structure using the cell counter plugin (Image J) and dividing this by the ROI area. Three separate ROI's were measured per whole mount and the mean TEB number calculated per mammary gland for each folic acid treatment.

Table 2.5 Reagent/Chemical/Material For qRT-PCR of Mammary Tissue from FA Treated C57BL/6 Mice.

Reagent/Chemical/Material	Supplier
DNase 1	Sigma-Aldrich, Dorset, UK
Acid Phenol Chloroform	Invitrogen, Paisley, UK
DNase/RNase Free H ₂ O	Fisher, Loughborough, UK
DNase Stop Solution	Sigma-Aldrich, Dorset, UK
dNTP Set	Promega, Southampton, UK
Primer Sets (Quantitect)	QIAGEN, Limberg, Netherlands
Custom Made Primer Sets	Primer Design, Southampton, UK
Primer Sets (TaqMan)	Invitrogen, Paisley, UK
Random Nonomers	Sigma-Aldrich, Dorset, UK
Gel Red	Sigma-Aldrich, Dorset, UK
Gel Ladder 1 KB	Sigma-Aldrich, Dorset, UK
mirVANA miRNA Isolation Kit	Invitrogen, Paisley, UK
M-MLV Reverse Transcriptase	Promega, Southampton, UK
M-MLV Buffer	Promega, Southampton, UK
Primer Sets (QIAGEN, Quantitect)	QIAGEN, Limberg, Netherlands
Trizol	Invitrogen, Paisley, UK
Taqman Gene Expression Mastermix	Invitrogen, Paisley, UK
384 Well PCR Plate (White)	Invitrogen, Paisley, UK
96 Well Non Skirted PCR Plate	Fisher, Loughborough, UK
TRIS Acetate EDTA Buffer	Fisher, Loughborough, UK
Sodium Acetate	Invitrogen, Paisley, UK
Light Cycler 480	Roche Diagnostics, Sussex, UK
Syber Green	Sigma-Aldrich, Dorset, UK
Quantifast Syber Green	Qiagen, Limberg, Netherlands

2.3 Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.3.1 Total RNA Extraction

Total messenger ribonucleic acid (mRNA) was analysed in folic acid and non-folic acid supplemented C57BL/6 mice in order to gain an insight into the transcriptome at specific time points post folic acid supplementation. Initial experiments were conducted to ascertain the most effective method for RNA extraction from murine mammary tissue. Three methods of extraction were compared; TRIZOL™ (Invitrogen), All Prep RNA/DNA Mini Kit (Qiagen, UK) and mirVana miRNA Extraction Kit (Invitrogen, UK). MirVana miRNA Extraction Kit was selected as it was shown to isolate high quality, pure RNA (Figure 2.5). Furthermore, this method preserved the population of micro RNA's in each sample for future downstream analysis. Additional optimisation experiments were performed to assess baseline gene expression levels between different mammary glands (10) in adult non-supplemented C57BL/6 mice (n=6).

2.3.2 Total RNA Extraction from Mammary Glands

RNA was extracted from mouse mammary gland (2nd and 3rd thoracic) tissue sections (50 mg) crushed to powder in liquid nitrogen, suspended in 500 µl of denaturing lysis solution (Invitrogen, UK) to stabilise RNA and deactivate RNase's and vortexed for 30 seconds. Lysates were homogenised using a 200 µl pipette tip to further disrupt fatty deposits, 50 µl of miRNA homogenate (Invitrogen, UK) was added and samples were incubated for 10 minutes on ice. An equal volume (500 µl) of Acid Phenol Chloroform (Invitrogen, UK) was added to each sample, vortexed for 30-60 seconds and centrifuged at maximum speed (10,000 g) for 5 minutes at room temperature. The upper aqueous phase was collected and combined with 1.25 volumes of 100 % Et OH (room temperature). Samples were added to a mirVana filter column (Invitrogen, UK), centrifuged for ~15 seconds (10,000 g) and the flow-through discarded. The filter column was washed by adding 700 µl of wash solution 1 (Invitrogen, UK) and centrifuged for ~15 seconds. This was repeated twice using 500 µl of miRNA wash solution 2/3 (Invitrogen, UK) and discarding the flow-through. The spin filter was centrifuged at maximum speed (10,000 g) for 1 minute to remove residual wash solutions. The spin filter was transferred to a new RNAase free collection tube and RNA was eluted in 50 µl of heated (95°C) RNAase free H₂O (Fisher Scientific, UK) by

spinning at 10,000 g for 1 min. This was repeated in a second collection tube to ensure that residual RNA was eluted from the column filter and maximise total RNA yield.

2.3.3 RNA Quantification

Total RNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). 1 µl of RNA was analysed by measuring the light absorbance at 260 nm (OD_{260}). RNA quality and purity was assessed by comparing light absorption values at 260 nm (OD_{260}) to 280 (OD_{280}) and 230 (OD_{230}). An OD_{260}/OD_{280} ratio of ~2 is generally accepted as pure RNA, free from contamination (Thermo Scientific, USA). OD_{260}/OD_{230} ratios, used as a secondary measure of nucleic acid purity < 1.7 are considered to have low contamination of organic compounds or guanidium salts found in purification columns.

2.3.4 Agarose Gel

RNA quality was assessed by running RNA samples on an agarose gel (1%). 1 gram of agarose (Sigma-Aldrich, UK) was added to 100 ml of Tris- Acetate EDTA (TAE) buffer (Fisher, UK), heated for 2 minutes and allowed to cool. Once cooled 7 µl of Gel Red intercalating agent (Sigma-Aldrich, UK) was added, mixed and the agarose/Gel Red solution was poured into a gel casting tray and allowed to cool and set for ~30 minutes.

2.3.5 RNA Gel Electrophoresis

RNA was resolved using gel electrophoresis. RNA samples (100-300 ng) were mixed with gel loading dye (1-2 µl) (Thermo Fisher, UK) and added to individual wells within an agarose gel. A 1 KB DNA ladder (O'gene Ruler, Thermo Fisher, UK) was run on each gel. Agarose gels containing RNA samples were loaded into a gel electrophoresis tank (BIO RAD, UK), submerged in TAE Buffer (40 mM Tris Base, 1 mM EDTA pH 8.0 and 20 mM acetic acid) and run at 130 V for between 10 and 20 minutes at room temperature. Resolved gels were visualised on a UV transilluminator.

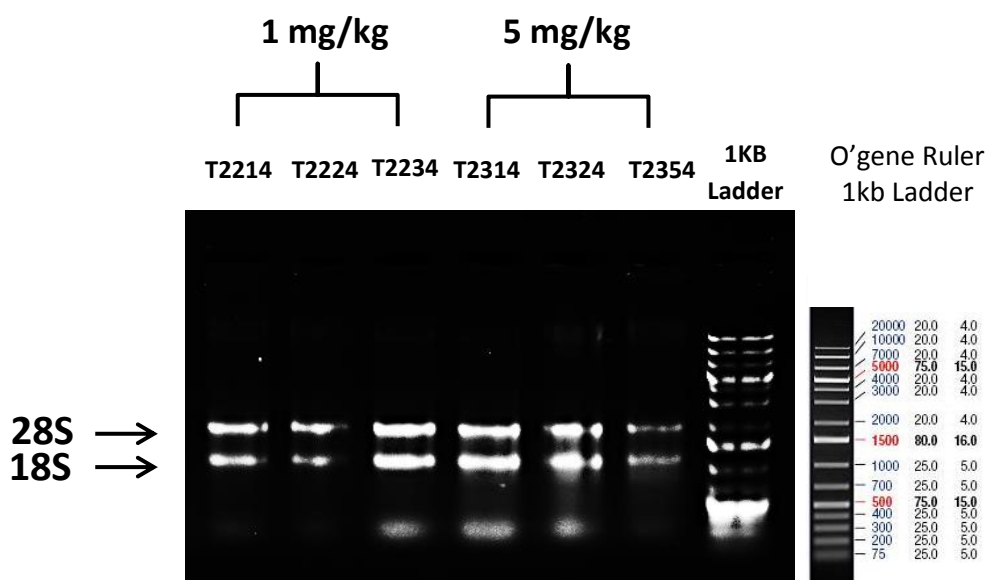


Figure 2.5. Mammary mRNA Samples Resolved Using Gel Electrophoresis.

RNA samples (100-300 ng/μl) extracted from the mammary glands of C57BL/6 mice supplemented with either 1 mg/kg or 5 mg/kg FA (PND 74-102) followed by a maintenance diet of 2.1 mg/kg (PND 102-130) were run on a 1 % agarose gel at 130 V for ~20 minutes. Both 28S (~2000 bp) and 18S (~1200 bp) bands are clearly visible and intact.

2.3.6 Total RNA Extraction from Mouse Livers

RNA was extracted from mouse liver tissue (C57BL/6 Virgin female, 7 week old, non-supplemented) using Trizol reagent (Invitrogen, UK). Liver (100 mg) was crushed to powder in liquid nitrogen and suspended in 1 ml of Trizol reagent. Samples were vortexed for 30 seconds and incubated for 5 minutes at room temperature. Samples were disrupted with a 200 μl pipette and centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant was isolated and transferred to a new 1.5 ml Eppendorf. 200 μl of phenol chloroform (Sigma-ALdrich, UK) was added; samples were shaken vigorously by hand and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 rpm for 15 minutes at 4°C and the aqueous phase isolated. RNA was precipitated from the aqueous phase by addition of 500 μl of isopropyl alcohol (Fisher, UK). Samples were incubated for a further 10 minutes at room temperature and centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was removed and the RNA pellet washed with 1 ml of 75% EtOH, vortexed and centrifuged at 7,500 rpm for 5 minutes at

4°C. This was repeated and the RNA pellet was air dried for 5 minutes. Pelleted RNA was dissolved in 50 µl of DNA/Raise free H₂O (Fisher, UK) and incubated at 55-60°C for 10 minutes. RNA was quantified using a Nanodrop 1000 (Thermo Scientific, USA) and stored at -80 °C. Selected samples were run on a 1% agarose gel to confirm RNA integrity. RNA from mouse livers was used for generation of cDNA standards for qRT-PCR mRNA quantification of target genes.

2.3.7 RNA Purification

Extracted RNA samples that exhibited A260/A280 absorbance ratios of > 1.5 were purified. RNA was diluted to 200 µl with RNase free sterile H₂O (Fisher H₂O), 1/10 volume of 3 M sodium acetate (Sigma-Aldrich, UK) and 1 volume of isopropanol (Fisher, UK) was added prior to incubation at -20°C for 1 hour (or overnight to further increase yield). Samples were centrifuged at maximum speed (20,000 x g) for 15 minutes, supernatant removed and the pellet washed with 500 µl of 70 % EtOH (4°C). Samples were centrifuged at maximum speed for 2 minutes and EtOH was removed. This process was repeated twice to ensure total removal of isopropanol, the pellet was air dried for ~10 minutes to remove residual EtOH and resuspended in 50 µl of RNase free, sterile H₂O (Fisher, UK).

2.3.8 Synthesis of Complimentary DNA (cDNA)

2.3.8.1 DNase Treatment

Total extracted RNA was diluted to a concentration of 125 ng/µl using RNase free H₂O (Fisher, UK). 1 µg (8 µl) of each RNA sample was added to an RNase-free 96 well PCR plate (Thermo Scientific, USA). 1 µl of amplification grade DNase 1 (1 unit/µl) (Sigma, UK) was added to each sample along with 1 µl of 10 x reaction buffer (Sigma, UK). Samples were mixed with a pipette and incubated for 15 minutes at room temperature. 1 µl of DNA Stop solution (Sigma-Aldrich, UK) was added to bind calcium and magnesium ions and inactivate DNase 1. Samples were heated at 70 °C for 10 minutes to denature DNase 1 and RNA was chilled on ice.

2.3.8.2 Reverse Transcription

Total DNase treated RNA was converted to complimentary DNA (cDNA). 1 µl of 10 mM dNTP mix (Promega, UK) was added, along with 1 µl of random nonomers (RN) (final concentration of 1-5 µM) (Sigma, UK), to each DNAase treated RNA sample, incubated at 70°C for 10 minutes and placed on ice. A master mix was formulated containing 5 µl of RNase free H₂O (Fisher Scientific), 4 µl of 10x reverse transcriptase buffer (Promega, UK) and 1 µl of Maloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega, UK) of which 10 µl was added to each sample. Samples were incubated in a Verity 96 well Fast Thermal Cycler (Thermo Fisher Scientific) at room temperature for 10 minutes followed by 37°C for 60 minutes to synthesize the CDNA strand. Finally, samples were heated at 90°C for 10 minutes to denature the M-MLV reverse transcriptase. Synthesised cDNA was stored at -20 °C short term or -80°C for prolonged periods.

2.3.8.3 Oligonucleotide Primers

Primers were commercially produced and supplied from Qiagen (UK) as a 10x mix of lyophilized forward and reverse primer (Table 2.6) that were reconstituted to 100 µM by adding 1.1 ml of Tris EDTA (TE), pH 8.0, aliquoted and stored at -20°C. Taqman (Invitrogen) primers assays were supplied commercially from Invitrogen (UK) reconstituted (240 µl). Custom made primers for variant transcripts were designed using Ensemble Release 83 (NCBI). Murine isoform DNA sequences were located for genes of interest and primers were designed within exons. Primer sequences were tested for correct alignment and non-specific binding using the Basic Local Alignment Search Tool (BLAST) (NCBI, UK). Custom primers were synthesised and supplied by Primer Design, UK as a mix of lyophilized forward and reverse primers and were reconstituted in 220 µl of RNA free H₂O (Fisher, UK).

2.3.8.4 Quantitative Real Time Polymerase Chain Reaction

Quantitative Real Time PCR (qRT-PCR) was performed using a LightCycler 480 Real-Time PCR System (Roche, UK). cDNA (1 µg) was diluted with RNase free H₂O (Fisher, UK) to 10 ng/µl (100 µl). cDNA samples that were less than 1 µg (RNA was below 125 ng/µl when 8 µl were converted to cDNA) were diluted to a concentration that equated to 10 ng/µl. qRT-PCR was carried out using Syber Green (Sigma, UK; Qiagen, UK) or TaqMan (Invitrogen) gene expression assays. Total

reaction volume for Syber Green assays was set at 25 µl. A master mix containing 4.5 µl of DNase/RNase free H₂O (Fisher, UK), 0.5 µl of reference dye (ROX) (Sigma, UK), 2.5 µl of primer mix (10 µM) and 12.5 µl of Syber Green (Sigma, UK) was formulated and 20 µl added to a 384 well PCR plate (Thermo, USA). 5 µl of cDNA was added to each reaction and samples were centrifuged (1 min) and analysed by qRT-PCR.

Table 2.6. Gene Primers used in qRT-PCR for quantification of mRNA expression.

Abbreviations used: *Brca1*, breast cancer 1 early onset; *Oct-4*, octamer-binding transcription factor 4; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Ubc*, ubiquitin C; *Ezh2*, enhancer of zeste homologue 2; *Suz12*, polycomb repressive complex 2 subunit; *Bmi1*, polycomb group RING finger protein 4; *Rpl*, ribosomal protein; *Cyc*, cyclophilin; *Act*, actin like protein; *B2m*, beta 2 microglobulin.

NA = Not Commercially Available

Gene Primer	Forward Primer (5' → 3') Reverse Primer (3' → 5')	Reference	Product Size (bp)	Annealing Temp. (°C)
<i>Brca1</i>	QIAGEN Quantitect™ Primer Assay	QT00145810	62	55
<i>Oct-4</i>	QIAGEN Quantitect™ Primer Assay	QTOO109186	101	55
<i>Gapdh</i>	QIAGEN Quantitect™ Primer Assay	QT01658692	144	55
<i>Ubc</i>	QIAGEN Quantitect™ Primer Assay	QT00245189	75	55
<i>Ezh2</i>	QIAGEN Quantitect™ Primer Assay	QT01063671	119	55
<i>Suz12</i>	QIAGEN Quantitect™ Primer Assay	QT00160790	100	55
<i>Bmi1</i>	QIAGEN Quantitect™ Primer Assay	QT00165298	179	55
<i>Brca1</i>	TAQMAN™ Gene Expression Assay	Mm01249840-m1	75	60
<i>Oct-4</i>	TAQMAN™ Gene Expression Assay	Mm 03053917-gl	139	60
<i>Gapdh</i>	Primer Design™ Assay	Ge-SY-6	NA	60
<i>Ubc</i>	Primer Design™ Assay	Ge-SY-6	NA	60
<i>Rpl</i>	Primer Design™ Assay	Ge-SY-6	NA	60
<i>Cyc</i>	Primer Design™ Assay	Ge-SY-6	NA	60
<i>Act</i>	Primer Design™ Assay	Ge-SY-6	NA	60
<i>B2m</i>	Primer Design™ Assay	Ge-SY-6	NA	60

2.3.8.5 PCR Cycling Condition

PCR cycling conditions for Quantitect Syber Green (QIAGEN, UK) primer assay samples comprised a holding stage of 95 °C for 2 mins followed by cycles (40) of primer denaturing, specific primer annealing and primer elongation (Table 2.7). A melt curve step comprised 95 °C for 15 seconds, 60 °C for 1 min and 95 °C for 15 seconds. TaqMan cycling conditions comprised holding stages of 50 °C for 2 minutes and 95 °C for 10 minutes followed by cycles (40 x) of primer denaturing, primer annealing and specific primer elongation (Table 2.7).

Table 2.7. Conditions for qRT-PCR Quantification of mRNA Expression.

Abbreviations used: *Brca1*, breast cancer 1 early onset; *Oct-4*, octamer-binding transcription factor 4; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Ubc*, ubiquitin C; *Ezh2*, enhancer of zeste homologue 2; *Suz12*, polycomb repressive complex 2 subunit; *Bmi1*, polycomb group RING finger protein 4; *Rpl*, ribosomal protein; *Cyc*, cyclophilin; *Act*, actin like protein; *B2m*, beta 2 microglobulin.

Gene Primer	Reference	Denature Temp (°C)	Time (s)	Anneal Temp (°C)	Time (s)	Extension Temp (°C)	Time (s)	Cycles
<i>Brca1</i>	Quantitect™	95	30	55	60	72	60	40
<i>Oct-4</i>	Quantitect™	95	30	55	60	72	60	40
<i>Gapdh</i>	Quantitect™	95	30	55	60	72	60	40
<i>Ubc</i>	Quantitect™	95	30	55	60	72	60	40
<i>Ezh2</i>	Quantitect™	95	30	55	60	72	60	40
<i>Suz12</i>	Quantitect™	95	30	55	60	72	60	40
<i>Bmi1</i>	Quantitect™	95	30	55	60	72	60	40
<i>Brca1</i>	Taqman™	95	15	60	60	60	60	40
<i>Oct-4</i>	Taqman™	95	15	60	60	60	60	40
<i>Gapdh</i>	geNORM™	95	30	60	60	60	60	50
<i>Ubc</i>	geNORM™	95	30	60	60	60	60	50
<i>Rpl</i>	geNORM™	95	30	60	60	60	60	50
<i>Cyc</i>	geNORM™	95	30	60	60	60	60	50
<i>Act</i>	geNORM™	95	30	60	60	60	60	50
<i>B2m</i>	geNORM™	95	30	60	60	60	60	50

2.3.8.6 Characterisation of Optimal Genes for Normalisation

In order to accurately quantify changes in gene expression using qRT-PCR, it is necessary to normalise Ct values from a gene of interest to a ubiquitously expressed reference gene that is not affected by experimental treatment conditions (housekeeping gene). This accounts for variations in cDNA synthesis efficiency, cDNA loading and tissue specific treatment, allowing accurate quantification of Ct data to analyse changes in levels of mRNA expression. Characterisation of optimal genes for normalisation is dependent on experimental design, tissue specificity and dietary intervention which require tissue specific optimisation for individual treatments.

Six reference genes, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), ubiquitin C (*Ubc*), actin like protein (*Act*), beta-2 microglobulin (*B2m*), ribosomal protein (*Rpl*) and cyclophilin (*Cyc*) were selected from a geNorm primer (Primer Design, UK) expression profile based on research conducted by Han *et al.*, (2010) investigating the selection and use of reference genes in mouse mammary glands. The expression of these genes in C57BL/6 mammary tissue (n=3) from each FA treatment group (total n=12) was measured using qRT-PCR and analysed using geNorm primer normalisation software (Primer Design, UK) (Figure 2.6).

2.3.8.6.1 geNorm Protocol

Lyophilised Primers (Primer Design, UK) were reconstituted in 220 µl of DNase/RNase free H₂O (Fisher, UK). Three samples were selected from each folic acid treatment group (0.2 mg/kg, 1 mg/kg, 5 mg/kg, 20 mg/kg) and qRT-PCR was performed using the protocol outlined in section 2.3.8.4, with the number of cycles adjusted to 50. All samples, standards and NTC's were run in duplicate. Values were extrapolated and analysed using qBase+ software (Biogazelle, Belgium). The average expression stability value (M) and levels of variation in reference gene stability was calculated for each gene and genes were ranked based on the levels of variation between treated samples (expressed in geNorm M values) from the most unstable reference genes (high M value) to the most stable reference genes (Low M value) (Figure 2.6).

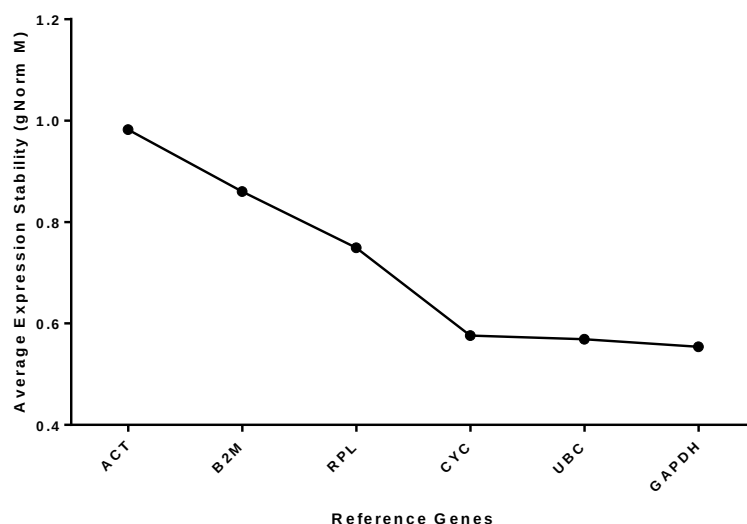


Figure 2.6. gNorm Analysis of Optimal Reference Genes for qRT-PCR Expression Analysis in Folic Acid Supplemented C57BL/6 Mammary Tissue.

Average expression stability value (M) for 6 reference genes expressed in mouse mammary glands treated with four different concentrations of folic acid. Genes are ranked according to expression stability, with the least stable gene, *Act* (highest M value) on the left of the x-axis and the most stable gene, *Gapdh* (lowest M value) on the right. *Gapdh* and *Ubc* were selected as reference genes for expression analysis in folic acid treated mammary tissue.

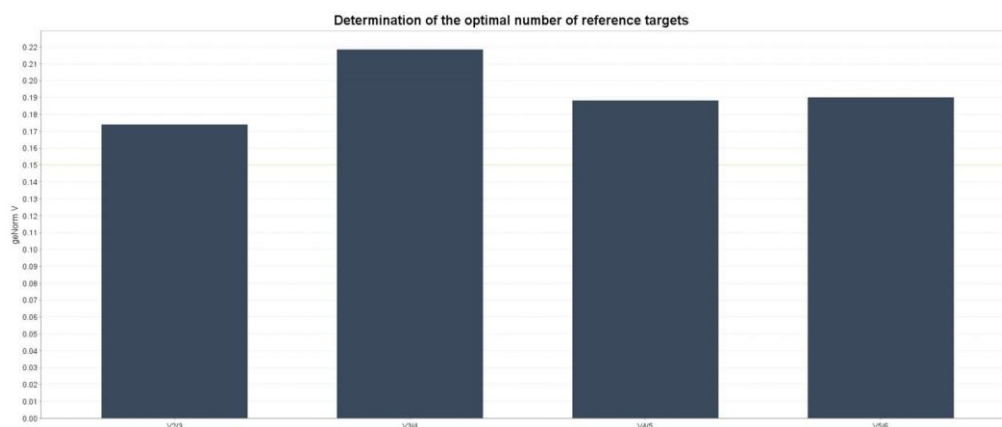


Figure 2.7. gNorm Analysis for the Determination of the Optimal Number of Reference Targets to be used in Gene Expression Normalisation.

A gNorm $V_n/n+1$ value is calculated for individual comparisons between two consecutive numbers (n and $n+1$) of candidate reference genes. If the $V_n/n+1$ value drops below the GNorm V threshold of 0.15 then addition of further reference genes ($n+1$) is considered limited.

Determination of the optimal number of reference targets to be used in gene expression normalisation was calculated using gNorm V (Figure 2.7). A gNorm $V_{n/n+1}$ value is calculated for individual comparisons between two consecutive numbers (n and $n+1$) of candidate reference genes. If the $V_{n/n+1}$ value drops below the GNorm V threshold of 0.15 then addition of further reference genes ($n+1$) is considered limited. Two reference genes (*GAPHD* and *UBC*) that exhibited the highest stability were selected for qRT-PCR normalisation (Figure 2.6). This allowed the calculation of a gene expression normalisation factor for individual samples by using the geometric mean (GM) of two selected reference genes. Cyclophilin (CYC), a reference gene used for preliminary qRT-PCR optimisation experiments was also found to be stably expressed (Figure 2.6).

2.3.8.7 Quantification of Gene Expression from Cycle Threshold (Ct)

Levels of mRNA within samples were quantified from Ct values using either standard curve analysis, or comparative Ct ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001) and normalised to two reference genes, *Gapdh* and *Ubc*, selected from gNorm analysis. Standard curve analysis was used for qRT-PCR using Syber Green chemistry (Sigma, UK). Syber Green binds to newly formed double stranded DNA (dsDNA) synthesised during PCR amplification, resulting in a net increase in fluorescence which is directly proportional to the amount of PCR product available. Syber Green dye binds to all dsDNA, which can include nonspecific dsDNA sequences. To ensure amplification of the desired amplicon, it is necessary to ensure that primers are functioning optimally. The incorporation of standard curve analysis takes into account primer efficiency and, combined with melt curve analysis, provides accurate assessment of gene amplification.

Comparative Ct analysis was used for qRT-PCR using Taqman probes. Taqman chemistry uses fluorogenic-labeled probes that utilise the 5' nuclease activity of Taq DNA polymerase. Typical probes contain a fluorescent reporter dye located at the 5' end and a quencher dye at the 3' end. The close proximity of reporter and quencher dyes limit the levels of fluorescence emitted by the reporter, however, during PCR amplification the probe anneals downstream to the specific primer sequence and during extension is cleaved by the 5' nuclease activity of Taq DNA polymerase, separating the reporter and quencher, resulting in a net increase in fluorescence. This method is highly specific, due to the specific hybridization required between probe and target to generate signal and demonstrates increased detection sensitivity without the potential

for non-specific binding. For this reason, Taqman was selected for amplification of *genes* that demonstrated low levels of expression (*Brca1* and *Oct-4*).

2.3.8.7.1 Standard Curve Method

Standard curve analysis was calculated from a serial dilution of six standards (250, 100, 50, 25, 10 and 1 ng) generated from mouse liver RNA, reverse transcribed to cDNA and run in parallel to test samples on each PCR plate (table 2.8). Sample mRNA concentrations were calculated by plotting Log^{10} of each standard concentration (ng) against the average Ct (run in duplicate) value from each qRT-PCR experiment. The equation of the line ($y = mx + c$) was then used to extrapolate the gradient (m) and the y intercept (c) values, of which the Ct value was subtracted from the gradient (m), divided by the y intercept (c) and multiplied by the power of 10 ($= 10^{\frac{((\text{CT}-\text{M})}{-\text{c}})}$) (Figure 2.8). The mean mRNA values (from two duplicates) were then normalised to the geometric mean (GM) of the mRNA concentrations from the two normalised housekeeping genes (GAPDH and UBC). The geometric mean was calculated by taking the square root ($\sqrt{}$) of the sum of each average ct or calculated mRNA value for each housekeeping gene multiplied together.

Table 2.8. Example of Standard Curve Calculation for the Reference Gene BMI1 in Female Juvenile FA supplemented Mice.

cDNA standards were prepared from RNA extracted from mouse liver and the Log^{10} cDNA concentration calculated along with Average Ct values (mean from two duplicates Ct 1 and Ct 2) calculated from qRT-PCR analysis.

Standard cDNA Concentration (ng per 5 μ l)	Log 10 Con	Ct1	Ct2	Average	Standard Deviation (SD)
250	2.39	24.35	24.53	24.44	0.13
100	2	25.4	25.36	25.38	0.02
50	1.69	26.65	26.46	26.55	0.13
25	1.39	27.35	27.31	27.31	0.02
10	1	29.8	30.24	30.24	0.13
1	0.69	33.75	35.4	35.4	1.16

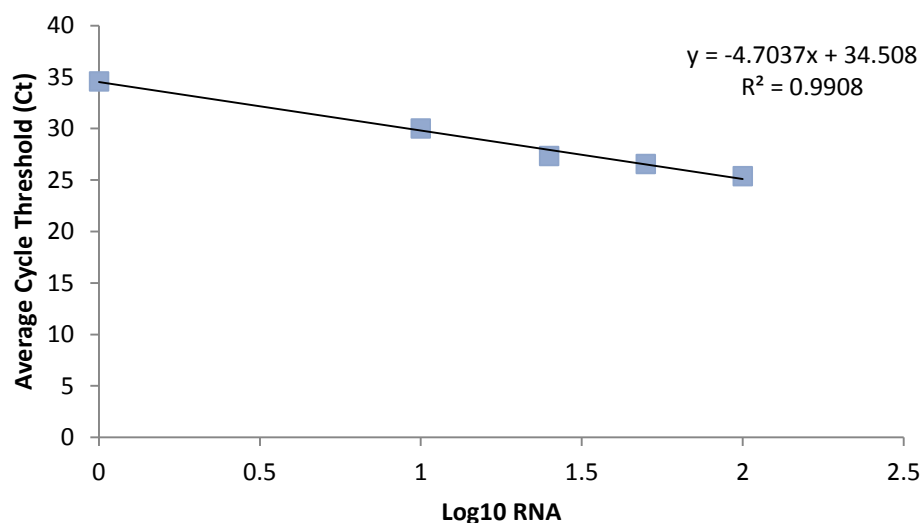


Figure 2.8. Example of a Standard Curve Using qRT-PCR for the reference gene BMI1 in Juvenile Folic Acid Supplemented C57BL/6 mice.

cDNA standards (250, 100, 50, 25, 10, and 1 ng) were prepared from RNA extracted from mouse liver and analysed by qRT PCR in conjunction with FA treated mammary samples.

2.3.8.7.2 Comparative CT Analysis

Comparative Ct analysis ($\Delta\Delta\text{CT}$) was calculated by directly comparing the difference in Ct values of target genes between treatments and normalising to two internal housekeeping genes (*Gapdh* and *Ubc*) (Figure 2.9). Changes in Ct were first calculated from the average (two duplicates) of two normalised housekeeping genes (*Gapdh* and *Ubc*) and the GM determined. The relative change in Ct ($^{\Delta}\text{Ct}$) of the target gene was then calculated by subtracting the GN from each sample respectively. The $^{\Delta}\text{Ct}$ for each sample was then subtracted from the $^{\Delta}\text{Ct}$ within the treatment group that exhibited the largest change in Ct value formulating $\Delta\Delta\text{Ct}$. Relative gene expression was calculated using the equation $\text{Power}(2^{-\Delta\Delta\text{CT}})$ and taking the logarithmic value of this output (Log^{10}) (Livak and Schmittgen, 2001).

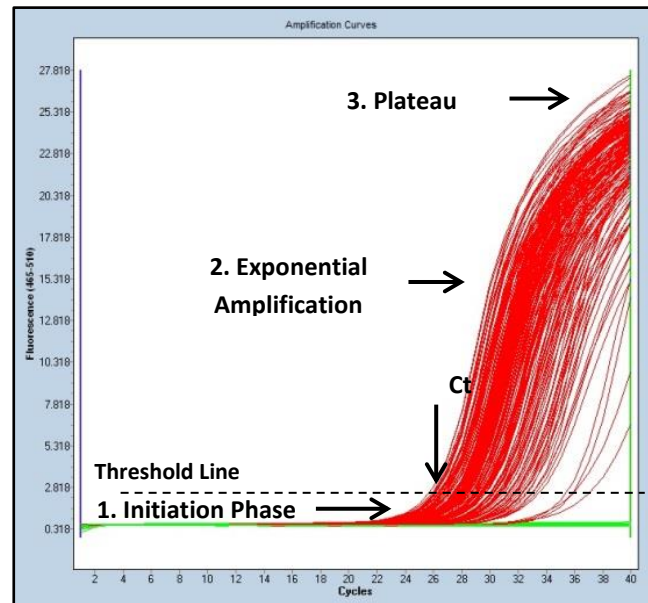


Figure 2.9 Amplification Plot from qRT-PCR analysis of EZH2 in Adult FA supplemented C57BL/6 mice.

Plot shows fluorescence (465 – 510 nm) which is proportional to the amount of amplified product in each sample (Y-axis) plotted against the number of amplification cycles (X-axis). **1:** During the initiation phase fluorescence remains at background levels. **2** Exponential amplification of PCR product yields a detectable increase in fluorescence signal which crosses the threshold line (Ct). Measurement of the Ct value within the exponential phase accurately calculates the initial amount of template within each reaction. The greater the amount of product available at the beginning of each reaction the earlier the amplified product will cross the detectable threshold and the lower the Ct value. **3:** As the number of cycles increases and reagents become limited, exponential amplification slows and begins to plateau (Figure 2.9).

2.3.8.7.3 Melt Curves Analysis

qRT-PCR using Syber Green intercalating dye (Sigma-Aldrich, UK) detects total double stranded DNA, which can include gene primers that have annealed to themselves (primer dimers), DNA contamination and PCR product from misaligned primers. To ensure accurate detection and amplification of the required amplicon, melt curves were generated for each sample and analysed for amplicon specificity. At the end of each qRT-PCR run the temperature was gradually increased and the change in fluorescence recorded. When the temperature reached the specific

melting point of the PCR product the double stranded DNA denatured and the fluorescence signal was rapidly lost (2.10). Quantitation of the melting point for each sample and analysis of the shape of the melt curve was used to ensure that qRT-PCR Ct values for each gene were representative of the desired single amplicon.

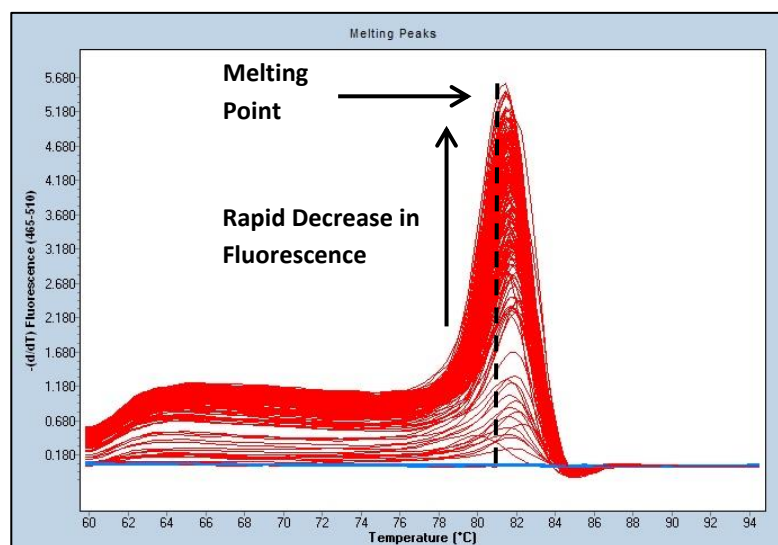


Figure 2.10. qRT-PCR Melt Curve analysis of *Ezh2* expression in C57BL/6 mice (200) supplemented during juvenile pre-pubertal period or adulthood.

Melt curves were calculated using software version 2.11. The melting temperature (T_m) of the amplified double-stranded fragment is calculated by plotting the negative derivative of the change in fluorescence divided by the change in temperature in relation to the temperature $[(d/dT) \text{ fluorescence (465-510)}]$. The peak of this converted melting curve is representative of the fragment specific T_m . Melting peaks (T_m) for *Ezh2* were observed at $\sim 82^\circ\text{C}$ in all adult and juvenile samples demonstrating target specific amplification of the *Ezh2* amplicon.

2.3.8.8 Characterisation of Gene Expression between Mammary Glands in C57BL/6 Mice

To perform the desired number of techniques and analysis in individual animals it was necessary to utilise multiple different mammary glands from each animal in order to acquire adequate quantities of tissue. Optimisation experiments were performed to ascertain if differences in gene expression were present between individual mammary glands. qRT-PCR analysis of two genes were selected to compare glands, *Brca1* and Estrogen Receptor (*ER1*). *Brca1* is ubiquitously and generally lowly expressed in normal mammary tissue. *ER1*, a gene involved in hormonal cycling in female mice is critically implicated in mammary gland function (Figure 2.11).

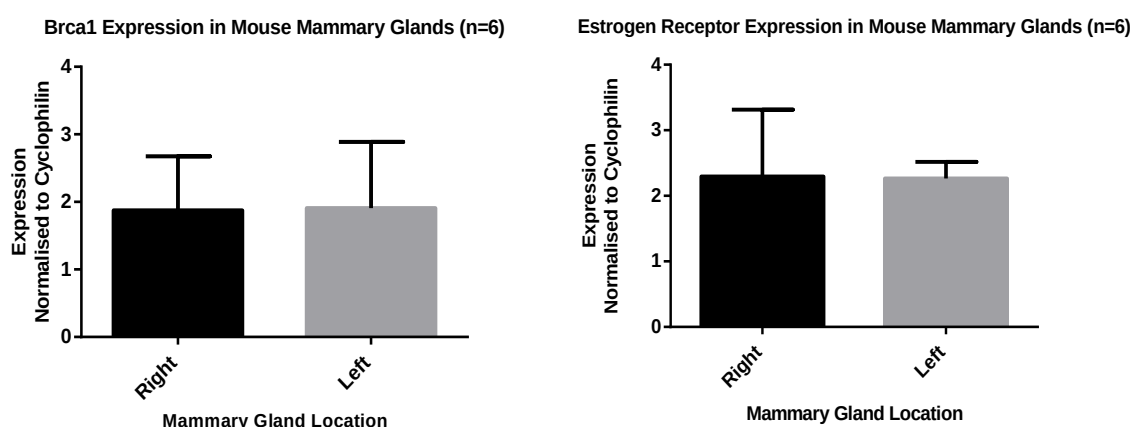


Figure 2.11. qRT-PCR Gene Expression Analysis of *Brca1* and *ER1* in Female C57BL/6 Mouse Mammary Glands.

Brca1 and *ER1* gene expression was not significantly different between mammary glands (n=5) located left or mammary glands (n=5) located right of the midline in PND 49-56 (7 week) female C57BL/6 mice (n=6) fed a normal RM1 diet.

Table 2.9 Reagent/Chemical/Material for Analysis of Protein Expression from Folic Acid Treated C57BL/6 Mice.

Reagent/Chemical/Material	Supplier
Methanol	Fisher, UK
Ethanol 100%	Fisher, Loughborough, UK
Nonidet (NP) 40	Sigma-Aldrich, Dorset, UK
Ponceu Red	Sigma-Aldrich, Dorset, UK
Dried Skimmed Milk Powder (Marvel)	Premier Foods, UK
Amonium Persulphate (APS)	Sigma-Aldrich, Dorset, UK
Peirce BCA Protein Assay	Thermo Fisher Scientific, UK
Comassie Brilliant Blue	GE Healthcare Bucks, UK
Tris Base	Fisher, Loughborough, UK
Tween 20	Sigma-Aldrich, Dorset, UK
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Dorset, UK
Protease Inhibitor Cocktail	Sigma-Aldrich, Dorset, UK
Ponceau S Solution	Sigma-Aldrich, Dorset, UK
Prism Ultra Protein Ladder (10 kDa – 245 kDa)	Abcam, UK
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, Dorset, UK
Glycerol	Sigma-Aldrich, Dorset, UK
Glycine	Sigma-Aldrich, Dorset, UK
Bromophenol Blue	Sigma-Aldrich, Dorset, UK
3mm Filter Paper	Whatman, UK
PVDF Membrane	Millipore, UK
Phosphate Buffered Saline	Sigma-Aldrich, Dorset, UK
Dithiothreitol (DTT)	Sigma-Aldrich, Dorset, UK
Acrylamide	Sigma-Aldrich, Dorset, UK
Sodium Deoxycholate	Sigma-Aldrich, Dorset, UK
Supersignal West Femto	Thermo Fisher Scientific, UK
Versadoc MP 5000	BIO-RAD Berkely, CA
Veriti Thermal Cycler	Thermo Fisher Scientific, UK
Phosphate Buffered Saline	Sigma-Aldrich, Dorset, UK
Mini Protein Electrophoresis Cell	BIO-RAD Berkely, UK
HeLa Cell Extract	Active Motif, UK

2.4 Analysis of Protein Expression

Protein expression levels were assessed in mammary tissue in which folic acid treatment induced significant changes in mRNA expression or were associated with pathways linked to differential gene expression.

2.4.1 Measurement of Protein Expression by Western Blotting

2.4.2 Tissue Processing, Protein Extraction and Optimisation

Initial experiments were conducted to ascertain the quantity of mammary tissue that would yield an appropriate level of protein lysate for subsequent western blot analysis. Mammary glands (2nd -3rd thoracic) were divided into 10, 20 or 30 mg volumes, crushed in liquid nitrogen and protein lysates were prepared in RIPA buffer containing 50mM Tris (pH8), 150mM NaCl, 0.5% sodium deoxycholate and 1% nonidet-P40 with 1% Protease Inhibitor Cocktail (Sigma, UK). Crushed mammary tissue was homogenised in RIPA buffer (BioVortexer laboratory tube homogeniser) for 5 minutes, vortexed for 1 minute to aid tissue lysis, placed on ice for 10 minutes and centrifuged (10,000 rpm) for 10 minutes at 4°C. The supernatant was removed, aliquoted (20 µl) and stored at -80°C.

2.4.3 Determination of Total Protein Concentrations

Total protein lysate concentrations were determined using a Pierce BCA Colorimetric Protein Quantitation Assay (Thermo Scientific, UK). Diluted albumin (BSA) standards were prepared using a serial dilution from final BSA standard concentrations (0 – 2 µg/µl). 1 µl of mammary protein lysate added to 9µl of RNA/DNA free H₂O, or 10 µl of protein lysate was added undiluted to 200µl of BCA working reagent, mixed and incubated at 37 °C for 30 mins in the dark with gentle agitation. Samples were allowed to cool to room temperature and the absorbance at 540 nm measured on an ELX800 Universal Micro Plate Reader (Biotek Instruments INC, USA). All samples were measured in triplicate. Total protein concentration was determined by extrapolating sample values from a BSA protein standard curve (Thermo Scientific, UK) (Table 2.10) (Figure 2.12). Optimisation of this protocol showed that the addition of 1 µl of protein lysate in comparison to 10 µl was sufficient to generate a signal that could be accurately

extrapolated from a BSA standard curve, thus, minimising the volume of protein lysate that was required for BCA quantification and increasing the amount available for use in western blotting. Furthermore, 30 mg of mammary tissue was deemed optimal to yield sufficient levels of protein lysate for further downstream analysis. All samples were therefore prepared using 30 mg of mammary tissue and using 1 μ l (in triplicate) of protein lysate for total protein quantification.

Table 2.10 BSA Standards and Absorbances for Protein Standard Curve Analysis.

BSA standards were serially diluted from a BSA stock solution. Absorbance (540 nm) values (in triplicate) were calculated and normalised to a blank reading (0 μ g/ μ l BSA).

BSA μ g/ μ l	Volume of Assay (μ l)	Total BSA (μ g)	Abs 1	Abs 2	Abs 3	Abs Mean	Abs Mean - Blank
2	10	20	0.918	0.888	0.845	0.884	0.819
1.5	10	15	0.664	0.724	0.688	0.692	0.628
1	10	10	0.509	0.485	0.503	0.499	0.435
0.75	10	7.5	0.375	0.384	0.365	0.375	0.310
0.5	10	5	0.281	0.245	0.259	0.262	0.197
0.25	10	2.5	0.166	0.161	0.159	0.162	0.098
0.125	10	1.25	0.118	0.117	0.117	0.117	0.053
0.025	10	0.25	0.074	0.074	0.073	0.074	0.009
0	10	0.25	0.064	0.066	0.063	0.064	0.000

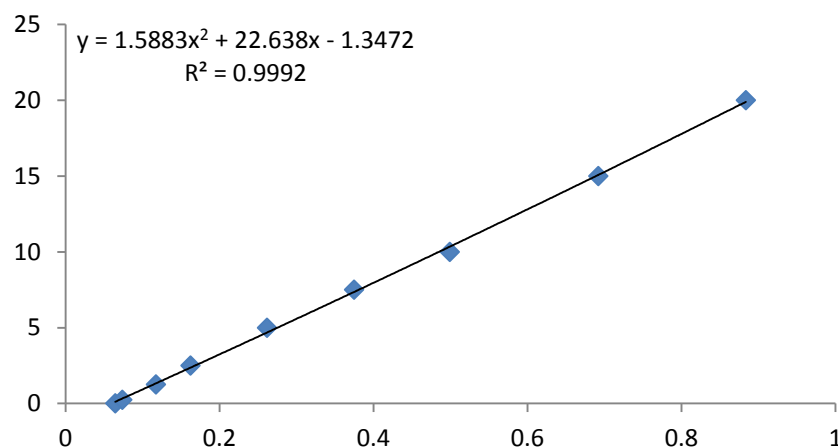


Figure 2.12 Standard Curve Used to Determine Total Protein Concentration of Mammary Gland Samples.

Standards were serially diluted from a BSA stock solution along with mammary gland samples and absorbance (540 nm) was analysed on an ELX800 Universal Micro Plate Reader (Biotek Instruments INC, USA). Standards were plotted as a polynomial type 2 correlation between absorbance (540 nm) and total BSA (µg) allowing total protein concentrations of mammary samples to be extrapolated from the standard curve.

2.4.4 SDS Page Gel Electrophoresis

Western blot analysis of total protein levels was performed using an SDS PAGE polyacrylamide gel (10% or 15%) combined with gel electrophoresis. 25 µg of mammary protein lysate was added to an equal volume of loading buffer containing 125 mM Tris (PH 6.8), 200 mM DTT, 4% SDS, 20% Glycerol and 0.2% bromophenol blue. Samples were loaded into 500 µl PCR tubes, heated to 95°C for 5 minutes in a PCR thermal cycler (Applied Biosystems, UK), cooled to 4°C and placed on ice. Denatured protein lysate containing an equal volume of loading buffer was resolved by SDS PAGE using a 10% or 15% polyacrylamide resolving gel and 5% polyacrylamide stacking gel (Table 2.11). Briefly, a resolving gel solution was made and pipetted between assembled glass mini gel plates (Bio Rad, UK) to ~ 5 mm below a gel comb line, 500 µl of 100% isopropanol added and the gel left to set (~20 mins). Once the resolving gel was set, the isopropanol was removed and the top of the gel was washed with ddH₂O. Finally a stacking gel solution (5%) (Table 2.11) was added on top of the resolving gel and a gel comb inserted. Once dry, gels were immediately placed into a mini gel assembly unit (Bio Rad, UK) and submerged in

1 x running buffer diluted 1/10 from a 10 x running buffer stock containing 4% Tris Base, 14.4 % Glycine and 1% SDS.

Table 2.11. Components of SDS Polyacrylamide Resolving and Stacking Gels for Western Blot
Buffer B; 187g Tris Base (1.5 M), 4.0g SDS (0.4 %), ddH₂O to 1 litre, pH 8 with HCL. Buffer C; 60.5g Tris Base (0.5 M), 4.0 g SDS (0.4 %) ddH₂O to 1 litre. PH 6.8 with HCL

Solution	10% Resolving Gel (ml)	15% Resolving Gel (ml)	Stacking Gel (5 ml)
H ₂ O	5	3.75	3.25
40 % Acrylamide Mix	2.5	3.75	0.5
Buffer B	2.5	2.5	-
Buffer C	-	-	1.25
10 % APS	0.1	0.1	0.05
TEMED	0.01	0.01	0.005

Protein lysate samples (20 or 25 µg), were loaded into the polyacrylamide gel and run at 80 volts until the dye front had run through the stacking gel. The voltage was subsequently increased to 160 volts until the dye front reached the bottom of the gel. 10 µl of 10-245 kDa protein ladder (Prism ultra-protein ladder, Abcam, UK) was added to each gel to enable quantification of protein size (kDa). Furthermore, 25 µg of Human Cervix Adenocarcinoma (HeLa) nuclear extract (Active Motif, UK) in 20 mM Hepes (pH 7.9), 100 mM KCL, 1 mM MgCL₂, 20% glycerol, 0.5 mM PMSF and 0.5mM DTT was added to each gel as a positive control (EZH2, H3K27 tri methyl).

2.4.5 Protein Transfer

SDS polyacrylamide gels were removed from the electrophoresis cassette, and separated from the glass gel casts. The stacking gel was cut away and removed along with any remaining dye present at the bottom of the gel. The gel was submerged in 1 x PBS for approximately 5 minutes to remove residual running buffer. A gel sandwich was assembled using two blotting sponges, two 5mm sections of blotting paper (Whatman, UK) and a single section of 0.45 µm pore Polyvinylidene Difluoride (PVDF) membrane, (Immobilon, Millipore, UK). Separated proteins

were transferred to PVDF membrane, preincubated in 100% methanol for 1 minute and washed in 1 x PBS (1 min). Protein transfer was performed in buffer containing 25 mM Tris, 192 mM glycine, 20 % (v/v) methanol and 0.1% (w/v) SDS for 60 mins at 100 V (4°C). The membrane was blocked with 5% (w/v) dried skimmed milk powder (Marvel, UK) in 1 x PBS containing 0.05% (v/v) Tween-20 (Sigma, UK) for 1 hour at room temperature with gentle agitation.

2.4.6 Protein Transfer Efficiency

The efficiency of protein transfers were assessed post transfer using Comassie Blue gel stain (0.25% Comassie Brilliant blue, 50% methanol, 10% acetic acid), or by staining the PVDF membrane with Ponceau S solution (0.1% w/v in 5% acetic acid, Sigma) and by visualisation of the levels of protein bands that were present within the gel, or on the membrane respectively. Comassie Blue stain was added directly to the polyacrylamide gel immediately post transfer and incubated for ~2 hours at room temperature or overnight at 4°C. Excess stain was eluted and the gel de-stained in a solvent solution containing 10% glacial acetic acid (Fisher, UK) with 40% methanol and protein bands within the gel were visualised by eye. Efficient protein transfer to PVDF membrane was achieved when no protein was observed within the gel. PVDF membranes were also stained with Ponceau S (Sigma, UK) to detect protein bands that had successfully transferred to the membrane. All protocols were optimised using both methods to ensure protein transfer from gel to membrane was optimally efficient prior to running test samples.

2.4.7 Protein Detection Using Antibodies

Blocked (milk/PBS/Tween) PVDF membranes (60 mins) were cut into sections that corresponded to the correct sizes for interrogated proteins. Membrane sections were incubated overnight at 4°C with primary antibodies specific to proteins of interest in 3 ml of 5% (w/v) skimmed milk powder containing 1 x PBS and 0.05% Tween-20. Membranes were washed four times for 8 minutes in PBS/0.05% Tween-20 and incubated in horseradish peroxidase-conjugated secondary antibodies at optimised concentrations in 5% (w/v) skimmed milk powder containing PBS and 0.05% Tween-20 for 1 hour at room temperature with gentle agitation.

Blocked (milk/PBS/Tween) PVDF membranes (60 mins) were cut into sections that corresponded to the correct sizes for interrogated proteins. Membrane sections were incubated overnight at 4°C with primary antibodies specific to proteins of interest in 3 ml of 5% (w/v) skimmed milk powder containing 1 x PBS and 0.05% Tween-20. Membranes were washed four times for 8 minutes in PBS/0.05% Tween-20 and incubated in horseradish peroxidase-conjugated secondary antibodies at optimised concentrations (Table 2.12) in 5% (w/v) skimmed milk powder containing PBS and 0.05% Tween-20 for 1 hour at room temperature with gentle agitation.

Table 2.12 Primary and Secondary Antibodies used for Western Blotting.

Antibody Name (Primary)	Reference	Species Raised in	Working Concentration
EZH2	ABCAM, Polyclonal (AB3748)	Rabbit	1:1000
H3K27 (Tri-methyl)	ABCAM, Polyclonal (AB6002)	Mouse	1:1000
β-actin	ABCAM, Polyclonal (AB8227)	Rabbit	1:1000
GATA3	ABCAM, Polyclonal (AB106625)	Rabbit	1:500
Antibody Name (Secondary)	Reference	Species Raised in	Working Concentration
HRP Conjugated	ABCAM, Polyclonal (AB3748)	Goat	1:1000
HRP Conjugated	Sigma, Polyclonal	Rabbit	1:50,000

2.4.8 Protein Visualisation

Membranes were washed 4 times for 8 min in TBS/0.05% Tween-20 (Sigma, UK) and protein bands detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, UK). 1ml of Luminol/Enhancer Solution was added to an equal volume of Stable Peroxide Solution and mixed. Each section of PVDF membrane was incubated in 500 µl of luminal enhancer/peroxide solution cocktail for a period of 5 minutes. Membranes were blotted dry and visualised on a VersaDoc 4000MP imaging system (Bio-Rad, UK). Protein molecular weights were determined using a Prism Ultra Protein Ladder (10 – 250kDa) (Abcam, UK) and protein band intensities were analysed using Quantity One software (Bio-Rad, UK) from raw data images. β -actin was used as ubiquitously expressed reference protein to allow normalisation of protein loading (Table 2.13).

2.4.9 Validation of Protein Bands

Specific protein bands were identified based on size (kDa) corresponding to known protein standards loaded onto each gel (Abcam, UK). In cases where blots highlighted more than one band in close proximity to the required size (kDa), it was necessary to confirm which band was specific to the protein of interest and identify possible post transcriptional modifications such as glycosylation or phosphorylation that may alter protein size.

2.4.10 Positive Control (Ezh2, H3K27 tri-methyl)

Mammary tissue lysate samples were run in conjunction with a sample that was known to express the protein of interest at a concentration that was readily quantifiable (positive control sample). HeLa nuclear extract (Active Motif, UK), prepared from a cell culture of the HeLa human epithelial carcinoma cell line was used as a positive control for Ezh2 and H3K27 (tri-methyl) western blots to assess protein band specificity. HeLa nuclear extract (20 or 25 µg) containing lysis buffer (20 mM Hepes (pH 7.5), 350 mM NaCl, 20% glycerol, 1% Igepal, 1 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT) and protease inhibitors were added to each SDS polyacrylamide gel and run in line with mammary samples using the protocol outlined above (2.4.4).

2.4.11 Blocking with Immunizing Peptide

To determine H3K27 (tri-methyl) antibody binding specificity, immunizing peptide blocking experiments were performed. Primary antibody (H3K27 tri- methyl) at a concentration that was optimised to show positive staining (1 µg/ml) was incubated with an immunizing peptide specific to the epitope recognised by the antibody (H3K27 tri - methyl) (ABCAM, UK) (5 µg/ml) for 30 minutes at room temperature. Folic acid treated mammary gland total protein lysate samples (20 µg); previously shown to express specific protein bands of interest were incubated with either primary antibody and peptide (+ peptide) or non-immunised primary antibody (- peptide) and resolved by Western Blotting (see section). Bands that were absent in the + peptide samples and present in the – peptide samples were considered specific for the protein of interest (H3K27 tri-methyl) (Figure 2.13). Immunizing peptide was also run with a reference protein (loading control) for each blot (β-actin) to ensure that protein loading was consistent between samples and also that the peptide did not inhibit a protein that was non-specific to its target.

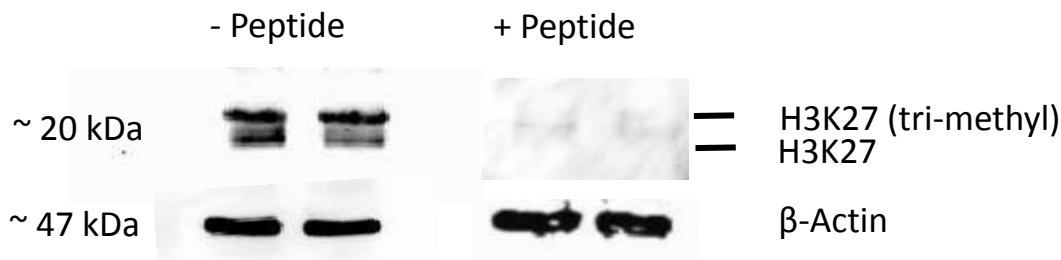


Figure 2.13 Protein Blocking with an Immunizing Peptide against H3K27 (tri-methyl) in Folic Acid Supplemented C57BL/6 Mouse Mammary Glands.

Total protein lysate extracted from mammary glands (2nd and 3rd thoracic) was resolved via Western Blotting against the antigen H3K27 (tri-methyl) in the absence (- peptide) or presence (+ peptide) of an H3K27 (tri-methyl) blocking peptide. Absence of the H3K27 (tri-methyl) band in the presence of blocking peptide confers correct specificity of the antibody validating the blot.

Table 2.13 Reagent/Chemical/Material For Analysis of Protein Expression and Cellular Localisation using Immunohistochemistry in FA Treated C57BL/6 Mouse Mammary Glands.

Reagent/Chemical/Material	Supplier
Ethanol 100%	Fisher, Loughborough, UK
Clearine	Leica, Solms, Germany
Pertex	Leica, Solms, Germany
Citrate Monohydrate	Fisher, Loughborough, UK
ABC Complex's	Vector Laboratories, UK
3'3 Diaminobenzadine (DAB)	Vector Laboratories, UK
Culture Medium	UOS, Southampton, UK
Vectasheild Hardmount with DAPI	Vector Laboratories, UK
Vectasheild Mounting medium	Vector Laboratories, UK
Nail Varnish	Various
Pressure Cooker	Tefal, Windsor, UK
Surgipath X-tra Adhesive Slides	Leica, Solms, Germany
Coverslips	Leica, Solms, Germany
Methanol	Fisher, Loughborough, UK
Hydrogen Peroxide	Fisher, Loughborough, UK
Incubator	Leec, Nottingham, UK
Microtome	Leica, Solms, Germany
Neutral Buffered Formalin	Fisher, Loughborough, UK
Parrafin	Leica, Solms, Germany
Goat Serum	Dako, Cambridgeshire, UK
Triton X 100	Sigma Aldrich, Dorset, UK
Tris	Fisher, Loughborough, UK
Sodium Chloride (NaCl)	Fisher, Loughborough, UK
Hydrochloric Acid (HCL)	Fisher, Loughborough, UK
Sodium Hydroxide (NaOH)	Fisher, Loughborough, UK
Meyers Heamatoxyln	Leica, Solms, Germany
Antibodies	Abcam, UK, Dako, UK

2.5 Protein Visualisation using Immunohistochemistry

2.5.1 Tissue processing and optimisation

Excised mammary glands (2nd and 3rd thoracic) were fixed (24 hours) in 10% neutral buffered formalin (NBF) and embedded in paraffin. Paraffin blocks were cooled on an ice block and mounted onto a microtome (RM225, Leica, UK). Mammary glands were sectioned to 4µm and floated in a water bath (45 °C) prior to being mounted on Surgipath X-tra Adhesive pre-cleaned micro glass slides, 26 x 76 x 1.0 mm (Leica, UK) and dried in an incubator (Leec, UK) at 40 °C for a minimum of 24 hours prior to immunohistochemical staining. A single section of mammary tissue and a section of positive control tissue suitable for the primary antibody (human polyp, tonsil or murine fetus) were included on every slide. All tissue sections were processed using the Avidin Biotin-Peroxidase technique for paraffin sections (Histochemistry Research Unit (HRU), University of Southampton) or immunofluorescence detection. Slide mounted mammary sections were deparaffinised in Clearline (Leica, UK) solution (2 x 5 minutes) and rehydrated through a series of graded ethanols (100%, 90% 70%) for 5 minutes each. Sections were incubated in 0.5% hydrogen peroxide (H₂O₂) with methanol for 10 minutes to inhibit endogenous peroxidase activity and washed (3 x 2 minutes) in tris buffered saline (TBS) solution.

2.5.2 Antigen Retrieval

All mammary tissue sections were subjected to antigen retrieval based on the specification for optimisation of primary antibodies. In the case of the primary antibodies used in the present study, sections were pre-treated using either pressure cooking or microwave based heat mediated antigen retrieval.

2.5.2.1.1 Heat Mediated Pressure Treatment

0.01 M citrate buffer (pH 6.0) was formulated using citric acid monohydrate crystals (4.2 g) (Fisher, UK) in 2000 ml of ddH₂O and adjusted to pH 6.0 with 1 M sodium hydroxide (~25 ml). Citrate buffer (2000 ml) was added to a pressure cooker chamber (Tefal, UK) and heated to 100°C. Paraffin-cleared, rehydrated mammary sections were placed in stainless steel racks (5 mm spacers), submerged fully in boiling citrate buffer and heated to 13lb of pressure for 2

minutes. Pre-treated tissue sections were cooled in a bath of cold water and washed (2 x 5 mins) in TBS.

2.5.2.1.2 Heat Mediated Microwave Treatment

0.01M citrate buffer (pH 6.0) was formulated using citric acid monohydrate crystals (2.1 g) (Fisher, UK) in 1000 ml of ddH₂O and adjusted to pH 6.0 with 1 M sodium hydroxide (~ 25 ml). Citrate buffer (1000 ml) was equally divided (~333.3 ml) between three plastic containers. Paraffin cleared rehydrated mammary sections were added to three plastic slide racks (24 slides per rack) and any spaces filled with blank slides. Sections were submerged in citrate buffer (1 rack per container) and heated by microwave on 50% power for 25 minutes. Pre-treated tissue sections were cooled in a bath of cold running tap water and washed (2 x 5 mins) in TBS. All sections were then then run through the Avidin Biotin-Peroxidase technique for paraffin sections (HRU) or used for immunofluorescence staining protocols.

2.5.3 Antibodies

All antibodies for immunohistochemistry were purchased from ABCAM, (UK) or DAKO (Agilent Technologies, UK) (Table 2.14). Antibodies were titrated in mammary and control tissue at a range of concentrations based on antibody specifications and current literature, to establish antibody efficiency, appropriate antigen retrieval, optimal functional concentration and reduce non-specific binding.

2.5.4 Avidin Biotin Peroxidase/DAB Treatment

Mammary sections were drained and incubated in Avidin Solution (Vector Laboratories, CA) (20 minutes), washed (3 x 2 minutes) in TBS, incubated in Biotin Solution (Vector Laboratories, CA) (20 minutes) and washed (3 x 2 minutes) in TBS. Sections were incubated in blocking culture medium containing 80% Dulbecco's Modified Eagles Medium (DMEM) (Sigma), 20% Fetal Calf Serum (PAA), 1% Bovine Serum Albumin (BSA) for 20 minutes. For immunostaining utilising a secondary antibody raised in goat, 5% Goat Serum (DAKO) was added to the culture media prior

to incubation. Sections were drained and incubated in primary antibodies at appropriate dilutions overnight at 4°C in either TBS or 0.1% Triton X 100 (nuclear staining). The following day, sections were drained, primary antibody was removed (3 x 5 minutes wash in TBS) and secondary horseradish peroxidase (HRP) conjugated secondary antibodies were added at appropriate dilutions (Table 2.14) and incubated at room temperature for 30 minutes. Secondary antibodies were removed (3 x 5 mins wash in TBS) and sections were incubated in ABC complexes (Vector Laboratories, CA) for a further 30 mins. Sections were then incubated with 3'3 Diaminobenzadine (DAB) solution (5 mins), washed and stained in Mayer's Haematoxylin (1 min), prior to being dehydrated through a series of graded ethanols (70%, 90%, 100%) and 100% Clearine solution (3 x 2 mins). Finally, slides were cover-slipped using Pertex (Leica, UK) and visualised (microscope). All mammary sections included a positive control (on the same slide) and were repeated using serial sections on a second slide, with the primary antibody incubation step replaced with TBS (no primary control).

Table 2.14 Primary and Secondary Antibodies Used for Immunohistochemistry

Antibody Name (Primary)	Reference	Species Raised in	Working Concentration
Oct-4	Abcam, Polyclonal (AB19857)	Rabbit	1:250
Cd44	Abcam, Polyclonal (AB119863)	Rabbit	1:100
Ki67	DAKO, Polyclonal	Rabbit	1::400
α SMA	Abcam, Polyclonal (AB5694)	Rabbit	1:400
Brca1	Abcam, Polyclonal (AB191042)	Rabbit	1:1000
Cytokeratin 8 (Phospho S431)	Abcam, Polyclonal (AB59434)	Rabbit	1:50
Gata3	Abcam, monoclonal (AB199428)	Rabbit	1:250
Ezh2	Abcam, Polyclonal (AB3748)	Rabbit	1:100
Antibody Name (Secondary)	Reference	Species Raised in	Working Concentration
HRP Conjugated	Abcam, Polyclonal (205718)	Goat	1:1000
HRP Conjugated	Abcam, Polyclonal (AB6002)	Rabbit	1:50,000
Biotin Pre-absorbed	Abcam, Polyclonal (AB7096)	Rat	1:200
Alexa Fluor 488	Abcam, Polyclonal (AB)	Goat	1:200
Alexa Flour 647	Abcam, Polyclonal (AB)	Rat	1:100

2.5.5 Immunofluorescence

Indirect Immunofluorescence was used as an alternative to DAB to identify protein markers of interest. This method utilised primary antibodies that bind to the antigen of interest, combined with secondary antibodies (indirect method) that recognise the primary antibody and are chemically conjugated to a fluorescent dye. This allows a high level of sensitivity in detection of lowly expressed proteins, dual markers, to be stained in single tissue sections and the identification of markers that co-localise within the mammary gland structure.

Mammary gland sections were mounted onto slides and subjected to appropriate heat mediated antigen retrieval. Sections were blocked in culture medium containing 80% Dulbecco's Modified Eagles Medium (DMEM) (Sigma), 20% Fetal Calf Serum (PAA), 1% Bovine Serum Albumin (BSA) for 20 minutes. For immunostaining utilising a secondary antibody raised in goat. 5% Goat Serum (DAKO) was added to the culture media prior to incubation. Sections were drained and incubated in primary antibodies at appropriate dilutions for 2 hours at room temperature or overnight (~12 hours) at 4°C. For staining of nuclear proteins, primary antibodies were diluted in 0.1% Triton X 100 to aid nuclear membrane permeabilisation. Sections were drained, primary antibody was removed (3 x 5 mins wash in TBS) and secondary fluorescent labelled antibodies were added at appropriate dilutions and incubated at room temperature for 1.5 hours. Secondary antibodies were removed (3 x 5 mins wash in TBS) and slides were rinsed in distilled H₂O and dehydrated through a series of graded ethanols (70%, 90%, 100%) and 100% Clearline solution (3 x 2 mins). Slides were briefly dried and coverslips were added using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualisation (Vectasheild, CA). Sections were visualised on a Zeiss Axio Observer Z1 fluorescent microscope. Fluorescence readings were adjusted by subtracting background from serial no primary (TBS only) control slides, taken from test slide images to account for variations in levels of tissue background auto-fluorescence.

2.5.6 Quantification of Immunoreactive Cells

To establish cellular expression of proteins of interest, it was necessary to quantify the number of immuno-reactive (IR) cells, or the quantity of immuno-positive (IP) staining within mammary epithelial cells in response to FA supplementation.

2.5.6.1 Linear Unmixing

In order to quantify numbers of IR cells within tissue sections stained with DAB (brown) and counterstained with haematoxylin (blue) it was necessary to separate the DAB and haematoxylin and generate an image containing only DAB colour output. Mammary images were loaded into Time Resolved Imaging 2 (TRI2) Software Package (Medical Research Council, Oxford Institute, UK) and processed using the linear unmixing function. Positive DAB staining was manually selected from each image in channel 1 and haematoxylin in channel 2 with the background set as white. Images were unmixed and generated containing only DAB staining patterns (Figure 2.14). Images were processed using image J software.

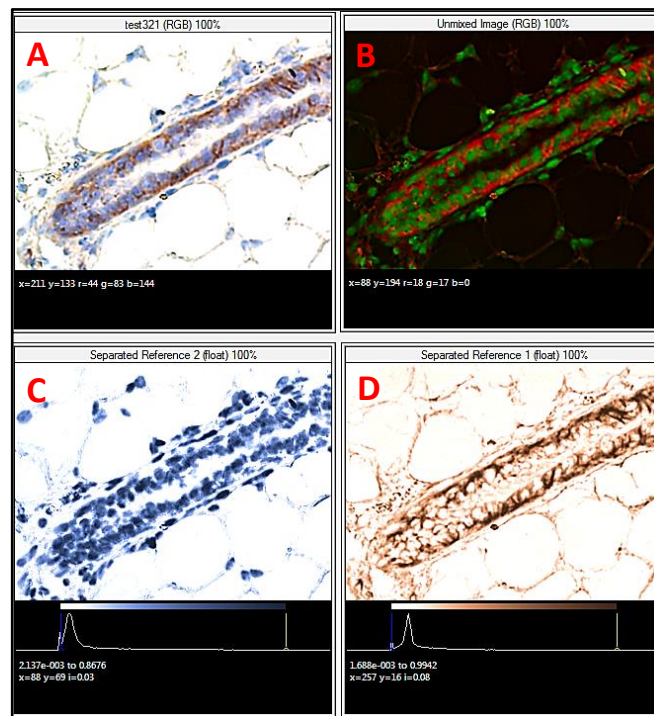


Figure 2.14. Linear Unmixing of DAB Stained Epithelial Mammary Ducts.

(A) Female C57BL/6 mammary duct stained with DAB (Brown) and counterstained with haematoxylin (Blue). (B) Unmixed image with red channel identifying DAB staining pattern and green channel identifying haematoxylin. (C) Linear unmixed separated reference showing only haematoxylin (blue) staining. (D) Linear unmixed separated reference showing only DAB (brown) staining pattern.

1.5.6.2 Immuno-reactive Cell Number (DAB and Fluorescence)

IR cell numbers were calculated from DAB (Figure 2.15) or immunofluorescent (Figure 2.16) stained mammary images using Image J analysis software. Three Images (x 40 magnification) were taken (brightfield) of mammary ducts from individual tissue sections per slide. The area of each duct was measured and calculated by drawing around the epithelial structure and generating an ROI. Individual IR cells were counted per ROI using the Cell Counter plug in function (Image J). IR positive cell numbers were normalised to the area of each mammary structure to calculate the number of IR positive cells per structure. Three ROI's were counted for each sample and an average was recorded). Quantification of IR cells was performed from mammary sections collected from adult (PND 130) FA treated (PND 74-102) C57BL/6 mice.

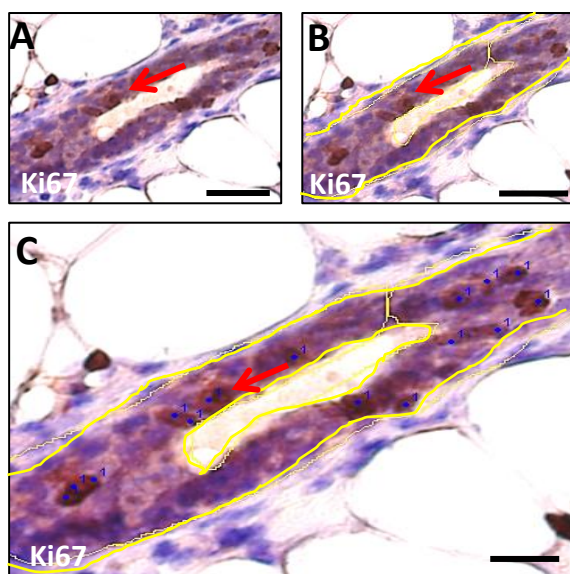


Figure 2.15 Calculation of Immunoreactive (IR) Staining Using DAB in C57BL/6 Mammary Glands.

(A) Ki67 IR staining (brown) in the mammary gland. **(B)** Generated ROI surrounding duct (yellow)
(C) Generated ROI (ROI1) selecting IR positive Ki67 stained cells (blue dots) using cell counter plug in (image J)

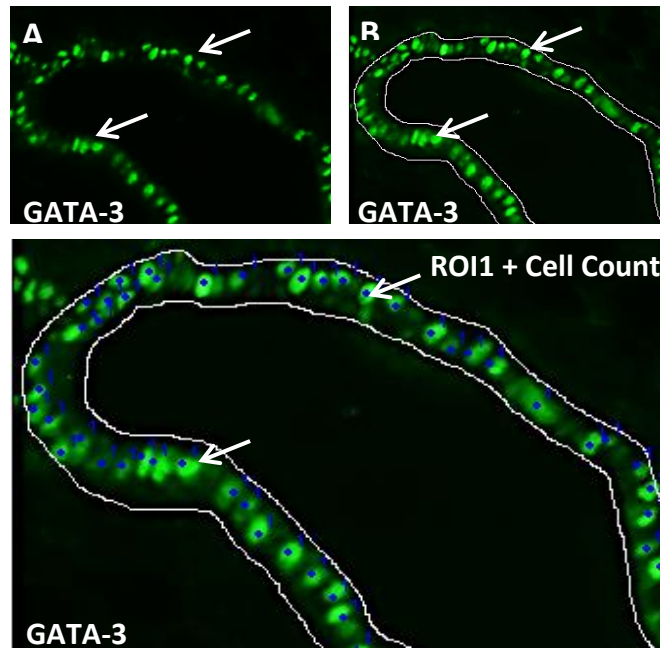


Figure 2.16. Calculation of Immunoreactive (IR) Staining Using Immunofluorescence in C57BL/6 Mammary Glands.

(A) GATA3 IR staining (green) in the mammary gland. **(B)** Generated ROI surrounding duct (yellow) **(C)** Generated ROI (ROI1) selecting IR positive GATA3 stained cells (blue dots) using cell counter plug in (image J).

2.5.6.2 Quantity of Immuno-Reactive Positive Staining (DAB)

Total IR staining (DAB) was calculated in structures where counting individual cells was not possible. Linear unmixed DAB images were generated and quantified using image J software. Individual ROI's were generated by drawing around mammary ducts and copying the selection. The area of the selected ROI was recorded for each structure measured. ROI's were thresholded and converted to 8-Bit images. A mask was generated which correlated to the area of the structure that was IR positive with a DAB staining pattern. The area of the mask for each structure was recorded and divided by the original area of the ROI. This calculated the total area of the staining pattern for each epithelial structure. Measurements were repeated using three different ROI's for each section and an average was taken.

2.5.6.3 Quantity of Immuno-reactive Positive Staining (Fluorescence)

Total IR staining (Alexa Fluor 488) was calculated in mammary epithelial structures. Images (TIF) were taken of individual tissue sections and analysed using image J software (Figure 2.17). To normalise for changes in levels of tissue autofluorescence, images were first acquired from serial no primary (TBS only) control samples run in conjunction with each immunohistochemistry experiment. The background levels of fluorescence were recorded and images adjusted so that background signal was minimal. The same settings were then used when imaging IR positive sections generating images that display fluorescence output above the level of control samples. Total fluorescence of individual structures was calculated by first converting the fluorescence image to 16 BIT binary file and drawing around mammary ducts. The selected ROI was then used to measure the area and integrated density of the image. A separate ROI was generated that selected only background and measured in the same way. Corrected total cellular fluorescence (CTCF) was calculated using the following equation:-

$$\text{CTCF} = \text{Integrated Density} - (\text{area of structure} \times \text{mean fluorescence of background reading})$$

Three readings were recorded for each tissue section and averaged to give overall mean fluorescence as a measure of integrated density. The change in fluorescence was then calculated between FA supplemented groups.

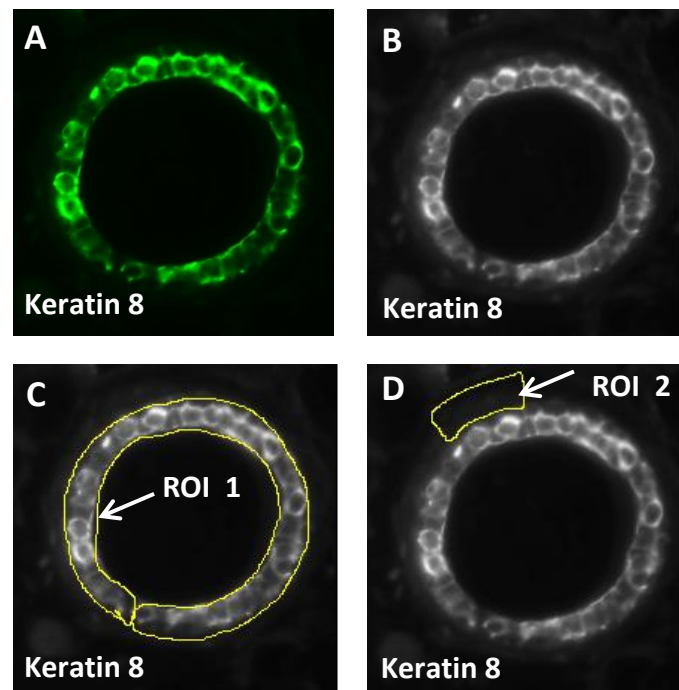


Figure 2.17. Calculation of Total Immuno-reactive Staining using Fluorescence in C57BL/6 Mammary Glands.

(A) Cytokeratin 8 (phospho S431) IR cytoplasmic staining (Alexa 488) in the mammary gland. **(B)** 16 BIT converted image (image J). **(C)** Generated ROI (ROI1) selecting IR positive K8 staining localised in the luminal epithelial cell population. **(D)** Generated ROI (ROI 2) selecting representative background (IR negative) staining.

Table 2.15. Reagent/Chemical/Material For Whole Transcriptome Shotgun Sequencing in Folic Acid Treated C57BL/6 Mouse Mammary Glands.

Reagent/Chemical/Material	Supplier
miRVana RNA isolation Kit	Invitrogen, Paisley, UK
Nanochip 6000	Leica, Solms, Germany
RNA Free H ₂ O	Fisher, Loughborough, UK
DNase 1 Solution	Sigma-Aldrich, Dorset, UK
DNTP's	Promega, Southampton, UK
MMLV Reverse Transcriptase	Promega, Southampton, UK
384 well PCR Plate	Sigma-Aldrich, Dorset, UK
Random Nonomers	Sigma-Aldrich, Dorset, UK
Acid Phenol	Invitrogen, Paisley, UK
RNA/DNase Free H ₂ O	Fisher, Loughborough, UK
Nanodrop Spectrophotometer	Thermo Scientific, UK
Light Cyclor 480	Roche, West Sussex, UK
Bioanalyser 2100	Agilent, Uk
IPA Software	Qiagen, UK

2.6 Whole Transcriptome Shotgun Sequencing of Female Mammary Glands from Folic Acid Supplemented C57BL/6 Mice

Whole Transcriptome Shotgun Sequencing (RNA-seq) analysis was carried out on a select group of samples that were shown to demonstrate differential mRNA expression in genes of interest from qRT-PCR analysis (Chapter 3). This allowed an in-depth snapshot of the whole transcriptome in response to two different concentrations of folic acid supplementation. C57BL/6 mice supplemented with either 1 mg/kg (1 x BDR) or 5 mg/kg (5 x BDR) of folic acid during the juvenile period (PND 28-56) or adulthood (PND 74-102) followed by an AIN-93M maintenance diet (2.1 mg/kg) for 4 weeks (PND 56-84 juvenile) (PND 102– 130 adult) were selected for RNA-seq analysis.

2.6.1 RNA Extraction

A total of 6 samples from each treatment group were randomly selected (n = 6) and RNA was extracted and quantified in line with the methodology outlined in chapter 2.3.1. RNA samples that displayed good Nanodrop ratios (280/230 ratio) and clear, intact 18S and 28S ribosomal subunit bands on an agarose gel (Figure 2.5) were selected for further quality control checks.

2.6.2 Bioanalyser

RNA samples selected for RNA-seq analysis were assessed for sizing, quantitation and quality control using a Bioanalyser 2100 (Agilent Technologies, UK). 1 µl of sample RNA was loaded onto a microfluidic Nanochip 6000 (Agilent Technologies, UK) and run in conjunction with an RNA ladder. Total RNA integrity was assessed using a RIN (RNA Integrity Number) value assigned via 2100 expert software (Agilent Technologies, UK). RIN scores unambiguously assess RNA sample quality in terms of degradation. Total RNA quality was displayed via electrophelogram showing the integrity of both 18S and 28S subunits and levels of degradation present. Samples with a RIN value of greater than 7 were considered high enough quality for RNA-seq analysis (Figure 2.18).

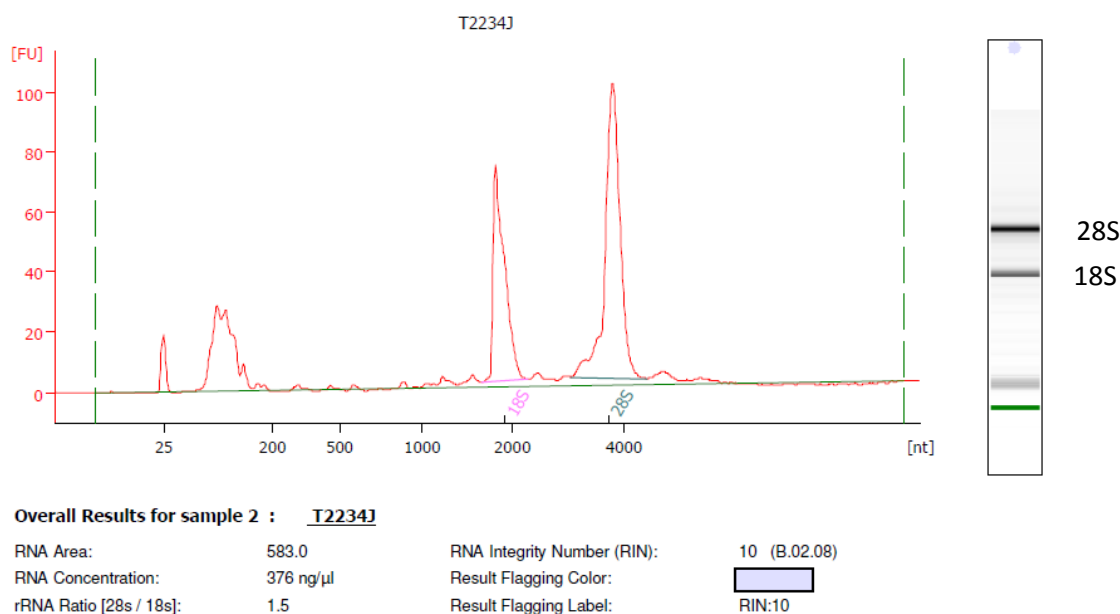


Figure 2.18 Example of Analysis by Agilent 2100 Expert Bioanalyser of a Single RNA Sample Extracted from the Mammary Gland of a Female C57BL/6 Mouse Supplemented with Folic Acid during the Juvenile/Pre-pubertal Period.

1 μl of RNA (376 ng/μl) extracted from mammary gland tissue (50 mg) using a mirVana isolation kit was loaded onto a microfluidic Nanochip 6000 (Agilent Technologies, UK) and run in conjunction with a RNA ladder. 28S and 18S bands were quantified and an RNA integrity number (RIN) score was calculated for each sample (1-10, 1= poor, 10 = high quality). RIN scores >7 were considered high enough quality for next generation sequencing (NGS) platforms. Sample above = RIN of 10.

2.6.3 RNA-seq

RNA-seq analysis was performed by Oxford Gene Technology (OGT, Oxford, UK) or by Oxford Genomics Center (OGC, Oxford, UK). All samples were prepared using the Illumina TruSeq RNA Sample Prep Kit v2 (Illumina, UK). Total RNA was extracted and mRNA captured with oligo-dT coated magnetic beads, fragmented and then randomly primed. First strand cDNA synthesis was initiated from the random primers, followed by second strand synthesis, end-repair, phosphorylation and A-tailing, adapter ligation. PCR amplification was performed to prepare the library for sequencing (Figure 2.19). All sequencing was performed on the Illumina HiSeq2000

platform using TruSeq v3 chemistry (Illumina). Paired end sequencing was performed over 100 cycles with the generation of Read files (Fastq) from the sequencing platform via the manufacturer's proprietary software. Read level QC metrics were provided by FastQC.

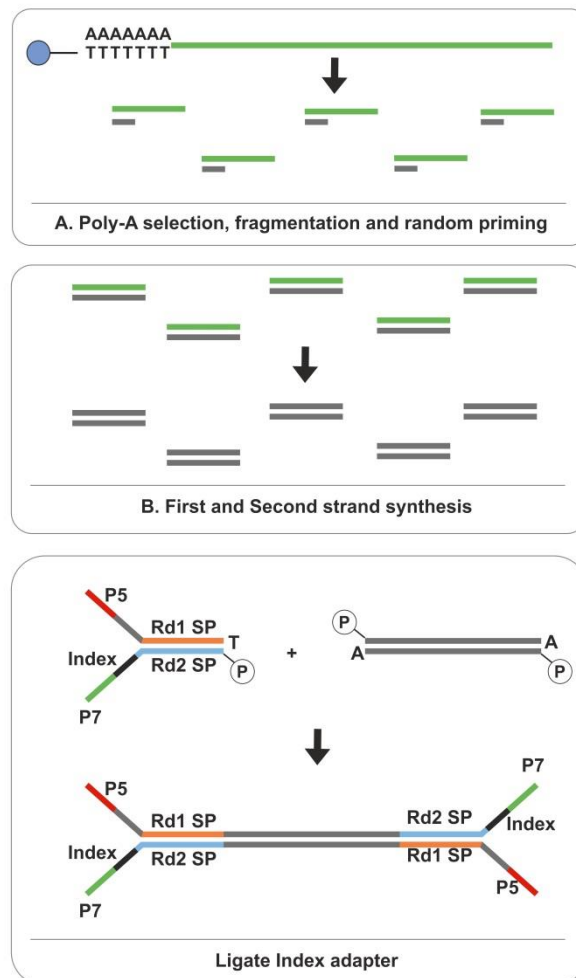


Figure 2.19. Illumina TruSeq RNA Sample Preparation for RNA-sequencing

(A) mRNA is poly-A selected, fragmented and undergoes random priming. **(B)** Selected mRNA then undergoes first and second strand synthesis. cDNA fragments go through end repair via the addition of a single 'A' base to the 3' end and ligation of index adaptors. Products are purified and enriched using PCR to generate a double stranded cDNA library.

All Fastq files were processed in a Linux environment (Version 3.2.1) run on Iridis 4 (University of Southampton super computer), a compute cluster composed of 12320 cores (250 TFlops).

Raw sequencing data (Fastq) files were quality checked using FastQC, version 0.11.4 (Andrews, 2010) to ensure that raw data was accurate and no biases were present that would affect subsequent data analysis. Samples with poor read qualities were removed from analysis. Reads were aligned to the current mouse Mm10 (UCSC) build using Tophat 2.0.14, part of the RNA-seq Tuxedo suite. Aligned BAM files were used to generate count matrices using HTSeq version 0.6.1. In order to differentiate between reads that overlap more than one gene, the union overlap resolution mode in HTSeq count software was utilised. Union restricts reads to those that overlap a single gene, avoiding reads that map to sequences in multiple genes (Figure 2.20).

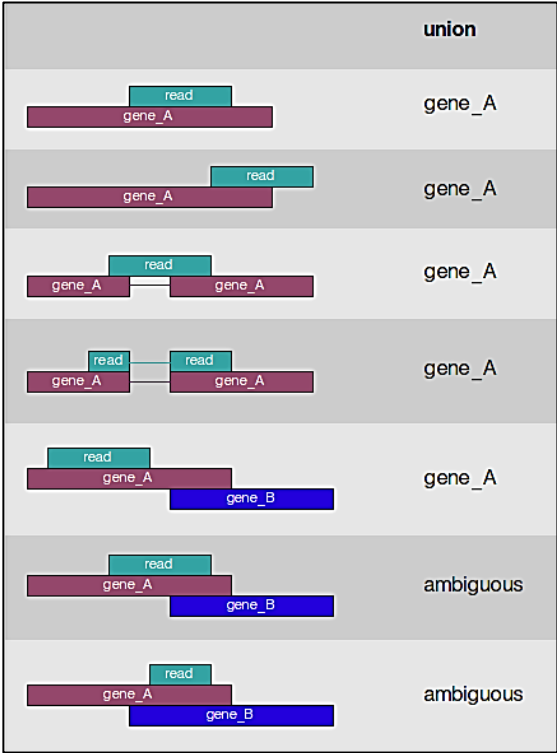


Figure 2.20. HT-Seq Count Script Showing Union Overlap Alignment of Sequencing Reads.

For each position in the read (P), a set $S(p)$ is defined as the set of all genes that overlap position P . If S only contains a single gene then the read is counted, if it contains more than one gene the read is considered ambiguous and excluded.

2.6.3.1 Median Absolute Deviation (MAD)

For each sample, the correlation difference was calculated by taking the average of all pairwise correlations involving the sample (within the same group) and the average of all pairwise correlations that do not include that sample. This vector of values was converted to a MAD score (robust Z-score) by subtracting the medians and dividing this value by the median absolute deviations (MAD) using the following equation:

$$\text{Median Absolute Deviation} = \text{median} (|Y_i - \text{Median Correlation Differences}|)$$

The MAD score was then calculated using the following equation:

$$\text{MAD Score} = (\text{Correlation Difference Sample} - \text{Median Correlation Differences}) / (\text{MAD} * 1.46138189)$$

Sample MAD scores were compared and outliers removed from further analysis. HT-seq files were annotated to ensemble annotations GRCm38.83 (gene location). Principle Component Analysis (PCA) was used to observe correlations between samples from the same and different treatment groups, both before and after MAD outliers were removed (Figure 2.21).

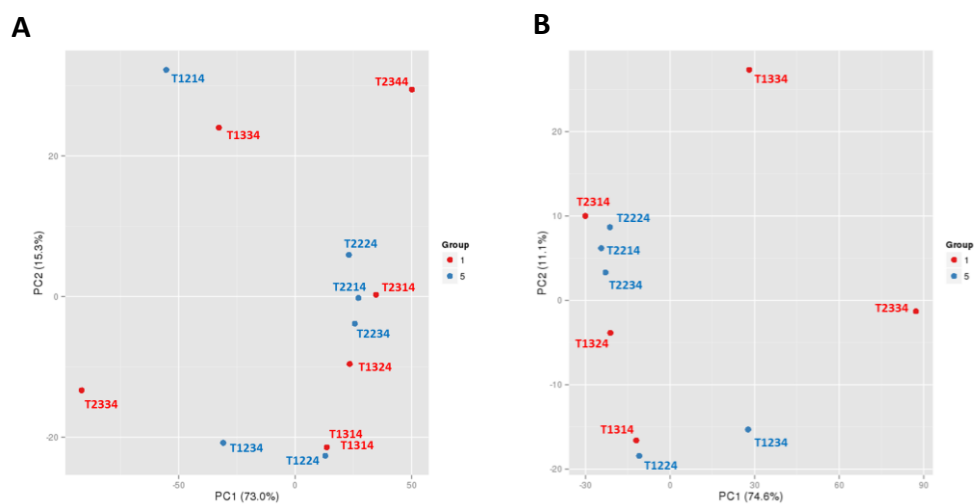


Figure 2.21. PCA Plots for Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Plots show (A) original 12 samples measured and (B) 10 samples used for analysis after MAD calculation and sample adjustment.

2.6.3.2 Differential Gene Expression Analysis

Differential gene expression was calculated using EdgeR (Version 3.10.5). Samples were discarded if they exhibited very low levels of expression over 5 samples (threshold count per million set to 2). A generalised linear model was generated ($\sim$ groups x cage) to account for variation in sample reads between animals from the same groups housed in different cages.

2.6.3.3 Gene Ontology (GO)

Gene ontology (GO) analysis was facilitated by TopGO software R package Version 2.22.0 (Alexa and Rahnenfuhrer, 2010). GO analysis allows known relationships between biological terms and genes that function within these terms to be investigated. This provides detailed information that allows associations between groups of genes that demonstrate similar functional roles. An input of normalised gene expression measurements was used for enrichment analysis of GO into three categories or ontologies identified as cellular components, molecular function or biological process.

2.6.3.4 Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis was performed on sequencing data obtained from RNA-seq analysis using GSEA version 2.2.2 from the Broad Institute, MIT and Harvard (Subramanian et al., 2005, Mootha et al., 2003). GSEA allows the analysis of a pre-defined set of genes that demonstrate statistically significant concordant differences between treatment groups. RNA-seq data was investigated using gene sets from the Molecular Signatures Database (MSigDB) which is explicitly designed for the provision of gene sets specific for enrichment and analysis methods (Liberzon et al., 2011). MSigDB data sets used for enrichment analysis consisted of Hallmark (H) gene sets consisting of expressed signatures derived from the aggregation of MSigDB gene sets to represent well defined biological processes; curated (C1) gene sets acquired from PubMed publications, online pathway databases and domain experts; motif gene sets which are based on cis-regulatory motifs that are conserved and identified by comparative analysis of mouse, human, rat and dog genomes and GO gene sets annotated by GO terms. GSEA was performed using the GSEA pre-rank module Version 1 (Gene pattern Broad Institute) which enables the generation of a pre-validated gene list from RNA-seq data that was ranked based on

fold change and P values. Genes from the MSigDB were matched with genes from the ranked list and enrichment scores (ES) were calculated.

2.6.3.5 Pathway Analysis

Pathway analysis of differential expression was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (National Institute of Allergy and Infectious Diseases (NIAID) and Ingenuity Pathway Analysis (IPA) Qiagen, UK.

2.6.3.6 Validation of RNA-seq using qRT-PCR

RNA-seq differential gene expression was validated using qRT PCR. Two genes that demonstrated increased differential expression, and two genes that demonstrated decreased differential expression, were selected based on significantly different FDR scores. RNA from the same samples sent for RNA-seq analysis was converted to cDNA and qRT-PCR was performed as outlined in Section 2.3 using commercially available primer sets (Qiagen UK). Differential gene expression calculated from RNA-seq data was correlated to changes in expression observed through qRT-PCR to test for concordance between techniques.

2.7 Statistical Analysis

Statistical analysis was performed using SPSS statistics 21.0 (IBM, USA) and Graph Prism6 (CA, USA). Data sets were tested for normal distribution using a Kolmogorov-Smirnov, and Schapiro-Wilk test with the statistical significance limit set to $P < 0.05$. Normal Q-Q plots of expected vs observed values were plotted to assess if samples demonstrated a linear relationship (Figure 2.22). Data sets that were not normally distributed were $\log^{10}$ transformed.

Differences between folic acid treated groups were calculated using a 1 way ANOVA with post hoc Dunnett test, post hoc Tukey's test or an unpaired two tailed t test. Linear regression was also used to asses if there were correlations between data sets. Statistical significance limit was set to $P < 0.05$. All data is presented as mean $\pm$ standard error of the mean (SEM).

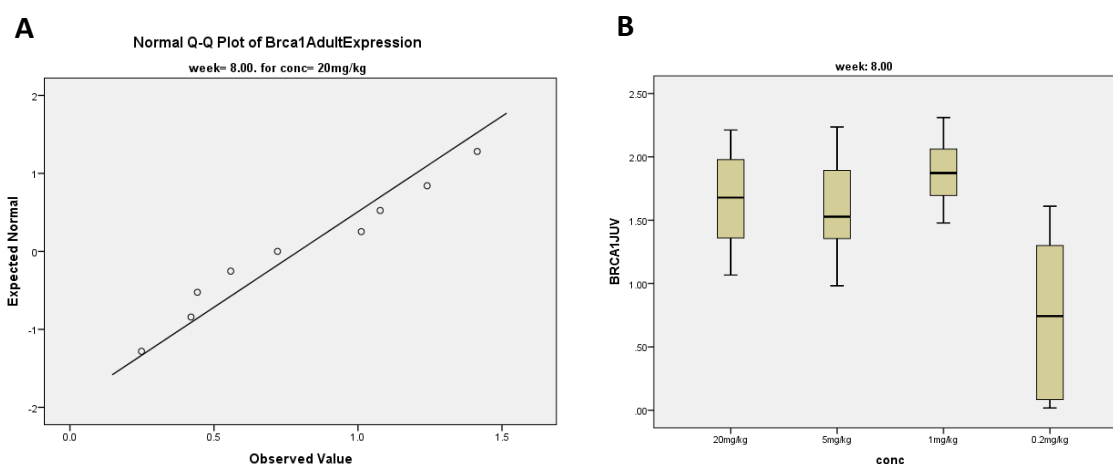


Figure 2.22. Example of a Normal Q-Q Plot and Box Plots of *Brca1* Gene Expression in Mammary Glands of Adult Folic Acid Supplemented C67BL/6 Mice.

(A) Q-Q plot shows data plotted as expected normal vs observed values and shows normal distribution with a linear relationship between grouped samples. **(B)** Box plots show normal distribution between sample sets.

2.7.1 Power Calculations

Sample sizes were calculated using the 1-sample Z-test method from Statistical Solutions (LLC). Retrospective calculations were performed on data collected from different experimental parameters and compared to sample sizes used.

2.7.1.1 5-mTHF

Retrospective sample size calculations for plasma 5-mTHF in juveniles with a mean folate concentration of 104.87 nM (1 mg/kg), expected mean folate concentration (5 mg/kg) and standard deviation (SD) 6.95 (two sided test) show that a sample size of 5 is required to provide statistical power of 80 % with a $p=0.05$. In adults a sample size of 28 is required to provide statistical power of 80%.

2.7.1.2 Gene Expression

Retrospective sample size calculations for relative expression of *Brca1* normalised to *Gapdh* and *Ubc*, 4 weeks after folic acid supplementation with mean expression of 1.88 (1 mg/kg), expected mean expression of 1.58 (5 mg/kg) and SD of 0.378 (two sided test) shows that a sample size of 10 is sufficient to provide statistical power of 70% with a $p=0.05$.

2.7.1.3 Mammary Morphology

Retrospective sample size calculations for mammary morphology (number of epithelial branches) with mean expression of 58.79 (1 mg/kg), expected mean expression of 67.37 (5 mg/kg) and SD of 9.34 (two sided test) shows that a sample size of 8 is sufficient to provide statistical power of 70% with a $p=0.05$.

Chapter 3: The Effects of Folic Acid Supplementation during the Juvenile Period or Adulthood on *Brca1* and *Oct-4* expression in the Female C57BL/6 Mouse Mammary Gland

3.1 Introduction

The role of folate and its synthetic form, folic acid, in cancer development and progression is highly controversial. A number of epidemiological studies demonstrate an inverse relationship (and in some cases dose dependent) between folate status (either as dietary intake of folate or blood folate concentrations) and cancer risk (Kim, 2007). However, more recently an increasing number of studies have linked high dietary folic acid intake with increased breast cancer risk, suggesting a U shaped relationship (Chen et al., 2014). Consistent with this U shaped relationship, current animal studies have shown that either low or high folic acid intake is associated with increased levels of mammary tumorigenesis (Deghan Manshadi et al., 2014). Furthermore, in many of the animal models, effects of folic acid supplementation are dependent not only upon the level of supplementation but also the timing and exposure (Deghan Manshadi et al., 2014). Increased tumorigenesis was observed in mice supplemented with folic acid at doses 5 times BDR and equivalent in humans (1000 µg) to those obtainable through dietary folate combined with folic acid fortified foods or levels recommended during pregnancy (Deghan Manshadi et al., 2014).

The mechanism by which folic acid may influence cancer risk is currently not fully understood. Folate plays a major role in the synthesis of RNA and DNA, with tetrahydrofolate, the biologically active metabolite of folic acid, an essential cofactor in the supply of methyl groups in DNA methylation reactions. DNA methylation patterns are generally established in early life and stably maintained. However, there is now substantial evidence that early life nutritional environment can modify the epigenome, inducing persistent epigenetic and phenotypic changes in the offspring (Sie et al., 2013, Ly et al., 2011). The epigenome is susceptible to changes throughout the life course, however, research has shown that the prenatal and pre-pubertal periods of development are most critical and can shape future disease risk (Hanson and Gluckman, 2014). The mammary gland undergoes profound development and remodelling during puberty until adulthood where morphological changes slow down and respond to cyclic hormonal cues (Macias and Hinck, 2012). Dramatic remodelling further occurs during pregnancy, followed by a period of involution (Macias and Hinck, 2012). With increased levels of dietary folic acid intake over the past decade due to food fortification in a number of countries, increased use of supplements containing folic acid and recommendations for peri-conceptional folic acid intake, it is likely that individuals at all stages of the life course are exposed to elevated levels of folic acid during key stages of mammary development.

Folic acid may influence cancer risk through epigenetic mechanisms which induce persistent changes in levels of gene expression. Preliminary studies in our research group have shown that folic acid supplementation during the juvenile period leads to persistent changes in the expression and methylation of *Brca1* and *Oct-4* in muscle and adipose tissue. *BRCA1* is implicated in both inherited and sporadic forms of breast cancer, regulation of DNA repair capacity and has been shown to orchestrate mammary stem cell differentiation. Overexpression of the pluripotency gene *Oct-4* has also been linked to breast cancer progression and thus, variations in folic acid intake or folate status particularly during early life, a period of increased plasticity, may act through the altered epigenetic regulation of genes such as *Brca1* and *Oct-4* in the mammary gland to modify long term cancer risk. Thus, the aim of the current chapter is to investigate the effect of different levels of folic acid supplementation during the juvenile period or during adulthood on the long term expression of both *Brca1* and *Oct-4* in the female mouse mammary gland.

Currently, little is known about the tissue-specific physiological consequences of high levels of folic acid intake on the expression of genes related to breast carcinogenesis. Furthermore, little is known about how the specific function, concentration ranges and timing of folic acid supplementation may contribute to breast cancer risk. To address these questions initial measurements were performed to assess the effects of varying concentrations of dietary folic acid intake on body weight and energy metabolism at age specific time points throughout dietary intervention as well as the levels of plasma 5 methyl tetrahydrofolate (5-meTHF). Research by Green et al., (2011) suggests that measurement of folate in the blood plasma compartment is a reliable indicator of dietary folate intake (Green, 2011). This was further necessary to calculate folic acid intake between supplemented groups, to ensure mice consumed the modified AI93M diets, metabolised dietary folic acid and maintained normal growth patterns. The effect of folic acid supplementation on the expression of key genes involved in mammary stem cell regulation and differentiation which are implicated in cancer risk within the mammary glands of female C57BL/6 mice supplemented with different folic acid diets during either the juvenile period or adulthood was then investigated. This will inform on the effect of both the level and timing of dietary folic acid supplementation on the expression of key genes implicated in breast cancer risk.

3.2 Aims

1. To determine if folic acid supplementation at high (5 mg/kg) or supra-physiological (20 mg/kg) levels for 28 days during either the juvenile period or adulthood induces persistent changes in the levels of *Brca1*, *Oct-4*, *Ezh2*, *Bmi1* and *Suz12*.
2. To determine whether folic acid supplementation affects the expression of polycomb genes *Ezh2*, *Suz12* and *Bmi1*, which along with *Brca1* and *Oct-4* play a key role in mammary stem cells differentiation.
3. To investigate whether changes in *Ezh2* mRNA expression is accompanied by a change in levels of *Ezh2* and levels of H3K27 (tri-methyl) protein expression.

3.3 Methods

Female C57BL/6 mice (10 animals in each experimental dietary group) were fed modified A193M folic acid diets containing basal (1 mg/kg), high (5 mg/kg) or supra-maximal (20 mg/kg) levels for 4 weeks followed by a maintenance diet for either 4 weeks or 12 weeks to examine the persistent effects of folic acid supplementation. These concentrations were selected to reflect the increase in folic acid intake through mandatory dietary fortification within the population and supplementation along with concentrations recommended prior to and during pregnancy to women with a history of neural tube defects, respectively. Body weight (g), food intake (g) and energy intake (kJ per 10 g of mouse) were recorded initially at day 10, followed thereafter at 7 day intervals throughout the duration of both the juvenile and adult folic acid dietary interventions. Mice supplemented with folic acid for 4 weeks only, or for 4 weeks followed by a 4 week maintenance diet were fasted for 6 hours (7.30 – 1.30) to minimise diurnal changes, culled and blood was removed via cardiac puncture and analysed for levels of plasma 5-meTHF by Bevitall (Bergen, Norway) using the *Lactobacillus Casei* microbiological assay (Horne and Patterson, 1988). Plasma estradiol-17 β and progesterone were quantified using hormone specific ELISA analysis (Enzo Lifesciences, SWE). qRT-PCR was used to measure levels of gene expression in the mammary glands of female virgin C57BL/6 mice in response to folic acid supplementation during either the juvenile period or adulthood. Total RNA was extracted from the right thoracic mammary glands and was reverse transcribed to cDNA. qRT-PCR was performed and mRNA expression was normalised to two reference genes selected using gNorm analysis (Primer Design, UK). Statistical analysis of weight and energy intake levels was compared using a 1 way ANOVA with Tukey's post hoc test. Statistical analysis of plasma folate and gene expression levels was compared using 1 way ANOVA with Dunnett post hoc test or independent t test with the P value set to $P < 0.05$.

3.4 Results

3.4.1 Weight and Energy Intake Measurements

To ensure mice from the present study consumed the modified AI93M diets and maintained normal growth and feeding patterns, body weights, food intake and energy intakes were recorded weekly throughout the duration of the dietary intervention study.

3.4.1.1 Juvenile Supplemented Mouse Weights

Juvenile weights (g) were measured from PND 28 to PND 129 (Figure 3.1). Weight rapidly increased in all groups (1 mg/kg = 4.905g, 5 mg/kg = 5.732g, 20 mg/kg = 5.269g) between day 28 (weaning onto folic acid modified diets) and day 38 (Figure 3.1 A). Weight steadily increased from day 38 to day 129 (Figure 3.1 B). No significant differences in weight were identified between folic acid supplemented groups at each time point apart from at day 45 (1 mg/kg and 5 mg/kg) and day 129 (1 mg/kg and 5 mg/kg).

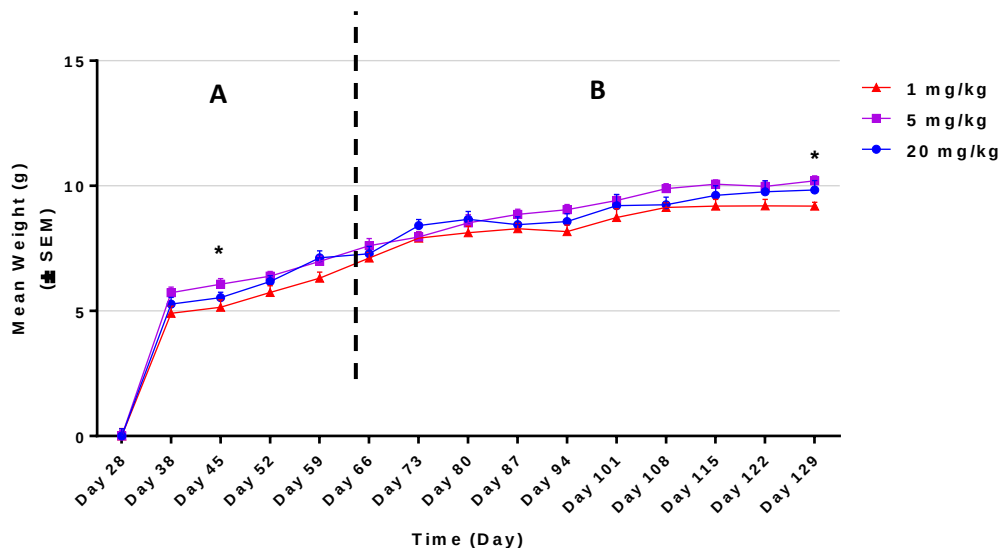


Figure 3.1 Mean Weights of Mice Supplemented during the Juvenile period.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5 mg/kg or 20 mg/kg folic acid from PND 28 to PND 56 (A). Diets were modified to AI93M maintenance diets (2.1 mg/kg folic acid) from PND 56 to PND 140. (B) Weights were recorded from PND 28 to 129. Data was baseline adjusted and displayed as mean weight (g) $\pm$ standard error of the mean (SEM). * $P < 0.05$.

3.4.1.2 Juvenile Supplementation Energy Intakes

Juvenile energy intakes (kJ per 10g of mouse) were measured from PND 28 to PND 129 (Figure 3.2). Energy intakes rapidly decreased in all groups (1 mg/kg = 6.933 kJ, 5 mg/kg = 10.073 kJ, 20 mg/kg = 9.030 kJ) between day 28 (weaning onto folic acid modified diets) and day 38, before stabilising at day 45. Significant differences were observed at day 38 (1 mg/kg and 5 mg/kg, 1 mg/kg and 20 mg/kg), and day 59 (1 mg/kg and 5 mg/kg folic acid concentrations (Figure 3.2, **B**).

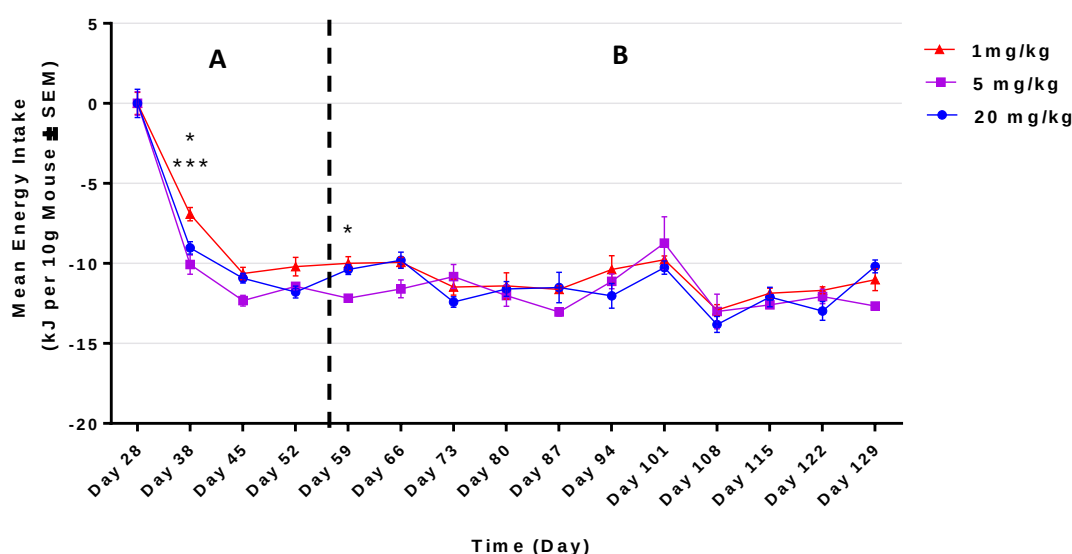


Figure 3.2. Mean Energy Intakes of Mice Supplemented during the Juvenile Period.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5mg/kg or 20 mg/kg of folic acid from PND 28 to PND 56 (**A**). Diets were modified to A193M maintenance diets (2.1 mg/kg folic acid) from PND 56 to PND 140 (**B**). Energy intakes were recorded from PND 28 to 129. Energy intakes were baseline adjusted and displayed as mean energy intake (kJ per 10 g of mouse) $\pm$ standard error of the mean (SEM).

3.4.1.3 Adulthood Supplemented Mouse Weights

Adult weights were measured from PND 74 to PND 181 (Figure 3.3). Weight increased in all groups from day 74 to day 102 (modified folic acid diets, Figure 3.3 A) and day 102 to day 181 (maintenance diet, Figure 3.3 B). No significant differences were observed between groups (folic acid concentrations) at each time point.

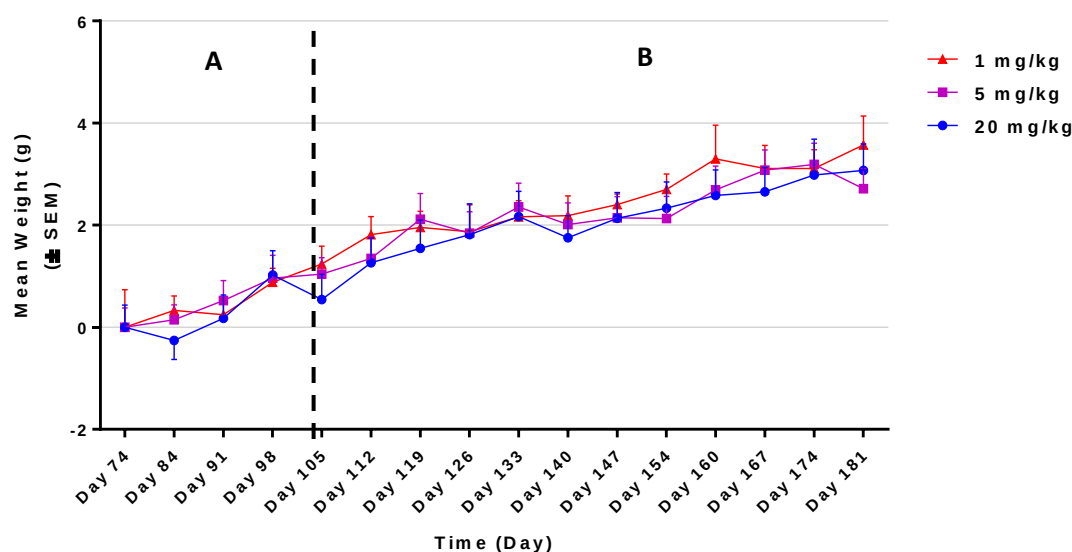


Figure 3.3. Mean Weights of Mice Supplemented during Adulthood.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5mg/kg or 20 mg/kg of folic acid from PND 28 to PND 56. Diets were modified to A193M maintenance diets (2.1 mg/kg folic acid) from PND 102 to PND 181. Weights were recorded from PND 74 to 181. Weights were baseline adjusted and displayed as mean weight (g) $\pm$ standard error of the mean (SEM).

3.4.1.4 Adulthood Supplementation Energy Intakes

Adult energy intakes (kJ per 10g of mouse) were measured from PND 74 to PND 181 (Figure 3.4). Energy intakes showed initial variance between groups during modified folic acid dietary supplementation (Figure 3.4, PND 74 to 102, A). Variance between groups was maintained from day 102 (start of maintenance diet, Figure 3.4, B) to day 181. Statistical significance (2 way ANOVA, $P < 0.05$, with post hoc Tukey's Test) between 1 mg/kg and 5 mg/kg folic acid was observed at day 91, 98, 140, 154, 160 and 167. Statistical significance was observed between 1 mg/kg and 20 mg/kg folic acid at all time points apart from day 98, 91 and 74 (Figure 3.4).

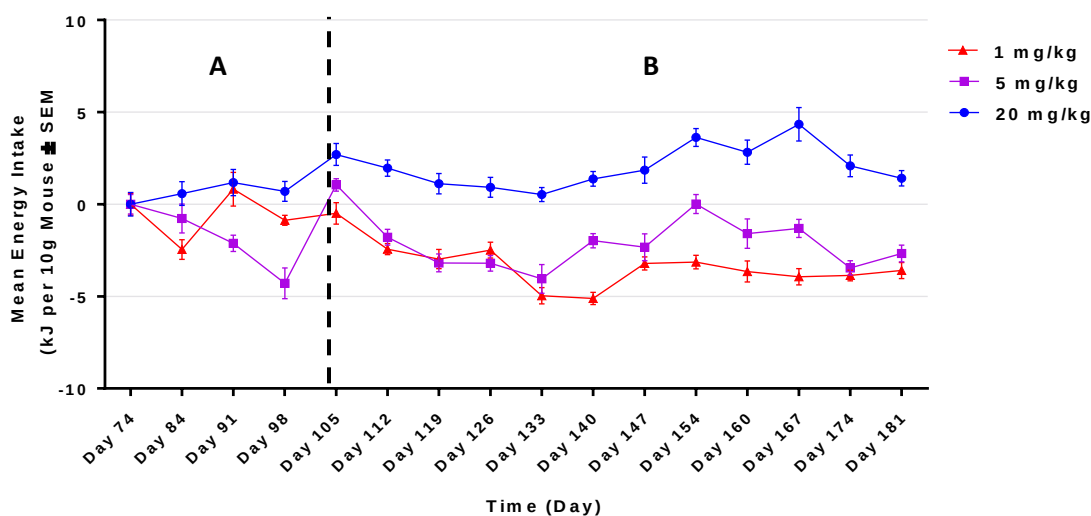


Figure 3.4 Mean Energy Intakes of Adult C57BL/6 Mice.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5mg/kg or 20 mg/kg of folic acid from PND 28 to PND 56 (A). Diets were modified to A193M maintenance diets (2.1 mg/kg folic acid) from PND 56 to PND 140 (B). Energy intakes were recorded from PND 28 to 129. Energy intakes were baseline adjusted and displayed as mean energy intake (kJ per 10 g of Mouse) $\pm$ standard error of the mean (SEM).

3.4.1.5 Calculation of Dietary Folic Acid Intake

Based on findings showing changes in adult energy intake in folic acid supplemented mice, levels of folic acid intake were calculated directly in both juvenile and adult supplemented mice to establish if animals consumed different dietary amounts. Folic acid intake (μ g of folic acid per mouse) calculated using levels of food consumed, showed a dose response increase between groups (Table 3.1 A and C) which levelled out once mice were transferred to a 2.1 mg/kg maintenance diet after the folic acid dietary interventions (Table 3.1 B and D).

Table 3.1 Example of Average Food Consumption and Estimated Total Folic Acid Intake in Mice Supplemented with Folic Acid during the Juvenile Period or Adulthood.

Folic acid intake was calculated in mice fed 1 mg/kg (control), 2.1 mg/kg (maintenance diet), 5 mg/kg or 20 mg/kg at (A) end of the juvenile supplementation period (PND 56); (B) 4 weeks after the juvenile supplementation period (PND 84); (C) end of the adult supplementation period (PND 102); (D) 4 weeks after the adult supplementation period (PND 130).

	Dietary Folic Acid Concentration		
	1 mg/kg	5 mg/kg	20 mg/kg
A	End of Juvenile Supplementation		
Average food consumed per mouse mg/24 hours	2.39 (SD:0.16)	2.49 (SD:0.00)	2.34 (SD: 0.09)
Total folic acid intake per mouse (µg of FA per Mouse)	2.74	12.395	48.24
B	4 weeks after Juvenile Supplementation		
	2.1 mg/kg	2.1mg/kg	2.1 mg/kg
Average food consumed per mouse mg/24 hours	2.472 (SD:0.52)	2.202 (SD: 0.27)	2.215 (SD: 0.14)
Total folic acid intake per mouse (µg of FA per Mouse)	5.19	4.62	4.65
C	End of Adult Supplementation		
	1 mg/kg	5 mg/kg	20 mg/kg
Average food consumed per mouse mg/24 hours	2.66 (SD: 0.01)	2.35 (SD: 0.39)	2.64 (SD: 0.22)
Total folic acid intake per mouse (µg of FA per Mouse)	2.93	11.75	52.86
D	4 weeks after Adult Supplementation		
	2.1 mg/kg	2.1 mg/kg	2.1 mg/kg
Average food consumed per mouse mg/24 hours	2.57 (SD: 0.00)	2.71 (SD: 0.30)	2.73 (SD: 0.15)
Total folic acid intake per mouse (µg of FA per Mouse)	5.39	5.69	5.73

SD = Standard Deviation. Maintenance diet contained 2.1 mg/kg FA for all post supplementation diets

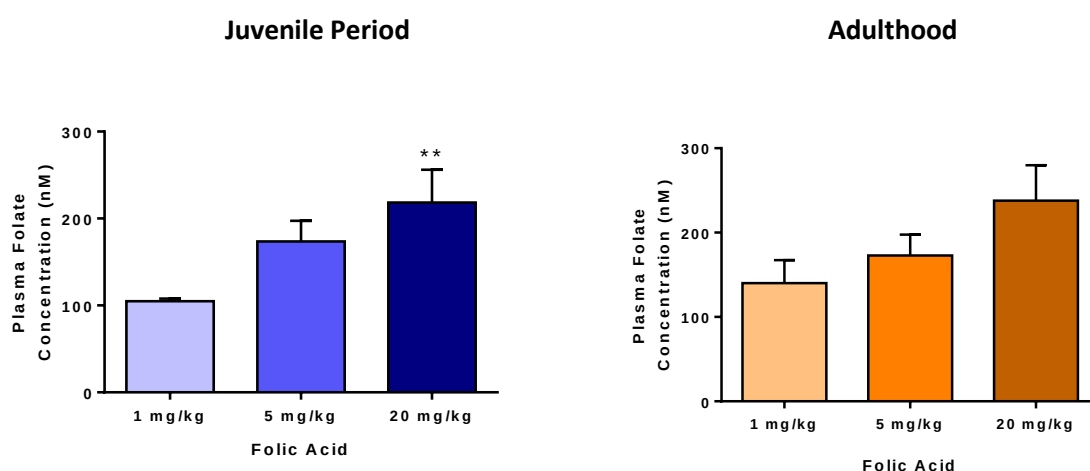
3.4.2 Blood Folate Metabolites

To assess levels of metabolised folic acid, plasma 5-methyl tetrahydrofolate (5-meTHF) levels were measured in the plasma of mice directly after 4 weeks of folic acid supplementation during the juvenile period or adulthood (Table 3.2). 5-meTHF was selected for measurement as opposed to folic acid as this represents the metabolised form of folic acid which demonstrates increased stability and is easier to measure in the small volumes of plasma that were extracted from mice. However, measurement of 5-meTHF in plasma immediately after the end of the supplementation period may not reflect the level of metabolised folate in the mammary gland, long term folate status or measure the level of unmetabolised folic acid.

Average folate concentrations (nM) increased in a dose response manner as levels of folic acid supplementation increased (Table 3.2) 5-meTHF concentrations were significantly different in mice fed a supramaximal folic acid diet during the juvenile period but did not reach significance at 5 mg/kg or in mice fed during adulthood (Figure 3.5). Linear regression analysis of folate concentrations showed a significant effect in both juvenile and adult supplemented mice with 37% of change in folate concentration attributed to diet in juveniles ($P = 0.0170$) and 26% in adults ($P = 0.043$), respectively (Figure 3.6).

Table 3.2. 5-meTHF Blood Plasma Concentration in Mice Supplemented with Different Concentrations of Folic Acid for 4 Weeks during the Juvenile Period or Adulthood.

Period of supplementation	Supplemented Folic Acid Concentrations					
	1 mg/kg		5 mg/kg		20 mg/kg	
	Average Blood Plasma Folate Concentration (nM)					
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Juvenile (PND 28-56)	104.8	6.9	173.6	53.3	218.2	84.4
Adulthood (PND 74-102)	140.2	60.5	172.8	60.9	237.9	93.6
Statistical Analysis (1 Way Anova with post-hoc Dunnett test)						
p<0.05, p = 0.0282						
Juvenile 1 mg/kg vs 20 mg/kg						

**Figure 3.5 Plasma Folate Concentrations of Mice Fed Different Levels of Folic Acid during the Juvenile Period or Adulthood.** Data presented as mean $\pm$ SEM. n = 5-6 per group. * p < 0.05.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5mg/kg or 20 mg/kg of folic acid for 4 weeks (PND 28 to PND 56) during the juvenile period (A) or during adulthood (PND 74-102) (B). Plasma folate levels were measured at the end of the supplementation periods (PND 56 for juvenile and PND 102 for adults). Plasma folate concentrations are displayed as Mean $\pm$ SEM. n = 5-6. Statistical significance was observed between 1 mg/kg and 20 mg/kg juvenile supplemented groups ** P < 0.01 (1 way ANOVA with Post Hoc Dunett test).

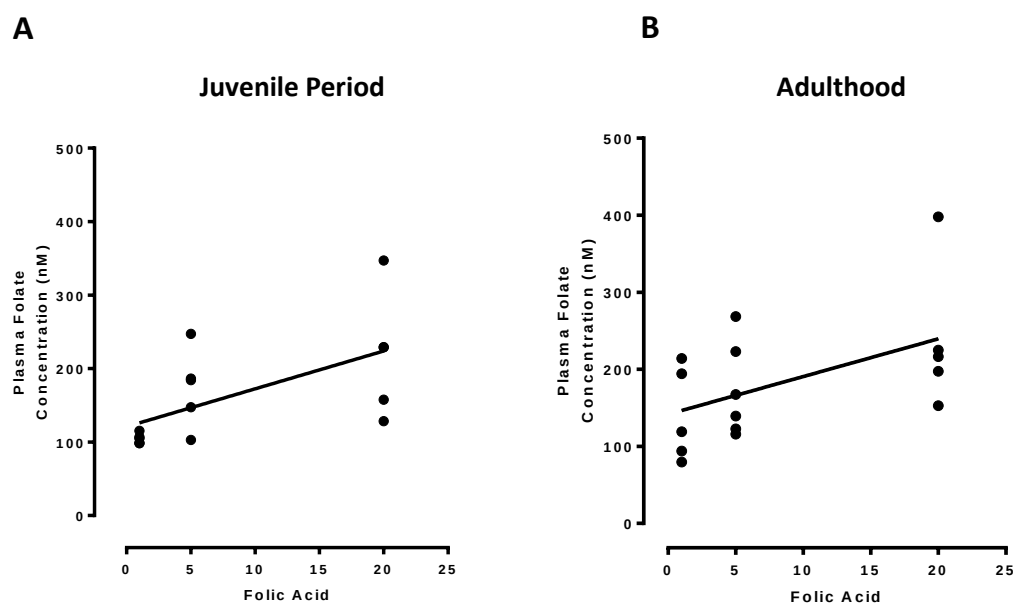
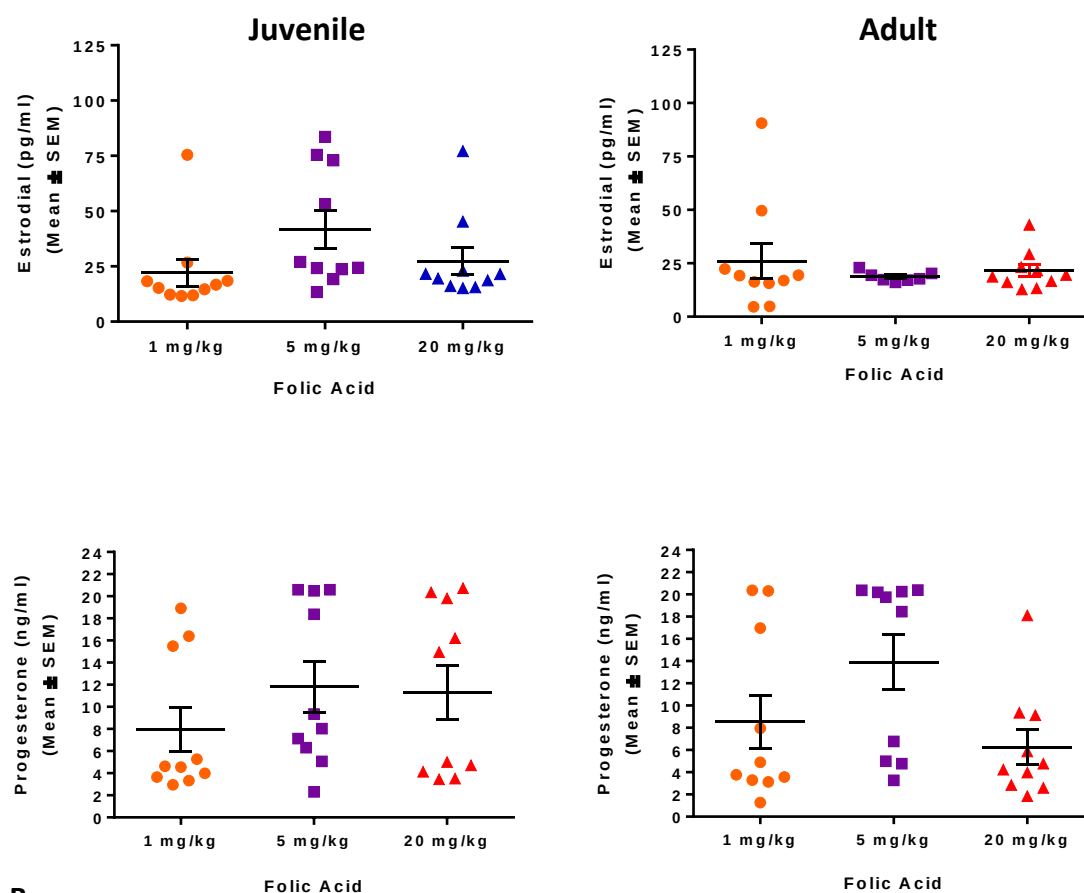


Figure 3.6 Linear Regressions of Plasma Folate Concentrations in Mice Supplemented with Different Levels of Folic Acid during the Juvenile Period or Adulthood.

Female C57BL/6 mice were supplemented with 1 mg/kg, 5mg/kg or 20 mg/kg of folic acid for 4 weeks (PND 28 to PND 56) during the juvenile period (**A**) or during adulthood (PND 74-102) (**B**). Plasma folate levels were measured at the end of the supplementation periods (PND 56 for juvenile and PND 102 for adults). Plasma folate concentrations are displayed as individual measurements. $n = 5-6$.

3.4.3 Plasma Hormone Levels

In order to establish whether phases of the reproductive cycle (estrus cycle) were consistent between folic acid supplemented groups and if any differences in hormone levels affected primary results, levels of progesterone and estradiol 17- β , key hormonal regulators of the estrus cycle, were measured in mice supplemented during the juvenile period or adulthood 4 weeks after the period of supplementation. Hormone concentrations in C57BL/6 from Fata et al., (2001) were used as a reference to identify stages of estrus cycle (Fata et al., 2001) (Figure 3.7 B). In juvenile mice plasma, progesterone concentrations were widely distributed and representative of both proestrus and diestrus (Figure 3.7A). Estradiol concentrations were mainly representative of proestrus/metestrus with a small number of samples in the 1mg/kg, 5 mg/kg and 20 mg/kg folic acid groups having higher levels of estradiol, consistent with estrus. However, there were no significant differences in the estradiol or progesterone concentrations between the groups. Within adults, findings were similar, with the majority of mice demonstrating progesterone concentrations consistent with proestrus or diestrus and estradiol-17 β concentrations consistent with proestrus/metestrus (Figure 3.7B). Further research is required to ascertain if folic acid deficiency affects hormone status in mice after the period of supplementation and the implications on disease risk.

A**B**

Estrous stage	Estradiol* (pg/ml)	Progesterone* (ng/ml)
Proestrus	47.3 ± 2.1	8.1 ± 1.8
Estrus	66.0 ± 3.2	1.2 ± 0.5
Metestrus	50.3 ± 3.2	7.7 ± 0.9
Diestrus	51.3 ± 3.2	18.4 ± 3.6

* Values are presented as mean ± SEM, n = 8 mice/estrous stage.

Figure 3.7. Plasma Hormone Concentrations in Female Mice fed Folic Acid during the Juvenile Period or Adulthood.

(A) Estradiol-17 β and progesterone concentrations were measured 4 weeks after folic acid supplementation. Original non-log transformed graphs shown for comparison of concentrations. (B) Reference concentrations of Estradiol-17 β and progesterone with corresponding estrus stages in female mice, adapted from Fata et al., (2001).

3.4.4 Folic Acid Supplementation during the Juvenile Period Induces Dose Specific Changes in Levels of Expression of Stem Cell Regulator Genes

3.4.4.1 *Brca1* and *Oct-4* gene Expression

Brca1 gene expression did not differ in mice supplemented during the juvenile period 4 or 12 weeks after the end of the supplementation period at PND 84 or PND 140 (Figure 3.8). There was a significant decrease ($P = 0.014$) in *Oct-4* mRNA expression at PND 84 in mice fed 20 mg/kg folic acid but this effect did not persist 12 weeks after folic acid supplementation at PND 140 (Figure 3.8).

3.4.4.2 Polycomb Gene Expression

Polycomb group proteins play a fundamental role in the regulation of cellular differentiation by silencing differentiation associated genes and maintaining the expression of pluripotency genes (Prezioso and Orlando, 2011). Recent studies have shown that EZH2, along with BRCA1, play a fundamental role in the orchestration of mammary cell differentiation and EZH2 overexpression has been closely linked with development and progression of breast cancer (Yamaguchi and Hung, 2014). Thus, to determine if changes in levels of folic acid supplementation also affects the expression of polycomb genes which work in concert with BRCA1 as key regulators of mammary cell differentiation, the expression of *Ezh2* (catalytic subunit of PRC2), *Bmi1* (integral component of PRC1), and *Suz12* (essential for PRC2 activity) were investigated.

Ezh2 and *Suz12* gene expression did not differ in mice supplemented during the juvenile period at PND 84 or PND 140 (Figure 3.9). *Bmi1* gene expression showed no effect of folic acid supplementation at PND 84 in the 5 mg/kg groups, however, at 20 mg/kg a trend towards significance was observed ($P = 0.064$) at PND 84 but this effect did not persist 12 weeks after folic acid supplementation at PND 140 (Figure 3.9).

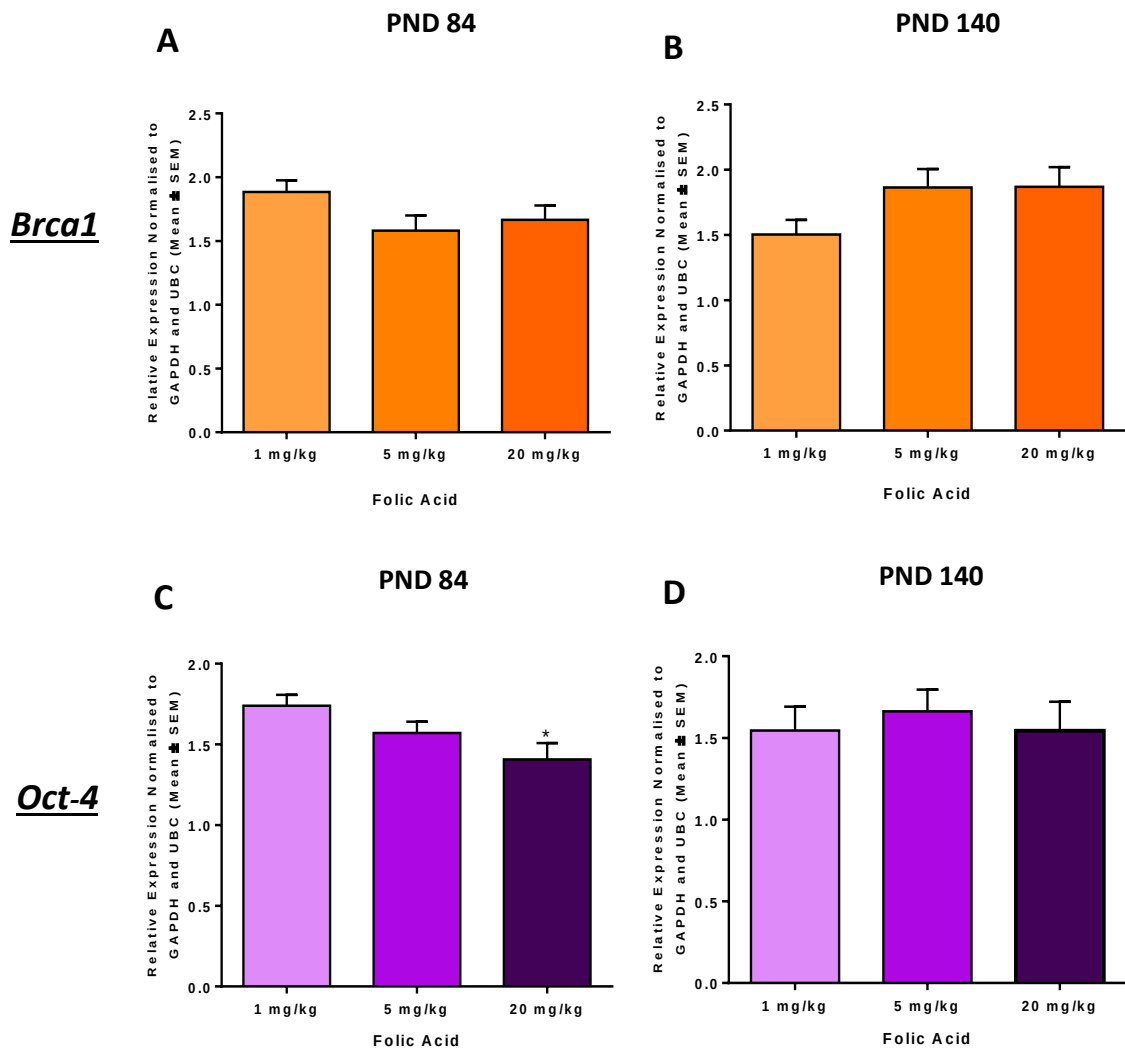


Figure 3.8 Relative mRNA Expression of *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with FA during Juvenile Development.

Relative expression of *Brca1* (A, B) and *Oct-4* (C, D) in mice supplemented with 1 mg/kg (1 x BDR - control), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56 – 84) (A – *Brca1*, C – *Oct-4*) or 12 weeks maintenance diet (PND 56 – 140) (B – *Brca1*, D – *Oct-4*). Data presented as mean ± SEM. n = 9-10 per group. * p < 0.05.

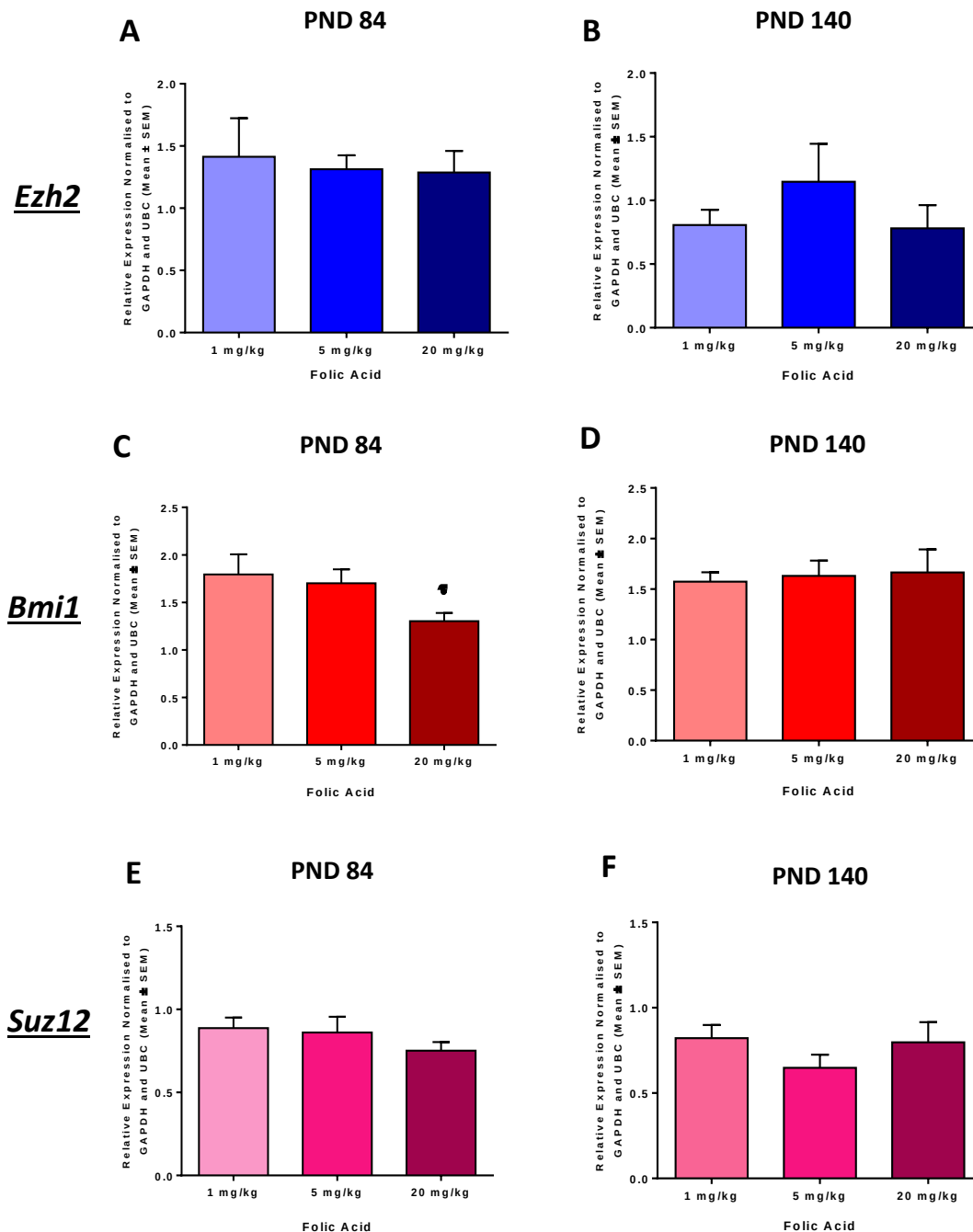


Figure 3.9 Relative mRNA Expression of Polycomb Genes *Ezh2*, *Bmi1* and *Suz12* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with Folic Acid during Juvenile Development.

Relative expression of *Ezh2* (A, B) *Bmi1* (C, D) and *Suz12* (E, F) in mice supplemented with 1 mg/kg (1 x BDR - control), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56 – 84) (A – *Ezh2*, C – *Bmi1*, E – *Suz12*) or 12 weeks maintenance diet (PND 56 – 140) (B – *Ezh2*, D – *Bmi1*, F – *Suz12*). Data presented as mean ± SEM. n = 6-10 per group. * p < 0.05.

3.4.5 Folic Acid Supplementation during the Juvenile Period induces Changes in Levels of Stem Cell Regulator genes with Ageing.

To determine how folic acid supplementation affects expression over time, levels of mRNA were compared between 4 weeks (PND 84) and 12 weeks (PND 140) post folic acid supplementation. There was a significant decrease ($P = 0.018$) in *Brca1* mRNA expression between PND 84 and PND 140 in the 1 mg/kg control group, but this decrease in expression with age was not observed in the 5 mg/kg or 20 mg/kg supplemented groups. Similarly, a significant decrease ($P = 0.003$) in *Oct-4* mRNA was observed between PND 84 and PND 140 in the 1 mg/kg supplemented control group but not in mice fed 5mg/kg or 20 mg/kg of folic acid (Figure 3.10). A non-significant trend towards a decrease in *Ezh2* gene expression (1 mg/kg $P = 0.074$, 20 mg/kg $P = 0.058$) in mice fed 1 mg/kg and 20 mg/kg between PND 84 and PND 140 was observed. However, no effect of folic acid supplementation on *Ezh2* expression was observed in mice fed 5 mg/kg. No differences in *Bmi1* or *Suz12* expression were observed between PND 84 and PND 140 (Figure 3.10).

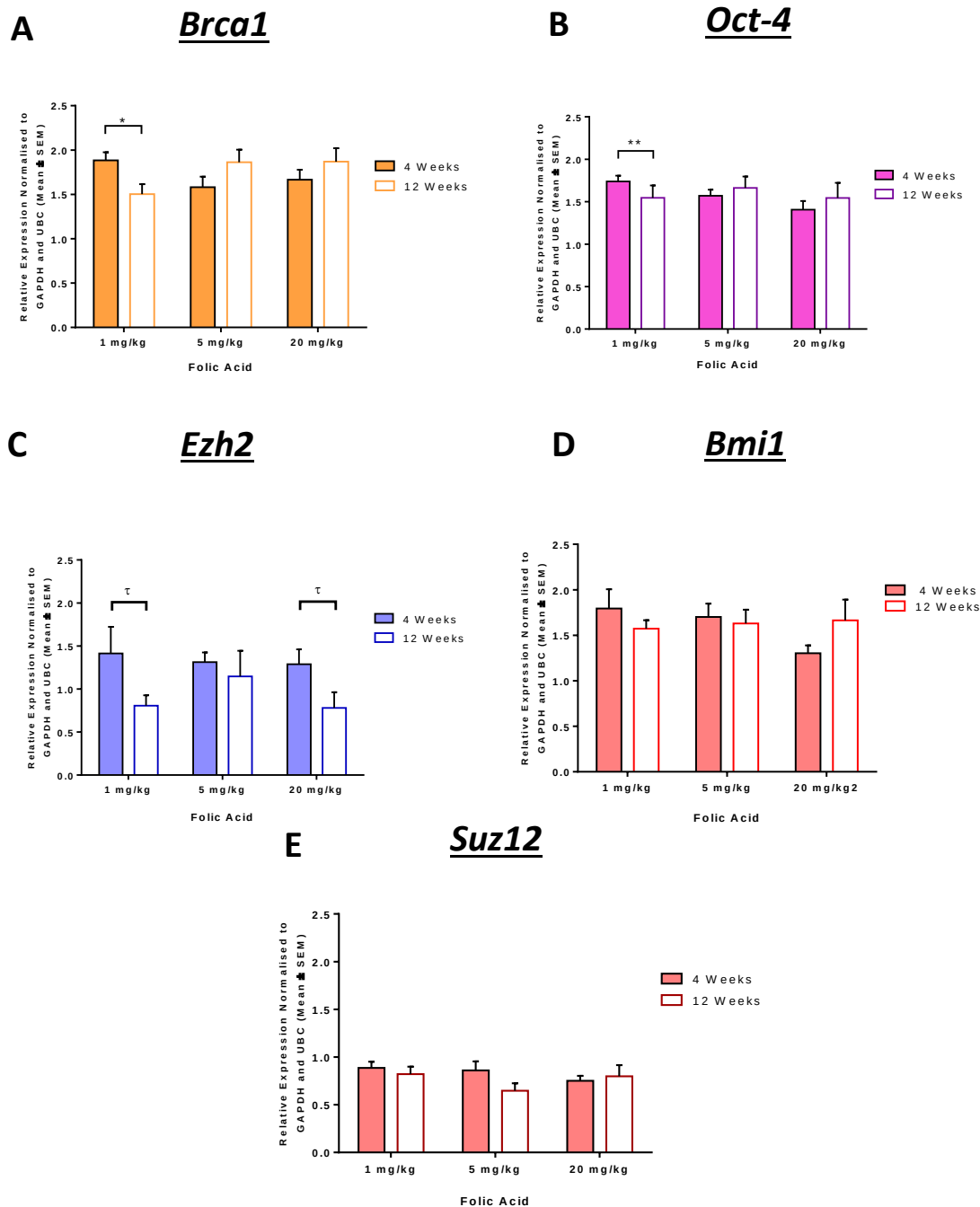


Figure 3.10. Comparison between Relative mRNA Expression of Polycomb Genes *Ezh2*, *Bmi1*, *Suz12* and *Brca1* and *Oct*-in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with Folic Acid during the Juvenile Period.

Relative expression of *Brca1* (A), *Oct-4* (B), *Ezh2* (C), *Bmi1* (D) and *Suz12* (E) in mice supplemented with 1 mg/kg (1 x BDR control), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28–56) followed by a 4 week maintenance diet (PND 56–84) or 12 week maintenance diet (PND 56–140). Data presented as mean $\pm$ SEM. n = 8–10 per group. * p < 0.05, ** p < 0.01).

3.4.6 Folic Acid Supplementation during Adulthood Induces Dose Specific Changes in Levels of Expression of Stem Cell Regulator Genes

3.4.6.1 *Brca1* and *Oct-4* Gene Expression

Brca1 expression significantly increased ($P = 0.025$) at PND 130 in mice fed 5 mg/kg folic acid and *Oct-4* mRNA significantly increased in mice fed 5 mg/kg ($P = 0.032$) and 20 mg/kg ($P = 0.01$) at PND 130, but this effect did not persist after 12 weeks at PND 186 (Figure 3.11).

3.4.6.2 Polycomb Gene Expression

Polycomb genes *Ezh2*, *Bmi1* and *Suz12* expression significantly increased ($P = 0.002$), ($P = 0.005$) and ($P = 0.007$), in mice fed 5 mg/kg folic acid 4 weeks (PND 130) after supplementation but, in line with both *Brca1* and *Oct-4* expression, did not persist at PND 186 (Figure 3.12).

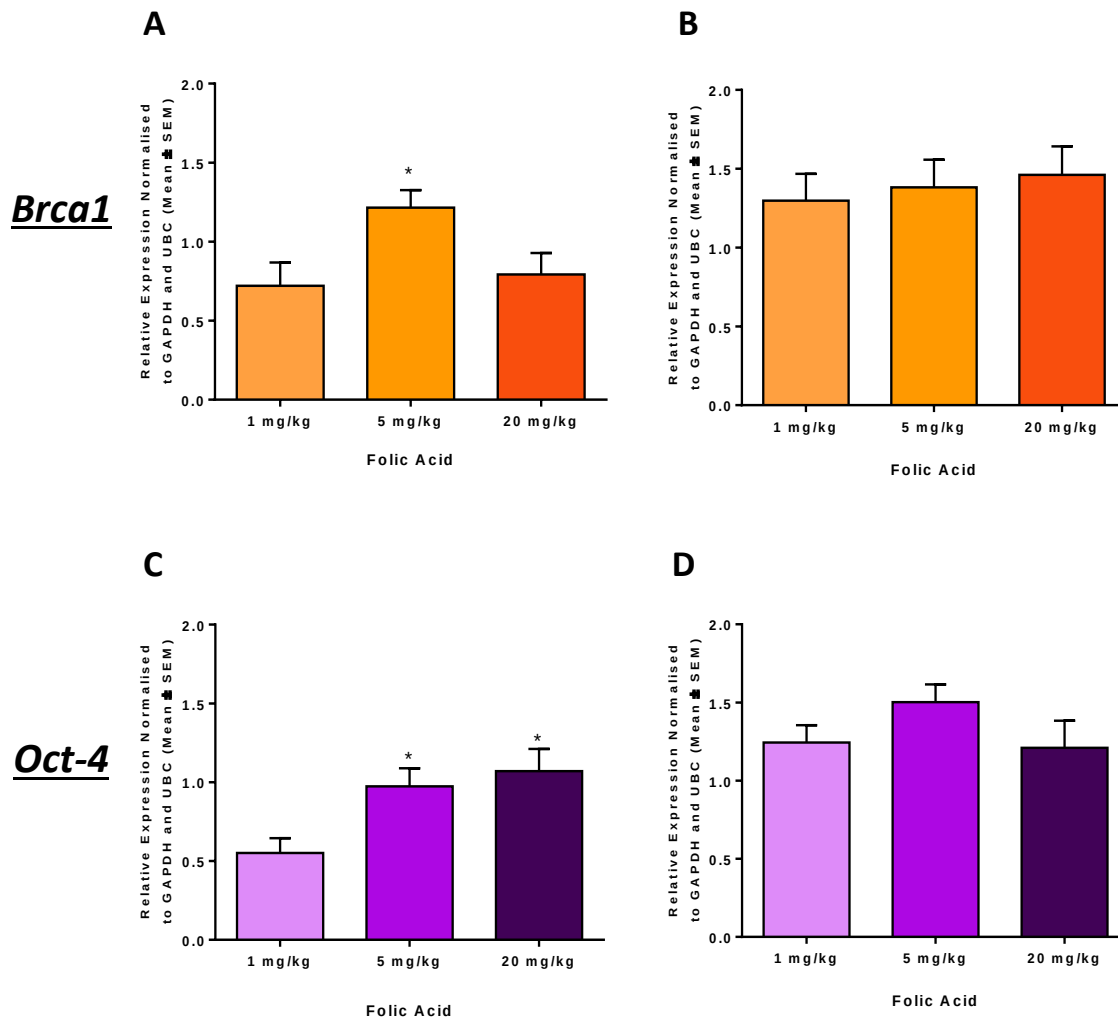


Figure 3.11 Relative mRNA Expression of *Brca1* and *Oct-4* in Female C57BL/6 mammary glands (2nd and 3rd thoracic) Supplemented with Folic Acid during Adulthood.

(A, B) Relative expression of *Brca1* in adult mice supplemented with 1 mg/kg (1 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) folic acid for 4 weeks (PND 74 – 102) followed by a 4 week maintenance diet (A – *Brca1*, C – *Oct-4*) (PND 102-130) or 12 week (B – *Brca1*, D – *Oct-4*) maintenance diet (PND 102-186). Data presented as mean ± SEM. n = 9-10 per group. * P < 0.05

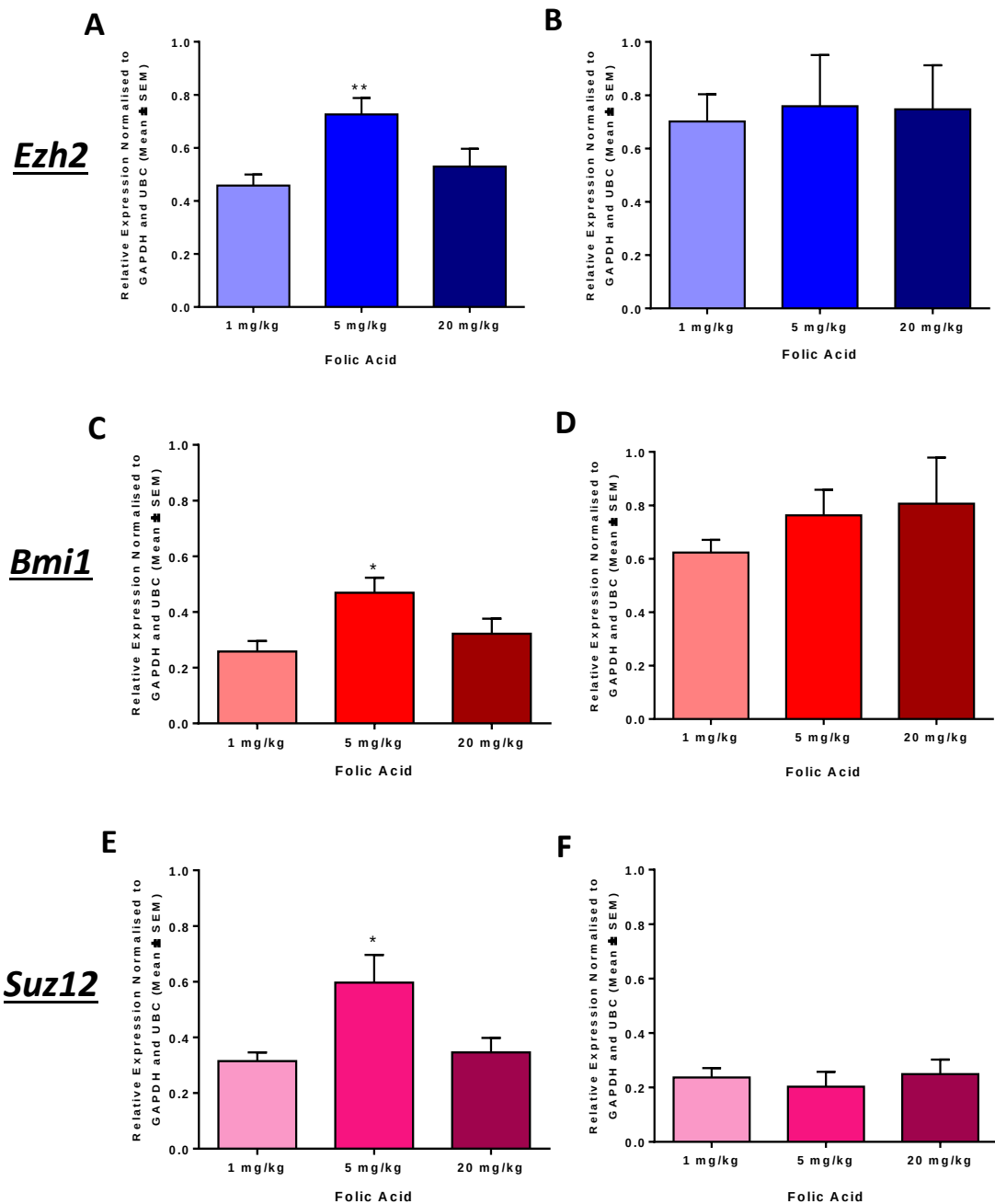


Figure 3.12 Relative mRNA expression of Polycomb Genes *Ezh2*, *Bmi1* and *Suz12* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with FA during Adulthood

(A) Relative expression of *Brca1* in adult mice supplemented with 1 mg/kg (1 x BDR - control), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 74 – 102) followed by a 4 week maintenance diet (PND 102-130) (A – *Ezh2*, C – *Bmi1*, E – *Suz12*) or 12 week maintenance diet (PND 102-186) (B – *Ezh2*, D – *Bmi1*, F – *Suz12*). Data presented as mean ± SEM. n = 8-10 per group. * P < 0.05, ** P < 0.01.

3.4.7 Folic Acid Supplementation during Adulthood Induces Changes in Levels of Stem Cell Regulator Genes with Ageing.

Brca1 mRNA expression was significantly increased in mice fed 1 mg/kg ($p = 0.021$) or 20 mg/kg ($p = 0.009$) folic acid between 4 weeks (PND 130) and 12 weeks (186) after supplementation. This significant increase in *Brca1* in response to ageing was not observed in mice fed 5 mg/kg (Figure 3.13). *Oct-4* mRNA expression was significantly increased in mice fed 1 mg/kg ($p = 0.000$) or 5 mg/kg ($p = 0.004$) between PND 130 and 186. This effect was not observed in the 20 mg/kg group (Figure 3.13) suggesting the increase in *Oct4* expression observed with age is modified in response to high concentrations of folic acid.

Ezh2 mRNA expression significantly increased in mice fed 1 mg/kg ($P = 0.025$) folic acid at PND 186, however, this increase in expression with age was not observed in the 5 mg/kg or 20 mg/kg supplemented groups. *Bmi1* gene expression showed a significant increase in mRNA expression in mice fed 1 mg/kg ($p = 0.000$) folic acid, with this age dependent increase in expression also observed in 5 mg/kg ($P = 0.013$) and 20 mg/kg (0.009) groups between PND 130 and PND 186. *Suz12* mRNA expression did not differ in mice fed 1 mg/kg or 20 mg/kg group, but at 5 mg/kg *Su12* expression was significantly increased between PND 130 and PND 186 ($P = 0.004$).

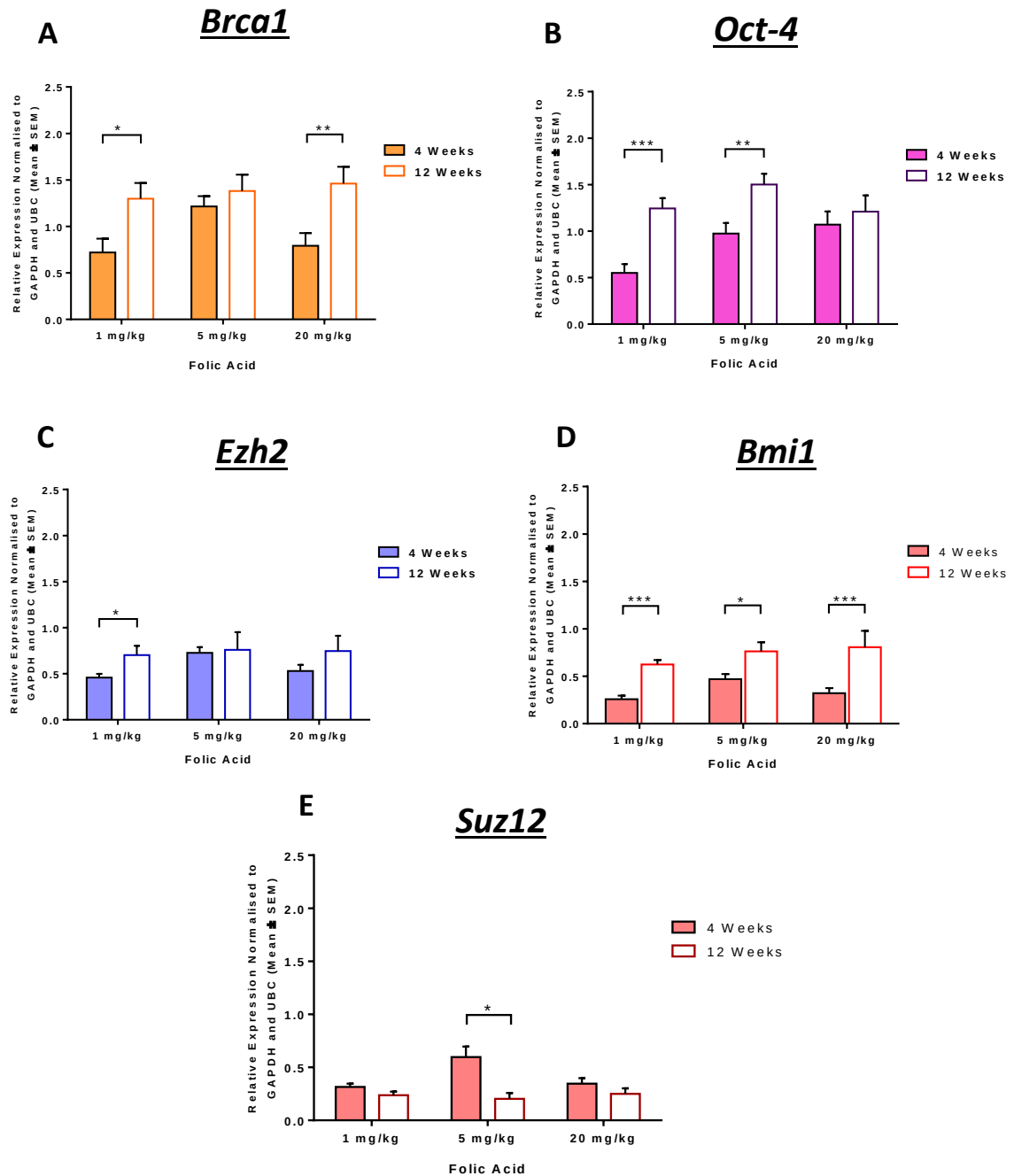


Figure 3.13 Comparison between Relative mRNA Expression of Polycomb Genes *Ezh2*, *Bmi1*, *Suz12* and *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with Folic Acid during Adulthood.

Relative expression of *Brca1* (A), *Oct-4* (B), *Ezh2* (C), *Bmi1* (D) and *Suz12* (E) in mice supplemented with 1 mg/kg (1 x BDR - control), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 74 – 102) followed by a 4 week maintenance diet (PND 102 - 130) or 12 week maintenance diet (PND 102 - 186). Data presented as mean $\pm$ SEM. n = 8-10 per group. * p < 0.05, ** p < 0.01, *** p < 0.001).

3.4.8 Plasma Hormone Levels do Not Modulate Folic Acid Mediated Changes in Gene Expression in Mouse Mammary Glands

Linear regression was used to ascertain if changes in estradiol-17 β or progesterone at all concentrations of folic acid supplementation affect key gene expression findings in levels of *Brca1*, *Oct-4* and *Ezh2* (Table 3.3). As the majority of changes were observed 4 weeks after supplementation regression analysis was investigated using gene expression values from this time point.

No significant effects ($P > 0.05$) of hormone status on *Brca1*, *Oct-4* or *Ezh2* gene expression data were observed, suggesting levels of plasma estradiol-17 β or progesterone which are reflective of estrus cycle, do not significantly affect mammary gene expression in relation to changes in levels of folic acid supplementation.

Table 3.3 Linear Regression Analysis of Plasma Hormone Levels and Gene Expression Data.

Gene	Estradiol-17 β					Progesterone				
	R ²	Adj R ²	Standardised Coefficient β	t	Sig	R ²	Adj R ²	Standardised Coefficient β	t	Sig
Juvenile Supplemented Mice										
<i>Brca1</i>	0.002	-0.025	-0.047	-0.287	0.775	0.051	0.026	-0.227	-1.416	0.165
<i>Oct-4</i>	0.003	-0.024	-0.051	-0.316	0.753	0.003	-0.023	-0.056	-0.348	0.730
<i>Ezh2</i>	0.030	0.004	0.004	-0.173	0.287	0.013	-0.013	-0.114	-0.707	0.484
Adult Supplemented Mice										
<i>Brca1</i>	0.003	-0.028	-0.055	0.759	-0.009	0.076	0.049	0.275	1.692	0.099
<i>Oct-4</i>	0.088	0.059	-0.297	-1.734	0.093	0.018	-0.011	0.135	0.792	0.434
<i>Ezh2</i>	0.009	-0.022	-0.097	-0.550	-0.586	0.010	-0.019	0.100	0.584	0.563

3.5 Folic Acid Supplementation induces Changes in Levels of Ezh2 Protein Expression in Female C57BL/6 Mice Supplemented during Adulthood.

As *Ezh2* plays a critical role in mammary stem cell differentiation and in the regulation of *Oct-4* and *Brca1* expression, it was investigated whether changes in *Ezh2* gene expression observed in adulthood after 5 mg/kg folic acid is mirrored by a change in protein levels in mice supplemented during adulthood (PND 74-102) followed by a 4 week maintenance diet (PND 130).

Ezh2 protein expression was assessed by western blot in mice supplemented with folic acid during adulthood (PND 74-102) 4 weeks after the supplementation period (PND 130) (Figure 3.14 A). Levels of *Ezh2* protein was significantly higher ($p = 0.0023$) in mice supplemented with 5 mg/kg when compared to a 1 mg/kg control diet (Figure 3.14 B). As *Ezh2* is the catalytic subunit of the polycomb PRC2 complex, responsible for the methylation of lysine 27 on histone 3 (H3K27), western blots were performed to investigate whether levels of H3K27 (tri-methyl) were also increased in the 5mg/kg folic acid supplemented group at PND 130 in mice supplemented during adulthood (Figure 3.15 A, B). Relative ratios of methylated H3K27 to unmethylated showed a decrease in mice fed 1 mg/kg and an increase in mice fed 5 mg/kg.

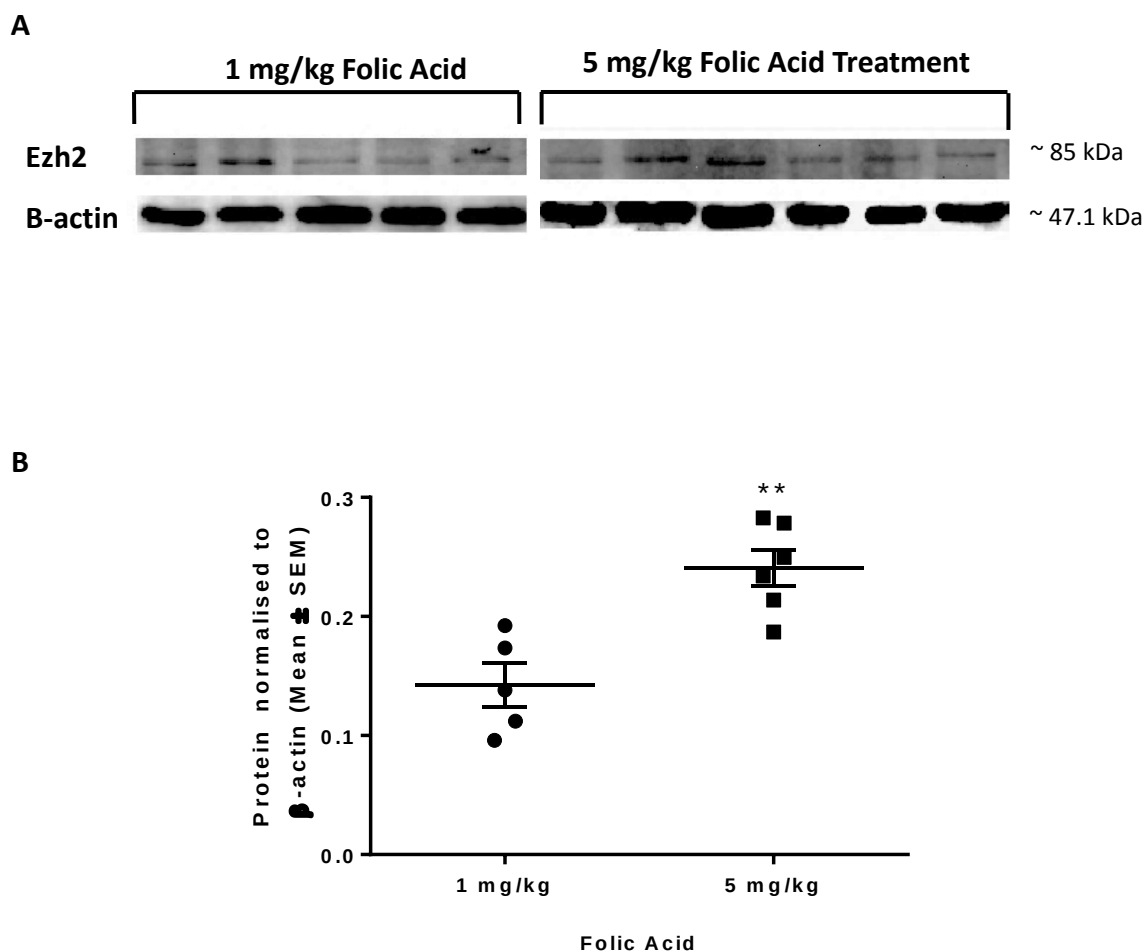


Figure 3.14. Protein Expression of Ezh2 in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with FA during Adulthood.

(A) Western Blot showing expression of Ezh2 protein in adult mice supplemented with 1 mg/kg (1x BDR control) or 5 mg/kg (5 x BDR) folic acid for 4 weeks (PND 74 – 102), followed by a 4 week maintenance diet (PND 102-130). Human HeLa cell lysate was run as a positive Ezh2 control (not shown). (B) Quantification of protein expression by densitometry analysis. Data was analysed using an independent t test and presented as mean $\pm$ SEM. n = 5-6 per group. ** p < 0.01.

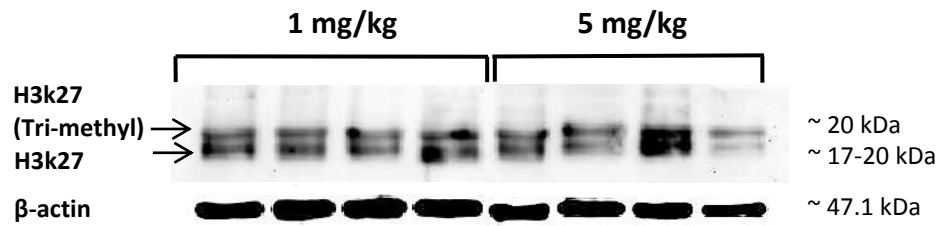
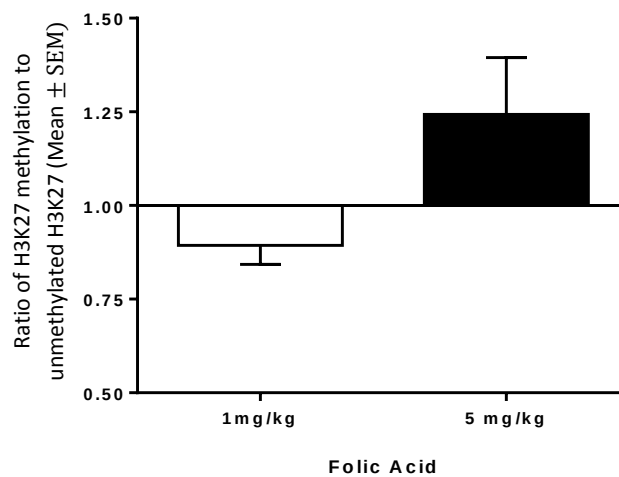
A**B**

Figure 3.15. Protein expression of H3K27 (Tri-methyl) in Female C57BL/6 mammary glands (2nd and 3rd thoracic) supplemented with FA during adulthood.

(A) Western Blot showing expression of H3K27 (Tri-methyl) protein in adult mice supplemented with 1 mg/kg (1x BDR control) or 5 mg/kg (5 x BDR) folic acid for 4 weeks (PND 74 – 102), followed by a 4 week maintenance diet (PND 102-130). (B) Relative ratios of unmethylated H3K27 to methylated H3K27. Relative ratio of H3K27 methylation to unmethylated H3K27. Data presented as mean $\pm$ SEM. n = 4 per group.

3.6 Discussion

Results from the present study demonstrate that folic acid supplementation in female C57/BL6 mice, during either the juvenile period (PND 28-56) or adulthood (PND 74-102) induce concentration, timing and duration specific changes in gene expression within the mammary glands. Gene expression changes are summarised in Table 3.4.

Table 3.4. Summary of Gene Expression Changes in Mice Supplemented with Folic Acid for 28 days during the Juvenile period or Adulthood Followed by 4 week or 12 Week Maintenance Diet.

Folic Acid Supplementation during the Juvenile Pre-pubertal period (PND 28-56)				
	4 weeks after supplementation (PND 84)		12 weeks after supplementation (PND 140)	
	5 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg
<i>Brca1</i>	-----	-----	-----	-----
<i>Oct-4</i>	-----	↓	-----	-----
<i>Ezh2</i>	-----	-----	-----	-----
<i>Bmi1</i>	-----	t ↓	-----	-----
<i>Suz12</i>	-----	-----	-----	-----
Folic Acid Supplementation during the Adulthood (PND 74-102)				
	4 weeks after supplementation (PND 130)		12 weeks after supplementation (PND 186)	
	5 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg
<i>Brca1</i>	↑	-----	-----	-----
<i>Oct-4</i>	↑	↑	-----	-----
<i>Ezh2</i>	↑	-----	-----	-----
<i>Bmi1</i>	↑	-----	-----	-----
<i>Suz12</i>	↑	-----	-----	-----

Energy intake did not differ significantly between groups supplemented during the juvenile period, consistent with no change in body weight between groups. However, in adult supplemented mice energy intake differed significantly between folic acid groups (Figure 3.4). Mice fed a supramaximal (20 mg/kg) concentration of folic acid demonstrated increased energy intake compared to baseline (1 mg/kg) or high (5 mg/kg) supplemented concentrations (Figure 3.4). There were minimal differences (PND 140 to PND 167) between mice supplemented with 1 mg/kg or 5 mg/kg folic acid. The increase in energy intake in mice supplemented during adulthood (PND 74 to PND 102) was not accompanied by a significant increase in body weight between groups, suggesting energy is not being stored in the form of muscle or adipose tissue; rather, energy is being expended at an increased rate either metabolically or physically. These changes in energy intake in adult mice were observed throughout the duration of the supplemental period and persisted until 12 weeks after end of supplementation (Figure 3.4). This suggests that effects of folic acid intake on energy metabolism are concentration specific, persistent and reflect changes in metabolism within adult mice. At present, no literature has reported modified levels of energy intake in adult mice supplemented with 20 mg/kg folic acid both during and up to 12 weeks post supplementation. Interestingly it does appear that energy intake is significantly affected in mice fed a supramaximal concentration of folic acid (20 mg/kg) suggesting that an extreme deviation from baseline levels of folic acid intake may perturb energy metabolism. However, further research is required to understand the mechanisms associated with these observable changes. This could include analysis of levels of physical activity in individual mice, allowing the calculation of metabolic expenditure through exercise and calculation of individual food intakes (not averaged) to ascertain the exact quantities of food each individual mouse consumes, feeding behaviour, time of feeding and if this changes based on folic acid diet.

Average plasma folate (5me-THF) increased in a dose response manner at the end of juvenile and adult supplementation between basal (1 mg/kg), high (5 mg/kg) and supramaximal (20 mg/kg) levels of folic acid intake. However, apart from a significant difference between control and 20 mg/kg in juvenile supplemented mice, these changes did not reach significance. These findings may be explained by the relatively small numbers of plasma samples analysed. Retrospective power calculations using current data showed that 5 samples were sufficient to provide statistical power of 80% with a $p < 0.05$ in juvenile supplemented mice, however in adults a sample size of 28 was required to achieve 80% statistical power. This suggests that a larger

sample size would provide higher statistical power; however, linear regression analysis showed that changes in folic acid diet does explain 37% of variation in folate status observed in juveniles and 26% in adults. However there are issues in analysing 5-mTHF levels as a proxy for folic acid intake which may include differences in the conversion rate of folic acid to 5-mTHF and its turnover, which may vary depending on folic acid intake, as well as the age of life and tissue. Only a few other studies investigating the effects of folic acid intake on cancer risk in animal models have assessed levels of 5-mTHF after supplementation. Research by Mansahdi et al., (2014) found that at necropsy, the mean plasma folate concentrations were significantly different among folic acid dietary groups, increasing with increased levels of dietary folic acid. However, the mean folate concentrations in the mammary gland of three folic acid supplemented dietary groups were not significantly different from each other, but were found to be significantly higher than the control group and increased with increasing dietary folic acid intake (Deghan Manshadi et al., 2014). An alternative would be to measure folic acid, however, this is much more labile, demonstrates differences in stability compared to naturally occurring folate and does not inform on the levels of biologically active folate. If resources were available, measurement of 5me-THF, folic acid and the ratio of SAM/SAR would have been performed immediately after supplementation and also both 4 and 12 weeks after supplementation in plasma, blood and mammary tissue. This would provide insight into the metabolism of excess folic acid, levels of tissue uptake, and implications for DNA methylation. Such insights into the mechanism of folic acid metabolism will be increasingly important having shown that folic acid intake can alter a number of key genes implicated in breast cancer risk.

Changes in the levels of folic acid supplementation during the juvenile period had no effect on the expression of polycomb genes *Ezh2*, *Bmi1* or *Suz12* either 4 weeks (PND 84) or 12 weeks (PND 140) after the period of supplementation. Folic acid supplementation at 5 mg/kg or 20 mg/kg during the juvenile period had no effect on the levels of *Brca1* gene expression either 4 weeks or 12 weeks after supplementation. Conversely, a decrease in *Oct-4* expression was observed in mice fed 20 mg/kg folic acid 4 weeks after supplementation which was not maintained after 12 weeks. This data shows that folic acid supplementation during the juvenile period modifies *Oct-4* expression only at a supra-maximal concentration (20 mg/kg) and these effects persist for a minimum of 4 weeks beyond the period of supplementation, suggesting the effects of folic acid are both concentration and duration specific.

Research by Lillycrop *et al.*, (unpublished) has reported that high folic acid supplementation (5 mg/kg) during the juvenile period in rats decreases *Oct-4* expression in liver (43%, $p = 0.009$) and adipose tissue (30%, $p = 0.0001$), suggesting a folic acid mediated decrease in *Oct-4* gene expression is specific to different tissues. Furthermore, these changes were shown to correspond with significant changes in CpG methylation within each of these genes. *Oct-4* is detected in stem cells and multipotent progenitor cells present within adult tissues and has been shown to play a critical role in self-renewal of the stem cell pool and the regulation of genes that control cellular differentiation and repopulation of specific cell types in adult tissues or organs (Tai *et al.*, 2005, Diaz-Flores *et al.*, 2006). Decreased *Oct-4* expression as a result of high folic acid intake in juvenile supplemented mice may have a direct effect on the stem cell pool and regulation of cellular differentiation within multipotent adult stem cells which may influence mammary gland cell populations. Furthermore, these persistent changes in gene expression observed after the period of supplementation may in turn be regulated through DNA methylation reactions modulated by changes in folic acid mediated one carbon metabolism and be reflective of increased levels of plasticity within the developing mammary gland.

In adult supplemented mice, expression of polycomb genes *Ezh2*, *Bmi1* and *Suz12* increased only in mice fed 5 mg/kg folic acid 4 weeks after supplementation (PND 130) but this effect did not persist after 12 weeks (PND 186). The specificity of expression changes observed at 5 mg/kg and not at 20 mg/kg in these genes suggests that folic acid functions to modulate gene expression at physiological as opposed to supramaximal concentrations without a specific dose response effect. Research by Manshadi *et al.*, (2014) investigating the effects of folic acid supplementation on mammary tumour growth in rats identified a similar effect, with folic acid at 5 mg/kg as opposed to 8 mg/kg or 10 mg/kg demonstrating the most significant increase in development of pre-existing neoplasms with no overall dose response effects (Deghan Manshadi *et al.*, 2014). Manshadi *et al.*, (2014) suggests that these findings may occur due to enzymatic constraints, specifically the fact that folate accumulation in tissues is limited by folypolyglutamate synthase activity which may be limited at high substrate concentrations (Shane, 2010). Furthermore, the inhibition of dihydrofolate reductase (DHFR), which critically methylates folic acid, by high levels of unmetabolised folic acid may decrease the intracellular pool resulting in a reduced effect that could modify levels of gene expression (Bailey and Ayling, 2009). In addition, research by Neuhouser *et al.*, (2011) using mathematical modelling of folate biochemistry to predict the function of folate excess, suggests non-linearity in folate effects based on underlying enzyme activity (Neuhouser *et al.*, 2011).

Folic acid mediated increase in expression of *Ezh2*, *Bmi1* and *Suz12* suggests an increase in levels of PRC1 and PRC2 complex activity. Research has shown that lineage-specific proliferation and differentiation are orchestrated by PRC complexes through the suppression of transcriptional pathways (Ezhkova et al., 2009). Increased levels of *Ezh2* and *Bmi1* would suggest the recruitment of PRC2 and subsequent increase in methyltransferase activity on H3K27 in mammary epithelial cells which may function to repress transcriptional gene regulation. Within the present study, levels of Ezh2 protein in adult supplemented mice were found to be higher in mice fed 5 mg/kg of folic acid, 4 weeks (PND 130) after supplementation. This was in line with the gene expression changes that showed increased expression of *Ezh2* at this time point and concentration. Therefore, changes in *Ezh2* expression translates to functional changes in levels of protein which are modified by folic acid intake and persist up to 4 weeks after the end of the modified diet. Furthermore, protein analysis to assess if increased levels of Ezh2 modified the levels of its target protein, H3K27 (tri-methyl), showed that the ratio of H3K27-tri methyl to H3K27 was higher in mice fed 5 mg/kg folic acid compared to those fed 1 mg/kg, suggesting folic acid supplementation increases the levels of H3K27-tri methyl (Figure 3.14). This suggests that Ezh2 protein is functioning as a methyltransferase modifying its target H3K27 in response to high (5 mg/kg) folic acid intake and that this effect has persisted 4 weeks (PND 130) after the period of supplementation. This shows a folic acid mediated increase in Ezh2 expression and PRC2 activity. Methylation of H3K27 is associated with transcriptional repression which controls gene expression patterns (Schwartz and Pirrotta, 2007). Therefore, increased levels of methylated H3K27 may function in the repression of genes associated with breast cancer risk through Ezh2 mediated histone modifications.

Ezh2 overexpression has been linked to breast cancer development specifically through increased cell proliferation by mechanisms associated with mammary stem cell regulation (Wu and Crowe, 2015). High levels of EZH2 has been shown to promote mammary stem cell and luminal progenitor cell expansion, increase metastasis and inhibit estrogen receptor-positive cellular differentiation increasing breast cancer development (Wu and Crowe, 2015). Thus, a folic acid mediated increase in Ezh2 expression may function to modulate mammary luminal progenitor cell expansion and increase breast cancer risk long after the initial period of supplementation during adulthood. Interestingly a recent study by Yoo et al., (2015) has proposed an alternative method in that *EZH2* functions to control a defined differentiation program in mammary alveolar epithelium and enables the recruitment of EZH1, STAT5 and RNA Pol 2 to mammary specific genes, which is independent of the genomic H3K27me3 status (Yoo

et al., 2015). Therefore, increased *Ezh2* expression in combination with *Suz12* and *Bmi1* is likely to be associated with an expansion of mammary cells regulated by either epigenetic modifications to histones or through regulation of genes such as *Brca1* or *Oct-4*.

Changes in the levels of *Brca1* and *Oct-4* gene expression in adult supplement mice demonstrated almost identical effects to those observed in polcomb *Ezh2*, *bmi1* and *Suz12* with increased expression 4 weeks after dietary intervention (PND 130). This effect was not maintained 12 weeks after supplementation (PND 186). No changes in *Brca1* expression were observed in mice fed 20 mg/kg of folic acid at PND 130 or PND 186. Folic acid therefore induces a dose dependent effect on *Brca1* gene expression that persists for up to 4 weeks after the period of supplementation. Furthermore, expression changes observed at 5 mg/kg were not mirrored or increased at 20 mg/kg, suggesting folic acid does not produce a dose response effect on *Brca1* gene expression. As previously discussed this may be directly related to enzyme constraints associated with supramaximal levels of folic acid intake or other mechanisms that currently have yet to be elucidated.

Brca1's role as a critical regulator of DNA repair, cell cycle, transcriptional regulation and regulation of stem cell function is tightly regulated in the developing breast (Mueller and Roskelley, 2003). Upregulation of *Brca1* has been shown to be required for controlled proliferation and differentiation of mammary cells (Suba, 2015). Furthermore, research using different human tumour cell lines suggests that increased *Brca1* mRNA and protein is upregulated after cell proliferation, linking increased *Brca1* transcription with cancer risk (Vissac et al., 2002). Increased *Brca1* could also be linked to its role in DNA repair suggesting a folic acid mediated increase in DNA repair capacity. Research has further shown that EZH2 directly modulates BRCA protein levels and is required for the proliferative effects of EZH2 (Gonzalez et al., 2009). Furthermore, *Ezh2* can phosphorylate *Brca1*, reducing its activity; therefore, increased levels of *Brca1* may not prove protective if inhibited by high levels of *Ezh2*. In addition, changes in levels of stem cell regulator gene expression of *Oct-4* has been linked to the modulation of *Ezh2* and also *Brca1* through ER mediated mechanisms (Wang and Di, 2014).

Folic acid supplementation in adult mice at both 5 mg/kg and 20 mg/kg increased *Oct-4* gene expression 4 weeks after supplementation at PND 130; however, this effect did not persist after 12 weeks at PND 186. This contrasts the folic acid mediated change in *Oct-4* gene expression observed in juvenile supplemented mice which decreased in the 20 mg/kg group at PND 84, suggesting the timing of folic acid supplementation plays a critical role in the direction of *Oct-4*

gene expression. Increased transcriptional expression of *Oct-4* functions specifically to modulate levels of cellular proliferation and gene regulation during lactation and mammary development in adult stem cell populations (Hassiotou et al., 2013a). Increased levels of Oct-4 expression and its associated gene network are implicated in malignant mammary gland transformation (Cai et al., 2016). Research has shown that *OCT-4* expression is significantly higher in breast cancer tissues in humans and therefore a folic acid mediated increase in *Oct-4* could increase breast cancer risk (Cai et al., 2016).

There are multiple splice variants of Oct-4, the two major being Oct-4A and Oct-4B which both function in the regulation of mammary stem cells. Oct-4A functions as a regulator of stemness in ESCs and the exact function of OCT4B is not clear, however, it has recently been shown to regulate OCT4A expression in an miRNA-dependent manner in cancer cell lines (Li et al., 2015). Gene expression analysis in the present study used primers that did not discriminate between Oct-4A and Oct-4B isoforms, thus changes observed represent total Oct-4 mRNA. However, future research could investigate the effects of folic acid on expression of individual Oct-4 isoforms within the mammary gland.

Polycomb genes *Ezh2*, *Bmi1* and *Suz12* along with *Brca1* and *Oct-4* expression levels were compared for the effect of aging in mice supplemented during the juvenile period. No folic acid mediated differences in expression with ageing were observed in *Ezh2*, *Bmi1* or *Suz12* between 4 and 12 weeks post folic acid supplementation. In mice fed a control diet (1 mg/kg), *Brca1* expression decreased 12 weeks (PND 140) after supplementation in comparison to 4 weeks (PND 84). This suggests that levels of *Brca1* expression decreases as mice age during juvenile development to young adult. Previous literature on gene expression profiling in humans has suggested that BRCA1 expression may decrease in older individuals post menopause, however, is it currently unclear if changes in BRCA1 expression may occur at an earlier age (Loredo-Pozos et al., 2009). In contrast, mice fed 5 and 20 mg/kg folic acid showed no corresponding changes in *Brca1* expression over time, suggesting that folic acid supplementation may prevent any decrease in *Brca1* expression that occurs with ageing. This change could be related to levels of DNA methylation within *Brca1*, with folic acid intake modulating normal levels of hypermethylation which usually cause a decrease in *Brca1* expression as mice age. Levels of *Brca1* expression increased after 12 weeks in mice fed both 5 mg/kg and 20 mg/kg folic acid, the opposite effect of what was observed in control groups. *Oct-4* expression was unchanged between 4 and 12 week post folic acid supplementation in 1 mg/kg, 5 mg/kg and 20 mg/kg

groups suggesting that levels of *Oct-4* expression in response to folic acid supplementation do not differ from changes that occur naturally with ageing.

In adult folic acid supplemented mice, polycomb gene *Ezh2* showed significantly increased expression with ageing at 1 mg/kg; however, this effect was lost in mice fed 5 mg/kg and 20 mg/kg folic acid. Similar to observations in *Brca1* and *Oct-4*, folic acid supplementation appears to increase the levels of *Ezh2* to levels nearer what would be normally observed at 12 weeks after supplementation. These findings suggest a folic acid mediated change in stem cell regulation and proliferation or that methyltransferase activity may occur prematurely in mice supplemented during adulthood which may pose a risk towards neoplastic development. In contrast *Bmi1* demonstrated an increase with age which was unaffected by folic acid supplementation. Conversely, *Suz12* decreased with age which was not affected by folic acid treatment. *Brca1* expression was increased in 1 mg/kg and 20 mg/kg folic acid supplemented groups in response to ageing; the opposite was observed in the juvenile supplemented group. These changes may reflect, in part, the differences in growth and plasticity within mammary tissue between juvenile development and adulthood and differences in *Brca1* methylation and gene expression patterns during breast development. This effect was also observed in mice fed 20 mg/kg suggesting a supramaximal concentration of folic acid during adulthood does not affect *Brca1* expression as mice age. No differences in *Brca1* expression were observed in mice fed 5 mg/kg, with levels after 4 weeks at a similar level to that observed after 12 weeks. It appears 5 mg/kg of folic acid increases *Brca1* expression 4 weeks after supplementation to levels in line with those observed after 12 weeks, increasing levels of *Brca1* prematurely compared to control groups. This shows that folic acid induces dose dependent changes on *Brca1* expression and accelerates the natural increase in *Brca1* expression that occurs with ageing within the mammary gland of adult mice.

Research has shown that *Brca1* is expressed during differentiation in rapidly proliferating cell types within the mammary gland and has been linked to mammary stem cell function (Marquis et al., 1995a). Folic acid at 5 mg/kg may therefore modulate proliferation in mammary tissue by provision of nucleotide precursors or changes in levels of DNA methylation 4 weeks after supplementation to levels that would normally be observed after 12 weeks, resulting in an increase in *Brca1* expression at this time point. *Oct-4* expression was shown to increase with ageing in adult supplemented control groups. This was the opposite of what was observed in juvenile supplemented mice. *Oct-4* significantly increased in mice fed both 1 mg/kg and 5 mg/kg

folic acid, however, no increase was observed in the 20 mg/kg groups. Folic acid therefore increases levels of *Oct-4* expression 4 weeks after supplementation to what is normally observed after 12 weeks. This provides parallels to observations with *Brca1* expression in mice supplemented with 5 mg/kg of folic acid and suggests changes in gene expression with ageing are concentration specific. Increased levels of *Oct-4* could infer changes in stem cell differentiation and prematurely increasing levels of expression may reflect dysregulated mammary stem cell programming and proliferation at an incorrect stage of mammary development. Interestingly, analysis of gene expression data compared with levels of estrus hormones in individual mice showed no influence of both estradiol-17 β and progesterone on folic acid mediated changes in gene expression. This suggests that the stages of the estrus cycle (which are reflective of hormone status) do not influence primary data findings from the present study.

Methylation of H3K27 is associated with transcriptional repression which controls gene expression patterns (Schwartz and Pirrotta, 2007). Therefore, increased levels of methylated H3K27 may function in the repression of genes associated with breast cancer risk through *Ezh2* mediated histone modifications.

3.7 Conclusion

In conclusion, this study has investigated the effects of varying concentrations of folic acid supplementation at two different time points of the life-course on the expression of key genes implicated in breast cancer aetiology, namely polycomb genes *Ezh2*, *Bmi1* and *Suz12*, tumour suppressor *Brca1* and pluripotency regulator *Oct-4*. Key findings from this study have identified that in mice supplemented during the juvenile period, a supra-maximal level of folic acid (20 mg/kg) increases *Oct-4* gene expression in both a dose and duration dependent manner. Furthermore, high levels of folic acid supplementation (20 mg/kg) prevent the age related decrease in *Brca1* expression 12 weeks after the modified diet observed in the control group (1 mg/kg). Interestingly, all changes in the levels of gene expression observed in response to folic acid supplementation during the juvenile period showed decreased levels of gene expression as opposed to the changes during adulthood that showed increased expression.

In mice supplemented during adulthood, all genes showed increased levels of expression only in the 5 mg/kg groups 4 weeks after supplementation, with the exception of *Oct-4* which was also increased in the 20 mg/kg group. Folic acid modifies *Ezh2*, *Bmi1*, *Suz12*, *Brca1* and *Oct-4* in a dose and duration specific manner. In line with expression, levels of *Ezh2* protein were significantly increased and its target H3K27-me3 was increased (non-significantly). Furthermore, comparisons between levels of *Ezh2* expression both 4 and 12 weeks after supplementation with the control group (1 mg/kg) show that folic acid supplementation at 5 mg/kg appears to increase levels of gene expression after 4 weeks to levels normally observed after 12 weeks. This suggests a potential role for folic acid in the regulation of gene expression that occurs with aging. Interestingly, all changes in levels of gene expression in response to folic acid supplementation during adulthood showed increased levels of gene expression, the opposite to that observed in mice supplemented during the juvenile period. This data shows for the first time that folic acid supplementation can induce opposite effects on levels of expression in key genes implicated in breast cancer risk and is dependent on both the dose and the timing of intake during the life-course.

Chapter 4: The Effects of Folic Acid Supplementation during the Juvenile Period or Adulthood on Mammary Gland Morphology in Female C57BL/6 mice

4.1 Introduction

Having observed changes in the expression of genes related to mammary gland differentiation and development it was necessary to ascertain the effects of folic acid supplementation on mammary gland morphology. Changes in mammary gland morphology are generally considered reliable biomarkers of mammary tumour risk (Thurfjell 2002). During mammary development, hormonal cycling, pregnancy, post-lactational involution and aging, the mammary gland undergoes physiological remodelling, a process which is disrupted in breast cancer (Parashurama et al., 2012). Development of normal mammary epithelium in mice consists of terminal end bud (TEB) invasion and elongation of ductal structures during the juvenile period and later side branching and alveoli development during adulthood (Parashurama et al., 2012). Epithelial and fibrous stromal tissues, collectively referred to as mammographic density, are considered the strongest risk factor for breast cancer in humans (McCormack and dos Santos Silva, 2006, Thurfjell 2002). In mice, research using Tamoxifen a potent protector against chemical induced mammary carcinogenesis, has shown both mammary gland mass and density are indicative of mammary cancer risk (McGinley and Thompson, 2011). In addition to mammary density, previous research has identified TEBs as structures that form mammary tumours when exposed to chemical carcinogens (Hilakivi-Clarke et al., 2006). TEBs extend into the mammary fat pad during puberty forming the epithelial tree and are considered the most actively growing ductal structures involved in cellular proliferation and differentiation. Furthermore, TEBs are thought to contain populations of stem cells and progenitor cells that can give rise to either luminal or basal/myoepithelial cell lineages and cancer stem cells that are thought to contribute to breast cancer development and persistence (Wiseman and Werb, 2002). A further morphological feature implicated in breast cancer consists of aberrant ductal remodelling (Li et al., 2000). In mouse models of breast cancer, such as the Mouse Mammary Tumour Virus-Wnt1 Oncogene breast cancer model (MMTV-Wnt1), increased numbers of branched ducts are indicative of maladaptive remodelling (Li et al., 2000). These changes have been associated with extracellular matrix modulators that include matrix metalloproteinases (MMPs), inhibitors of matrix metalloproteinases (TIMPs), cell surface proteins and soluble factors/co-receptors (Blavier et al., 2006, Kapoun and Shackleford, 1997, Ettenberg et al., 2010).

Exposure to dietary factors during prenatal and postnatal development has been shown to induce persistent changes in mammary gland morphology which may modify breast cancer risk (Sie et al., 2009). Currently, it is unclear exactly how changes in levels of dietary folic acid intake

affect mammary gland morphology with several studies providing contrasting results. Research by Meher et al., (2013) has shown that folic acid deficient mice demonstrate an absence of lactating ducts in their mammary glands (Meher et al., 2013). Furthermore, a study by Sie *et al.*, (2009) investigating the effects of folic acid supplementation in utero and during lactation, has shown that murine pups, demonstrate reduced numbers of TEBs and a lower breast cancer risk (Sie et al., 2009). Research suggests that dysregulated mammary remodelling is a major risk factor for breast cancer and that folic acid supplementation at physiological concentrations, equivalent to those recommended during pregnancy, functions to modify TEB numbers in offspring and enhance the development of pre-existing neoplastic lesions in rats (Deghan Manshadi et al., 2014, Sie et al., 2009). This suggests that folic acid plays a role in mammary gland remodelling and also in breast cancer progression. However, currently it is unclear as to the effects of varying concentrations of supplemental folic acid during different periods of mammary gland development and maturation on gland morphology and the long term implications on breast health. Furthermore, little is known about the effects of folic acid supplementation on mammary morphology after the period of supplementation, a question that is highly relevant to women currently recommended to supplement with high levels of folic acid during pregnancy and who discontinue intake postnatally.

With folic acid intake increasing globally due to mandatory fortification and supplementation it is necessary to clarify how concentration ranges and timing of folic acid supplementation may affect mammary structure and morphology. To address these questions mammary whole mounts were used to assess the effects of varying concentrations of folic acid (1 mg/kg, 5 mg/kg, 20 mg/kg) supplemented during either juvenile development or adulthood on mammary epithelial density, number of epithelial ductal branches, number of epithelial junctions and the number of TEBs after a four or twelve week maintenance diet (2.1 mg/kg). Whole mount morphological analysis is considered an optimal method to assess changes in murine mammary morphology (de Assis et al., 2010). From this it will be possible to determine if changes in dietary folic acid concentrations during the juvenile stage of mammary gland development or adulthood induce changes in mammary morphology that persist for either 4 or 12 weeks after the period of supplementation. This will inform on the effect of both the level and timing of dietary folic acid supplementation on aspects of mammary morphology associated with breast cancer risk.

4.1.1 Aims

- 1) To investigate if variations in concentrations of dietary folic acid intake supplemented for 28 days during the juvenile period or adulthood leads to changes in epithelial density, the number of epithelial branches, epithelial junctions or TEBs within the murine mammary gland that persist for 28 or 84 days after the period supplementation.

4.2 Methods

Female virgin C57BL/6 mice (10 animals in each experimental dietary group) were fed modified A193M diets containing basal (1 mg/kg), high (5 mg/kg) or supra-maximal (20 mg/kg) concentrations of folic acid in line with the detailed experimental design outlined in chapter 2.1.3. The second and third thoracic mammary glands were excised, whole mounted onto glass slides, fixed in Carnoy's solution and stained in carmine alum solution. Mammary epithelial density, number of epithelial branches, number of junctions and the number of TEBs were calculated (Chapter 2.2). Data was used to compare markers of mammary development and remodelling associated with breast cancer risk. Statistical analysis was performed using 1 way ANOVA with Dunnett post hoc test or independent t test with the P value set to $P < 0.05$.

4.3 Results

4.3.1 Folic Acid Supplementation during the Juvenile Period Induces dose Specific Changes in Mammary Gland Morphology

4.3.1.1 Mammary Epithelial Density and Number of Epithelial Branches

Mammary epithelial density was measured in female mice in response to folic acid supplementation during juvenile development (Figure 4.1). Statistical analysis showed no significant differences in epithelial density in the medial or distal regions of the mammary glands in mice fed 5mg/kg or 20 mg/kg 4 weeks after supplementation (PND 84) (Figure 4.1). A significant increase ($P = 0.039$) in epithelial density was observed at 5 mg/kg in the medial sections of the mammary glands 12 weeks after supplementation. This shows that folic acid induces changes in epithelial tree density occur long after the period of supplementation. No significant differences in epithelial density were observed in distal sections (Figure 4.1). Numbers of mammary epithelial branches were calculated after supplementation with each folic acid concentration (Figure 4.2). Statistical analysis showed no significant differences in branch number in the medial or distal sections of the mammary glands in mice fed 5 mg/kg or 20 mg/kg folic acid 4 weeks (PND 84) after supplementation (Figure 4.2). Furthermore, no significant differences in numbers of epithelial branches were observed in mice fed 5 mg/kg or 20 mg/kg folic acid 12 weeks (PND 140) after supplementation (Figure 4.2).

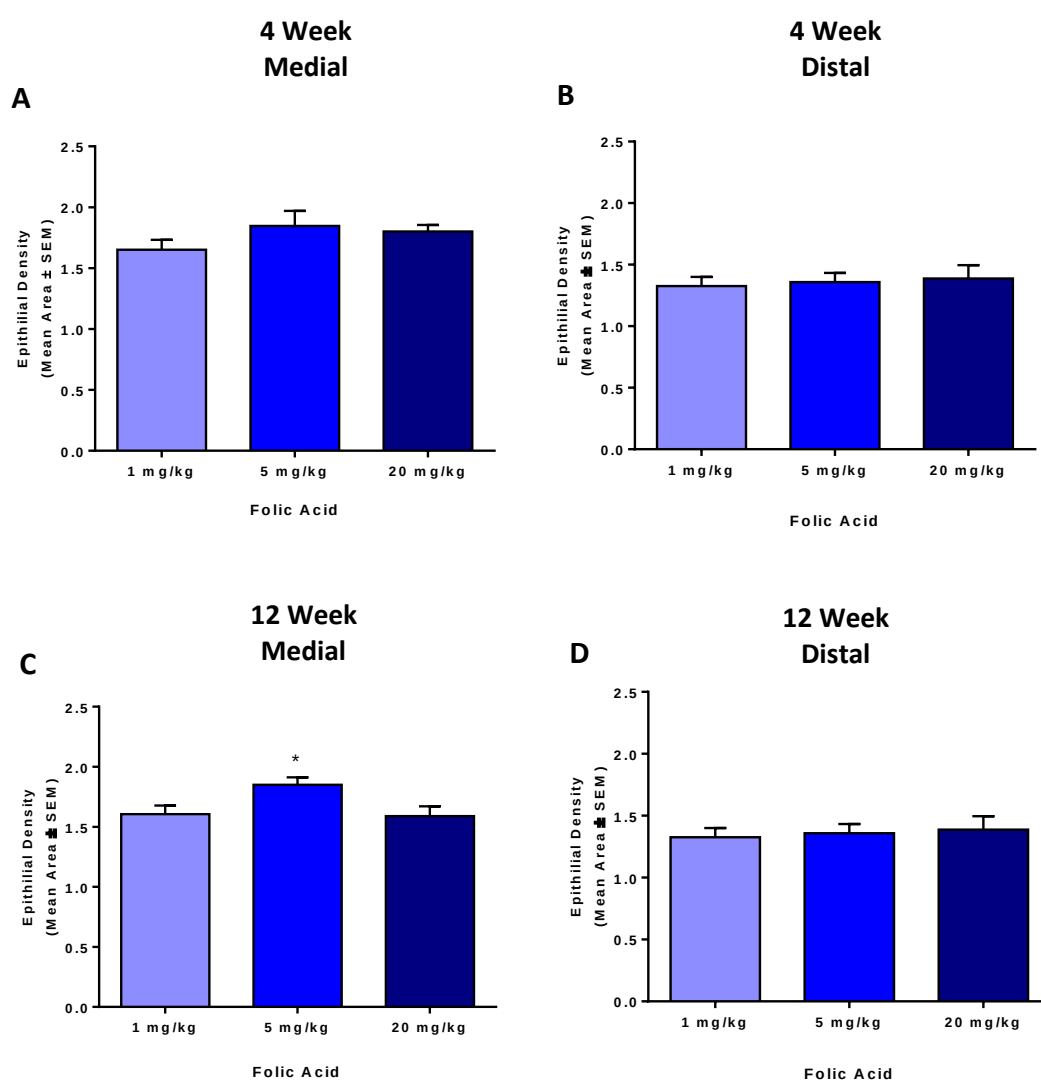


Figure 4.1. Epithelial Density of Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during the Juvenile Period.

(A, B) Mammary epithelial density in mice supplemented with 1 mg/kg (1 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) folic acid for 4 weeks (PND 28 – 56) followed by a 4 week (A, B) maintenance diet (PND 56-84) or (C, D) 12 week maintenance diet (PND 56-140). Data presented as mean ± SEM. n = 8-10 per group.

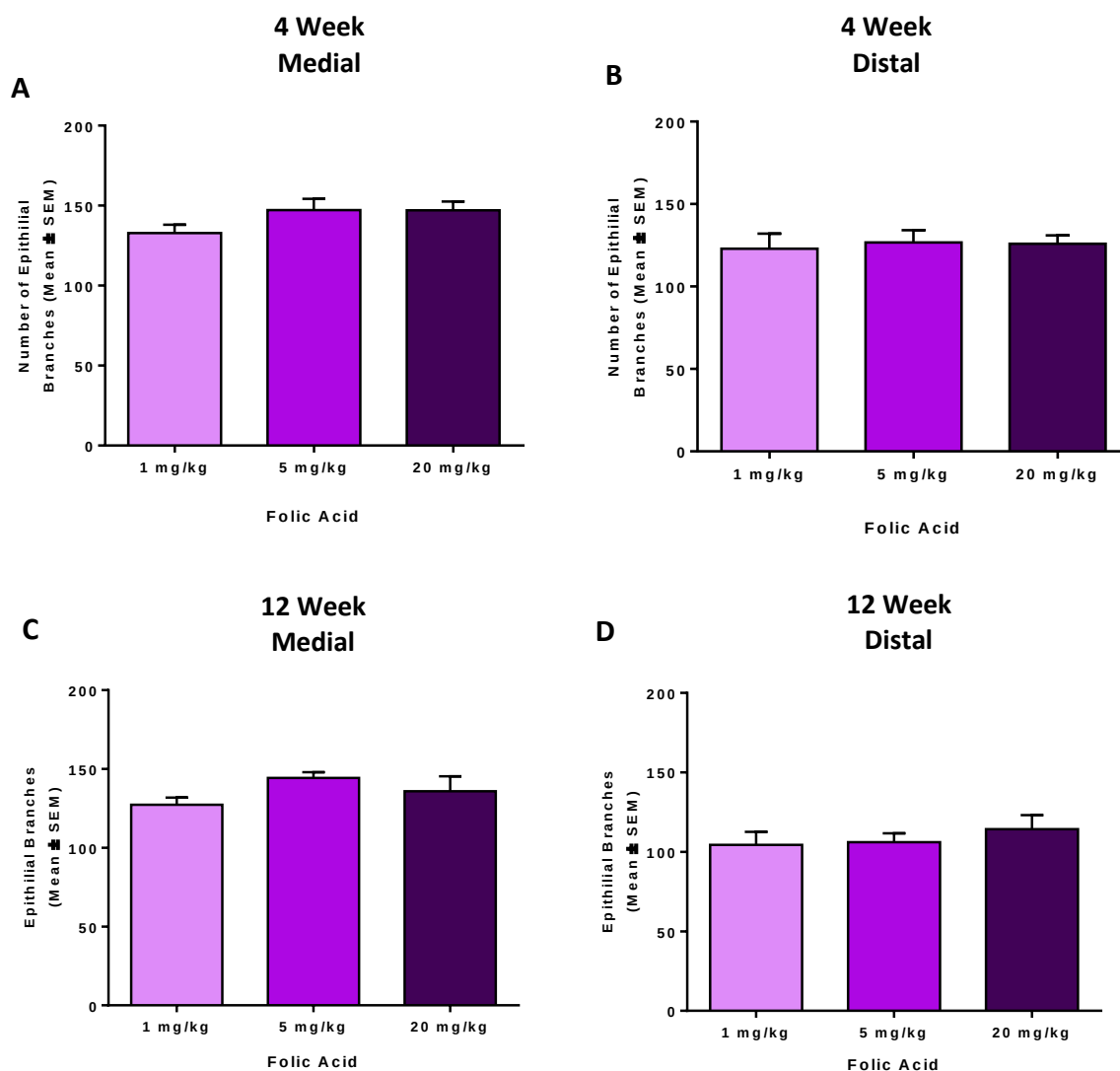


Figure 4.2. Number of Epithelial Branches in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during the Juvenile Period.

(A, B) Number of epithelial branches in mice supplemented with 1 mg/kg (1 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) folic acid for 4 weeks (PND 28 – 56) followed by a 4 week (A, B) maintenance diet (PND 56-84) or (C, D) 12 week maintenance diet (PND 56-140). Data presented as mean ± SEM. n = 8-10 per group.

4.3.1.2 Number of Mammary Epithelial Junctions and Terminal End Buds

Numbers of mammary epithelial junctions were calculated from branching structures within the mammary epithelial tree (Figure 4.3). Statistical analysis showed a non-significant trend towards an increase ($P = 0.081$) in the number of epithelial junctions in mice fed 5 mg/kg in the medial section of the mammary gland at PND 84. No significant differences were observed in the distal regions of the mammary glands in mice fed 5 mg/kg or 20 mg/kg folic acid for 4 weeks after supplementation (PND 84) (Figure 4.3). Furthermore, no significant differences in numbers of junctions were observed in mice fed 5 mg/kg or 20 mg/kg folic acid 12 weeks (PND 140) after supplementation (Figure 4.3). TEB analysis showed a significant increase ($P = 0.004$) in the medial section of the mammary glands in mice supplemented with 20 mg/kg folic acid at PND 84, however, this effect did not persist 12 weeks after supplementation (PND 140). A significant increase ($P = 0.025$) in TEBs was observed in the medial section of the glands in mice fed 5 mg/kg 12 weeks after supplementation (PND 140). No significant differences were observed in the distal sections of the glands 4 or 12 weeks after supplementation (Figure 4.4).

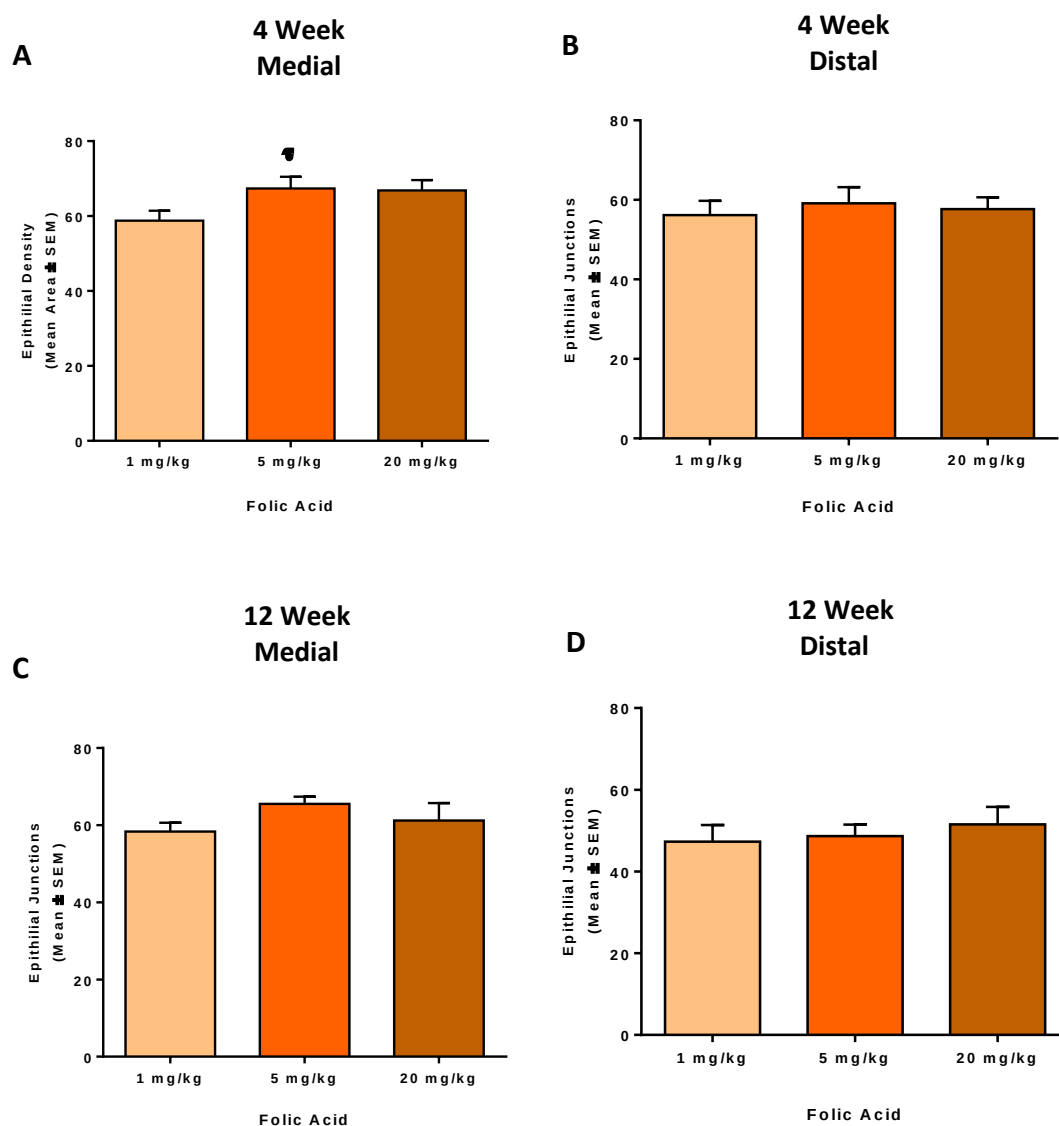


Figure 4.3. Number of Epithelial Junctions in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during the Juvenile/Pre-Pubertal period (A, B).

Mammary epithelial density in mice supplemented with 1 mg/kg (1 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) or (C, D) 12 week maintenance diet (PND 56-140). Data presented as mean $\pm$ SEM. n = 8-10 per group.

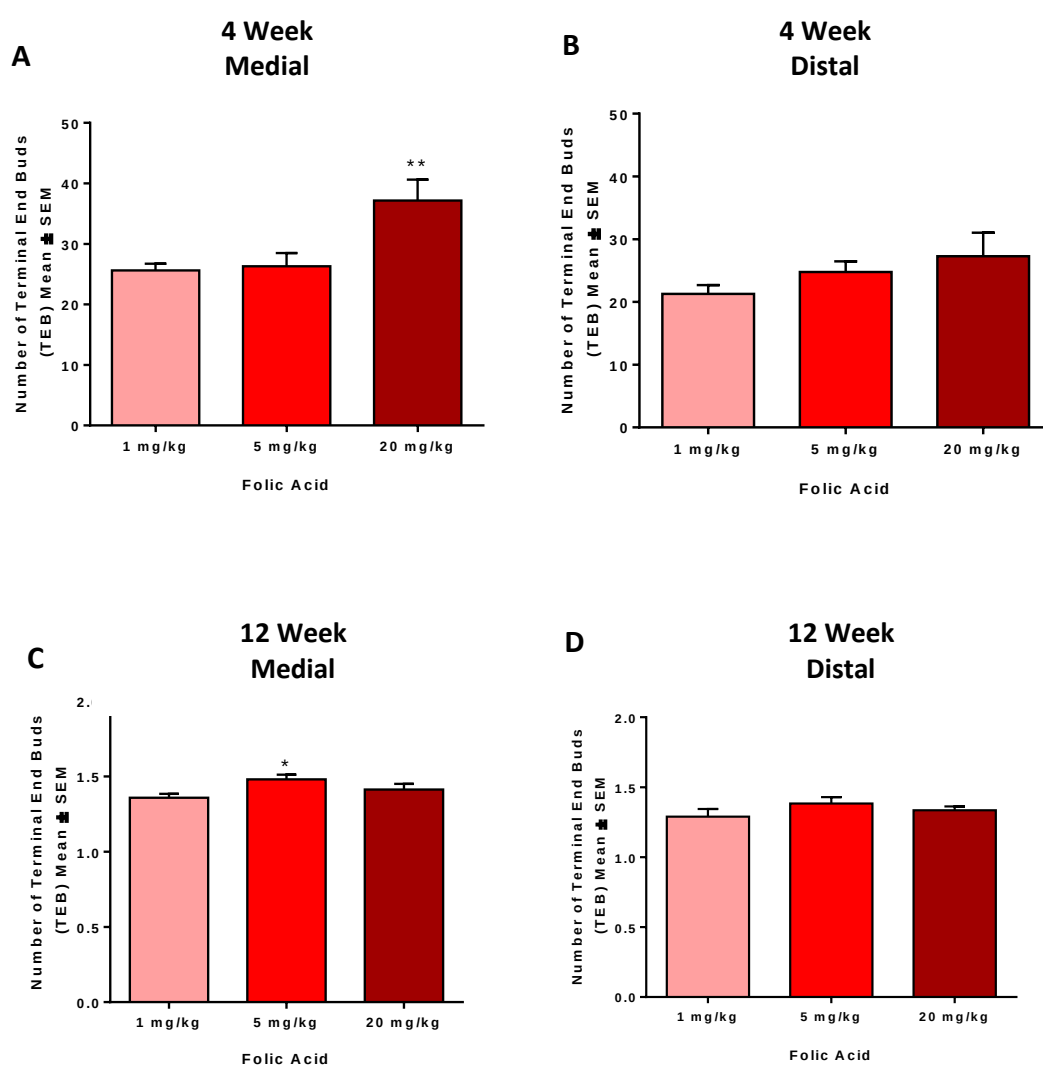


Figure 4.4. Number of TEBs in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Folic Acid during the Juvenile Period.

(A, B) TEBs in mice supplemented with 1 mg/kg (1 x BDR), 5mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) folic acid for 4 weeks (PND 28-56) followed by a 4 week maintenance diet (PND 56-84) or (C, D) 12 week maintenance diet (PND 56-140). Data presented as mean ± SEM. n = 7-10 per group.

4.3.2 Folic Acid Supplementation during Adulthood Induces dose Specific Changes in Mammary Gland Morphology

4.3.2.1 Epithelial Density and Number of Epithelial Branches

Statistical analysis showed no significant increase in epithelial density in the medial or distal region of the mammary glands in mice fed 5 mg/kg or 20 mg/kg compared to 1 mg/kg 4 weeks (PND 130) or 12 weeks (PND 186) after folic acid supplementation (Figure 4.5). Furthermore, no statistically significant differences in the number of epithelial branches were observed at 4 weeks (PND 130) or 12 weeks (PND 186) after supplementation (Figure 4.6).

4.3.2.2 Number of Epithelial Junctions and Terminal End Buds

Epithelial Junctions ($p = 0.038$) significantly increased in mice fed 20 mg/kg folic acid in the distal section of the mammary gland at PND 130. However, this effect did not persist after 12 weeks. No significant differences in the number of epithelial junctions were observed in the medial regions of the mammary glands 4 weeks (PND 130) or the distal and medial regions 12 weeks (PND 186) after folic acid supplementation (Figure 4.7).

TEBs in the distal section of the mammary glands significantly increased in mice supplemented with 20 mg/kg of folic acid 12 weeks after supplementation (PND 186). No significant differences were observed in the medial and distal regions 4 weeks (PND 130) after supplementation or the medial region 12 weeks (PND 186) after supplementation (Figure 4.8).

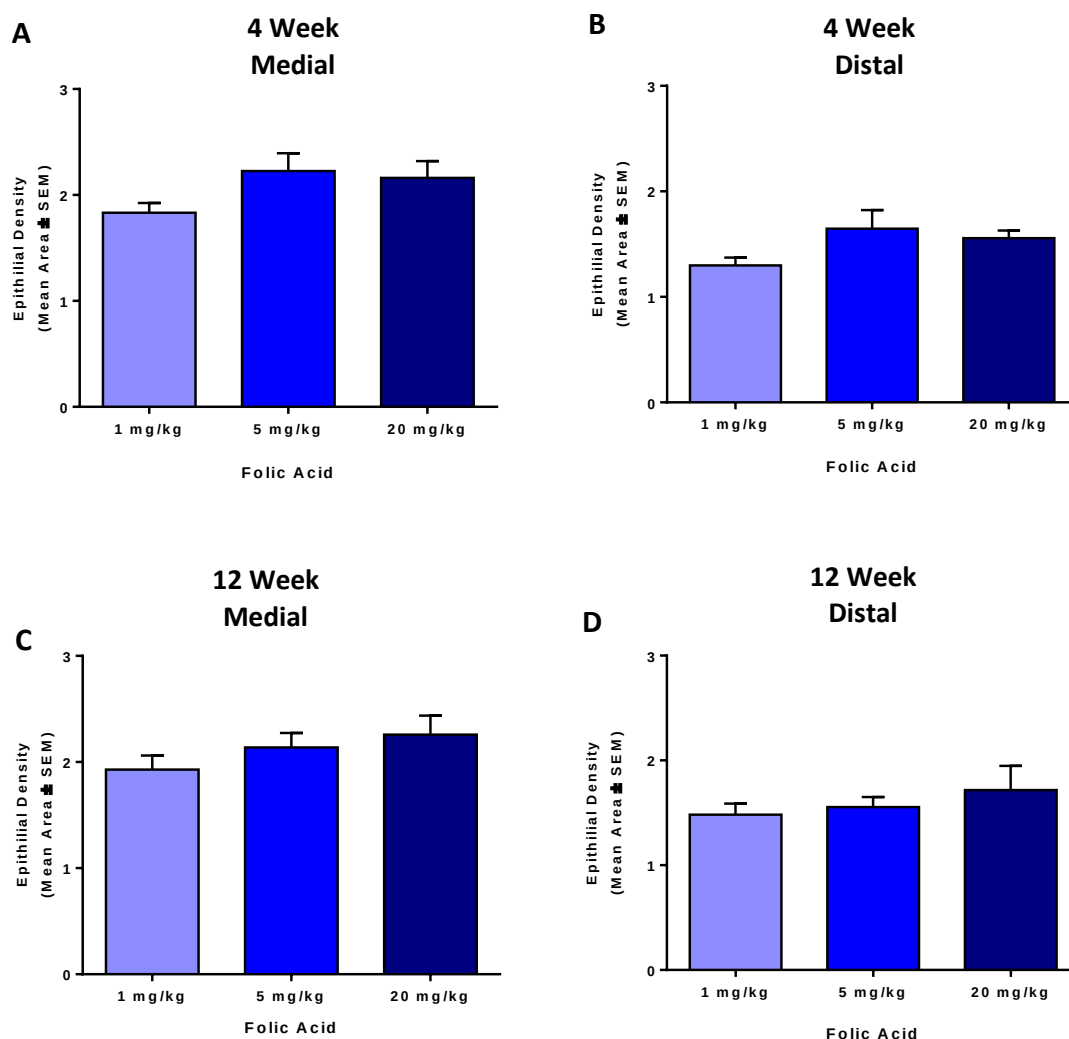


Figure 4.5. Epithelial Density in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during Adulthood

(**A, B**) Mammary epithelial density in mice supplemented with 0.2 mg/kg (0.2 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) or (**C, D**) 12 week maintenance diet (PND 56-140). Data presented as mean ± SEM. n = 8-10 per group.

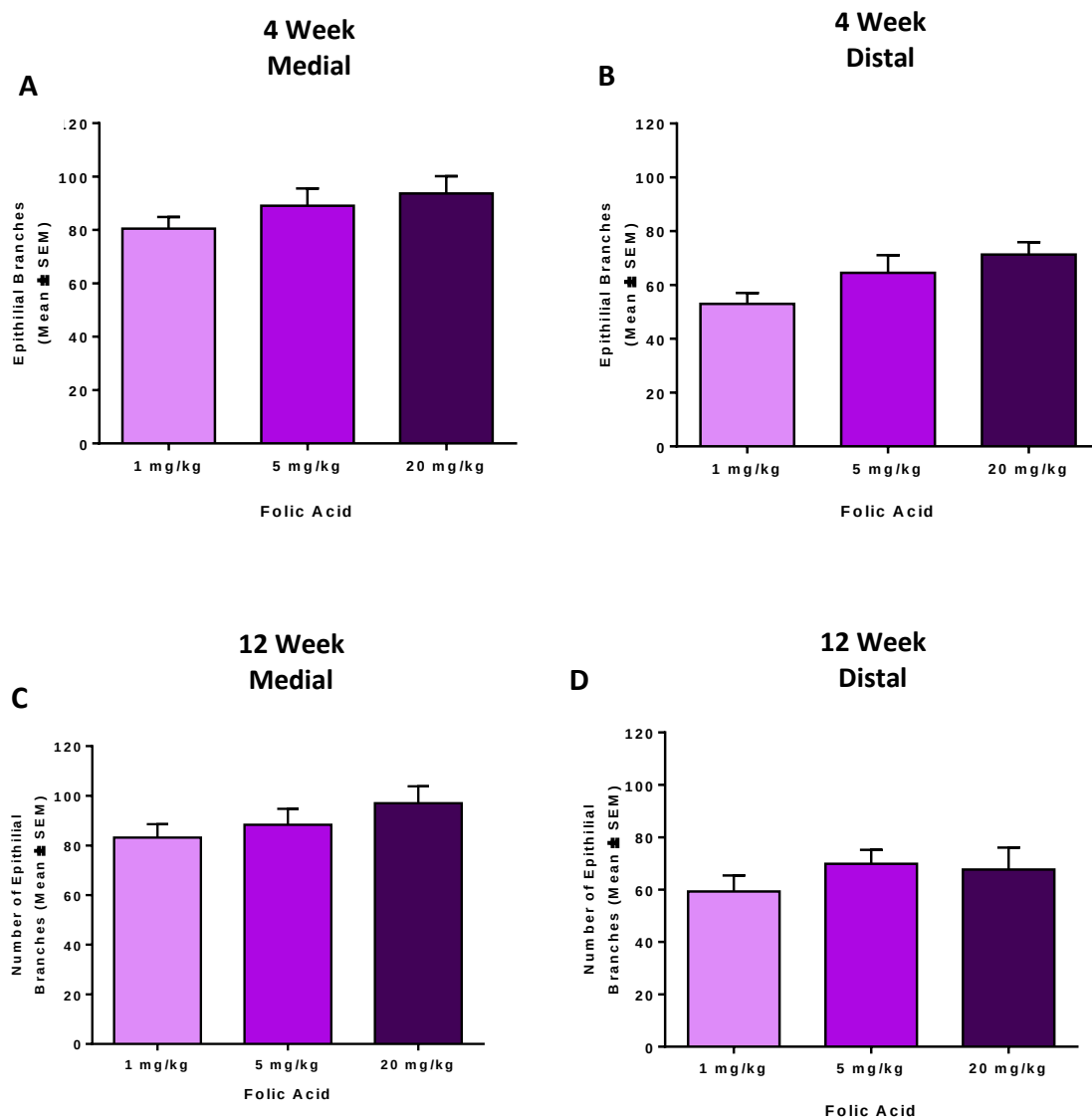


Figure 4.6. Epithelial Branches in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during Adulthood.

(A, B) Mammary epithelial density in mice supplemented with 0.2 mg/kg (0.2 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) FA for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) or (C, D) 12 week maintenance diet (PND 56-140). Data presented as mean $\pm$ SEM. n = 8-10 per group.

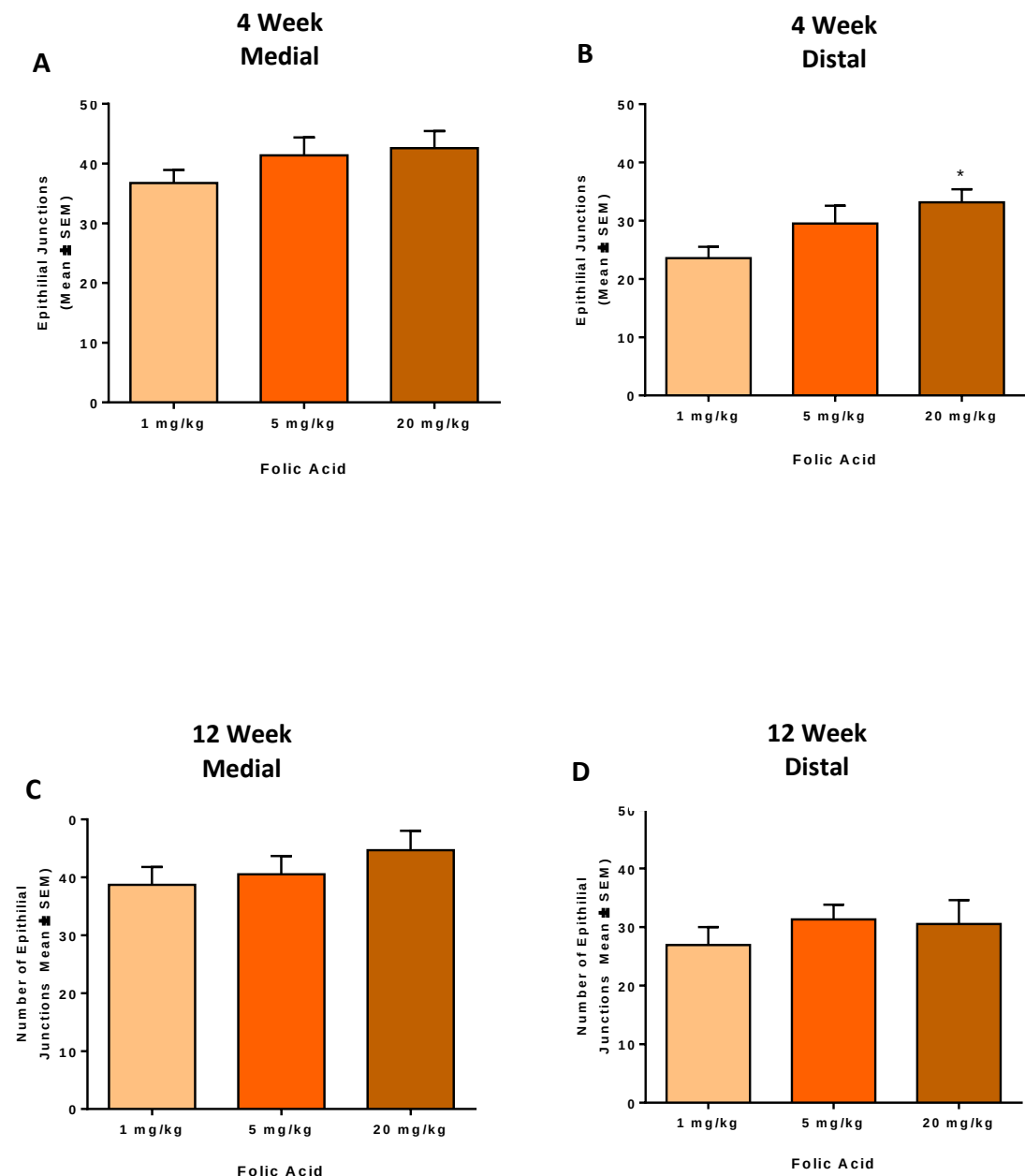


Figure 4.7. Number of Epithelial Junctions in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Increasing Concentrations of Folic Acid during Adulthood (A, B) Epithelial junctions in mice supplemented with 1 mg/kg (1 x BDR), 5 mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) Folic Acid for 4 weeks (PND 74 – 102) followed by a 4 week maintenance diet (PND 102-130) or (C, D) 12 week maintenance diet (PND 102-186). Data presented as mean $\pm$ SEM. n = 8-10 per group.

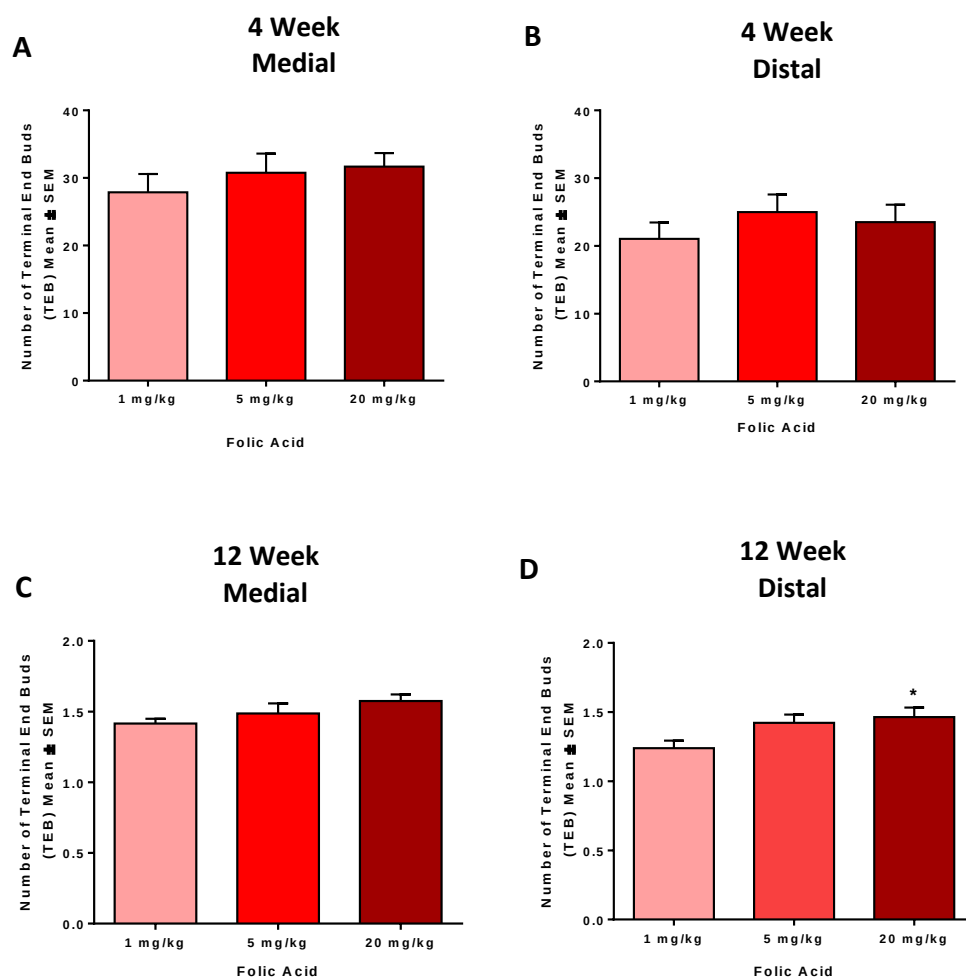


Figure 4.8. Number of TEBs in Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Supplemented with Folic Acid during Adulthood:

(A, B) TEBs in mice supplemented with 1 mg/kg (1 x BDR), 5mg/kg (5 x BDR) or 20 mg/kg (20 x BDR) folic acid for 4 weeks (PND 74-102) followed by a 4 week maintenance diet (PND 102-130) or (C, D) 12 week maintenance diet (PND 102-186). Data presented as mean ± SEM. n = 7-10 per group.

4.3.3 Plasma Hormone Levels Do Not Effect Folic Acid Mediated Changes in Mammary Morphology

Plasma hormone levels of estradiol-17 β and progesterone measured 4 weeks after folic acid supplementation were analysed using linear regression analysis against epithelial density and TEB data to ascertain if hormone levels which correspond to estrus status effect folic acid mediated changes in morphology (Table 4.1).

No significant effects were observed between plasma levels of estradiol-17 β and progesterone on epithelial density and TEB number in juvenile or adult supplemented mice suggesting hormone levels and estrus status does not effect changes in morphology in response to folic acid.

Table 4.1. Linear Regression Analysis of Plasma Hormone Concentration and Mammary Gland Morphology.

Morph	Estradiol-17 β					Progesterone				
	R ²	Adj R ²	Standardised Coefficient β	t	Sig	R ²	Adj R ²	Standardised Coefficient β	t	Sig
Juvenile Supplemented Mice										
Epithelial Density	0.000	-0.031	0.020	0.114	0.910	0.069	0.040	0.262	1.539	0.134
TEBs	0.060	0.032	0.244	1.468	0.151	0.001	-0.028	-0.036	-0.211	0.834
Adults Supplemented Mice										
Epithelial Density	0.001	-0.031	0.037	0.204	0.839	0.001	-0.030	-0.023	-0.135	0.894
TEBs	0.021	-0.013	-0.145	-0.789	0.436	0.063	0.034	0.252	1.472	0.151

4.4 Discussion

Results from the present study demonstrate that supplementing female C57BL/6 mice with folic acid for a period of 4 weeks during either the juvenile/pre-pubertal period (PND 28-56) or adulthood (PND 74-102) induces changes in mammary gland morphology that include increased density of the epithelial tree (juvenile supplemented mice), increased number of epithelial junctions (adult supplemented mice) and an increase in TEBs (adult and juvenile supplemented mice). Changes in morphology are both concentration and timing specific and in some cases persist for either 4 or 12 weeks after the period of supplementation (Table 4.2).

Table 4.2. Summary of Morphological Changes in Mice Supplemented with Folic Acid for 28 days during the Juvenile Period or Adulthood followed by a 4 week Maintenance Diet.

Morphology	Folic Acid Supplementation during the Juvenile Pre-pubertal period (PND 28-56)							
	4 weeks after supplementation (PND 84)				12 weeks after supplementation (PND 140)			
	5 mg/kg		20 mg/kg		5 mg/kg		20 mg/kg	
	Medial	Distal	Medial	Distal	Medial	Distal	Medial	Distal
Epithelial Density	-----	-----	-----	-----	↑	-----	-----	-----
Number of Branches	-----	-----	-----	-----	-----	-----	-----	-----
Number of Junctions	-----	-----	-----	-----	-----	-----	-----	-----
Number of TEBs	-----	-----	-----	↑	↑	-----	-----	-----
Morphology	Folic Acid Supplementation during the Adulthood (PND 74-102)							
	4 weeks after supplementation (PND 130)				12 weeks after supplementation (PND 186)			
	5 mg/kg		20 mg/kg		5 mg/kg		20 mg/kg	
	Medial	Distal	Medial	Distal	Medial	Distal	Medial	Distal
Epithelial Density	-----	-----	-----	-----	-----	-----	-----	-----
Number of Branches	-----	-----	-----	-----	-----	-----	-----	-----
Number of Junctions	-----	-----	-----	↑	-----	-----	-----	-----
Number of TEBs	-----	-----	-----	-----	-----	-----	-----	↑

4.4.1 Mammary Gland Morphology in Mice Supplemented during the Juvenile Period

Minimal changes were observed in mammary gland morphology in response to variations in the level of folic acid supplementation during the juvenile period. Epithelial density was increased in the medial region of the mammary gland in mice fed 5 mg/kg 12 weeks (PND 140) after the period of supplementation when compared to the 1 mg/kg control group (Figure 4.1). However, no changes in mammary epithelial density were observed 4 weeks after supplementation (PND 84) suggesting that 5 mg/kg of folic acid supplemented for 4 weeks during mammary development induces changes in morphology that become evident long after the initial period of supplementation (Figure 4.1). This suggests folic acid may induce modifications in the programming of epithelial cells during juvenile development that result in a change in density once the mammary gland has reached maturity. Furthermore, this suggests that the effects of folic acid on mammary morphology are both concentration and duration specific when supplemented during a period of increased mammary plasticity and development. Interestingly changes observed in density at 5 mg/kg do not correlate with changes in polycomb, *Brca1* or *Oct-4* gene expression suggesting regulation through alternative mechanisms. Research has linked changes in mammary morphological regulation with expression of estrogen genes, specifically estrogen receptor alpha ($Er\alpha$) and beta ($Er\beta$) (Cabanes et al., 2004). These genes would therefore provide putative targets to investigate regulation of morphology in response to folic acid intake.

The numbers of TEBs were found to be increased in the distal section of mammary glands in mice fed 20 mg/kg compared to 1 mg/kg 4 weeks (PND 84) after supplementation. This effect did not persist 12 weeks (PND 140) after supplementation, suggesting changes in TEB development in response to folic acid persist for a minimum of 4 weeks post supplementation but are remodelled after 12 weeks. Alternatively, changes that persist for 4 weeks after supplementation (PND 86) may not be visible at 12 weeks (PND 140) due to the control (1 mg/kg) group's epithelial development being able to catch up with increased development in response to high levels of folic acid during the supplementation period. Interestingly these effects were only observed in the distal section of the mammary gland suggesting mammary epithelial expansion into the fat pad as opposed to an increase in side branching from more developed epithelial ductal structures.

TEB numbers were further found to be increased in mice fed 5 mg/kg folic acid 12 weeks after supplementation with no effect observed after 4 weeks (PND 86). These findings are in line with

observations in epithelial density in mice fed 5 mg/kg suggesting folic acid induces an effect on morphology that occurs long after the period of supplementation. Furthermore, the increased epithelial density observed in juvenile mice fed 5 mg/kg 12 weeks (PND 140) after supplementation may be directly related to the increase in number of TEBs observed at this time point suggesting a folic acid mediated increase in mammary ductal development and expansion.

4.4.2 Mammary Gland Morphology in Mice Supplemented during Adulthood

Mice supplemented during adulthood showed changes in the number of epithelial junctions and terminal end buds post maintenance diet. The number of epithelial junctions was increased in the distal mammary section of mice fed 20 mg/kg compared to 1 mg/kg 4 weeks (PND 130) after supplementation. This effect did not persist after 12 weeks (PND 186) and was not related to an increase in epithelial density, number of epithelial branches or TEB numbers within the mammary gland. This suggests that high levels of folic acid modulates the existing epithelial tree structure but does not indicate new epithelial development or expansion into the fat pad. Interestingly, high levels of folic acid therefore may modulate the structure of the existing mammary epithelial tree during the 4 week period after supplementation.

The numbers of TEBs were found to be increased in the distal section of mammary glands in mice fed 20 mg/kg compared to 1 mg/kg 12 weeks (PND 186) after supplementation. This effect was not observed 4 weeks (PND 130) after supplementation, suggesting changes in TEB development in response to folic acid affect epithelial development long after supplementation. This mirrors effects observed in juvenile supplemented mice fed 5 mg/kg where changes in numbers of TEB were also observed 12 weeks after supplementation. The lack of changes observed in epithelial density, epithelial branches and junctions suggests that increased numbers of TEBs may indicate the initiation of development of new epithelial structures. The locations of increased TEBs within the distal section of the mammary gland suggests a folic acid mediated expansion of the mammary tree into the fat pad via TEB mediated ductal cellular proliferation.

4.5 Conclusion

In conclusion, this study has investigated the effects of varying concentrations of folic acid supplementation at two different time points of the life-course on mammary gland morphology, namely epithelial density, the number of epithelial branches, epithelial junctions and TEBs. Key findings from this study have identified that in mice supplemented for 4 weeks during the juvenile period, a supra-maximal level of folic acid (20 mg/kg) increases the number of TEBs, distinct markers of mammary epithelial ductal expansion and development, which persist for up to 4 weeks (PND 84) after the supplementation period (Hinck and Silberstein, 2005). In addition to changes observed 4 weeks after supplementation, folic acid further induces changes in epithelial density (equivalent to mammographic density in humans) and TEBs when supplemented at 5 mg/kg 12 weeks (PND 140) after supplementation, suggesting modified numbers of TEBs may contribute to an increase in epithelial density modulated by folic acid intake.

Supplementation with a supramaximal level of folic acid (20 mg/kg) for 4 weeks during adulthood increases the number of epithelial junctions which persists for up to 4 weeks (PND 130) post supplementation, suggesting epithelial tree remodelling. Furthermore, at the same concentration (20 mg/kg) folic acid increases the number of TEBs 12 weeks (PND 186) after supplementation. Interestingly, all changes in morphology observed in response to folic acid supplementation during the juvenile period or adulthood showed increased numbers of morphological markers suggesting folic acid functions to promote epithelial morphological development.

In summary, folic acid supplemented during juvenile development in mice therefore induces long term persistent effects on key markers of mammary cancer risk when supplemented in a dose (5 mg/kg) equivalent to that recommend to women who are pregnant or have had a history of neural tube defects in many countries (Deghan Manshadi et al., 2014). Furthermore, folic acid intake during adulthood induces changes in mammary gland modelling and markers of mammary cancer risk when supplemented with a supramaximal dose equivalent to what may be consumed with combined fortified food consumption and supplementation in countries that use mandatory fortification of folic acid in staple foods.

Chapter 5: The Effects of Folic Acid Supplementation during Adulthood on Protein Expression of Epithelial and Cell Differentiation Markers in the Female C57BL/6 Mouse Mammary Gland.

5.1 Introduction

In line with previous findings showing folic acid mediated changes in *Brca1*, *Oct-4*, polycomb regulator genes and mammary gland morphology, this Chapter investigates the cellular changes in mammary gland protein markers associated with mammary stem cell regulation, epithelial proliferation and cellular lineage. Studies in rodents have shown that folic acid increases the development of pre-existing mammary neoplasms and modulates levels of protein expression involved in apoptosis, cellular proliferation and breast carcinogenesis (Deghan Manshadi et al., 2014). Research suggests that during normal development mechanisms that regulate cellular differentiation are also implicated in tumorigenesis (Chou et al., 2010). Key protein differentiation and epithelial markers have been established that correlate with the metastatic potential of breast tumours and are implicated in breast cancer risk (Chou et al., 2010, Iyer et al., 2013). Mammary markers implicated in breast cancer risk may be regulated through epigenetic mechanisms that modulate gene and protein expression. Furthermore, folic acid, which plays a major role in the donation of one carbon groups to methylation reactions, may function in the epigenetic regulation of these protein markers.

The polycomb gene *Ezh2* is considered a marker of aggressive breast cancer and has been shown to promote the neoplastic transformation of breast epithelial cells. Both *EZH2* transcript and protein have been shown to be consistently elevated in invasive breast carcinoma and strongly correlated with breast cancer aggressiveness (Kleer et al., 2003). Data from the present study has shown that folic acid supplementation during adulthood induces a persistent increase in *Ezh2* expression. Research suggests that overexpression of *EZH2* is detected in invasive breast carcinoma tissue samples and also has been identified in human breast tissue samples up to 12 years prior to breast cancer development (Li et al., 2009a). Research by Li et al., (2009) using targeted over expression of *Ezh2* in transgenic mice has shown aberrant terminal end bud architecture, with 100% of female mouse mammary tumor virus-*EZH2* virgin mice developing intraductal epithelial hyperplasia. Mice also showed premature differentiation of ductal epithelial cells and up-regulation of the luminal cell marker GATA-3 (Li et al., 2009a). Increased levels of *Ezh2* in the present study may function through a mammary progenitor/stem cell pathway to directly modulate levels of luminal cells, which in turn modifies mammary epithelial architecture.

Along with *Ezh2* both *Brca1* and *Oct-4* proteins, involved in DNA repair and cellular pluripotency, respectively, are considered stem cell regulators and markers of breast cancer risk (Foulkes,

2004). Research has indicated Brca1 and Oct-4 as critical in the regulation of normal mammary gland development and maintenance, of which aberrant expression has been shown to modify levels of cellular differentiation and proliferation (Wang and Di, 2014, Hassiotou et al., 2013b). Furthermore, both Brca1 and Oct-4 have been linked with Ezh2 in mammary stem cell function and as markers of breast carcinogenesis (Wang and Huang, 2013, Bae and Hennighausen, 2014). The present study has shown persistently increased expression of Brca1 and Oct-4 in response to high levels of dietary folic acid. Induction of Brca1 expression in epithelial cell lines has been shown to modify expression of genes implicated in breast tumorigenesis including extracellular matrix protein laminin A3 (Welch et al., 2002). However, Ezh2 has also been directly linked to Brca1 protein function in mammary tissue. Research by Welch et al., (2002) using an EZH2 overexpressing breast cancer cell line, Cal51, proposes that Akt-1 (Ser473) is phosphorylated and activated by Ezh2 which represses nuclear localisation of BRCA1 protein (Welch et al., 2002). Thus, Ezh2 overexpression may repress Brca1 function which may result in changes in DNA repair capacity and these findings, combined with increased levels of *Oct-4* expression in the present study, further suggests a role in regulation of stem/progenitor cell function which may modulate mammary cell populations.

Key markers of the mammary gland basal/myoepithelial cell and luminal populations consist of α -SMA and cytokeratin 8, respectively. During postnatal mammary development, myoepithelial cells progressively acquire a differentiated phenotype and express α -SMA (Mikaelian et al., 2006). Furthermore, the luminal component of the mammary gland has been shown to heterogeneously express cytokeratin 8 (Mikaelian et al., 2006). A further marker of luminal differentiation is Trans-acting T-cell-specific transcription factor GATA-3, a transcription factor that regulates luminal epithelial cell differentiation and commitment within the mammary gland (Chou et al., 2010). Current research has identified GATA-3 as a strong predictor of clinical outcome in human luminal breast cancer (Chou et al., 2010). Furthermore, regulation of cell differentiation in mouse models of breast cancer has been directly linked with GATA-3 (Chou et al., 2010). Regulation of basal myoepithelial and luminal cell populations is, in part, controlled by proteins that regulate the epithelial stem cell pool, including Brca1, Oct-4 and Ezh2. Thus, mammary epithelial expression of both α -SMA, K8 and GATA-3 provide ideal markers to investigate basal/myoepithelial and luminal mammary cell populations in response to folic acid supplementation.

Currently, little is known as to the tissue specific effects of high folic acid intake after the period of supplementation on the localisation and expression of key markers implicated in mammary DNA repair, regulation of pluripotency, differentiation and cell lineage. Research from the present study has shown that in adult mice, high levels of folic acid intake persistently affects the expression of EZH2, Brca1 and Oct-4, which are regulators of mammary cell differentiation. It is therefore necessary to establish if folic acid supplementation during adulthood induces persistent differences in mammary cell populations which may influence breast cancer risk. To address these questions immunohistochemical analysis in mammary tissue of mice supplemented with high levels of folic acid during adulthood was performed. Brca1 and Oct-4 proteins were investigated for mammary localisation and expression along with the basal myoepithelial marker α -SMA and luminal marker Keratin 8. Furthermore, the cellular proliferation marker Ki67 and transcription factor GATA-3, a strong predictor of clinical outcome in human luminal breast cancer cells, were investigated (Chou et al., 2010).

5.2 Aims

- 1) To determine if folic acid supplementation at high (5 mg/kg) or supra-physiological (20 mg/kg) levels for 28 days during adulthood induces persistent changes in the localisation and expression of protein markers *Brca1*, *Oct-4*, *α SMA*, Keratin 8 or Gata-3 within the mammary gland

5.3 Methods

Immunohistochemistry was used to measure levels of protein expression in the mammary glands in response to folic acid supplementation during adulthood. Female C57BL/6 mice (10 animals in each experimental dietary group) were fed modified A193M folic acid diets containing basal (1 mg/kg), or high (5 mg/kg) folic acid in line with the detailed experimental design outlined in chapter 2. The inguinal mammary glands were excised, fixed in 10% NBF and paraffin embedded. Mammary glands (6 animals) were sectioned (4 μ m), mounted onto slides and stained for protein markers using antibodies specific to proteins of interest. Immunohistochemistry was performed using 3,3'-Diaminobenzidine or Immunofluorescence using fluorescently labelled secondary antibodies. Stained sections were imaged and analysed using an inverted Zeiss Axiovert 200 Microscope or an upright Leica microscope (Zeiss, UK). Immunostaining was quantified where possible using Image J software. Statistical analysis was performed using 1 way ANOVA with Dunnett post hoc test or independent t test with the P value set to $P < 0.05$.

5.3.1 High Folic Acid Intake during Adulthood Induces Changes in Mammary Differentiation Marker Proteins 4 Weeks after the period of Supplementation

5.3.2 Brca1 and Oct-4 Protein Expression

To assess the localisation of Brca1 and Oct-4 proteins within the mammary gland and to see if protein expression levels correlate with previous findings showing increased levels of gene expression in response to high level of dietary folic acid, Brca1 and Oct-4 proteins were investigated using immunohistochemistry.

Brca1 protein was localised predominantly in the cytoplasm of basal/myoepithelial and luminal mammary epithelial cells located in mammary ductal structures (Figure 5.1). Within mammary ducts Brca1 showed an increase in levels of expression in mice fed 5 mg/kg and 20 mg/kg. Within mammary alveoli Brca1 showed the same patterns of localisation expression as that identified within the ducts (Figure 5.2).

Oct-4 showed a speckled appearance localised within the cell nucleus and in line with Brca1 expression showed increased fluorescence in mice fed 5 and 20 mg/kg folic acid in alveoli and ductal structures (Figure 5.3, 5.4).

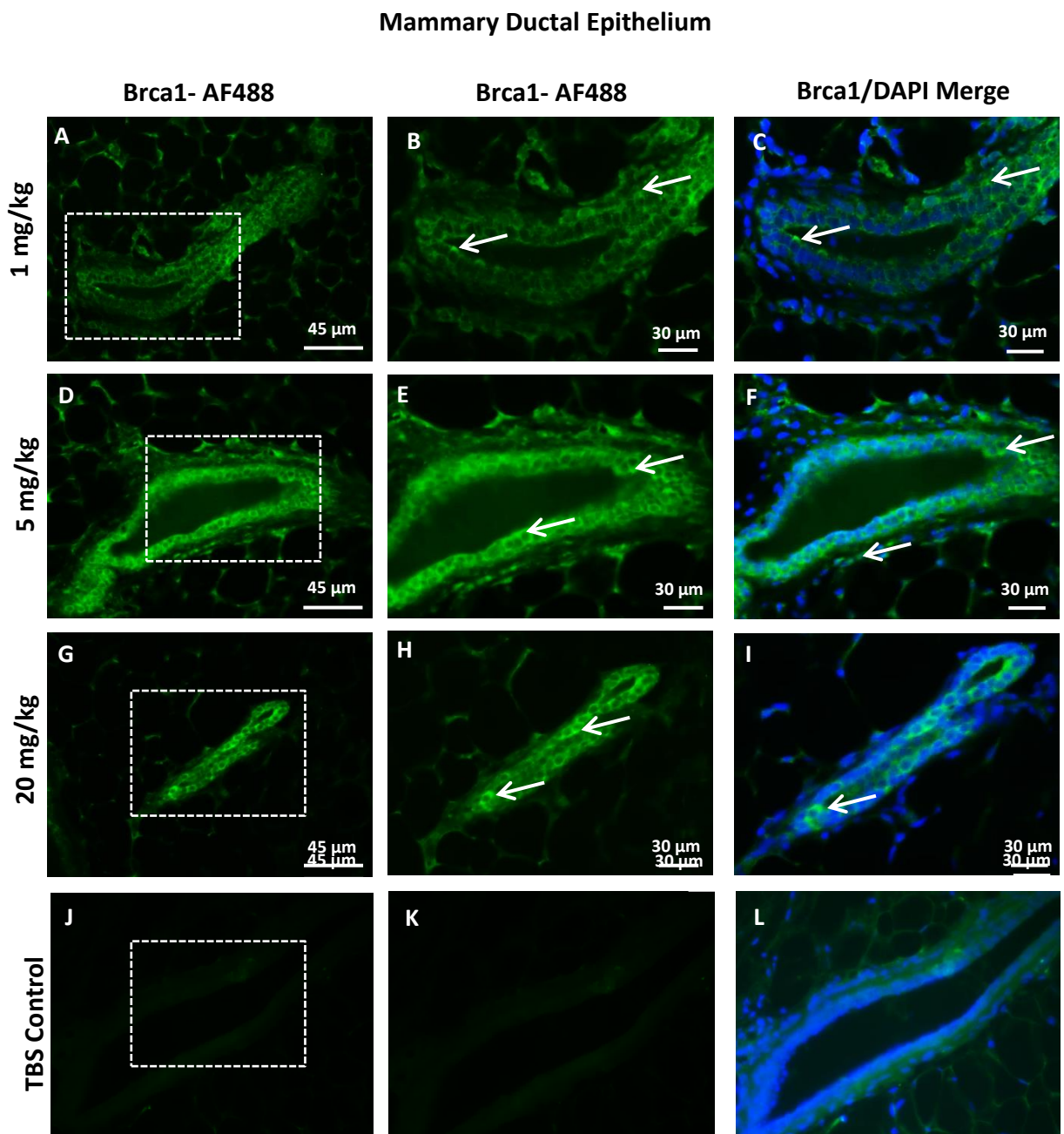


Figure 5.1 Brca1 Staining in Folic Acid Supplemented C57BL/6 Mouse Mammary Ductal Epithelium.

Representative images of Brca1 positive (green) mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (**A, B, C**), 5 mg/kg (**D, E, F**) or 20 mg/kg (**G, H, I**) folic acid for 4 weeks followed by a 4 week maintenance diet. Increased magnification of images (**B, E, H**). Merge, Brca1 (green) and DAPI (Blue) stain identifying location of epithelial cell nuclei (**C, F, I**). TBS no primary control (**J, K, L**).

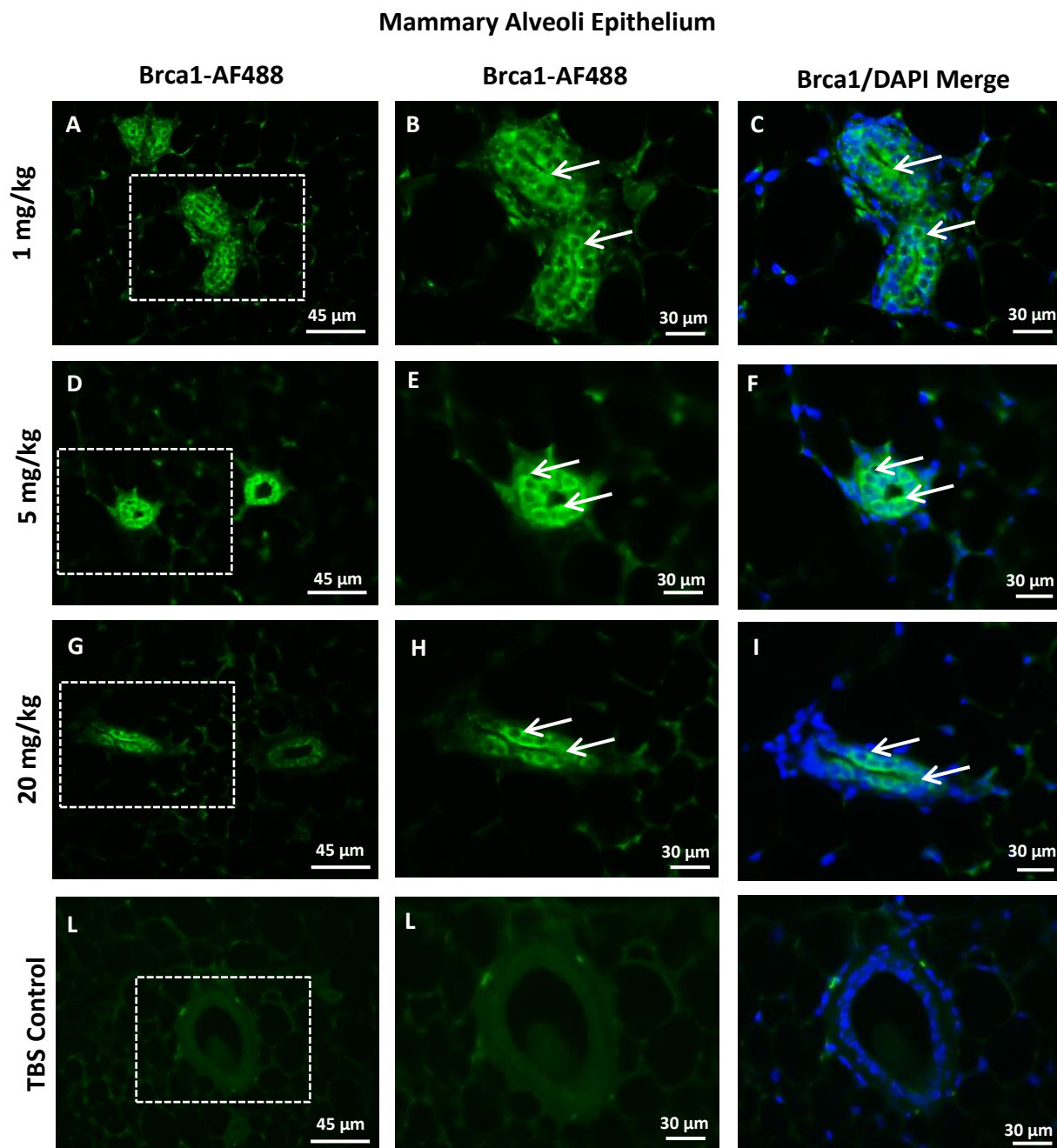


Figure 5.2 Brca1 Staining in Folic Acid Supplemented C57BL/6 Mammary Alveoli Epithelium.

Representative images of Brca1 positive (green) mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (**A, B, C**), 5 mg/kg (**D, E, F**) and 20 mg/kg (**G, H, I**) folic acid for 4 weeks followed by a 4 week maintenance diet. Increased magnification of images (**B, E, H**). Merge, Brca1 (green) and DAPI (Blue) stain identifying location of epithelial cell nuclei (**C, F, I**). TBS no primary control (**J, K, L**).

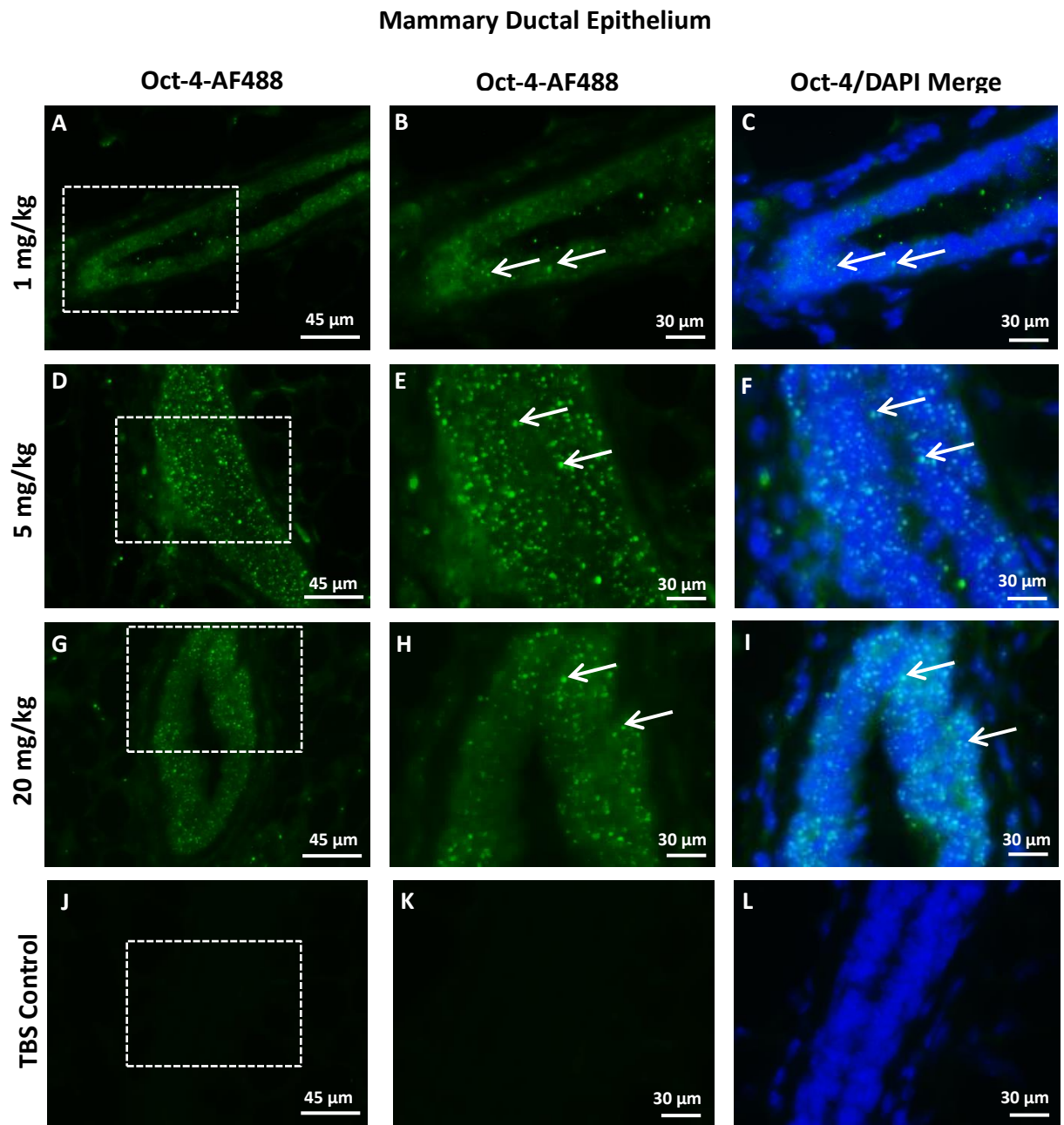


Figure 5.3 Oct-4 Staining in Folic Acid Supplemented C57BL/6 Mammary Ductal Epithelium.

Representative images of Oct-4 positive (green) mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (**A, B, C**), 5 mg/kg (**D, E, F**) and 20 mg/kg (**G, H, I**) folic acid for 4 weeks followed by a 4 week maintenance diet. Increased magnification of images (**B, E, H**). Merge, Oct-4 (green) and DAPI (Blue) stain identifying location of epithelial cell nuclei (**C, F, I**). TBS no primary control (**J, K, L**).

Mammary Alveoli Epithelium

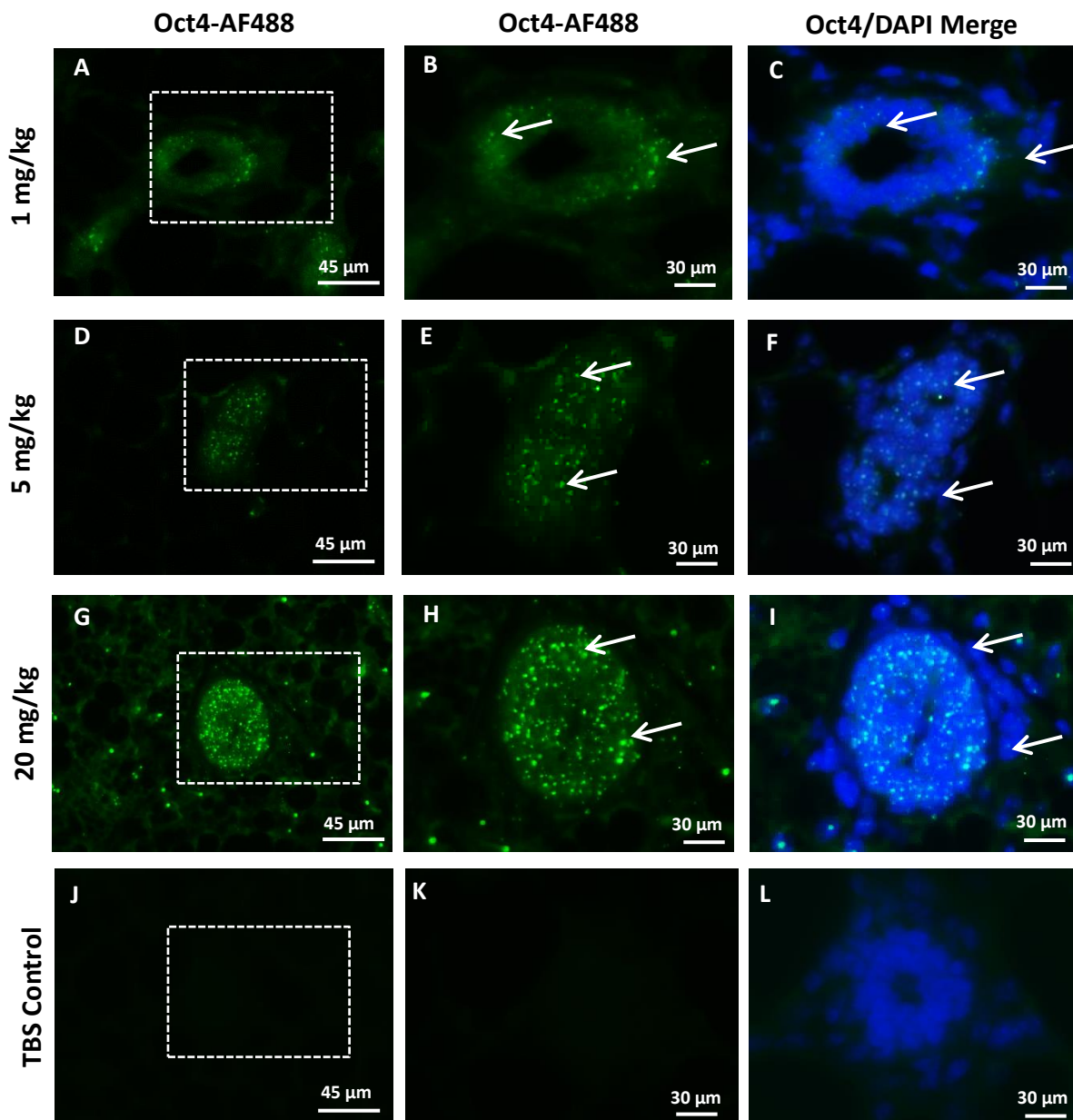


Figure 5.4 Oct-4 Staining in Folic Acid Supplemented C57BL/6 Mammary Alveoli Epithelium.

Representative images of Oct-4 positive (green) mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) or 20 mg/kg (G, H, I) folic acid for 4 weeks followed by a 4 week maintenance diet. Increased magnification of images (B, E, H). Merge, Oct-4 (green) and DAPI (Blue) stain identifying location of epithelial cell nuclei (C, F, I). TBS no primary control (J, K, L).

5.3.3 α -SMA

α -SMA and Keratin 8 (S431) localisation and expression were analysed to investigate basal/myoepithelial and luminal cell populations. α -SMA was localised in myoepithelial cells within mammary ducts and alveoli (Figure 5.5, 5.6). α -SMA showed no significant difference in DAB intensity in ducts or alveoli between mice fed 5 mg/kg or 20 mg/kg folic acid 4 weeks (PND 130) after supplementation (Figure 5.5, 5.6).

5.3.4 Ki67

Ki-67 a marker of cellular proliferation showed a distinct nuclear staining pattern in both mammary ductal and alveoli structures (Figure 5.7, 5.8). Within mammary ducts the number of Ki-67 positive cells increased in mice fed 5 and 20 mg/kg. Ki-67 positive staining did not significantly increase in mammary alveoli.

5.3.5 Keratin 8

Keratin 8 showed localised expression within the cytoplasm of ductal luminal cells (Figure 5.9). Cells positively stained with K8 showed a decrease in fluorescence intensity in mice fed 5 and 20 mg/kg folic compared to 1 mg/kg control (Figure 5.9).

5.3.6 Gata-3

Transcription factor Gata-3 positive staining was localised in the nucleus of luminal cells within mammary ducts. The number of Gata-3 positive cells significantly increased in mice fed 20 mg/kg folic acid (Figure 5.10).

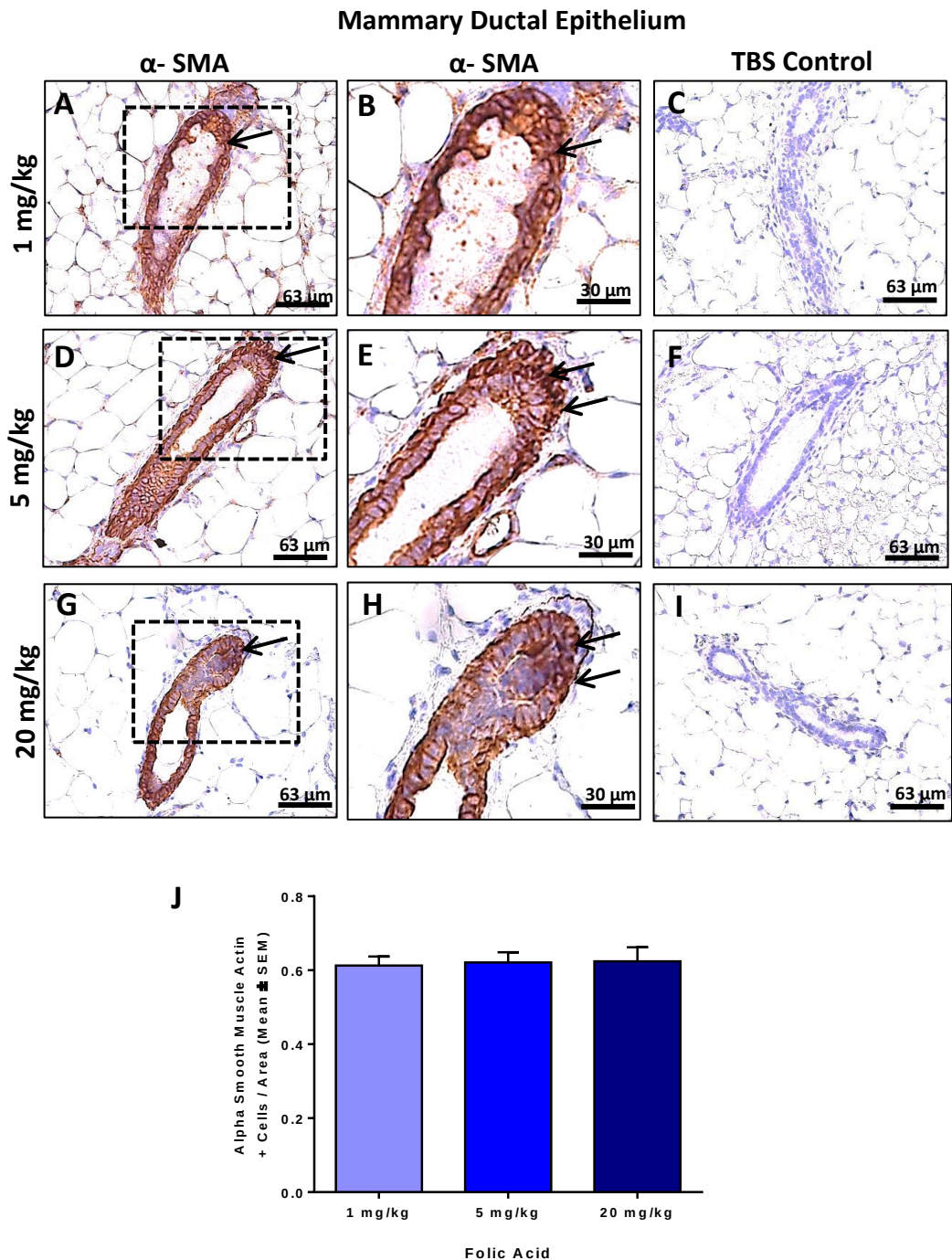


Figure 5.5 α -SMA Staining in Mammary Epithelial Ducts of Mice Supplemented with Folic Acid during Adulthood.

Representative images of α -SMA positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) folic acid for 4 weeks followed by a 4 week maintenance diet. α -SMA positive cells localised within the ductal epithelium (A, D, G). Increased magnification of images (B, E, I). TBS no primary control (C, F, I). Quantitative measurement of α -SMA positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

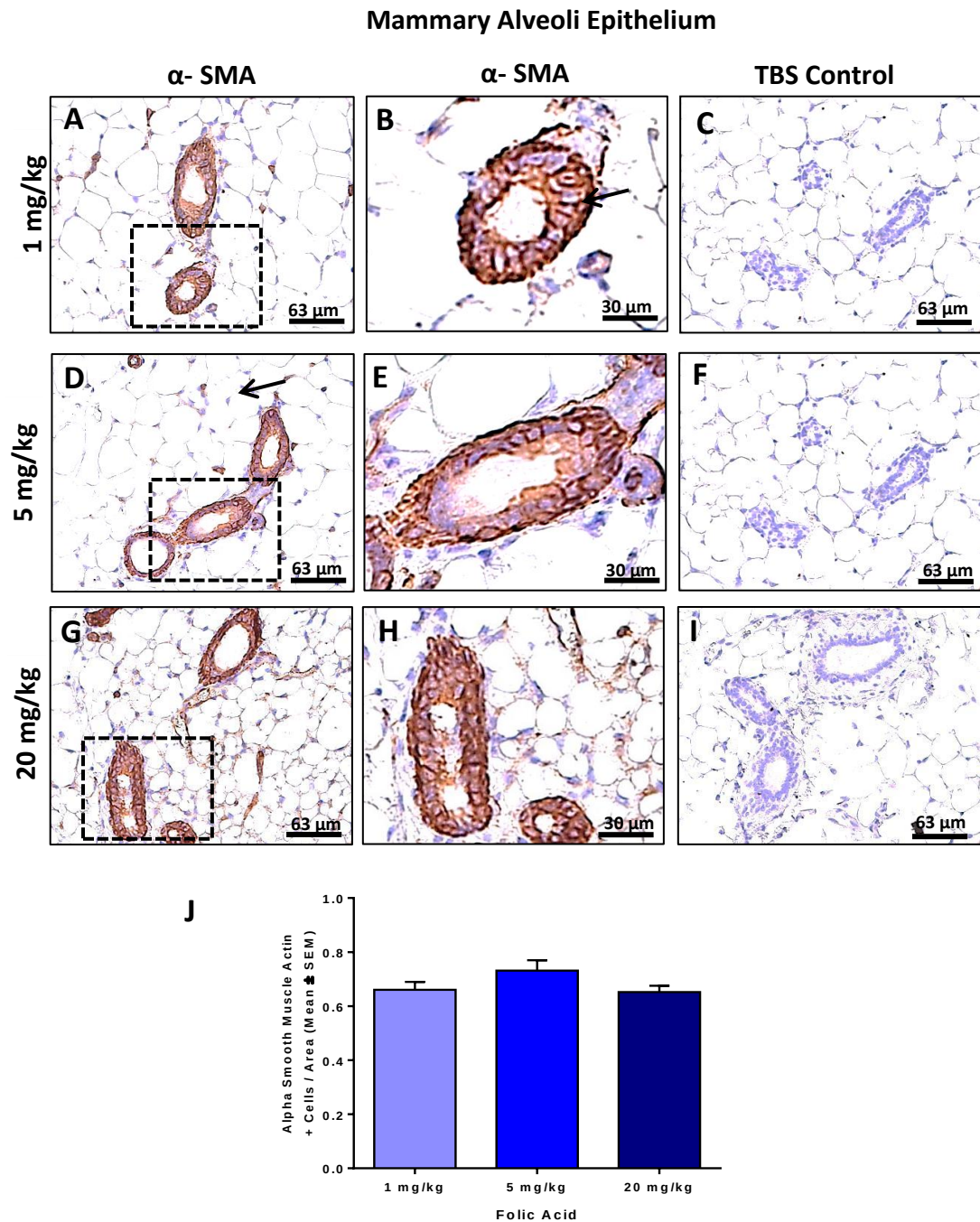


Figure 5.6 αSMA Staining in Mammary Alveoli Epithelium of Mice Supplemented with Folic Acid during Adulthood.

Representative images of α-SMA positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) folic acid for 4 weeks followed by a 4 week maintenance diet. α-SMA positive cells localised within the alveoli epithelium (A, D, G). Increased magnification of images (B, E, I). TBS no primary control (C, F, I). Quantitative measurement of α-SMA positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

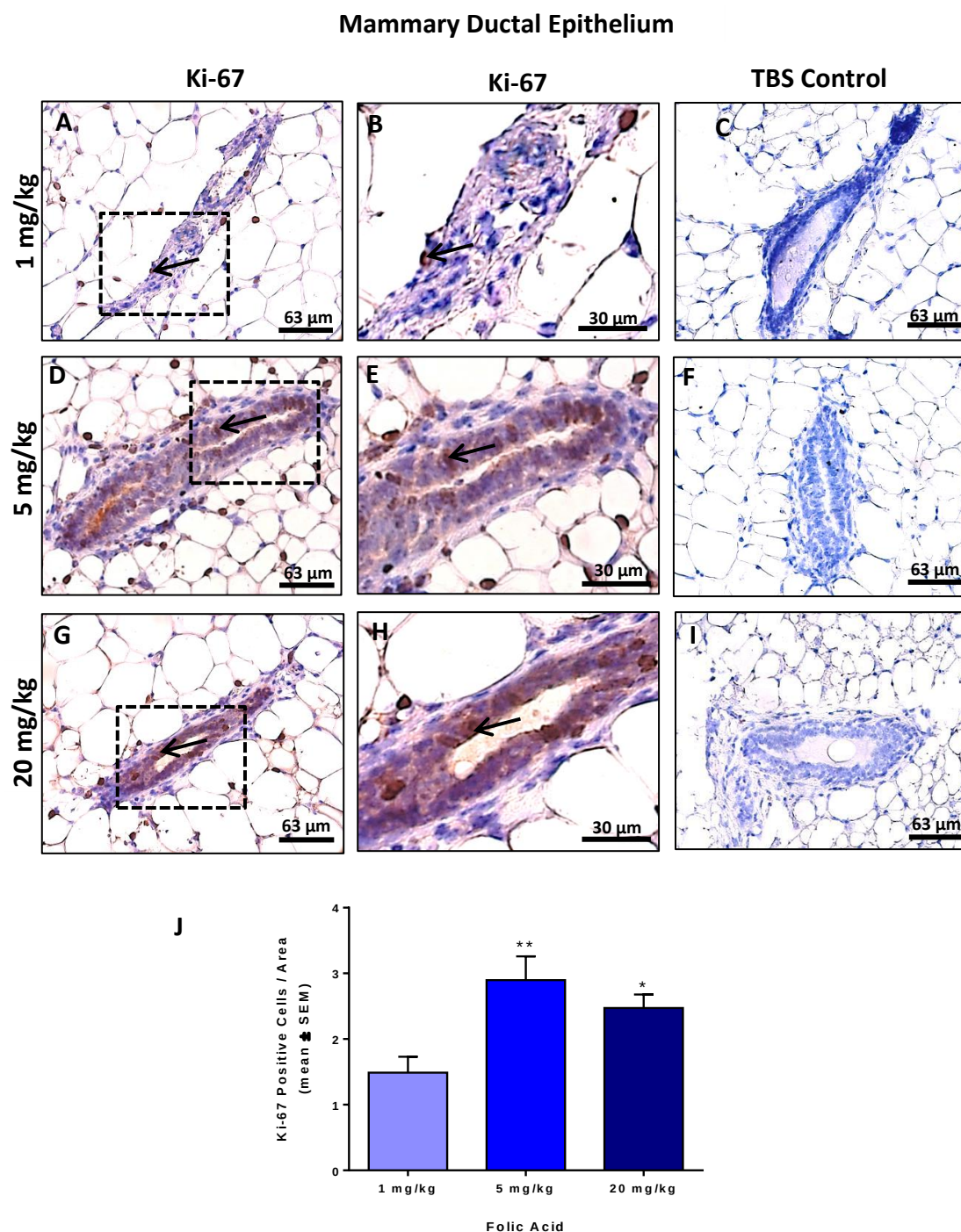


Figure 5.7 Ki-67 Staining in Mammary Ductal Epithelium of Mice Supplemented with Folic Acid during Adulthood.

Representative images of Ki-67 positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) FA for 4 weeks followed by a 4 week maintenance diet. Ki-67 positive cells localised within the ductal epithelium (A, D, G). Increased magnification of images (B, E, I) TBS no primary control (C, F, I). Quantitative measurement of Ki-67 positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

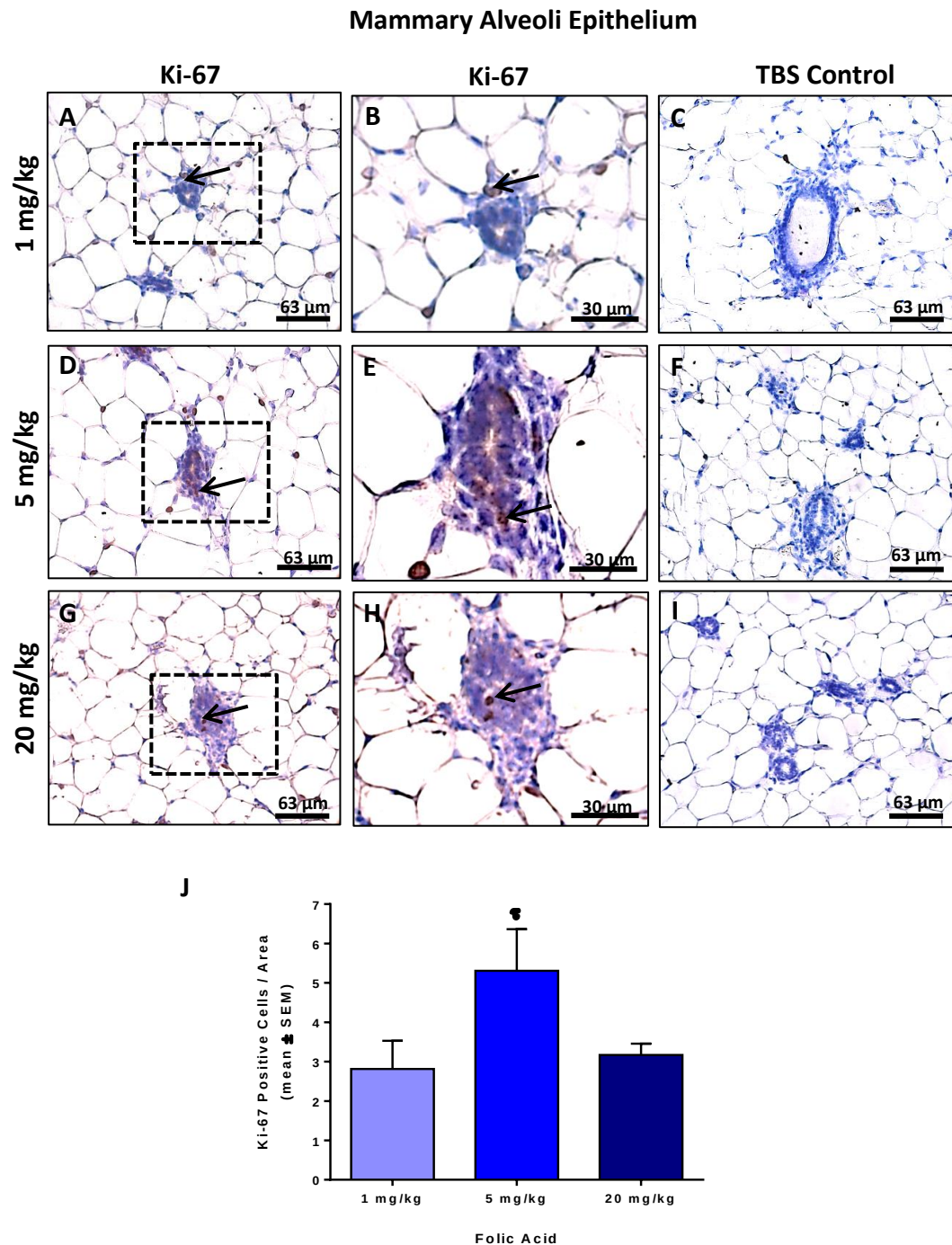


Figure 5.8 Ki-67 staining in Mammary Alveoli Epithelium of mice supplemented with folic acid during adulthood.

Representative images of Ki-67 positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) FA for 4 weeks followed by a 4 week maintenance diet. Ki-67 positive cells localised within the alveoli epithelium (A, D, G). Increased magnification of images (B, E, I) TBS no primary control (C, F, I). Quantitative measurement of Ki-67 positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

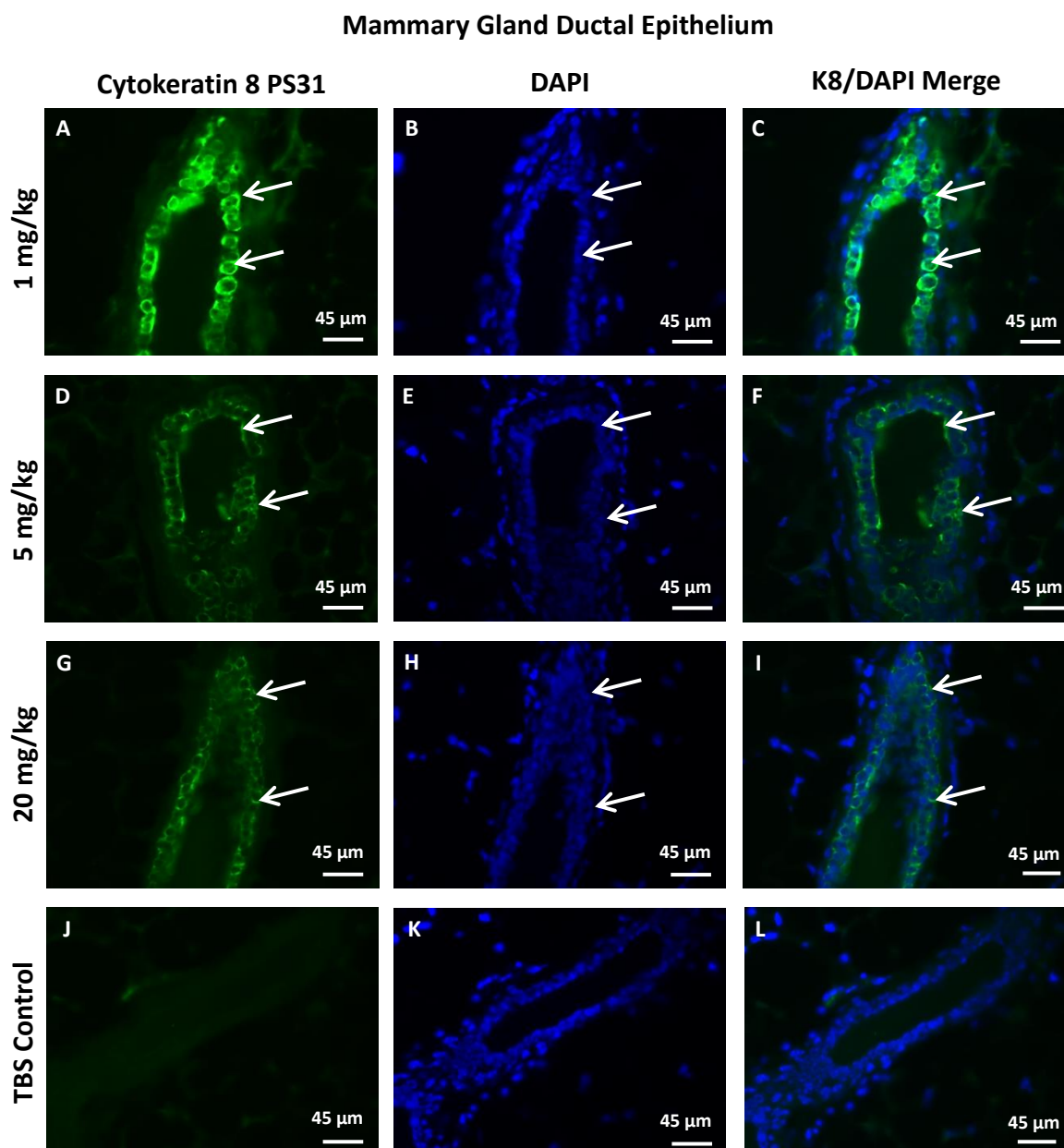


Figure 5.9 Cytokeratin 8 PS31 (K8) staining in Mammary Ductal Epithelium of Mice supplemented with folic acid during adulthood.

Representative images of K8 positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) FA for 4 weeks followed by a 4 week maintenance diet. K8 positive cells localised within the ductal epithelium (A, D, G). Increased magnification of images (B, E, I) TBS no primary control (C, F, I). Quantitative measurement of K8 positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

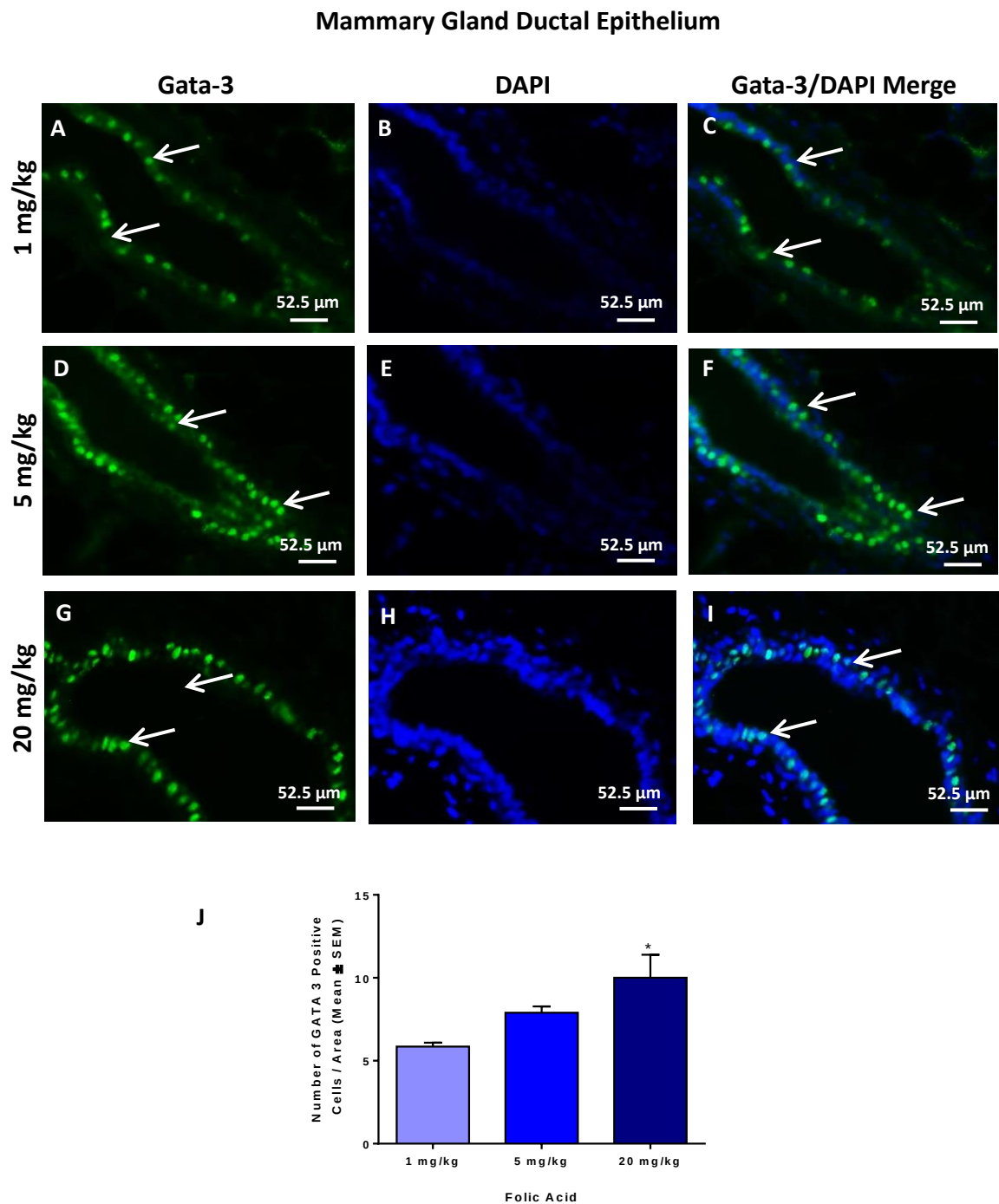


Figure 5.10 Gata-3 Positive Staining in the Mammary Gland of Mice Supplemented with Folic Acid during Adulthood.

Representative images of GATA3 positive mammary gland sections from adult mice supplemented (PND 74-102) with 1 mg/kg control (A, B, C), 5 mg/kg (D, E, F) and 20 mg/kg (G, H, I) FA for 4 weeks followed by a 4 week maintenance diet. GATA3 positive cells localised within the alveoli epithelium (A, D, G). Increased magnification of images (B, E, I) TBS no primary control (C, F, I). Quantitative measurement of GATA3 positive cells in ductal epithelium from folic acid supplemented mice (J) (n = 6 per folic acid concentration).

5.4 Discussion

Results from the present study demonstrate that high folic acid supplementation during adulthood induces dose specific changes in levels of protein expression within the mammary gland 4 weeks after the period of supplementation. Changes in positive staining are summarised in Table 5.1

Table 5.1. Summary of Protein Expression Changes in Mice Supplemented with Folic Acid for 28 days during Adulthood Followed by a 4 weeks Maintenance Diet.

Folic Acid Supplementation during Adulthood (PND 74-102)				
	Ductal		Alveoli	
	5 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg
Brca1	↑	↑	↑	↑
Oct-4	↑	↑	↑	↑
α-SMA	-----	-----	-----	-----
K8	↓	↓	-----	-----
Ki-67	-----	↑	-----	-----
Gata-3	-----	↑	-----	-----

Brca1 protein was expressed within mammary ducts and alveoli showing an intense positive staining pattern that was consistent with cytoplasmic localisation. Co-localisation of DAPI with low levels of Brca1 fluorescence suggests expression of Brca1 within the nucleus at lower levels. Within mammary ducts and alveoli, Brca1 positive staining showed increased fluorescence in mice fed 5 mg/kg and also a smaller increase at 20 mg/kg folic acid. These findings were consistent with gene expression changes (Chapter 4) that showed increased *Brca1* expression in mice fed 5 mg/kg 4 weeks after supplementation.

BRCA1 has been shown to be located within the cell nucleus and the cytoplasm (Wiener et al., 2015). Within the cytoplasm BRCA1 interacts with BARD1 and BRCA1/BARD1 shuttle between the nucleus and cytoplasm (Wiener et al., 2015). Within the nucleus Brca1 functions in the regulation of DNA repair, cell cycle progression and gene transcription. Research has shown that BRCA1/BARD1 colocalise mainly in the cytoplasm within breast tumours (Wiener et al., 2015). Furthermore, aberrant expression of BRCA1 within the cytoplasm is associated with breast cancer cells. Therefore, increased levels of cytoplasmic Brca1 in response to folic acid intake could provide a putative link to breast cancer risk. Increased levels of cytoplasmic Brca1 in response to folic acid supplementation is not documented, however, mechanisms to explain this observation could relate, in part, to modulation in the levels of Brca1 nuclear shuttling or relocation of Brca1 away from the nucleus. Furthermore, changes in BARD1 could modulate nuclear translocation and Brca1 nuclear retention (Fabbro et al., 2002). Increased levels of cytoplasmic, not nuclear Brca1, would suggest Brca1 is not functioning in a DNA-repair or cell checkpoint regulator capacity and may be implicated in another role; however, further investigation is required to understand the mechanisms involved.

Oct-4 positive staining was localised within the nucleus of mammary ducts and alveoli, with increased levels of fluorescence in mice fed 5 mg/kg and 20 mg/kg folic acid. These findings were consistent with gene expression changes shown in chapter 3, confirming changes in mRNA are translated into protein. Positive Oct-4 staining appeared to be located in both basal/myoepithelial and luminal cell populations within ducts and alveoli, forming a speckled appearance within the cell nuclei (Figure 5.3). This pattern is consistent with positive OCT-4 staining identified by Tai et al., (2005) in human breast epithelial cells (Tai et al., 2005). Oct-4 protein is expressed in adult pluripotent stem cells and tumour cells but is thought to be limited in normal differentiated cells or expressed at very low levels (Tai et al., 2005). Increased Oct-4 expression is associated with mammary development and lactation as well as malignant mammary transformation (Hassiotou et al., 2013b). Oct-4 functions in the regulation of cellular differentiation but also plays a role in the regulation of gene networks through indirect mechanisms (Cao et al., 2013). Research by Cao et al., (2013) has shown changes in Oct-4 expression may enhance or suppress BIRC5 expression through indirect regulation of the BIRC5 promoter, promoting cancer progression in hepatocellular carcinoma (Cao et al., 2013). Changes in the levels of Oct-4 within the mammary gland may therefore function not only in the regulation of pluripotency but in gene regulatory roles through indirect mechanisms within stem cell populations. As previously discussed in Chapter 4, mechanisms to account for persistent

changes in Oct-4 in response to folic acid supplementation may involve epigenetic regulation of gene expression.

Oct-4 protein has been shown to exist in multiple isoforms. The two main isoforms, namely Oct-4A and Oct-4B, function in stem cell regulation, with Oct-4A thought to be confined to undifferentiated embryonic stem cells and embryonic carcinoma cells. The function of Oct-4B is not currently clear, with some research suggesting Oct-4B functions in somatic cells, but may be limited to the cell cytoplasm; the variant Oct-4B1 is highly expressed in embryonic stem and carcinoma cells (Atlasi et al., 2008). Research in the present study used antibodies that are not specific to individual Oct-4 isoforms and therefore identify expression of both Oct-4A and Oct-4B. Nuclear localisation of Oct-4 within mammary ducts suggests the presence of Oct-4A, which is nuclear specific. For future investigation it would be necessary to investigate levels and localisation of individual isoforms of Oct-4 in response to folic acid supplementation. This would provide a greater insight into the effects of folic acid on Oct-4 function within the mammary gland.

Caveats associated with Brca1 and Oct-4 protein expression relate to the direct measurement of levels of fluorescence. Direct quantification of fluorescence within cytoplasmic compartments such as that observed in Brca1 expression, and also quantification of low levels of Oct-4 expression, is difficult using available imaging software. With additional time and resources it would be possible to develop a method to quantify both Oct-4 and Brca1 expression numerically to further assess expression differences.

α -SMA was localised in basal myoepithelial cells within mammary ducts and alveoli. α -SMA positive staining was consistent between control and folic acid treatments, showing no change in staining patterns. Research has shown that during post-natal mammary development, myoepithelial cells progressively acquire a differentiated phenotype and express smooth muscle markers (Tamiolakis et al., 2002). Furthermore, cellular differentiation towards myoepithelial cells occurs in a number of cancers, including myoepithelial-rich breast carcinoma (Tamiolakis et al., 2002). Consistent expression of α -SMA in mice fed high folic acid would therefore suggest no changes in myoepithelial cell differentiation in response to this dietary nutrient.

K8 (phospho-S431) showed decreased levels of fluorescence within the luminal component of mammary ducts in response to high and supramaximal concentrations of folic acid. Keratins are expressed in epithelial cells and are cytoplasmic intermediate filament proteins that function in

maintaining the structural integrity and mechanical properties of cells (Yamada 2002). Research has identified posttranslational modifications in K8, specifically serine 431, are critical within the regulation of cell structure and function (Busch et al., 2012). Increased levels of K8 phosphorylation have been linked to keratin cytoskeleton reorganisation and migration of human pancreatic tumour cells (Armaki). However, loss of K8 phosphorylation has been shown to increase tumour progression in squamous cell carcinoma. Furthermore, K8 phosphorylation has been shown to regulate keratin reorganisation and migration of epithelial cells (Busch et al., 2012). Research linking folic acid and K8 expression within the mammary gland is minimal; however, findings from the present study combined with previous literature suggest high folic acid intake affects levels of K8 which may modulate reorganisation of the cytoskeleton and the migration of epithelial cells through mechanisms that are currently unknown. As K8 is a marker of luminal cell populations, changes in levels of K8 expression can be an indicator of disorganisation of ductal luminal epithelial cells. Co-staining of K8 in combination with a basal/myoepithelial marker such as α -SMA could therefore be used to identify disorganisation in ductal structure in response to folic acid.

Numbers of Ki-67 positive cells showed a persistent increase in the ducts of mice fed 5 and 20 mg/kg of folic acid 4 weeks after the period of supplementation. Ki-67 has been shown to increase during mammary gland proliferation in rats (Hvid et al., 2012). Thus, a folic acid mediated increase in Ki-67 in the present study would suggest an increase in cellular proliferation within mammary ducts. High dietary folic acid intake has been linked to cellular proliferation and mammary tumour proliferation in the mammary gland of rats (Deghan Manshadi et al., 2014). Furthermore, high folic acid has been shown to increase cell turnover in human colon cells (Pellis et al., 2008). Mechanisms associated with folic acid intake and cell proliferation have been linked to dietary nucleotide provision (Jackson et al., 1997). Rats fed folate/methyl deficient diets produced an increased dUMP/dTTP ratio, a markedly reduced mitotic index (MI) and an increase in the proliferating cell index (PCI) to mitotic cells, suggesting a delay of cells in S-phase in hepatocytes (Jackson et al., 1997). High levels of folic acid may therefore modulate dUMP/dTTP ratios which may result in increased cellular proliferation.

Gata-3 positive staining increased within ductal luminal cell populations in response to supramaximal levels of dietary folic acid (20 mg/kg). Gata-3 is a critical regulator of mammary-gland morphogenesis and luminal-cell differentiation. Gata transcription factors bind to consensus sequences within the promoter regions of genes resulting in repression or activation

of target genes (Chou et al., 2010). Using conditional deletion of Gata-3 to investigate mammary epithelial development, Label et al., (2007) showed that Gata-3 plays an essential role in mammary gland morphogenesis in both the embryo and adult (Asselin-Labat et al., 2007). Furthermore, Gata-3 deficiency results in the expansion of luminal progenitors and a block in differentiation; however, introduction of Gata-3 into populations of enriched stem cells induces maturation along the alveolar luminal lineage (Asselin-Labat et al., 2007). A study by Pie et al, (2009) has shown that Gata-3 represses the cyclin inhibitor p18INK4C which functions to restrain progenitor cell proliferation (Pei et al., 2009). Thus, cells that express high levels of Gata-3 may repress p18INK4A, resulting in luminal progenitor proliferation. Furthermore, a study by Harb et al., (2015) has shown that very high levels of maternal folate were significantly associated with increased H3/H4 acetylation in the Gata-3 promoter region, suggesting folic acid intake may modify Gata-3 through post translational histone modifications (Harb et al., 2015). Interestingly, Ezh2 expression has been linked to Gata-3 within the murine mammary gland. Overexpression of Ezh2, as observed in the present study in response to high folic acid, has been shown to induce intraductal epithelial hyperplasia accompanied by upregulation of Gata-3 (Li et al., 2009b).

5.5 Conclusion

In conclusion, this study has investigated the effects of high (5 mg/kg) or supramaximal (20 mg/kg) folic acid intake during adulthood, 4 weeks after the period of supplementation on the localisation and expression of key proteins involved in mammary differentiation. Key findings from this study have identified persistent increases in Brca1 and Oct-4 positive staining in ducts and alveoli of mice fed 5 mg/kg and 20 mg/kg folic acid. These findings were consistent with gene expression changes previously investigated (Chapter 4). Basal/myoepithelial markers showed distinct localisation within ducts and alveoli and did not change in folic acid supplemented mammary glands. However, the luminal cell marker K8 (Phospho) showed decreased positive staining in response to high folic acid intake, suggesting a folic acid mediated change in phosphorylated K8 within the luminal population. Ki-67 showed a persistent increase in mammary ducts in response to high folic acid intake suggesting increased levels of proliferation, which was not observed in alveoli. Furthermore, levels of Gata-3 localised in luminal cells of ducts showed a persistent increase in numbers of positive cells in response to

high folic acid, suggesting folic acid may modulate the levels of differentiation within luminal breast epithelium (Miettinen et al., 2014).

Chapter 6: The Effect of Folic Acid Supplementation during the Juvenile Period or Adulthood on the Mammary Gland Transcriptome of Female C57BL/6 Mice. A Whole Transcriptome Shotgun Sequencing Approach

6.1 Introduction

The present study has identified changes in levels of expression of genes known to play key roles in mammary gland cell differentiation and morphology in response to an increase in dietary folic acid intake of 5 mg/kg compared to control values of 1 mg/kg during either the juvenile period or adulthood. In order to fully understand the effects of folic acid supplementation on genes associated with breast cancer risk, it is essential to investigate the transcriptome on a global scale to provide high resolution insight into folic acid induced changes in levels of mRNA expression across the genome and to be able to further investigate pathways associated with changes in differential gene expression.

Whole transcriptome shotgun sequencing, more commonly known as RNA-seq provides the capability to obtain single base-resolution on the entire genome scale and provides a snapshot of the transcriptome, enabling identification of differential gene expression, novel transcripts as well as intergenic regions (Wang et al., 2009). Current research by Dabydeen *et al.*, (2015) has utilised RNA-seq to provide a transcriptome predictive of tamoxifen resistance in mouse models of breast cancer (Dabydeen et al., 2015). Furthermore, a recent study by Laporta *et al.*, (2015) has investigated the transcriptome of the mammary gland during lactation utilising a global *Tph1* deficient mouse model to identify novel genes, pathways and processes that might be altered by the absence of serotonin (Laporta et al., 2015). Currently, no published research has utilised this approach to investigate the transcriptome wide effects of folic acid supplementation during different periods of the life-course in the mammary gland. Furthermore, it is currently not known how high levels of folic acid intake, equivalent to doses recommended during pregnancy or obtained through consumption of fortified foods and supplements, affects genome wide gene expression, nor is it known if folic acid mediated changes in levels of gene expression persist after the period of supplementation.

To address these questions RNA-seq analysis was performed on mammary glands from C57BL/6 mice fed a control (1 mg/kg) or high (5mg/kg) folic acid diet for 4 weeks during either the juvenile period or adulthood followed by a 4 week maintenance diet. Mice supplemented with 5 mg/kg of folic acid were selected for RNA-seq analysis based on the results from previous chapters which showed that 5 mg/kg of dietary folic acid supplemented either during the juvenile period or adulthood induced persistent changes in expression of the polycomb genes, known modulators of chromatin structure and gene expression as well as alterations in *Brca1* and *Oct-4* expression. Furthermore, folic acid supplementation at 5 mg/kg (5 x BDR) was selected to

reflect levels of folic acid that are 5 x BDR in humans (~1000 µg) which represent levels obtained through folic acid food fortification and supplementation as well as the levels that are taken by human periconceptionally and during pregnancy.

6.1.1 Chapter Aims

- 1) To investigate if dietary folic acid intake at 5 mg/kg (5 x BDR) supplemented for 28 days during the juvenile period or adulthood leads to differential gene expression across the transcriptome within mammary glands of female C57BL/6 mice.
- 2) To compare differentially expressed genes between mice supplemented during the juvenile period and adulthood to investigate if the timing of folic acid supplementation induces gene specific changes.

6.2 Methods

RNA-seq was used to identify differential gene expression, gene ontology and gene set enrichment in the mammary glands of female virgin C56BL/6 mice in response to high (5 mg/kg) dietary supplemented folic acid during either the juvenile period (PND 28-56) or adulthood (PND 74-102) followed by a 4 week maintenance diet. Female virgin C57BL/6 mice (6 animals in each experimental dietary group) were fed modified AI93M folic acid diets containing 1 mg/kg (1 x BDR) or 5 mg/kg (5 x BDR) in line with the detailed experimental design outlined in chapter 2. mRNA was extracted from the right thoracic mammary glands (50 mg) and six samples were randomly selected from each group (total 12 samples). RNA-seq was performed on the Illumina HiSeq2000 platform using TruSeq v3 chemistry (Illumina) with a total of ~20 million reads per sample. Analysis of read data was conducted by Elie Antoun (Epigen group, University of Southampton, UK). Sequenced reads were checked for quality using FastQC, aligned to the mouse genome (GRCm38) and transcript models were constructed from alignments using HT-Seq. Sample MAD scores were calculated and sample outliers removed from analysis. Differential gene expression, gene ontology (GO), gene set enrichment analysis (GSEA) and pathway analysis (IPA) were assessed between treatments.

6.3 Results

6.3.1 Folic Acid Supplementation during the Juvenile Period Induces Genome Wide Changes in Gene Expression

RNA-seq analysis was compared for differential gene expression between mice supplemented with 1 mg/kg (n=6) or 5 mg/kg (n=6) folic acid during the juvenile period. Mean absolute deviation scores were generated for each sample and the absolute deviation compared between samples within each group (Table 6.1). Samples that deviated from the adjusted mean by > 1.1 were excluded from the study which consisted of a single sample from the 1 mg/kg supplemented group and a single sample from the 5 mg/kg group. Total n numbers for each group were set at 5 (Table 6.1).

Table 6.1. MAD Score Values for Mice Supplemented with 1mg/kg (n=6) or 5 mg/kg (n=6) Folic Acid for 4 Weeks during the Juvenile Period followed by a 4 week Maintenance Diet.

Samples were removed that deviated from the adjusted mean by > 1.1 (sample 6, 1 mg/kg and sample 6, 5 mg/kg).

	1 mg/kg						5 mg/kg					
Sample	1	2	3	4	5	6	1	2	3	4	5	6
MAD	7.78x10 ⁻⁵						0.00067					
Absolute Deviation	1.09	-0.50	-0.19	0.86	0.19	-19.6	0.74	0.59	-1.0	-0.59	0.63	-1.2

Total gene expression data between samples was correlated and displayed as an MA plot and Volcano plot allowing visualisation of numbers of differentially expressed genes across the genome (Figure 6.1). 3005 genes were identified as significantly differentially expressed in the 5 mg/kg group using a false discovery rate (FDR) adjusted P value of < 1, or 1819 genes using an FDR adjusted P value of 0.05. Of the 1819 genes identified (FDR < 0.05) 758 were upregulated and 1060 were downregulated in response to folic acid supplementation (Table 6.2).

The top three genes significantly upregulated consisted of immunoglobulin kappa constant (Igkc) (LogFC of 6.56), glycosylation dependent cell adhesion molecule 1 (Glycam1) (LogFC of 6.56),

(LogFC of 6.56) and immunoglobulin heavy constant delta (Ighd) (LogFC of 6.22). The top three genes significantly downregulated consisted of ATP citrate lyase (Acly) (LogFC of -3.125), acyl-CoA thioesterase 11 (Acot11) (LogFC of -2.99) and protein phosphatase 1, regulatory (inhibitor) subunit 3B (Ppp1r3b) (LogFC of -2.89) (Table 6.2).

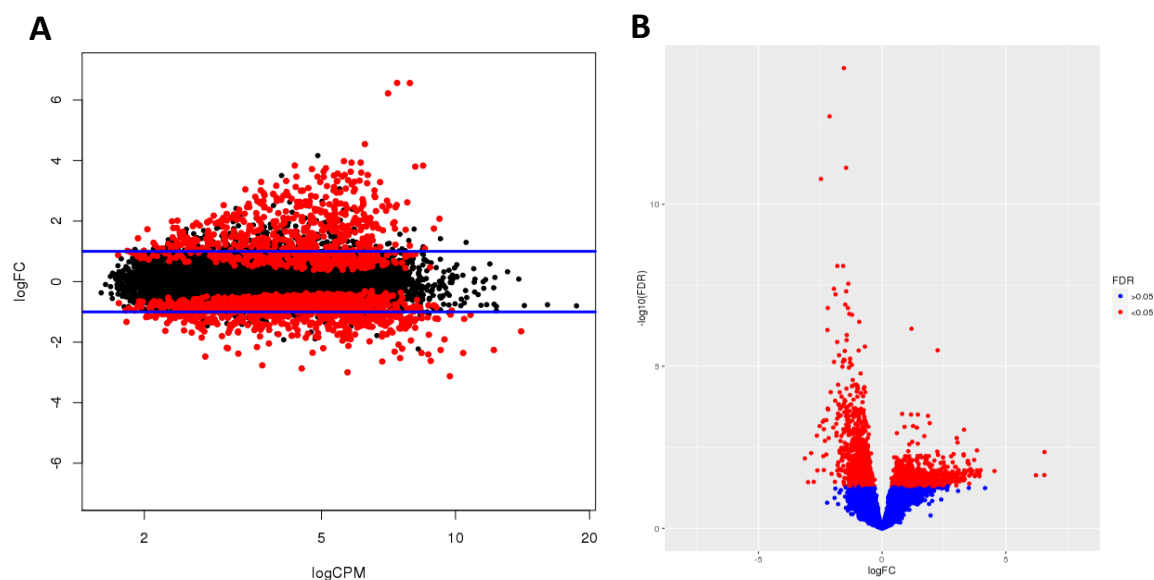


Figure 6.1 Differential gene expression in the mammary gland of female 57BL/6 mice supplemented with 1 mg/kg (1 x BDR - control) or 5 mg/kg (5 x BDR) folic acid for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) during the juvenile period. **(A)** MA plot showing average logCPM against logFC (change in log2 CPM). Individual genes are represented by single dots. Differentially expressed genes with adjusted p values (FDR) that are below 0.05 appear in red. The greater the levels of differential expression the further from the centre red dots appear. **(B)** Volcano plot of logFC against $-\log_{10}(\text{FDR})$. Genes that are dysregulated appear nearer the left and right hand sides of the plot. Genes that demonstrate significant differential expression (red) appear higher on the plot with highly significant changes located nearer the top. Lowly expressed genes were excluded after differential expression analysis using EdgeR. N = 5 per group.

Table 6.2. List of Top 15 Genes Differentially Regulated in the Mammary Gland of Female C57BL/6 Mice Supplemented with 1 mg/kg or 5 mg/kg Folic Acid for 4 Weeks (PND 28 – 56) followed by a 4 Week Maintenance Diet (PND 56-84) during the Juvenile Period.

Top 15 genes significantly upregulated (yellow) and downregulated (blue) (FDR = < 0.05, upregulated = + LogFC, downregulated = - LogFC.

Mgi Ref	LogFC	FDR	PVALUE	Description
Upregulated				
Igkc	6.56468	0.004422	0.000117	immunoglobulin kappa constant
Glycam				glycosylation dependent cell adhesion
1	6.56077	0.022728	0.001827	molecule 1
Ighd	6.22172	0.022999	0.001869	immunoglobulin heavy constant delta
Bank1	4.54327	0.017043	0.001037	B cell scaffold protein with ankyrin repeats 1
				T cell immunoglobulin and mucin domain
Timd4	3.98126	0.015901	0.000871	containing 4
Rac2	3.93223	0.024747	0.002216	RAS-related C3 botulinum substrate 2
Cd52	3.93066	0.020034	0.001411	CD52 antigen
				protein tyrosine phosphatase, non-receptor
Ptpn22	3.83613	0.003921	9.81E-05	type 22 (lymphoid)
Ptpnc	3.83246	0.016244	0.000931	protein tyrosine phosphatase, receptor type, C
Ighm	3.80012	0.020805	0.00155	immunoglobulin heavy constant mu
Sash3	3.74742	0.024615	0.002183	SAM and SH3 domain containing 3
Cxcr4	3.71905	0.007728	0.000272	chemokine (C-X-C motif) receptor 4
Arhgap				
4	3.63953	0.016715	0.000994	Rho GTPase activating protein 4
Epsti1	3.63009	0.01776	0.001116	epithelial stromal interaction 1 (breast)
Downregulated				
Acly	-3.1258	0.006938	0.000235	ATP citrate lyase
Acot11	-2.99763	0.037516	0.004865	acyl-CoA thioesterase 11
Ppp1r3				protein phosphatase 1, regulatory (inhibi)
b	-2.8694	0.004763	0.000129	subunit 3B
Aspg	-2.7664	0.036694	0.004698	asparaginase homolog (S. cerevisiae)
				acyl-CoA synthetase short-chain family
Acss2	-2.63932	0.001379	2.45E-05	member 2
				ELOVL family member 6, elongation of long
Elovl6	-2.61972	0.016314	0.000937	chain fatty acids (yeast)
Slc25a				solute carrier family 25 (mitochondrial
1	-2.52678	0.000701	9.32E-06	carrier, citrate transporter), member 1
Gsta3	-2.47654	1.67E-11	6.14E-15	glutathione S-transferase, alpha 3
Thrsp	-2.40495	0.000516	6.16E-06	thyroid hormone responsive
				ATPase, Na ⁺ /K ⁺ transporting, alpha 3
Atp1a3	-2.37909	0.005933	0.000182	polypeptide
Acaca	-2.35886	0.00087	1.25E-05	acetyl-Coenzyme A carboxylase alpha
				malic enzyme 1, NADP(+)-dependent,
Me1	-2.35783	0.015901	0.000872	cytosolic
Myoz2	-2.34864	0.000467	5.28E-06	myozenin 2
Pygl	-2.31461	0.001992	3.95E-05	liver glycogen phosphorylase
Tkt	-2.26003	0.005256	0.000147	transketolase

6.3.1.1 Gene Set Enrichment Analysis

Gene set enrichment was performed on the total number of genes expressed within RNA-seq analysis to identify the representation of specific classes of genes and to identify significantly enriched or depleted gene groups. RNA-seq genes were compared against gene sets that include Hallmark, C2 curated, C2 Kegg/Biocarter, C2 miRNA and C2 Transcription factor. The hallmark gene sets are coherently expressed signatures derived from MSigDB gene sets that represent biological states or processes. C2 curated gene sets are constructed from online pathway databases and publications in PubMed. C2 Kegg/Biocarter are derived from the KEGG/BioCarter pathway databases and C2 miRNA and transcription factor are gene sets that contain genes that share a 3'UTR microRNA binding motif and gene sets that share a transcription factor binding site (TRANSFAC v 7.4 database), respectively.

Within the Hallmark grouping, 33 gene sets were identified as statistically significantly enriched (FDRq value < 0.05) with 14 upregulated (+ NES score) out of a total of 24 and 19 downregulated (- NES score) out of a total of 25 (Table 6.3). The top 5 most significantly enriched gene sets upregulated consisted of G2M checkpoint (NES = 5.20, FDR < 0.001), E2F targets (NES = 4.97, FDR < 0.001), Interferon gamma response (NES = 4.56, FDR < 0.001), allograft rejection (NES = 4.53, FDR < 0.001) and Il6 Jak Stat3 signalling (NES = 3.80, FDR < 0.001) (Table 6.3). The top 5 downregulated enriched gene set consisted of oxidative phosphorylation (NES = -9.95692, FDR < 0.001), adipogenesis (NES = -8.89, FDR < 0.001), fatty acid metabolism (NES = -7.21, FDR < 0.001), bile acid metabolism (NES = -4.66, FDR < 0.001), peroxisome (NES = -4.51, FDR < 0.001) and myogenesis (NES = -4.07, FDR < 0.001) (Table 6.3).

C2 curated category showed a total of 1143 gene sets that were significantly enriched including 677 upregulated and 466 downregulated (FDR < 0.05). The top gene set upregulated was Zheng bound by Fox3 (NES = 7.07, FDR < 0.001) and further top 5 upregulated consisted of Lee differentiating T lymphocyte (NES = 6.90, FDR < 0.001), Pujana BRCA2 PCC network (NES = 6.05, FDR < 0.001), Reactome peptide chain elongation (NES = 5.76, FDR < 0.001) and Zhang TLX targets 36HR DN (NES = 5.72, FDR < 0.001) (Table 6.4). The top gene set downregulated was Mootha Human MITODB 6 2002 (NES = -10.50, FDR < 0.001) and further top 5 gene sets downregulated consisted of Mootha mitochondria (NES = -9.90, FDR < 0.001), Burton adipogenesis 6 (NES = -9.63, FDR < 0.001), Wong mitochondria gene module (NES = -8.89, FDR < 0.001) and Reactome TCA cycle (NES = -8.24, FDR < 0.001) (Table 6.4). Within the C2 KEGG/Biocarter category a total of 90 gene sets were significantly enriched including 50

upregulated and 40 downregulated. The top gene set upregulated in KEGG was Ribosome (NES = 5.65, FDR < 0.001) and the top downregulated enriched gene set was oxidative phosphorylation (NES = -7.07) (Table 6.5). The top gene set upregulated in Biocarter was the HIV NEF pathway (NES = 3.11, FDR = 0.001). The top gene set downregulated was Proteosome pathway (NES -3.11, FDR <0.001) (Table 6.5).

Of the 197 miRNA gene sets within the C2 Category 93 were significantly enriched for upregulated genes and 0 downregulated. The top upregulated miRNA gene set consisted of CTACTGT, MIR-199A (NES = 3.24, FDR < 0.001) (146 genes) of which 111 genes were enriched. Within the transcription factor gene sets the top upregulated enriched gene set consisted of V\$E2F1_Q6_01 (NES = 4.37, FDR < 0.001) and downregulated TGACCTTG_V\$SF1_Q6 (NES = -2.93, FDR <0.001) with 66.1 % and 45.3 % of gene sets significantly enriched respectively.

Table 6.3, Hallmark GSEA from Mice Supplemented with 1 mg/kg or 5 mg/kg of Folic Acid for 4 Weeks during the Juvenile Period (PND 74-102) followed by a 4 Week Maintenance Diet.

Top 20 enriched upregulated (+NES, yellow) and down regulated gene sets (-NES, blue). FDR 0 = < 0.001.

Hallmark Gene Set	Genes	En Score	NES	FDR
G2M CHECKPOINT	142	0.380648	5.200974	0
E2F TARGETS	139	0.358681	4.970934	0
INTERFERON GAMMA RESPONSE	161	0.308287	4.569857	0
ALLOGRAFT REJECTION	107	0.372155	4.531735	0
IL6 JAK STAT3 SIGNALING	57	0.431954	3.805374	0
INTERFERON ALPHA RESPONSE	83	0.322751	3.448822	0
MITOTIC SPINDLE	169	0.210059	3.168285	0
TNFA SIGNALING VIA NFKB	131	0.201225	2.688778	1.07E-04
INFLAMMATORY RESPONSE	107	0.214856	2.588125	2.97E-04
MYC TARGETS V2	50	0.239805	2.010998	0.011551
UNFOLDED PROTEIN RESPONSE	96	0.155376	1.801664	0.038361
APOPTOSIS	121	0.142108	1.789618	0.036746
DNA REPAIR	127	0.133535	1.749681	0.040516
MYC TARGETS V1	185	0.108317	1.729138	0.041964
COMPLEMENT	125	0.116329	1.510902	0.106363
IL2 STAT5 SIGNALING	141	0.102519	1.420312	0.146871
KRAS SIGNALING UP	134	0.097618	1.298903	0.225143
PI3K AKT MTOR SIGNALING	90	0.100075	1.101019	0.426904
TGF BETA SIGNALING	49	0.134544	1.099091	0.4064
HEDGEHOG SIGNALING	21	0.186893	1.033292	0.476013
OXIDATIVE PHOSPHORYLATION	192	-0.62461	-9.95692	0
ADIPOGENESIS	191	-0.57081	-8.89535	0
FATTY ACID METABOLISM	133	-0.54114	-7.21964	0
BILE ACID METABOLISM	71	-0.46139	-4.66359	0
PEROXISOME	81	-0.43315	-4.51513	0
MYOGENESIS	160	-0.27963	-4.07056	0
XENOBIOTIC_ METABOLISM	131	-0.28757	-3.85223	0
EPITHELIAL MESENCHYMAL TRANSITION	150	-0.22797	-3.21482	0
CHOLESTEROL HOMEOSTASIS	59	-0.33981	-3.05709	0
GLYCOLYSIS	151	-0.20503	-2.92599	0
MTORC1 SIGNALING	173	-0.17785	-2.63475	0
COAGULATION	78	-0.25789	-2.63355	0
REACTIVE OXYGEN SPECIES PATHWAY	44	-0.28702	-2.23288	0.001451
PROTEIN SECRETION	92	-0.17671	-1.92906	0.013549
HEME METABOLISM	149	-0.13024	-1.82969	0.021796
ESTROGEN RESPONSE LATE	137	-0.1305	-1.81897	0.021693
APICAL SURFACE	26	-0.28982	-1.8067	0.021998
SPERMATOGENESIS	25	-0.29207	-1.79086	0.022715
P53 PATHWAY	150	-0.12964	-1.77913	0.022998
UV RESPONSE UP	115	-0.12563	-1.57267	0.060575

Table 6.4. C2 GSEA from Mice Supplemented with 1 mg/kg or 5 mg/kg of Folic Acid for 4 Weeks during the Juvenile Period (PND 74-102) followed by 4 Week Maintenance Diet.

Top 20 enriched upregulated (+NES, yellow) and down regulated gene sets (-NES, blue) FDR 0 = < 0.001.

C2 All Gene Set	Genes	En Score	NES	FDR
ZHENG BOUND BY FOXP3	362	0.32317	7.074034	0
LEE DIFFERENTIATING T LYMPHOCYTE	137	0.513851	6.907414	0
PUJANA BRCA2 PCC NETWORK	297	0.30586	6.055378	0
REACTOME PEPTIDE CHAIN ELONGATION	63	0.613272	5.765909	0
ZHANG TLX TARGETS 36HR DN	134	0.429611	5.720336	0
DAZARD RESPONSE TO UV NHEK DN	262	0.305832	5.639997	0
QI PLASMACYTOMAUP	166	0.374296	5.637647	0
KEGG RIBOSOME	66	0.584352	5.634795	0
POOLA INVASIVE BREAST CANCER UP	128	0.414049	5.602195	0
REACTOME PROCESSING OF CAPPED INTRON CONTAINING PRE MRNA	123	0.421788	5.399849	0
PUJANA XPRSS INT NETWORK	129	0.404077	5.35488	0
REACTOME MRNA PROCESSING	140	0.391811	5.351311	0
CHEN HOXA5 TARGETS 9HR UP	160	0.361397	5.242026	0
OSMAN BLADDER CANCER DN	299	0.268279	5.238127	0
ZHANG TLX TARGETS 60HR DN	177	0.332973	5.18848	0
REACTOME NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	83	0.480555	5.124508	0
JISON SICKLE CELL DISEASE DN	138	0.372193	5.065168	0
BREAST CANCER 20Q13 AMPLIFICATION DN	136	0.360638	4.813508	0
REACTOME INFLUENZA LIFE CYCLE	109	0.391051	4.774934	0
REACTOME MRNA SPLICING	98	0.399526	4.733832	0
MOOTHA HUMAN MITODB 6 2002	365	-0.46789	-10.5083	0
MOOTHA MITOCHONDRIA	384	-0.45138	-9.90083	0
BURTON ADIPOGENESIS 6	172	-0.62519	-9.63612	0
WONG MITOCHONDRIA GENE MODULE	199	-0.54414	-8.89879	0
REACTOME TCA CYCLE	112	-0.65389	-8.24951	0
MOOTHA PGC	359	-0.37075	-8.04935	0
FLECHNER BIOPSY KIDNEY TRANSPLANT	424	-0.30109	-7.12314	0
REACTOME RESPIRATORY ET ATP SYNTHESIS	77	-0.69016	-7.03256	0
KEGG OXIDATIVE PHOSPHORYLATION	98	-0.60967	-6.98454	0
BURTON ADIPOGENESIS 5	112	-0.55321	-6.84886	0
MCBRYAN PUBERTAL BREAST 4 5WK DN	179	-0.43508	-6.76558	0
STEIN ESRRA TARGETS UP	311	-0.33846	-6.75426	0
RUAN RESPONSE TO TNF DN	82	-0.62207	-6.67135	0
REACTOME RESPIRATORY ELECTRON TRANSPORT	62	-0.7189	-6.57348	0
MOOTHA VOXPPOS	80	-0.63033	-6.52349	0
LANDIS ERBB2 BREAST TUMORS 324 DN	137	-0.1305	-1.81897	0.0216
WAKABAYASHI ADIPOGENESIS PPARG RXRA	26	-0.28982	-1.8067	0.0219
STEIN ESRRA TARGETS	25	-0.29207	-1.79086	0.0227
KEGG PEROXISOME	150	-0.12964	-1.77913	0.0229
KEGG_PARKINSONS_DISEASE	115	-0.12563	-1.57267	0.0605

Table 6.5 KEGG/Biocarter GSEA from Mice Supplemented with 1 mg/kg or 5 mg/kg Folic Acid for 4 Weeks during Juvenile Period (PND 74-102) followed by a 4 Week Maintenance Diet.

Top 20 enriched upregulated (+ NES, yellow) and top 20 down regulated gene sets (-NES, blue)

FDR 0 = < 0.001.

Kegg/Biocarter Gene Set	Genes	En Score	NES	FDR
RIBOSOME	66	0.584352	5.656341	0
SPLICEOSOME	111	0.348926	4.253642	0
INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION	19	0.694176	3.594788	0
LEISHMANIA INFECTION	47	0.415441	3.483613	3.08E-04
B CELL RECEPTOR SIGNALING PATHWAY	61	0.353398	3.263172	2.47E-04
BIOCARTA HIVNEF PATHWAY	54	0.355453	3.112948	2.06E-04
NATURAL KILLER CELL MEDIATED CYTOTOX.	67	0.304903	2.947777	1.76E-04
JAK STAT SIGNALING PATHWAY	74	0.279829	2.848132	1.54E-04
FC GAMMA R MEDIATED PHAGOCYTOSIS	78	0.268123	2.775027	6.84E-04
T CELL RECEPTOR SIGNALING PATHWAY	71	0.267188	2.655333	0.001708
RNA DEGRADATION	50	0.321174	2.617605	0.001758
APOPTOSIS	67	0.261083	2.607957	0.001612
BIOCARTA TNFR2 PATHWAY	16	0.528402	2.587922	0.001574
LEUKOCYTE TRANSENDOTH. MIGRATION	79	0.237953	2.536698	0.001622
DNA REPLICATION	29	0.385486	2.513112	0.001664
CHEMOKINE SIGNALING PATHWAY	118	0.198747	2.512709	0.00156
CYTOSOLIC DNA SENSING PATHWAY	31	0.375612	2.473374	0.001687
CELL CYCLE	97	0.199102	2.33183	0.005789
RIG I LIKE RECEPTOR SIGNALING PATHWAY	42	0.297877	2.321011	0.005807
P53 SIGNALING PATHWAY	50	0.277497	2.294674	0.006536
OXIDATIVE PHOSPHORYLATION	98	-0.60967	-7.07578	0
PEROXISOME	64	-0.60298	-5.75311	0
PARKINSONS DISEASE	94	-0.4957	-5.54637	0
VALINE LEUCINE AND ISOLEUCINE DEGR.	39	-0.742	-5.52808	0
ALZHEIMERS DISEASE	128	-0.39468	-5.17971	0
HUNTINGTONS DISEASE	146	-0.34729	-4.92022	0
FATTY ACID METABOLISM	33	-0.69248	-4.75769	0
CITRATE CYCLE TCA CYCLE	28	-0.72688	-4.60391	0
PROPANOATE METABOLISM	27	-0.72153	-4.45386	0
PPAR SIGNALING PATHWAY	46	-0.54372	-4.34847	0
BUTANOATE METABOLISM	24	-0.63993	-3.81343	0
KEGG DRUG METAB. CYTOCHROME P450	25	-0.58379	-3.41191	0
PYRUVATE METABOLISM	32	-0.48566	-3.30261	0
BIOCARTA PROTEASOME PATHWAY	28	-0.48918	-3.11941	0
METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	26	-0.50406	-3.09077	0
BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	15	-0.65044	-3.08123	0
GLYCOLYSIS GLUCONEOGENESIS	41	-0.38083	-2.89562	0
TRYPTOPHAN METABOLISM	22	-0.50452	-2.88749	0
ECM RECEPTOR INTERACTION	61	-0.30612	-2.84884	4.59E-05
PROTEASOME	115	-0.38159	-2.81073	8.92E-05

6.3.1.2 Gene Ontology

Gene ontology analysis was performed to describe differentially regulated genes in terms of three categories that include biological processes, molecular function and cellular component. This determines which pathways or gene networks might be altered. Within the biological processes category, 219 gene sets were identified as significantly changed in response to folic acid supplementation. The top three processes most significantly affected consisted of oxidation-reduction process (FDR, 2.40E-27), small molecule metabolic process (FDR, 2.00E-22) and lipid metabolic process (FDR, 8.90E-19) (Table 6.6). Within the molecular function category, 161 gene sets were identified as differentially regulated (FDR < 0.05). The top three functions changed consisted of oxidoreductive activity (FDR, 4.20E-26) cofactor binding (FDR, 1.30E-11) and coenzyme binding (FDR, 1.30E-10). Within the cellular compartment ontology, 79 gene sets were identified as significantly changed (FDR < 0.05). The top three compartments changed consisted of mitochondrion (FDR, 1.10E-29), mitochondrial part (8.70E-21) and mitochondrial envelope (FDR, 1.40E-17) with 1190, 495 and 391 significant number of genes in each set respectively (Table 6.6).

The most significantly affected pathway with the highest number of differentially expressed genes was located within cellular compartment ontology and identified as mitochondrion (GO: 0005739) which was investigated further. In total 384 genes within the mitochondrion grouping were identified as differentially expressed between 1 mg/kg and 5 mg/kg folic acid supplemented groups (FDR < 0.05), with 31 genes upregulated and 353 genes downregulated. The top three mitochondrion genes significantly upregulated consisted of *Gimap8* (3.01), *Kmo* (2.59) and *Hcls1* (2.33) (Table 6.7).

Table 6.6. Gene Ontology in the Mammary Gland of Female C57BL/6 Mice Supplemented with 1 mg/kg (1 x BDR - control) or 5 mg/kg (5 x BDR) Folic Acid for 4 weeks (PND 28 – 56) Followed by a 4 Week Maintenance Diet (PND 56-84) during the Juvenile Period.

Top 10 gene sets significantly changed for molecular processes, molecular function and cellular compartment ontology groups based on FDR values (FDR < 0.05).

No	GO-ID	Term	Sig	Expected	FDR
Biological Process					
1	GO:0055114	oxidation-reduction process	525	206	2.40E-27
2	GO:0044281	small molecule metabolic process	854	280	2.00E-22
3	GO:0006629	lipid metabolic process	587	202	8.90E-19
4	GO:0019752	carboxylic acid metabolic process	423	158	1.40E-18
5	GO:0006631	fatty acid metabolic process	183	87	4.80E-18
6	GO:0044710	single-organism metabolic process monocarboxylic acid metabolic process	2410	615	7.00E-18
7	GO:0032787	organic acid metabolic process	264	111	1.30E-17
8	GO:0006082	oxoacid metabolic process	457	164	2.70E-17
9	GO:0043436	organic acid catabolic process	451	162	3.90E-17
10	GO:0016054	organic acid catabolic process	94	53	2.00E-15
Molecular Function					
1	GO:0016491	oxidoreductase activity	395	166	4.20E-26
2	GO:0048037	cofactor binding	170	71	1.30E-11
3	GO:0050662	coenzyme binding	133	58	1.30E-10
4	GO:0016614	oxidoreductase activity, acting on CH-OH...	85	38	9.10E-08
5	GO:0016616	oxidoreductase activity, acting on the C...	78	34	8.90E-07
6	GO:0016627	oxidoreductase activity, acting on the C...	48	23	7.70E-06
7	GO:0016885	ligase activity, forming carbon- carbon b...	7	7	1.00E-05
8	GO:0050660	flavin adenine dinucleotide binding	53	24	1.60E-05
9	GO:0016903	oxidoreductase activity, acting on the a...	27	15	3.30E-05
10	GO:0016421	CoA carboxylase activity	6	6	5.40E-05
Cellular Compartment					
1	GO:0005739	mitochondrion	1190	384	1.10E-29
2	GO:0044429	mitochondrial part	495	183	8.70E-21
3	GO:0005740	mitochondrial envelope	391	147	1.40E-17
4	GO:0031966	mitochondrial membrane	371	139	1.60E-16
5	GO:0005743	mitochondrial inner membrane	290	115	6.70E-16
6	GO:0019866	organelle inner membrane	313	121	1.10E-15
7	GO:0005777	peroxisome	93	48	3.70E-12
8	GO:0042579	microbody	93	48	3.70E-12
9	GO:0031967	organelle envelope	593	180	4.40E-11
10	GO:0031975	envelope	594	180	5.10E-11

Table 6.7. Gene Ontology in Components of the Mitochondrion (GO analysis) in Mammary Tissue from Mice Supplemented with 1 mg/kg or 5 mg/kg Folic Acid for 4 Weeks during the Juvenile Period (PND 28-56), followed by a 4 Week Maintenance Diet (PND 56-84).

Top 30 genes upregulated (+ LogFC) shown.

Mgi_ref	LogFC	PValue	FDR	Description
Gimap8	3.013177	0.002217	0.024747	GTPase, IMAP family member 8 kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
Kmo	2.591989	0.003719	0.032678	hematopoietic cell specific Lyn substrate 1
Hcls1	2.338596	0.003959	0.033544	inhibitor of kappaB kinase epsilon
Ikbke	2.26283	0.00124	0.018821	clusterin
Clu	1.918308	0.00155	0.020805	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog
Lyn	1.6589	0.004841	0.037453	DNA-damage-inducible transcript 4
Ddit4	1.59664	0.005172	0.038771	cytochrome b-245, beta polypeptide
Cybb	1.560854	0.004453	0.035828	myelocytomatosis oncogene
Myc	1.439239	5.27E-05	0.002445	ceramide kinase
Cerk	1.380238	0.001855	0.022942	caspase 4, apoptosis-related cysteine peptidase
Casp4	1.307929	0.001554	0.020805	transformation related protein 53
Trp53	1.286205	0.001966	0.023335	uncoupling protein 2 (mitochondrial, proton carrier)
Ucp2	1.14685	0.000464	0.010769	Fyn proto-oncogene
Fyn	1.09612	0.003299	0.030536	chloride intracellular channel 1
Clic1	1.07016	0.007797	0.048164	PIN2/TERF1 interacting, telomerase inhibitor 1
Pinx1	1.024267	0.004411	0.035626	caspase 8
Casp8	0.966295	0.005528	0.040048	cAMP responsive element binding protein 1
Creb1	0.95252	0.002288	0.02523	myeloid cell leukemia sequence 1
Mcl1	0.945967	0.001945	0.023263	2'-5' oligoadenylate synthetase 2
Oas2	0.911087	0.000989	0.016689	tripartite motif-containing 39
Trim39	0.878092	0.006959	0.045391	RAB8B, member RAS oncogene family polymerase (DNA-directed), delta 3, accessory subunit
Rab8b	0.86481	0.005804	0.041025	cryptochrome 1 (photolyase-like)
Pold3	0.84336	0.001268	0.018979	solute carrier family 8 (sodium/calcium exchanger), member 1
Cry1	0.819085	0.006336	0.04312	receptor (TNFRSF)-interacting serine- threonine kinase
Slc8a1	0.799919	0.000414	0.009997	dynein light chain LC8-type 1
Ripk1	0.785644	0.000764	0.014673	ArfGAP with dual PH domains 2
Dynll1	0.785482	0.000858	0.015837	ELK3, member of ETS oncogene family
Adap2	0.732446	0.003362	0.030879	cytochrome P450, family 1, subfamily b, polypeptide 1
Elk3	0.706048	0.00793	0.04857	
Cyp1b1	0.70445	0.002855	0.028431	

6.3.1.3 Pathway Analysis

Pathway analysis was performed on differentially expressed genes from mice fed 5 mg/kg folic acid during the juvenile period to establish insight into regulators, networks and disease processes affected. Pathway analysis differs from GSEA and GO as differentially expressed genes are compared for pathway associations from large databases forming networks of associated genes and actual or predicted interactions which are otherwise unobtainable.

Top canonical pathways identified included mitochondrial dysfunction, oxidative phosphorylation, superpathway of cholesterol biosynthesis, fatty acid β oxidation 1 and leukocyte extravasation signalling (Figure 6.2). Of these pathways, mitochondrial dysfunction was the most significant pathway ($p = 4.09E-11$) with 43 out of a total of 158 genes (27.2%) shown to overlap (table 6.8).

The Top three upstream regulators were identified as *Map4k4*, *Ppara* and *Insr*. *Map4k4* was shown to have the most significant overlap ($8.22E-27$) with 52 of 53 genes shown to have expression direction consistent with activation of this gene (Table 6.9). Further regulators in order of significance included SCAP, PPARG, POR, INSIG1, N-Cor, TP53 and BSCL2. Transcription factor genes that were identified included TP53 of which predicted activation was shown to be inhibited, MED13 of which predicted activation was shown to be activated and FOXO1 (Table 6.9).

The most significantly changed disease pathway was identified as metabolic disease, with all 187 genes effected (p -value range $3.20E-4$ to $6.30E-15$) across 9 categories (Figure 6.3). Within the metabolic disease pathway the top three upregulated functions were glucose metabolism disorder ($z = 3.748$), disglycemia ($z = 3.485$) and hypoglycaemia ($z = 2.895$) (Table 6.10). The top function, glucose metabolism disorder showed 61 of 154 genes to have expression direction consistent with an increase in this disorder (Table 6.10). Cellular network analysis revealed a total of 25 gene networks that were affected by folic acid intake during the juvenile period (Figure 6.4). Of these, the top 3 networks all containing 35 genes consisted of 1) haematological disease, metabolic disease, DNA replication, recombination and repair; 2) lipid metabolism, molecular transport, and small molecule biochemistry; 3) energy production, lipid metabolism and small molecule biochemistry (Table 6.11).

Table 6.8 Top Canonical Pathways Identified from Ingenuity Pathway Analysis in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Juvenile Period.

Canonical Pathway	P-Value	Overlap (%)
Mitochondrial Dysfunction	4.09E-11	27.2
Oxidative Phosphorylation	3.66E-10	31.6
Super pathway of Cholesterol Biosynthesis	1.81E-07	48.1
Fatty Acid B-oxidation 1	2.70E-07	43.8
Leukocyte Extravasation Signalling	2.72E-06	19.7

Table 6.9 Top Upstream Regulators Identified in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Juvenile Period.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation Z Score	P-Value of Overlap
MAP4K4	Kinase	Activated	6.983	8.22E-27
PPARA	Ligand-dependent nuclear receptor	Inhibited	-3.530	1.36E-21
INSR	kinase	Inhibited	-4.947	7.86E-17
SCAP	other	Inhibited	-4.682	4.06E-15
PPARG	Ligand-dependent nuclear receptor	Inhibited	-6.022	4.20E-15
POR	Enzyme		1.545	8.22E-14
INSIG1	Other	Activated	4.175	1.35E-13
N-cor	Group	Inhibited	3.642	6.76E-12
TP53	Transcription factor	Inhibited	-3.755	3.80E-11
BSCL2	Other			1.93E-10

Table 6.10 Top Disease Pathways Identified in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Juvenile Period.

Disease and Disorders	P-Value Range	Number of Genes
Metabolic Disease	3.20E-04 – 6.30E-15	187
Immunological Disease	2.99E-04 – 6.28E-12	219
Endocrine System Disorders	3.18E-04 – 2.42E-11	139
Gastrointestinal Disease	5.12E-04 – 2.42E-11	205
Hepatic System Disease	5.12E-04 – 6.85E-10	133

Table 6.11 Top Networks Identified in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Juvenile Period.

Network Pathways	Score	Focus Molecules
Haematological Disease, Metabolic Disease, DNA Replication, Recombination and Repair	35	35
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry,	35	35
Energy Production, Lipid Metabolism, Small Molecule Biochemistry	35	35
Embryonic Development, Organismal Development, Tissue Morphology	30	33
Connective Tissue Disorders, Developmental Disorders, Neurological Disease	30	33

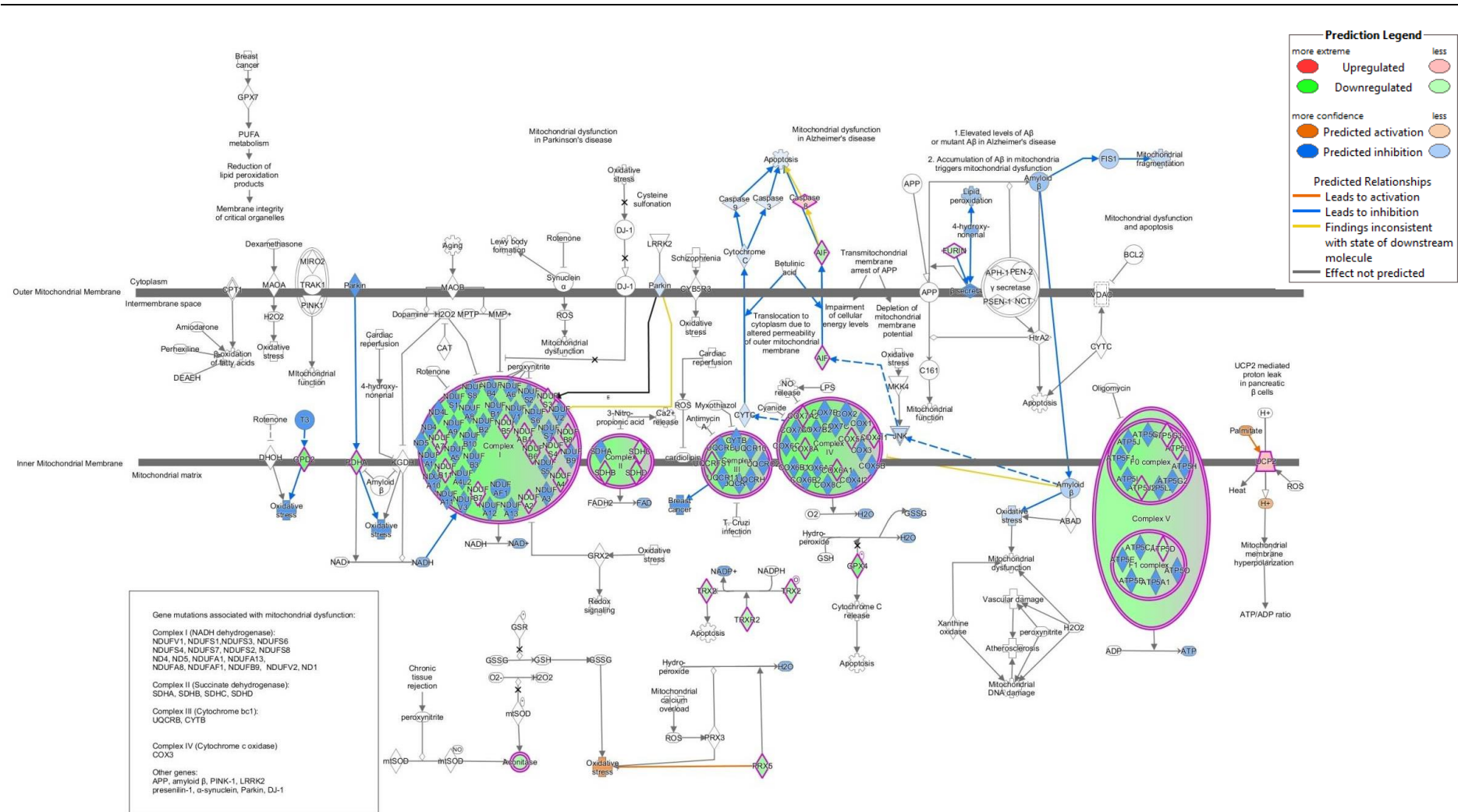


Figure 6.2 Top Disease Pathway of Mitochondrial dysfunction in mammary glands of mice supplemented with 5 mg/kg folic acid during the Juvenile Period.

Gene's upregulated (pink) and downregulated (green) are shown in respect of their interaction status.

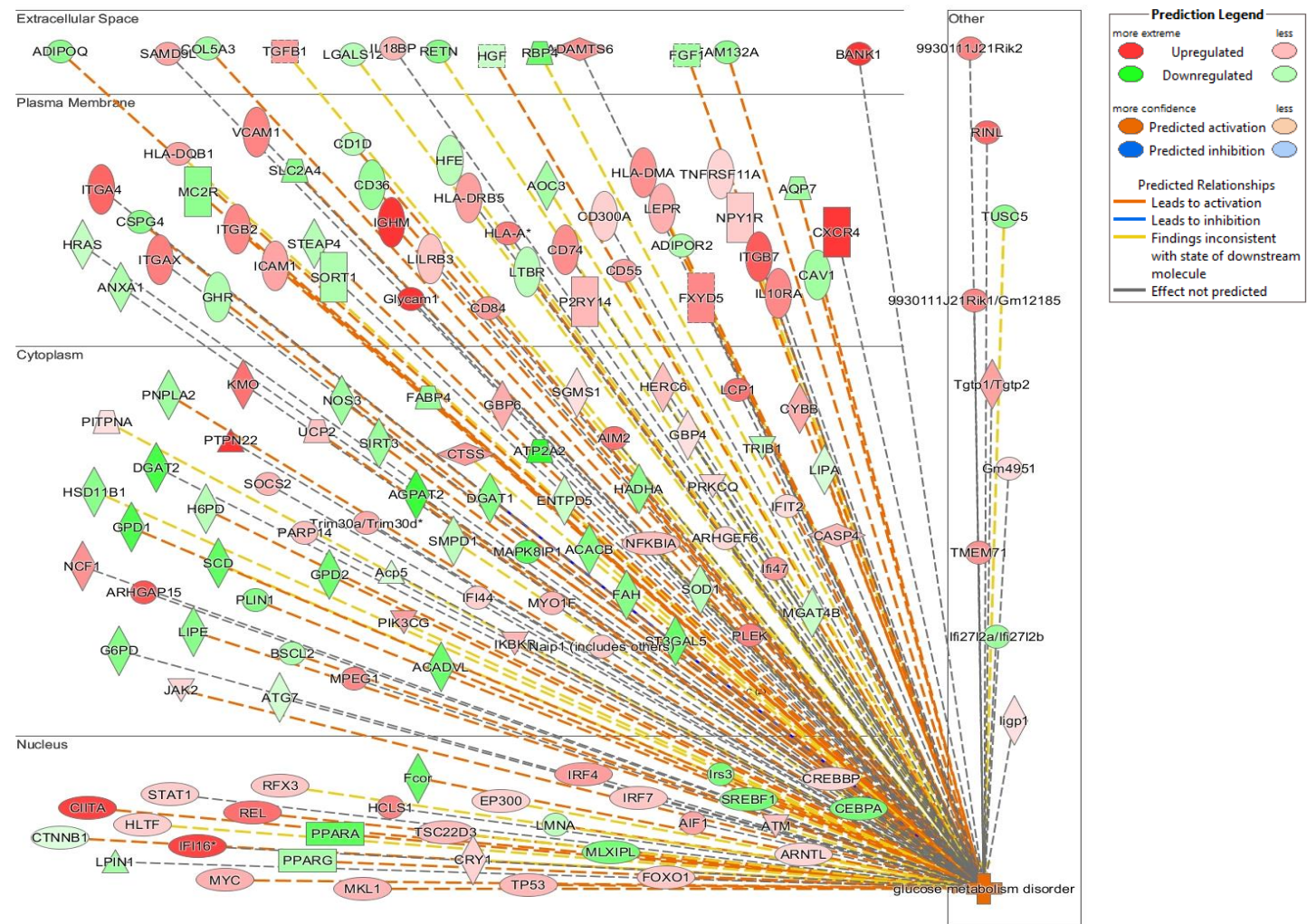


Figure 6.3 Top Network Pathway Metabolic Disease in mammary glands of mice supplemented with 5 mg/kg folic acid during the Juvenile Period. Gene's upregulated (pink) and downregulated (green) are shown in respect of their interaction status.

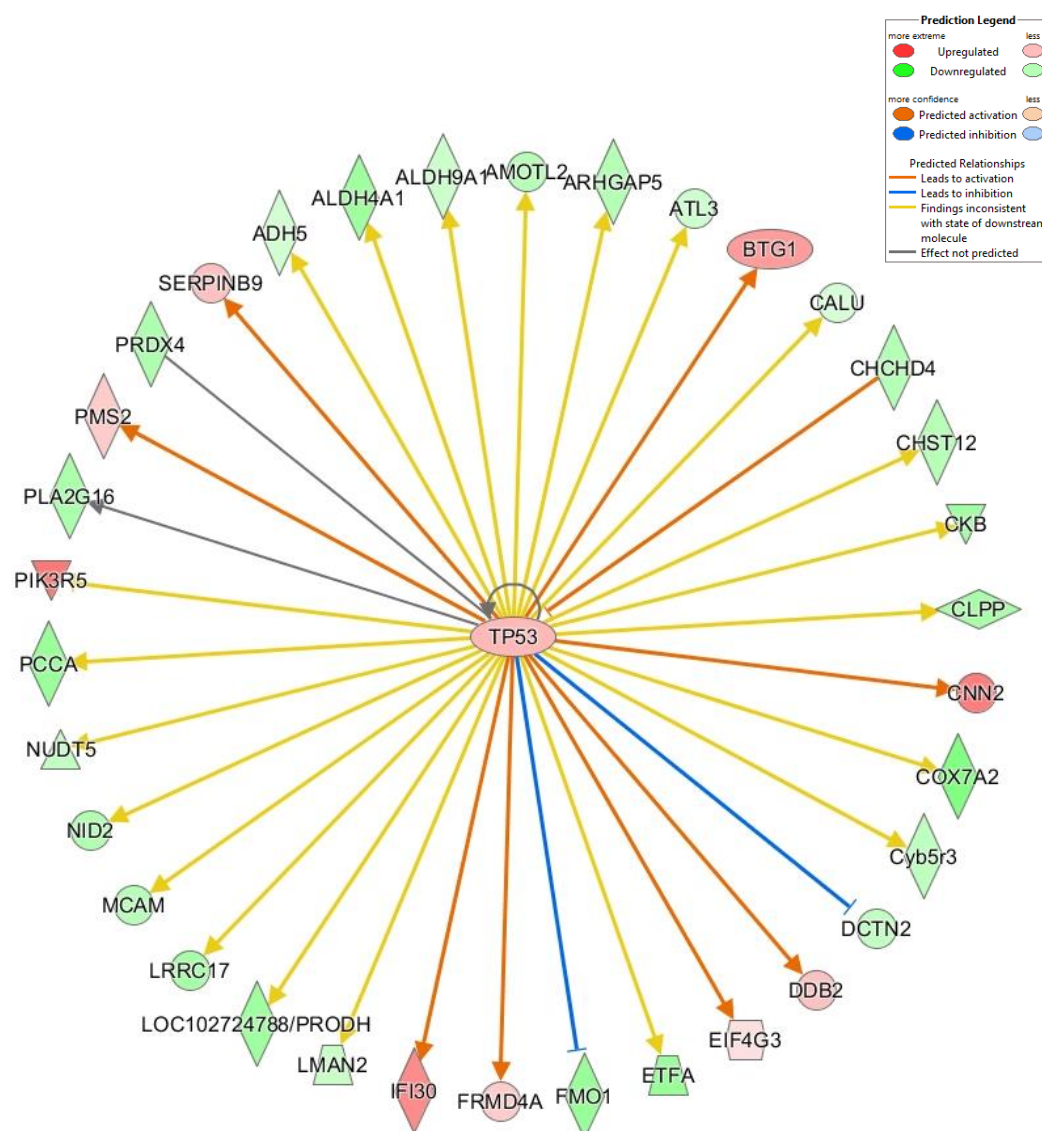


Figure 6.4 Top Disease Pathway Identified in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Juvenile Period.

Networks associated with Haematological Disease, Metabolic Disease, DNA Replication, Recombination and Repair. Genes upregulated (pink) and downregulated (green) are shown in respect of their interaction status.

6.3.2 Folic Acid Supplementation during Adulthood Induces Genome Wide Changes in Gene Expression.

RNA-seq analysis was compared for differential gene expression between mice supplemented with 1 mg/kg (n=6) or 5 mg/kg (n=6) folic acid during adulthood. QC analysis identified one sample (5 mg/kg) with poor read counts which was removed from analysis. Mean absolute deviation scores were generated for each sample and the absolute deviation compared between samples within each group (Table 6.12). Samples that deviated from the adjusted mean by > 2 were excluded from the study which consisted of a single sample from the 1 mg/kg supplemented group. Total n numbers for each group were set at 5 (Table 6.12).

Table 6.12. MAD Score Values for Mice Supplemented with 1mg/kg (n=6) or 5 mg/kg (n=5) Folic Acid for 4 Weeks during Adulthood Followed by a 4 Week Maintenance Diet.

Samples were removed that deviated from the adjusted mean by > 2 (sample 6, 1 mg/kg).

	1 mg/kg						5 mg/kg				
Sample	1	2	3	4	5	6	2	3	4	5	6
MAD	0.068						0.054				
Absolute Deviation	0.50	0.86	0.29	-0.29	-1.60	-4.15	1.09	0.68	0	0.799	-0.05

Total gene expression data between samples was correlated and displayed as an MA plot and Volcano plot allowing visualisation of numbers of differentially expressed genes across the genome (Figure 6.5). 372 genes were identified as significantly differentially expressed in the 5 mg/kg group using a false discovery rate (FDR) adjusted P value of < 1, or 222 genes using an FDR adjusted P value of < 0.05. Of the 222 genes identified (FDR < 0.05), 191 were upregulated and 31 were downregulated in response to folic acid supplementation.

The top three genes significantly upregulated consisted of sphingomyelin phosphodiesterase 3 (*Smpd3*) (LogFC of 3.75), peptidase inhibitor 16 (Pi16) (LogFC of 3.69) and CD248 antigen endosialin (CD248) (LogFC of 3.33) (Table 6.13). The top three genes significantly downregulated consisted of expressed sequence Al606181 (Al606181) (LogFC of -1.97), *Bcl2* binding component 3 (Bbc3) (LogFC of -1.76) and *Rpl22l1* (LogFC of -1.74) (Table 6.13).

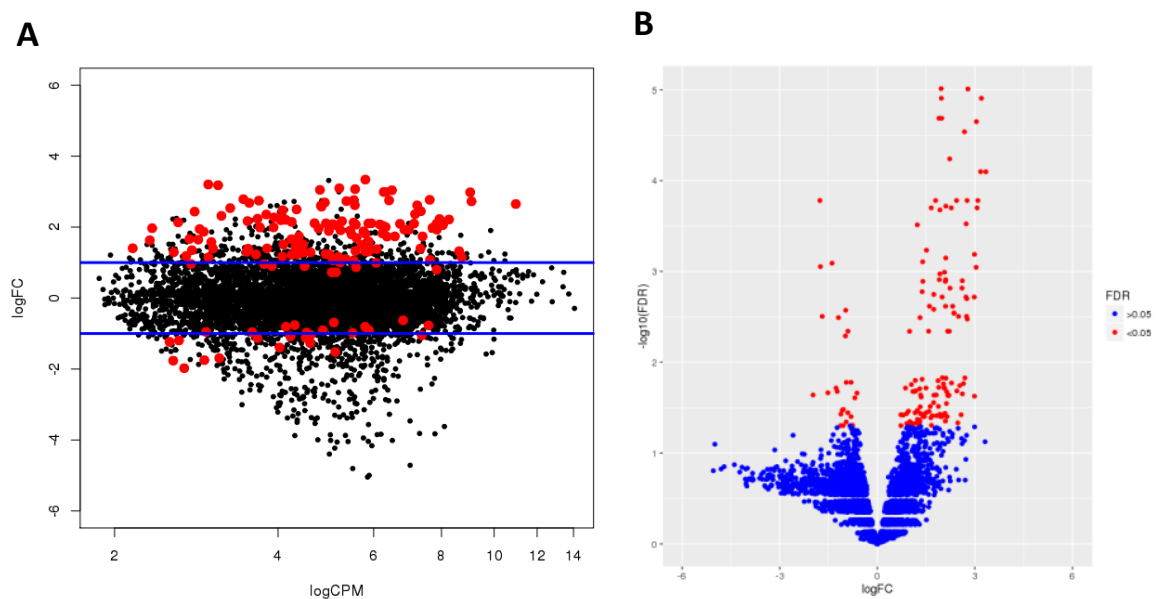


Figure 6.5 Differential Gene Expression in the Mammary Gland of Female 57BL/6 Mice Supplemented with 1 mg/kg (1 x BDR - control) or 5 mg/kg (5 x BDR) Folic Acid for 4 Weeks (PND 74 – 102) Followed by a 4 Week Maintenance Diet (PND 102-130) during Adulthood.

(A) MA plot showing average logCPM against logFC (change in log₂ CPM). Individual genes are represented by single dots. Differentially expressed genes with adjusted p values (FDR) that are below 0.05 appear in red. The greater the levels of differential expression the further from the centre red dots appear. (B) Volcano plot of logFC against – logFDR (–log₁₀ of the FDR). Genes that are dysregulated appear nearer the left and right hand sides of the plot. Genes that demonstrate significant differential expression (red) appear higher on the plot with highly significant changes located nearer the top. Lowly expressed genes were excluded after differential expression analysis using EdgeR. N = 5 per group.

Table 6.13 Differential Gene Expression in the Mammary Gland of Female C57BL/6 mice Supplemented with 1 mg/kg (1 x BDR - control) or 5 mg/kg (5 x BDR) Folic Acid for 4 Weeks (PND 74–102) Followed by a 4 Week Maintenance Diet (PND 102-130) during Adulthood.

Top 15 genes significantly upregulated (yellow) and downregulated (blue) (FDR = < 0.05, upregulated = + LogFC, downregulated = -LogFC). Total genes = 222 with 191 upregulated and 31 downregulated.

Mgi Ref	LogFC	FDR	PVALUE	Description
Upregulated Genes				
Smpd3	3.752212	0.005139	2.91E-05	sphingomyelin phosphodiesterase 3, neutral
Pi16	3.698289	0.009287	7.72E-05	peptidase inhibitor 16
Cd248	3.33967	7.99E-05	7.16E-08	CD248 antigen, endosialin
Gabra3	3.202282	1.24E-05	4.03E-09	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3
Itga11	3.179464	7.97E-05	6.50E-08	integrin alpha 11
Adgrd1	3.095523	0.000166	2.16E-07	adhesion G protein-coupled receptor D1
Cd163	3.070336	0.0002	3.18E-07	CD163 antigen
Cd209f	3.04923	2.24E-05	1.28E-08	CD209f antigen
F13a1	3.039735	0.0009	2.20E-06	coagulation factor XIII, A1 subunit
Ccl6	2.989764	0.000649	1.32E-06	chemokine (C-C motif) ligand 6
Fndc1	2.989204	0.023618	0.000316	fibronectin type III domain containing 1
Fn1	2.981304	0.001912	6.86E-06	fibronectin 1
Arsi	2.940951	0.005942	3.89E-05	arylsulfatase i
Cd209g	2.787528	9.80E-06	1.60E-09	CD209g antigen
Downregulated Genes				
Al606181	-1.97643	0.022917	0.000295	expressed sequence Al606181
Bbc3	-1.76456	0.000166	1.88E-07	BCL2 binding component 3
Rpl22l1	-1.74953	0.000885	2.09E-06	ribosomal protein L22 like 1
	-1.69341	0.003125	1.40E-05	mmu-mir-703
Gm5963	-1.51961	0.021709	0.000272	predicted pseudogene 5963
Snhg12	-1.39266	0.000811	1.85E-06	small nucleolar RNA host gene 12
Cd274	-1.26747	0.019244	0.000229	CD274 antigen
Setd4	-1.23327	0.020966	0.000258	SET domain containing 4
Tpt1	-1.20074	0.010028	8.66E-05	tumor protein, translationally-controlled 1
Mphosp6	-1.19544	0.003226	1.50E-05	M phase phosphoprotein 6
Dut	-1.12815	0.04987	0.000899	deoxyuridine triphosphatase
Slbp	-1.12055	0.037288	0.000565	stem-loop binding protein
Gm6158	-1.07958	0.03376	0.000476	predicted gene 6158
Ptma	-1.05222	0.04987	0.000897	prothymosin alpha
Cep57	-1.04211	0.033096	0.000464	centrosomal protein 57

6.3.2.1 Gene Set Enrichment Analysis

GSEA was performed on genes differentially expressed in response to 5 mg/kg folic acid in order to identify cumulative changes in groups of genes that have previously been biologically categorised. Within the Hallmark grouping, 29 gene sets were identified as statistically significantly enriched (FDRq value < 0.05) with 19 upregulated (+ NES score) out of a total of 29 and 10 downregulated (- NES score) out of a total of 20 (Table 6.14). This infers that the biological activity of these gene sets is directly correlated with the ranking of genes that were analysed, thus is affected by the folic acid diet. The top five highly significant enriched gene sets upregulated consisted of epithelial mesenchymal transition (EMT) (NES = 7.84, FDR < 0.001) (a process in which the loss of cell-cell adhesion and cell polarity within epithelial cells results in the transition to mesenchymal stem cells with migratory and invasive properties), myogenesis (NES = 6.926, FDR < 0.001), apical junction (NES = 3.784, FDR < 0.001), estrogen response early (NES = 3.671, FDR < 0.001) and KRAS signalling (NES = 3.348, FDR < 0.001) (Table 6.14). The top downregulated enriched gene set consisted of E2F targets (NES = - 6.67, FDR < 0.01), genes critically involved in the regulation of cell cycle progression and DNA regulation, MYC targets (NES = -4.817, FDR < 0.001), G2M checkpoint (NES = -4.370, FDR < 0.001), oxidative phosphorylation (NES = -4.102 FDR < 0.001) and allograft rejection (NES = -3.624, FDR < 0.001) (Table 6.14).

C2 curated category showed a total of 1080 gene sets that were significantly enriched including 560 upregulated and 520 downregulated (FDR < 0.05). The top gene set upregulated was NABA matrisome (NES = 11.47, FDR < 0.001), a classification of ECM related genes. The further top 5 gene sets upregulated included Lim mammary stem cell up (NES = 10.647, FDR < 0.001), Naba core matrisome (NES = 9.129, FDR < 0.001), Liu prostate cancer down (NES = 8.824, FDR < 0.001), Boquest stem cell up (NES = 8.388, FDR < 0.001) (Table 6.15). The top gene set downregulated was Pujana BRCA1 PCC network (NES = -7.10, FDR < 0.01). The further top 5 gene sets downregulated included Marson bound by E2F4 unstimulated (NES = -6.245, FDR < 0.001), reactome processing of capped intron containing pre Mrna (NES = -5.948, FDR < 0.01), reactome metabolism of RNA (NES = -5.893, FDR < 0.001) and Zheng bound by FOX3 (NES = -5.881, FDR < 0.001) (Table 6.15). Within the Kegg/Biocarter category a total of 91 gene sets were significantly enriched including 43 upregulated and 48 downregulated. The top gene set upregulated in KEGG was ECM receptor interaction (NES = 5.93, FDR < 0.001) and the top downregulated enriched gene set was Splicesome (NES = -5.88) (Table 6.16). The top gene set upregulated in Biocarter

was the AGR pathway (NES = 2.883, FDR < 0.001). The top gene set downregulated was MCM pathway (NES -3.48483, FDR <0.001) (Table 6.16). Gene sets implicated in breast cancer include *Wnt* signalling pathway and Hedgehog signalling pathways which function in signal transduction and stem cell function, respectively.

Of the miRNA gene sets within the C3 Category (205) six were significantly enriched for upregulated genes. The top upregulated miRNA gene set consisted of TGCCTTA MIR-124A (NES = 3.21, FDR < 0.001) (458 genes) of which 260 genes were enriched. Within the transcription factor gene sets the top upregulated enriched gene set consisted of CTAWWWATA_V\$RSRFC4_Q2 (NES = 5.68, FDR < 0.001) and downregulated V\$E2F_Q4 (NES = -4.35, FDR <0.001) with 31.5 % and 52.7 % of gene sets significantly enriched respectively.

Table 6.14. Hallmark GSEA of Samples from Mice Supplemented with 1 mg/kg or 5 mg/kg of Folic Acid for 4 Weeks during Adulthood (PND 74-102), Followed by a 4 Week Maintenance diet.

Top 20 enriched upregulated gene sets (+ NES, yellow), top 20 down regulated gene sets (-NES, blue) FDR 0 = < 0.001.

Hallmark Gene Set	Genes	En Score	NES	FDR
EPITHELIAL MESENCHYMAL TRANSITION	164	0.531408	7.848795	0
MYOGENESIS	174	0.449458	6.926522	0
APICAL JUNCTION	163	0.255228	3.78442	0
ESTROGEN RESPONSE EARLY	166	0.244345	3.671612	0
KRAS SIGNALING DN	83	0.313711	3.348661	0
HYPOXIA	169	0.221252	3.317838	0
COAGULATION	86	0.304144	3.266531	0
UV RESPONSE DN	132	0.232409	3.114483	0
ANGIOGENESIS	27	0.490317	3.047816	0
ESTROGEN RESPONSE LATE	153	0.206369	2.973775	0
ADIPOGENESIS	192	0.183214	2.950639	0
BILE ACID METABOLISM	77	0.272684	2.851362	9.67E-05
KRAS SIGNALING UP	145	0.195524	2.72491	2.20E-04
IL2 STAT5 SIGNALING	158	0.162494	2.385385	9.62E-04
GLYCOLYSIS	169	0.150861	2.243919	0.001825
HEME METABOLISM	157	0.150062	2.165965	0.003098
APICAL SURFACE	32	0.299885	2.05001	0.006681
XENOBIOTIC METABOLISM	138	0.138558	1.828775	0.019333
WNT BETA CATENIN SIGNALING	34	0.256811	1.611424	0.02178
FATTY ACID METABOLISM	127	0.11724	-0.87156	0.613245
E2F TARGETS	152	-0.46922	-6.67624	0
MYC TARGETS V1	186	-0.3011	-4.81767	0
G2M CHECKPOINT	153	-0.29988	-4.3706	0
OXIDATIVE PHOSPHORYLATION	190	-0.25466	-4.10262	0
ALLOGRAFT REJECTION	115	-0.28414	-3.6242	0
INTERFERON GAMMA RESPONSE	163	-0.24083	-3.6053	0
DNA REPAIR	133	-0.2506	-3.32683	0
MYC TARGETS V2	53	-0.24512	-2.12755	0.006921
TNFA SIGNALING VIA NFKB	151	-0.12676	-1.84845	0.027667
INTERFERON ALPHA RESPONSE	84	-0.15811	-1.71064	0.049992
UNFOLDED PROTEIN RESPONSE	104	-0.12996	-1.56582	0.093578
MTORC1 SIGNALING	185	-0.09111	-1.43869	0.155319
IL6 JAK STAT3 SIGNALING	61	-0.14803	-1.35932	0.19457
REACTIVE OXYGEN SPECIES PATHWAY	45	-0.17334	-1.35477	0.183986
COMPLEMENT	132	-0.0911	-1.21697	0.2877
PEROXISOME	83	-0.11237	-1.1807	0.30666
PI3K AKT MTOR SIGNALING	96	-0.09937	-1.11195	0.364146
SPERMATOGENESIS	62	-0.11007	-1.00084	0.484088
P53 PATHWAY	172	-0.06138	-0.92864	0.562207
UV RESPONSE UP	127	-0.06605	-0.87156	0.613245

Table 6.15. C2 GSEA of Samples from Mice Supplemented with 1 mg/kg or 5 mg/kg of Folic Acid for 4 Weeks during Adulthood (PND 74-102) Followed by a 4 Week Maintenance Diet.

Top 20 enriched upregulated gene sets (+ NES, yellow), top 20 down regulated gene sets (-NES, blue) FDR 0 = < 0.001.

C2 All Gene Set	Genes	En Score	NES	FDR
NABA MATRISOME	429	0.490619	11.47848	0
LIM MAMMARY STEM CELL UP	367	0.479857	10.64709	0
NABA CORE MATRISOME	155	0.644179	9.129037	0
LIU PROSTATE CANCER DN	357	0.395048	8.824186	0
BOQUEST STEM CELL UP	206	0.511573	8.38872	0
NABA ECM GLYCOPROTEINS	113	0.619614	7.761062	0
NABA MATRISOME ASSOCIATED	274	0.402452	7.745561	0
SCHUETZ BREAST CANCER DUCTAL INVASIVE UP	279	0.398097	7.552965	0
ONDER CDH1 TARGETS 2 UP	205	0.39839	6.732481	0
CANCER MESENCHYMALTRANSITION SIGNATURE	52	0.761852	6.575606	0
CHICAS RB1 TARGETS CONFLUENT	432	0.272712	6.426322	0
ANGIOIMMUNOBLASTIC LYMPHOMA UP	163	0.415806	6.217342	0
PASINI SUZ12 TARGETS DN	278	0.322574	6.205965	0
GOZGIT ESR1 TARGETS DN	487	0.241495	6.187787	0
VECCHI GASTRIC CANCER EARLY UP	127	0.477651	6.135502	0
SWEET LUNG CANCER KRAS DN	347	0.286855	6.127998	0
BENPORATH SUZ12 TARGETS	405	0.265996	6.021975	0
MARTENS TRETINOIN RESPONSE UP	300	0.301062	6.019284	0
BENPORATH ES WITH H3K27ME3	428	0.255513	5.966137	0
KEGG ECM RECEPTOR INTERACTION	64	0.627409	5.931865	0
PUJANA BRCA2 PCC NETWORK	322	-0.34389	-7.10385	0
MARSON BOUND BY E2F4 UNSTIMULATED	477	-0.25621	-6.24546	0
REACTOME PROCESSING OF CAPPED INTRON CONTAINING PRE MRNA	126	-0.45432	-5.94819	0
REACTOME METABOLISM OF RNA	212	-0.35828	-5.89353	0
ZHENG BOUND BY FOXP3	368	-0.26897	-5.88158	0
KEGG SPLICEOSOME	112	-0.47993	-5.84562	0
PUJANA XPRSS INT NETWORK	138	-0.42321	-5.82428	0
REACTOME MRNA PROCESSING	143	-0.41927	-5.76118	0
LEE DIFFERENTIATING T LYMPHOCYTE	141	-0.4093	-5.58447	0
REACTOME MRNA SPLICING	99	-0.47363	-5.46661	0
REACTOME METABOLISM OF MRNA	171	-0.34633	-5.34838	0
CAIRO HEPATOBLASTOMA CLASSES UP	481	-0.20954	-5.30911	0
KEGG RIBOSOME	54	-0.60524	-5.29248	0
ZHANG TLX TARGETS 36HR DN	141	-0.37175	-5.21137	0
GARY CD5 TARGETS DN	372	-0.22843	-5.16559	0
WONG EMBRYONIC STEM CELL CORE	271	-0.27178	-5.13655	0
MOOTHA HUMAN MITODB 6 2002	370	-0.22519	-5.10092	0
MUELLER PLURINET	231	-0.28913	-5.06638	0
REACTOME INFLUENZA LIFE CYCLE	99	-0.42996	-5.03735	0
REACTOME PEPTIDE CHAIN ELONGATION	52	-0.6037	-5.02837	0

Table 6.16. KEGG/Biocarter GSEA from Mice Supplemented with 1 mg/kg or 5 mg/kg of Folic Acid for 4 Weeks during Adulthood (PND 74-102) Followed by a 4 Week Maintenance Diet.

Top 20 enriched upregulated gene sets (+ NES, yellow), top 20 down regulated gene sets (-NES, blue) FDR 0 = < 0.001.

Kegg/Biocarter Gene Set	Genes	En Score	NES	FDR
ECM RECEPTOR INTERACTION	64	0.627409	5.932629	0
FOCAL ADHESION	171	0.340062	5.144737	0
ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY ARVC	55	0.423979	3.739462	0
HYPERTROPHIC CARDIOMYOPATHY HCM	58	0.380481	3.405544	0
DILATED CARDIOMYOPATHY	62	0.353771	3.344704	0
AXON GUIDANCE	96	0.272378	3.129538	1.71E-04
COMPLEMENT AND COAGULATION CASCADES	32	0.422703	2.909193	1.47E-04
BIOCARTA AGR PATHWAY	28	0.454549	2.883367	1.28E-04
CYTOKINE CYTOKINE RECEPTOR INTERACTION	104	0.238586	2.80364	2.12E-04
PATHWAYS IN CANCER	258	0.139832	2.609074	0.001021
MELANOGENESIS	67	0.2683	2.579481	0.001177
BASAL CELL CARCINOMA	32	0.394341	2.570325	0.001079
WNT SIGNALING PATHWAY	115	0.19853	2.541623	0.001274
REGULATION OF ACTIN CYTOSKELETON	159	0.173639	2.489758	0.002073
TIGHT JUNCTION	93	0.21943	2.443446	0.002399
CALCIUM SIGNALING PATHWAY	96	0.207098	2.351276	0.004144
GAP JUNCTION	66	0.230995	2.211586	0.008926
NEUROACTIVE LIGAND RECEPTOR INTERACTION	60	0.246729	2.207825	0.008721
STARCH AND SUCROSE METABOLISM	26	0.352598	2.13757	0.01315
GLYCOLYSIS GLUCONEOGENESIS	43	0.263751	2.100094	0.016475
SPLICEOSOME	112	-0.47993	-5.88582	0
RIBOSOME	54	-0.60524	-5.21229	0
DNA REPLICATION	32	-0.61022	-4.14199	0
OXIDATIVE PHOSPHORYLATION	96	-0.33552	-3.82092	0
RNA DEGRADATION	53	-0.43553	-3.70908	0
GRAFT VERSUS HOST DISEASE	15	-0.79802	-3.6954	0
ALLOGRAFT REJECTION	15	-0.79802	-3.64564	0
ANTIGEN PROCESSING AND PRESENTATION	42	-0.4658	-3.5749	0
AUTOIMMUNE THYROID DISEASE	16	-0.73551	-3.57309	0
MISMATCH REPAIR	20	-0.65757	-3.55789	0
KEGG HUNTINGTONS DISEASE	143	-0.24833	-3.492	0
BIOCARTA MCM PATHWAY	16	-0.73004	-3.48483	0
TYPE I DIABETES MELLITUS	17	-0.68034	-3.43198	0
BIOCARTA HIVNEF PATHWAY	56	-0.37176	-3.28429	0
PARKINSONS DISEASE	93	-0.29158	-3.26859	0
CELL CYCLE	105	-0.24847	-2.94739	6.04E-05
INTESTINAL IMMUNE NETWORK FOR IGA	19	-0.53621	-2.75557	4.64E-04
PYRIMIDINE METABOLISM	77	-0.24994	-2.64678	6.21E-04
NUCLEOTIDE EXCISION REPAIR	42	-0.34162	-2.61216	6.45E-04
B CELL RECEPTOR SIGNALING PATHWAY	64	-0.27197	-2.54969	8.83E-04

6.3.2.2 Gene Ontology

Differentially expressed genes were analysed for GO within three categories; biological processes, molecular function and cellular compartment ontologies (Table 6.17). Within the biological processes category 312 gene sets were identified as significantly changed in response to folic acid supplementation. The top three processes most significantly affected consisted of locomotion (FDR, $1.37\text{E-}27$), cell migration (FDR, $1.37\text{E-}27$) and cell motility (FDR, $1.37\text{E-}27$) (Table 6.17). Within the molecular function category 53 gene sets were identified as significantly changed (FDR < 0.05). The top three functions changed consisted of collagen binding (FDR, $7.80\text{E-}07$) metalloproteinase activity (FDR, $1.60\text{E-}06$) and glycosaminoglycan binding (FDR, $3.00\text{E-}06$). Within the cellular compartment ontology 33 gene sets were identified as significantly changed (FDR < 0.05). The top three compartments changed consisted of proteinaceous extracellular matrix (FDR, $6.00\text{E-}17$), extracellular matrix ($1.20\text{E-}15$) and extracellular space (FDR, $9.40\text{E-}14$) (Table 6.17).

The most significantly changed gene set within cellular compartment was identified as proteinaceous extracellular matrix (GO: 0005578) with extracellular matrix (GO: 0005739) identified as the second most significant. As extracellular matrix (ECM) contained a higher number of significantly categorised genes and there was overlap between both categories the ECM gene set was investigated further. In total 32 genes involved in ECM function were identified as differentially expressed between 1 mg/kg and 5 mg/kg folic acid supplemented groups (FDR < 0.05), with 32 genes upregulated and no genes downregulated. The top three ECM genes significantly upregulated consisted of Fibronectin 1 (LogFC 2.98), Lama2 (LogFC 2.752) and Emilin2 (LogFC 2.732) (Table 6.18).

Table 6.17. Gene Ontology in the Mammary Gland of Female C57BL/6 Mice Supplemented with 1 mg/kg (1 x BDR - control) or 5 mg/kg (5 x BDR) Folic Acid for 4 Weeks (PND 74 – 102) during Adulthood Followed by a 4 Week Maintenance Diet (PND 102-130).

Top 10 gene sets significantly changed for molecular processes, molecular function and cellular compartment ontology groups based on FDR values (FDR < 0.05).

No	GO-ID	Term	Sig	Expected	FDR
Biological Process					
1	GO:0040011	locomotion	145	44	3.50E-13
2	GO:0016477	cell migration	121	39	1.30E-12
3	GO:0048870	cell motility	129	40	2.70E-12
4	GO:0051674	localization of cell movement of cell or subcellular component	129	40	2.70E-12
5	GO:0006928	regulation of cell migration	159	45	3.00E-12
6	GO:0030334	regulation of cell motility	82	31	3.60E-12
7	GO:2000145	regulation of cellular component movement	86	31	1.60E-11
8	GO:0051270	regulation of locomotion	94	32	4.20E-11
9	GO:0040012	positive regulation of cell migration	90	31	6.30E-11
10	GO:0030335		42	20	2.80E-10
Molecular Function					
1	GO:0005518	collagen binding	17	10	7.80E-07
2	GO:0008237	metallopeptidase activity	18	10	1.60E-06
3	GO:0005539	glycosaminoglycan binding	27	12	3.00E-06
4	GO:0008201	heparin binding	23	10	2.70E-05
5	GO:0004222	metalloendopeptidase activity	12	7	4.50E-05
6	GO:0005125	cytokine activity	12	7	4.50E-05
7	GO:0005102	receptor binding	119	26	4.50E-05
8	GO:0008009	chemokine activity	6	5	5.20E-05
9	GO:0048407	platelet-derived growth factor binding	6	5	5.20E-05
10	GO:0032403	protein complex binding	100	23	5.60E-05
Cellular Compartment					
1	GO:0005578	proteinaceous extracellular matrix	59	31	6.00E-17
2	GO:0031012	extracellular matrix	68	32	1.20E-15
3	GO:0005615	extracellular space	106	38	9.40E-14
4	GO:0005576	extracellular region	366	72	1.00E-10
5	GO:0044421	extracellular region part	344	66	4.00E-09
6	GO:0005581	collagen trimer	12	10	5.50E-09
7	GO:0044420	extracellular matrix component	25	12	1.50E-06
8	GO:0005886	plasma membrane	305	54	4.50E-06
9	GO:0071944	cell periphery	313	54	1.10E-05
10	GO:0005583	fibrillar collagen trimer	6	5	6.00E-05

Table 6.18. Gene Ontology Analysis Within Components of the Extra Cellular Matrix (ECM) in Mammary Tissue from Mice Supplemented with 1 mg/kg or 5 mg/kg Folic Acid for 4 Weeks during Adulthood (PND 74-102), Followed by a 4 Week Maintenance Diet (PND 102-130).

Top 30 genes upregulated (+ LogFC) shown.

Mgi_ref	LogFC	PValue	FDR	Description
Fn1	2.981304	6.86E-06	0.001912	fibronectin 1
Lama2	2.75248	7.32E-06	0.001996	laminin, alpha 2
Emilin2	2.732957	5.37E-07	0.000299	elastin microfibril interfacer 2
Col1a1	2.726791	6.77E-06	0.001912	collagen, type I, alpha 1
Spon2	2.69445	0.000156	0.014871	spondin 2, extracellular matrix protein
Col3a1	2.651278	0.000192	0.01716	collagen, type III, alpha 1
Mmp3	2.61734	0.000283	0.022292	matrix metalloproteinase 3
Tnxb	2.614372	3.61E-06	0.001265	tenascin XB
Fbln1	2.476519	0.000791	0.046506	fibulin 1
Mmp2	2.447916	0.000251	0.020706	matrix metalloproteinase 2
Col5a1	2.366063	1.22E-05	0.002881	collagen, type V, alpha 1
Mmp9	2.355823	1.21E-05	0.002881	matrix metalloproteinase 9
Il1rl1	2.317838	9.45E-06	0.002416	interleukin 1 receptor-like 1
Col6a3	2.228025	4.22E-08	5.76E-05	collagen, type VI, alpha 3
Dpt	2.214016	2.26E-05	0.004558	dermatopontin
Adamts12	2.128152	0.00039	0.028502	motif, 12
Ecm1	2.105854	1.51E-06	0.000711	extracellular matrix protein 1 serine (or cysteine) peptidase inhibitor, clade F, member 1
Serpinf1	2.098949	9.34E-06	0.002416	
Lum	2.09554	0.000739	0.044462	lumican
Adamts2	2.034725	0.000195	0.017319	motif, 2
Fbln7	1.990433	0.000637	0.039699	fibulin 7
Col6a2	1.980787	1.01E-08	2.06E-05	collagen, type VI, alpha 2
Col6a1	1.929115	3.60E-07	0.00021	collagen, type VI, alpha 1 MGI
Vwf	1.928711	0.000565	0.037288	Von Willebrand factor homolog
Mamdc2	1.62894	0.000423	0.030646	MAM domain containing 2
Bmp1	1.593013	0.000502	0.03478	bone morphogenetic protein 1 procollagen C-endopeptidase enhancer protein
Pcolce	1.380253	5.44E-06	0.00167	a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 1
Adamts1	1.328471	0.000545	0.036758	
Hspg2	1.321204	0.000431	0.030939	perlecan (heparan sulfate proteoglycan 2) biglycan a disintegrin-like and metalloproteinase
Bgn	1.075354	0.000815	0.04727	

6.3.2.3 Pathway Analysis

Pathway analysis was performed on differentially expressed genes from mice fed 5 mg/kg folic acid during the adult period to establish insight into the molecular and chemical interaction and disease processes affected. Top canonical pathways identified included hepatic fibrosis/hepatic stellate cell activation, granulocyte adhesion, agranulocyte adhesion and inhibition of matrix metalloproteases (MMPs). Of these, inhibition of MMPs was the pathway with the highest number of differentially expressed genes that overlap with genes from the MMP pathway, with 7 out of a total of 36 genes (19.4%) shown to overlap in the grouping (Table 6.20). Genes affected included *Timp2*, *Timp3*, *Mmp2*, *Mmp9*, *Hspg*, and *Tsp2* which were shown to function in the extracellular space and induce downstream effects within the plasma membrane (Table 6.19). Top upstream regulators were identified as alpha catenin, *IL10RA*, *IGF2BP1*, *Col4a3* and *Fas*. Alpha catenin was shown to have the most significant overlap ($1.39\text{E-}20$) and in addition alpha catenin activity was predicted to be inhibited. Further regulators in order of significance included IFNG, TP53, RETNLB, NPR1 and IL6R.

The most significantly affected disease pathway was identified as cancer (p-value range $8.09\text{E-}3$ – $4.00\text{E-}10$) with a total of 56 genes affected. Within the cancer pathway the top three upregulated pathways were metastasis ($Z = 1.206$), mammary tumour ($Z = 1.118$) and growth of tumour ($Z = 0.592$) (Table 6.20). The top function, tumour growth, was shown to have a total of 30 genes differentially regulated. Cellular network analysis revealed 17 gene networks that were affected by folic acid intake during adulthood. Of these the top network containing 27 focus genes encompassed cell morphology, cellular assembly and organisation, cellular function and maintenance (Table 6.21).

Table 6.19. Top Upstream Regulators Identified from Ingenuity Pathway Analysis in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation Z Score	P-Value of Overlap
Alpha Catenin	Group	Inhibited	-4.502	1.39E-20
IL10RA	Transmembrane receptor	Activated	3.838	1.12E-12
IGF2BP1	Translation regulator	Activated	2.646	4.87E-11
COL4A3	Other			1.04E-09
FAS	Transmembrane receptor	Inhibited	-3.157	1.11E-09
IFNG	Cytokine		0.011	2.43E-08
TP53	Transcription Regulator		1.012	3.02E-08
RETNLB	Other	Activated	2.985	9.84E-08
NPR1	Enzyme	Inhibited	-2.415	2.16E-07
IL6R	Transmembrane receptor	Activated	2.219	3.78E-07

Table 6.20. Top Canonical Pathways Identified from Ingenuity Pathway Analysis in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Canonical Pathway	P-Value	Overlap (%)
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.87E-13	10.8
Granulocyte Adhesion and Diapedesis	2.35E-08	8.6
Agranulocyte Adhesion and Diapedesis	5.03E-08	8.0
Inhibition of Matrix Metalloproteases	1.54E-07	19.4
Atherosclerosis Signalling	8.03E-07	8.8

Table 6.21. Top Disease Pathways Identified from Ingenuity Pathway Analysis in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Disease and Disorders	P-Value Range	Number of Genes
Cancer	3.20E-04 – 6.30E-15	187
Organismal Injury and Abnormalities	2.99E-04 – 6.28E-12	219
Connective Tissue Disorders	3.18E-04 – 2.42E-11	139
Inflammatory Disease	5.12E-04 – 2.42E-11	205
Inflammatory Response	5.12E-04 – 6.85E-10	133

Table 6.22. Top Networks Identified from Ingenuity Pathway Analysis in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Adulthood.

Network Pathways	Score	Focus Molecules
Cell Morphology, Cellular Assembly and Organisation, Cellular Function and Maintenance	42	27
Cell Death and Survival, Cellular Movement, Hematological System, Development and Function	37	25
Cardiovascular Disease, Cardiovascular System Development and Function, Organ Morphology	21	17
Nervous System Development and Function, Organ Morphology, Tissue Morphology	19	16
Cancer, Organismal injury and abnormalities, tumour morphology	18	15

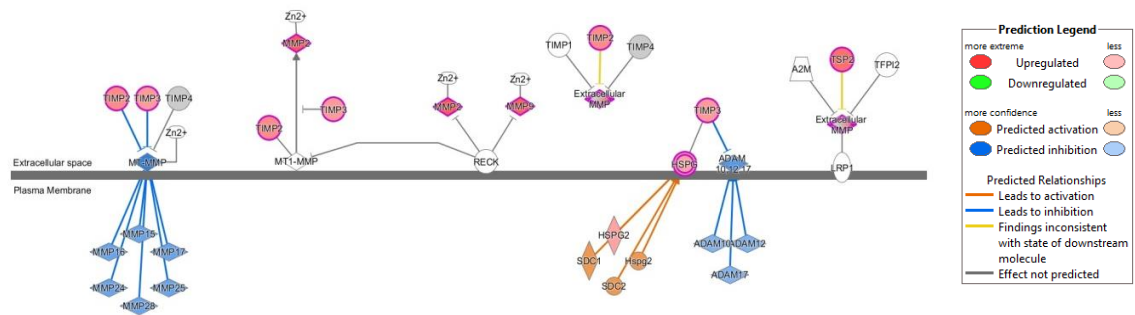


Figure 6.6. Top Disease Pathway (% overlap) of Inhibition of MMPs Identified in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during the Adulthood.

Gene's upregulated (pink) and downregulated (green) are shown in respect of their interaction status.

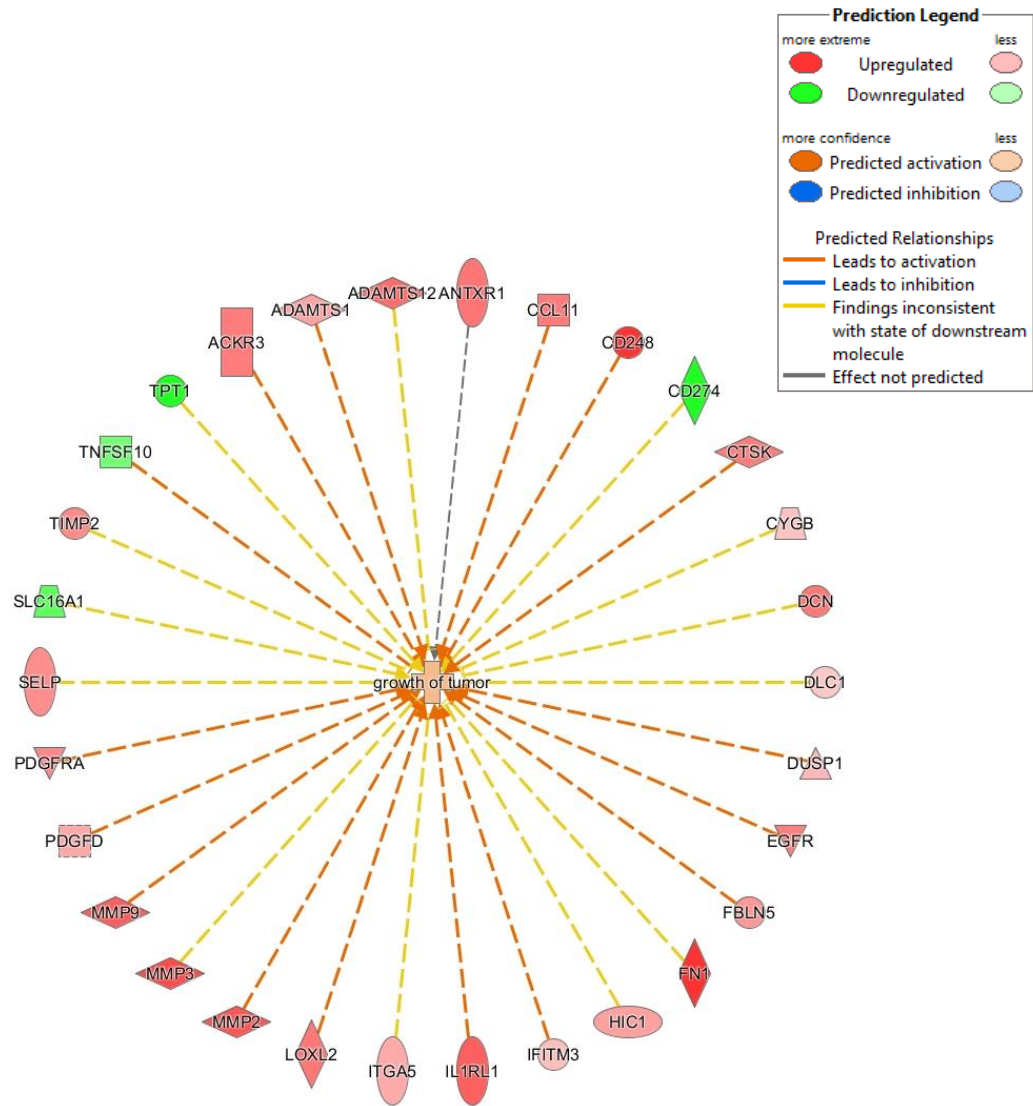


Figure 6.7. Top Disease Pathway in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Network associated with cancer tumour growth. Gene's upregulated (pink) and downregulated (green) are shown in respect of their interaction status, predicated activation (orange) and predicted inhibition (blue).

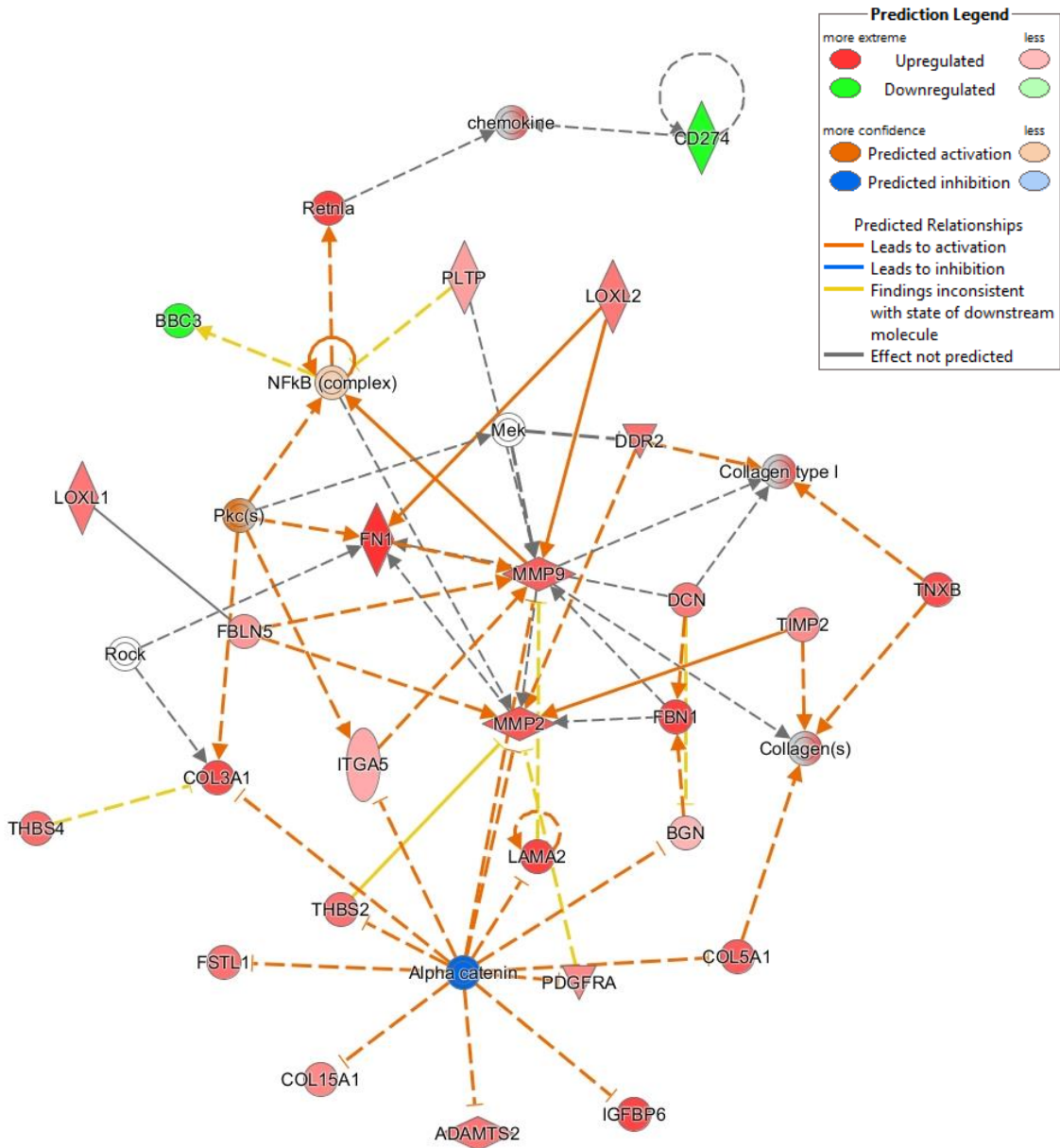


Figure 6.8 Top Network Pathway in Mammary Glands of Mice Supplemented with 5 mg/kg Folic Acid during Adulthood.

Network associated with cell morphology, cellular assembly and organisation and cellular function and maintenance. Gene's upregulated (pink) and downregulated (green) are shown in respect of their interaction status, predicated activation (orange) and predicted inhibition (blue).

6.3.3 Comparison between Juvenile and Adult Supplemented Mice

Differential gene expression was compared between RNA-seq outputs in mice supplemented during the juvenile period and mice supplemented during adulthood, followed by a 4 week maintenance diet. Mice supplemented during the juvenile period demonstrated a much larger total number of differentially expressed genes (1819) when compared to adults (222) with only 9 genes found to overlap between the two time points that include *Slc16a1*, *Ptp4a1*, *P3h1*, *Prkcdbp*, *Col6a2*, *Col5a3*, *Lum*, *Lrrc17* and *Ephb2* (Figure 6.9) Significantly upregulated genes were in much greater abundance in juvenile supplemented mice compared to adults, however interestingly no genes were found to overlap between the two time points (Figure 6.9). Genes that were significantly downregulated showed a similar trend with a greater abundance of genes observed in the juvenile supplemented mice when compared to the adults and only two genes shown to overlap between time points that include *Ptp4a1* and *Slc16a1* (Figure 6.9).

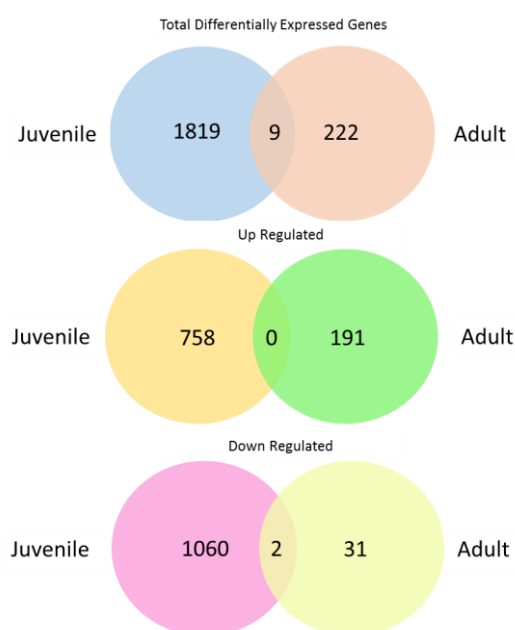


Figure 6.9 Venn Diagrams from RNA-seq Data of Differential Gene Expression in the Mammary Gland of Female C57BL/6 Mice Supplemented with 1 mg/kg or 5 mg/kg Folic Acid for 4 Weeks Followed by a 4 Week Maintenance Diet during the Juvenile Period or Adulthood.

Overlap between juvenile supplemented mice and adult supplemented mice for total number of differentially expressed genes up regulated genes and down regulated genes (FDR < 0.05). n = 5 mice per group.

6.3.4 Plasma Hormone Levels do not Effect Folic Acid Mediated Changes in Genes Associated with EMT, Extracellular Matrix, G2M Checkpoint or Mitochondrion Dysfunction.

Plasma Estradiol-17 β and progesterone levels were compared to normalised counts from genes that were identified as significantly differentially expressed or pathways that were enriched in response to high folic acid intake from RNA-seq analysis, to identify if changes in hormone level which are representative of estrus status effect gene expression data.

No significant differences were observed in adult or juvenile supplemented mice in genes associated with the G2M checkpoint (*Gimap8*) and mitochondrion (*Tp53*) or the extracellular matrix (*Mmp2*) and EMT (*Timp9*) respectively.

Table 6.16.23 Linear Regression Analysis of Plasma Hormone Levels and RNA-seq Data.

Gene	Estradiol-17 β					Progesterone				
	R ²	Adj R ²	Standardised Coefficient β	t	Sig	R ²	Adj R ²	Standardised Coefficient β	t	Sig
Juvenile Supplemented Mice										
<i>Gimap8</i>	0.002	-0.123	0.047	0.133	0.898	0.060	-0.057	0.245	0.715	0.495
<i>CDK14</i>	0.007	-0.117	0.084	0.239	0.817	0.002	-0.123	-0.045	-0.127	0.902
Adults Supplemented Mice										
<i>Mmp9</i>	0.076	-0.109	-0.275	-0.640	0.550	0.1720	0.068	-0.414	-1.297	0.234
<i>Timp2</i>	0.177	0.012	-0.420	-1.036	0.348	0.084	-0.031	-0.290	-0.856	0.417

6.3.5 Validation of RNA-seq

RNA-seq analysis was validated in mice supplemented with high folic acid (5 mg/kg) during the juvenile period or adulthood to assess if changes in gene expression correlated to changes in expression measured by qRT-PCR. Two genes were selected that were upregulated and two genes that were down regulated at both time points based on large fold change scores (FC).

6.3.5.1 Juvenile Validation

Directional changes in gene expression correlated with findings from RNA-seq data. Upregulated genes *Bank1* and *Epsti* showed fold change increases in gene expression of 2.29 and 2.33 respectively, however, did not reach significance between groups. Genes that were downregulated, *Ppp3* and *Aspg* showed fold change decreases in gene expression of -4.80 and -2.77 respectively and demonstrated significant differences between groups (Figure 6.10).

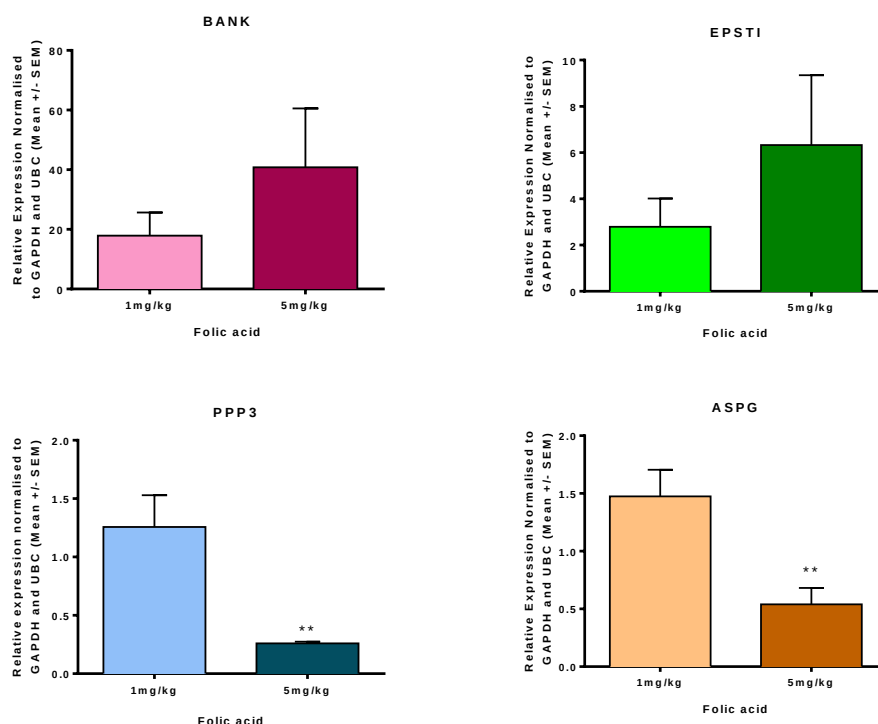


Figure 6.10 qRT-PCR Validation of RNA-seq data in Mice Supplemented with Folic Acid During the juvenile period followed by a 4 week maintenance diet.

Expression of 4 genes were measured that showed upregulation or downregulation within RNA-seq analysis in response to high folic acid intake (5 mg/kg).

6.3.5.2 Adult Validation

Directional changes in gene expression correlated with findings from RNA-seq data. Upregulated genes *Mmp3* and *Mmp2* showed fold change increases in gene expression of 3.75 and 1.40 respectively, however, did not reach significance between groups. Genes that were downregulated, *Spn1* and *Impg2* showed fold change decreases in gene expression of -3.54 and -2.63 respectively, however, did not reach significance between groups (Figure 6.11).

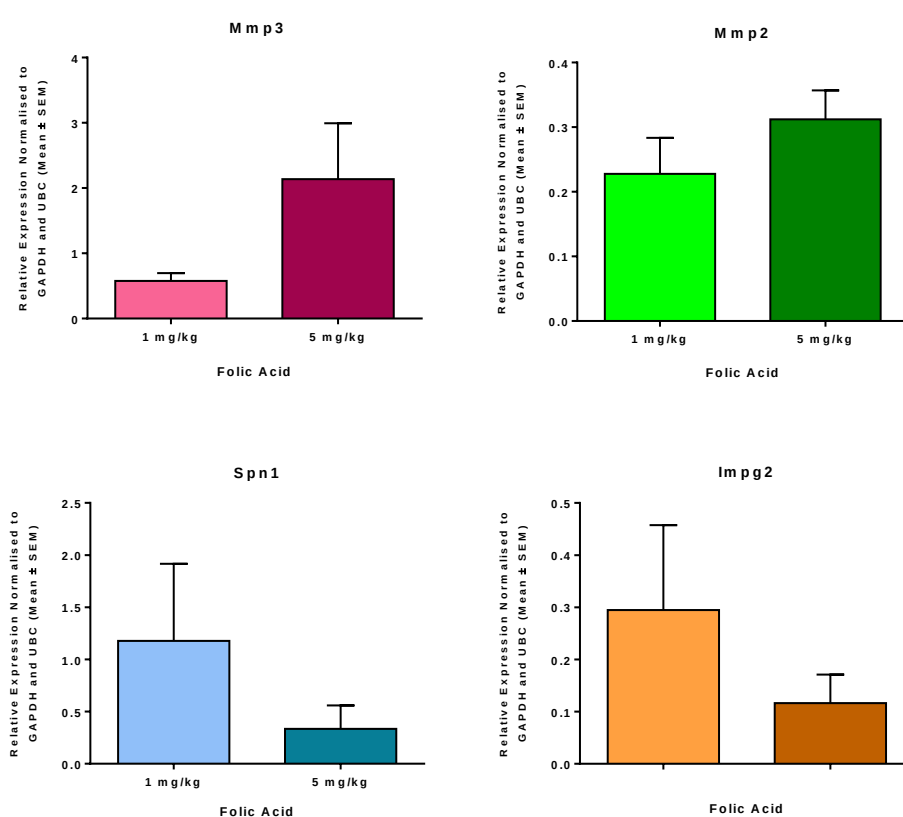


Figure 6.11. qRT-PCR Validation of RNA-seq data in Mice Supplemented with Folic Acid During Adulthood.

Expression of 4 genes were measured that showed upregulation or downregulation within RNA-seq analysis in response to high folic acid intake (5 mg/kg).

6.3.6 Discussion

It is well established that changes in the levels of nutritional intake can affect physiological and metabolic health. Of specific relevance is the dietary intake of folic acid which functions as a critical regulator of one carbon metabolism and has been associated with breast cancer risk. Fully understanding the molecular mechanisms associated with folic acid supplementation requires the analysis of differential gene expression on a global transcriptome scale. This data shows for the first time that the effect of folic acid supplementation at a physiologically relevant concentration (5 mg/kg) equivalent to that recommended during pregnancy on genome wide levels of gene expression, is specific to the timing of folic intake during either the juvenile period or adulthood. Mice supplemented with folic acid (5 mg/kg) during the juvenile period showed significant changes in the transcriptome with 1816 differentially regulated genes identified. The magnitude of differential expression within these genes was moderate (average of 1.46 fold change) with a higher percentage of genes (71.3%) upregulated compared to downregulated. The most highly differentially expressed upregulated gene, immunoglobulin kappa constant (*Igkc*) functions as an antibody within the immune response. Interestingly, research by Schmidt et al., (2012) has identified *Igkc* as a top biomarker with increased protein expression directly related to levels of metastasis free survival and treatment response in breast cancer patients (Schmidt et al., 2012). A folic acid mediated increase in levels of *Igkc* may therefore be considered protective within cancer tissue as it predicts metastasis free survival and also a favourable response to chemotherapy. Within the present study increased *Igkc* in response to folic acid supplementation during the juvenile period in non-cancer tissue may also be protective.

The most significantly downregulated gene, ATP citrate lyase (*Acly*) is an enzyme involved in the regulation of fatty acid biosynthesis, specifically the synthesis of cytosolic acetyl-CoA in many tissues. Research by Bauer et al., (2005) has identified *Atpcl* as an important component of cell growth and transformation in haematopoietic cells (Wellen et al., 2009). Wellen et al., (2009) has shown that histone acetylation in mammalian cells is dependent on *Atpcl* and that *Atpcl* activity is required to link growth factor-induced increases in nutrient metabolism with regulation of histone acetylation and gene expression (Wellen et al., 2009). Furthermore, Herberg et al., (1993) has shown *Acly*, extracted from mammary glands, to be phosphorylated by the catalytic subunit of CAMP. Interestingly, both protein kinase CAMP dependent catalytic alpha (*Prkaca*) and beta (*Prkacb*) expression in the present study were significantly downregulated in response to folic acid. Overall these changes suggest that folic acid modifies

both histone acetylation and phosphorylation events by the reduced expression of genes that function in the regulation of these processes.

GSEA of juvenile supplemented mice showed G2/M checkpoint as highly significantly enriched (NES 5.2009) with 80 % of genes upregulated in response to folic acid supplementation. G2/M checkpoint critically functions to prevent cells entering mitosis until they have had a chance to repair damaged DNA after cell replication. DNA damage induces four main responses within the cell that include removal of damaged DNA, activation of damage checkpoints, a transcriptional response and apoptosis to eliminate damaged cells (Sancar et al., 2004). Genes enriched in response to folic acid included cell cycle regulators CDKN1B, Cdk4 and Cdkn1b, suggesting modulation of cell cycle and activation of G2/M. Interestingly, both *Brca2* and *Ezh2* were both enriched within the G2/M gene set, suggesting a function for DNA repair and polycomb regulation.

Furthermore, both apoptosis and DNA repair gene sets were significantly enriched suggesting a requirement for DNA damage and apoptosis to remove dead cells. Research by Knock et al., (2006) has shown altered response of G2/M checkpoint regulators in response to low dietary folate that include *cdc25c* and *plk4* (Knock et al., 2006). Enriched G2/M gene sets in the present study contain *cdc25a*, *cdc25b* and *plk4*, suggesting a common pathway may exist for high folate intake. Cumulatively, GSEA within these processes implies a role for high folic acid intake in DNA damage and the upregulation or enrichment of associated genes. However, it is unclear exactly if enrichment of these genes occurs as a response to increased DNA damage or as a protective effect in cells with increased repair capacity. Further research is required to assess DNA damage and regulation of associated genes in response to high folic acid intake. This could be achieved using TUNNEL assays in paraffin embedded mammary tissue which detects DNA strand breaks through enzymatically labelling the free 3'OH termini with modified nucleotides. Light microscopy can then be used to identify DNA strand breaks and assess levels of DNA damage in response to folic acid intake (Baskin et al., 2010).

Mechanisms associated with the regulation of differentially expressed or enriched genes could involve folic acid mediated epigenetic changes to DNA methylation or imbalanced deoxyribonucleotide synthesis. Depletion of methyl donors has been shown to induce both gene specific hypermethylation and hypomethylation in different studies, however, currently there are few studies that have linked high folic intake, DNA methylation and changes in gene expression (Crider et al., 2012). Folate deficiency is associated with DNA damage through

decreased thymidylate synthesis from deoxyuridylate, resulting in deoxyribonucleotide imbalance, misincorporation of uracil into DNA (Umi) and the formation of double stranded breaks. However, a study by Van den Donk (2007) has shown high dose supplementation with 5 mg/day folic acid is associated with an increase in Umi in rectal biopsies (van den Donk et al., 2007). This suggests high folic acid intake may affect DNA integrity and related gene expression through different mechanisms.

Classification of genes using GO in juvenile supplemented mice identified a number of gene sets between classifications that cluster around oxidoreductive activity, lipid/fatty acid processes and mitochondrial function (Table 6.6). Of these processes the highly significantly enriched gene set in mitochondrion suggests that folic acid intake modulates cellular components involved in energy production within mammary tissue. Folate metabolism is compartmentalised in the cytoplasm, nucleus and mitochondrion (Ormazabal et al., 2015). The mitochondrial function of folate is linked to formate and CO₂ synthesis as well as the initiation of transfer RNA (tRNA) translation for protein synthesis (Ormazabal et al., 2015). Research has linked low folate status with mitochondrial DNA (mtDNA) instability, however, at present there is minimal research linking high folic acid intake with mitochondrial function in mammary tissue (Ormazabal et al., 2015). As a key compartment involved in folate metabolism and cellular energy provision, modulation of mitochondrial genes associated with high folic acid intake during juvenile development, a period of increased mammary development and plasticity, could be investigated further to establish how this affects mammary development.

Despite putative links between folic acid intake and mitochondrial disorders it is currently unclear as to the effects of high folic acid intake during the juvenile period on genes and gene networks, especially after the period of supplementation. Pathway analysis of juvenile supplemented mice showed the most significant effects in mitochondrial dysfunction which provides parallels with the results of GO. Furthermore, disease pathways most affected included glucose metabolism disorder, disglycemia and hypoglycaemia which can be linked directly to mitochondrial function. In addition, gene networks associated with lipid metabolism were shown to be effected. Research has linked folic acid with mitochondrial DNA (mtDNA) instability, showing no effects of high folic acid intake on mtDNA content of young mice; however, in old mice MtDNA content was 7-fold higher when compared to mice fed a control diet (Rakic et al., 2016). High mtDNA copy number can have beneficial effects on survival; however, very high levels have been linked to nucleotide enlargement, defective transcription and mtDNA

deletions. More importantly this demonstrates an age dependent effect of folic acid intake on mtDNA stability. Mitochondrial dysfunction has also been linked to breast cancer cell function through changes in reactive oxygen species (ROS), with high levels of ROS in breast cancer cells shown to promote cell mobility and invasion (Ma et al., 2013).

These changes could reflect, in part, the effects of folic acid during a period of plasticity, mammary remodelling and high energy requirements that occurs during juvenile mammary development. Previous research has linked maternal folic acid intake to the effects of glucose metabolism in the offspring, however there is limited research to suggest links between high folic acid intake in the juvenile period and genes implicated in glucose metabolism. This is also the case for genes involved in both dysglycemia and hypoglycaemia, shown to be altered in response to folic acid intake. Therefore, this data has identified novel pathways and disease states modulated by folic acid intake during juvenile development that warrant further investigation.

Mice supplemented with folic acid (5 mg/kg) during adulthood showed significant changes in the transcriptome with 222 differentially regulated genes identified. The magnitude of differential expression within these genes was moderate (average of 1.48 fold change) with a higher percentage of genes (14.9 %) upregulated compared to downregulated. The most highly differentially expressed (+LogFC) gene upregulated was sphingomyelin phosphodiesterase 2 (*Smad2*), a gene that encodes for an enzyme involved in the hydrolysis of sphingomyelin into phosphocholine and ceramide. Ceramide functions as a secondary messenger in the regulation of cell differentiation, cell proliferation, apoptosis and sterol homeostasis, suggesting a folic acid mediated role in cellular regulation of these processes. The most significantly categorised downregulated gene consisted of *Bcl-2* binding component 3 (PUMA), which encodes for a pro-apoptotic protein functioning to signal apoptosis to the mitochondria and is regulated by p53. Downregulation of PUMA is associated with cancer cells and research has shown that cells with p53 mutations exhibit significantly lower levels of PUMA, implicating it as a mark for p53 mutations. Interestingly, research by Hoeferlin *et al.*, (2013) investigating the role of ceramide in response to folate stress induced by *Aldh1l1*, a protein that catalyses the conversion of 10-formyltetrahydrofolate to tetrahydrofolate, has identified PUMA as a regulator of ceramide function. *Aldh1l1* which evokes metabolic stress and induces apoptosis in cancer cells similar to folate deficiency, results in elevated ceramide which was inhibited by the silencing of PUMA (Hoeferlin et al., 2013). Furthermore, folate withdrawal induced ceramide elevation and p53

accumulation, links folate, ceramide and PUMA pathways in the cell stress response (Hoeferlin et al., 2013). Within the present study increased folic acid supplementation (5 mg/kg) increases *Smad2*, possibly via changes in DNA methylation, which could facilitate an increase in levels of ceramide and the silencing of PUMA to regulate levels of apoptosis.

GSEA of adult supplemented mice showed EMT as highly significantly enriched (NES 7.848), with 71.9% of genes upregulated in response to folic acid supplementation. EMT, a process in which epithelial cells transition to mesenchymal stem cells that display increased migratory behaviour, increases invasiveness, motility and metastatic potential which is characteristic of BCSCs and implicated in breast cancer risk. Genes including Fibronectin, Vimentin and N-Cadherin, considered to be markers of EMT, were enriched in response to folic acid supplementation. The enriched gene set included *Snai2* (Slug), which encodes for a zinc finger transcription factor that functions as a transcriptional repressor of E-Cadherin, supporting mesenchymal phenotype in EMT. Furthermore, WNT- β -catenin gene set was significantly enriched. WNT/beta catenin is considered to play a role in EMT through the stabilisation and translocation of β -catenin (Lamouille et al., 2014). Upregulated target genes of Wnt/ β -catenin pathway that include fibronectin and MMPs have been associated with invasiveness and EMT phenotype. Furthermore, Notch genes, enriched in the *Wnt* gene set have been shown to induce EMT and a cancer stem cell phenotype in mammary epithelial cells (Jang et al., 2015). Notch 1 has been shown to regulate EMT in a slug dependent manner (Shao et al., 2015). Notch 1 positively regulates Slug expression through Slug promoter activation. Notch expression was also shown to be upregulated by Jagged1, also enriched in the WNT/ β -catenin pathway. Cumulatively, significantly enriched gene sets and gene components provide strong evidence for a folic acid mediated increase in EMT within adult mammary epithelium.

EMT is a complex process and requires both repression of epithelial genes and activation of mesenchymal genes. Research suggests that these processes are co-ordinated by epigenetic programming through DNA methylation and histone modifications (Wang and Shang, 2013). This provides a mechanism through which increased folic acid supplementation functions in one carbon metabolism as a methyl donor to facilitate methylation reactions that may modulate EMT associated genes and breast cancer risk.

Classification of genes using GO identified gene expression changes that cluster in terms of functions associated with ECM, cell migration and immune function. The predominance of

upregulated ECM relevant genes suggests changes in cellular structure are an important function in adult mammary glands modulated by folic acid intake. A total of 32 genes were upregulated, with no genes downregulated. Top upregulated genes including fibronectin 1 (Fn1), laminin (Lama2) and spondin 2 (Spon2) all share common roles in influencing cell differentiation, migration and adhesion, as well as being implicated in breast cancer. Upregulation of these genes would constitute mammary ECM remodelling towards differentiation and migration, suggesting folic acid supplementation may modify mammary gland morphologic structure through modulation of the ECM network.

Interestingly, within the ECM cluster the expression of three matrix metalloproteinase genes *mmp2*, *mmp3* and *mmp9* were increased, representing ~10 % of the total ECM GO output. *Mmp* genes, a large family of zinc-endopeptidases function to breakdown the ECM via the degradation of collagen in the regulation of processes that include tissue remodelling, mammary involution, bone development and wound healing (Fanjul-Fernández et al., 2010). Research suggests that upregulation of MMPs are implicated in a number of disease states that include cancer, vascular disease and inflammatory pathologies, suggesting a strong requirement for precise spatiotemporal regulation of *Mmp* genes in the homeostasis of the extracellular and pericellular environment (Fanjul-fernandez). Research by Williams *et al.*, (2011) has linked folic acid with MMP function in the regulation of placental trophoblast development, specifically with extracellular villus trophoblast invasion of placental explants. Folic acid mediated EVT invasion was directly correlated with increased placental explant secretion of MMP2, MMP3 and MMP9 (Williams et al., 2011). In addition to these findings a study by Wiesman *et al.*, (2003) has identified MMPs as refiners of the mammary branching pattern by distinct mechanisms during mammary gland branching morphogenesis in mice (Wiseman et al., 2003). Transient MMP activity is required for invasion and branch point selection in mammary branching morphogenesis. MMP2 was found to inhibit epithelial cell apoptosis, facilitating TEB invasion at the start of puberty. MMP2 was also found to repress precocious lateral branching during mid puberty. Furthermore, MMP3 was found to induces secondary and lateral branching of epithelial ducts (Wiseman et al., 2003).

Collectively these studies suggest that folic acid modulates expression of MMP2, MMP3 and MMP9 and a folic acid mediated increase in these genes as seen in the present study may function to increase epithelial branching morphogenesis within the mammary gland by functioning to facilitate the breakdown of the ECM and allow cellular invasion. Interestingly, in parallel with an

increase in Mmp expression, folic acid also appears to increase two *Timp* genes; *Timp2* and *Timp3*. Timp proteins are natural inhibitors of MMPs, however, in some cases can act as MMP activators (Wiseman et al., 2003). *Timp2* is thought to be part of an MMP-14 complex that activates MMP2 resulting in mammary ductal invasion (Wiseman et al., 2003). *Timp3* functions specifically within the ECM to inhibit MMPs and many ADAM genes specifically Adam17. Therefore, an increase in expression of *Timp3* may function to modulate a folic acid mediated increase in MMP genes expression.

Pathway analysis of adult supplemented mice showed the most significant overlap in the MMP genes which provides parallels with the results identified in the GO. As previously discussed, MMPs regulate ECM function and upregulation of these genes may suggest mammary gland branching morphogenesis or EMT (Radisky and Radisky, 2010). The top upstream regulator α -catenin was predicted to be inhibited. Research suggests that the biogenesis and function of mammary epithelium is dependent on functional α -catenin and loss can modulate epithelial expansion and functional differentiation (Nemade et al., 2004). Disease pathways affected included mammary morphogenesis and cancer, specifically metastasis and mammary tumour, suggesting high folic acid intake during adulthood modulates the expression of genes towards cancer risk.

The enrichment of genes within the EMT grouping, combined with changes in MMPs and ECM, provides links with gene expression changes in *Ezh2* and *Oct-4*, shown in chapter 3. *Ezh2* is a direct driver of EMT and increased *Ezh2* expression in response to high folic acid (5 mg/kg) in the present study may therefore increase EMT (Chang and Hung, 2012). Furthermore, the transdifferentiation from epithelial cells into mesenchymal stem cells with the capacity to differentiate into a variety of cell types can also be linked to *Oct-4*. Research by Tai et al., (2005) has identified *Oct-4* expression in mesenchymal cells (Tai et al., 2005). Increased *Oct-4* expression may therefore be linked to an increase in mesenchymal cells which possess enhanced migratory capacity, invasiveness and increased production of ECM components (Kalluri and Weinberg, 2009).

Interestingly, changes in reproductive hormones, specifically estrogen and progesterone, have been shown to play a role in the control of postnatal mammary development (Briskin and O'Malley, 2010). Analysis of normalised counts of genes associated with pro-estrus, estrus or di-estrus phases in the present study showed minimal changes, therefore, suggesting changes

observed in gene expression as well as morphology and protein expression within previous chapters are not due to differences in estrus cycles within the mice.

The timing of folic acid intake during either the juvenile period or adulthood clearly showed distinct differences in differential gene expression, with minimal overlap between timepoints. Research by Stute et al., (2011) has demonstrated characteristic patterns of gene expression during different stages of breast development in an animal model (Stute et al., 2012). Hierarchical cluster analysis revealed distinct separation in levels of gene expression between life stage groups, suggesting gene expression is dependent on the stage of mammary development. Within the present study, the high number of differentially expressed genes in juvenile supplemented mice compared to adults, in response to high folic acid, is likely to reflect increased levels of plasticity in the juvenile mammary gland, a period of intense mammary development. High intake of methyl donors in the form of folic acid that can directly affect levels of DNA methylation and chromatin regulation may function during this period in the modulation of gene expression which is critical in mammary development. Indeed, Stute *et al.*, (2012) identified imprinting effects in early life, with many genes associated with early breast differentiation persisting into later life. Furthermore, pathways activated during pubertal development have been implicated in cancer development and metastasis, suggesting programming during development may have application as biomarkers for breast cancer development later in life (Stute et al., 2012). Lower numbers of differentially expressed genes during adulthood in response to high folic acid intake could therefore be reflective of decreased plasticity and mammary development associated with the mature mammary gland. This may account for some of the differences in genes that were expressed between the two time points as well as the effects that high folic acid intake has on these genes. To further understand the effects of folic acid intake during juvenile and adult periods on levels of methylation, it would be useful to quantify the SAM: SAH ratio. A low ratio of SAH is considered a sensitive indicator of under methylation.

Validation of the RNA-seq data showed correct changes in direction of gene expression in upregulated and downregulated genes in both juvenile and adult supplemented mice. Furthermore, fold changes in levels of expression were in all but one case greater than 2. However, often differential expression between groups fell just short of significance. This may be due to subtle differences between the two methods of analysis and the statistical

measurements used to quantify expression. Future analysis could be performed to test the correlation between samples assessed using each method.

6.4 Conclusion

Currently, to date, no documented studies have investigated folic acid supplementation using a whole transcriptome approach. Results from the present study demonstrate that folic acid, supplementation at 5 mg/kg for a period of 4 weeks during either the juvenile period (PND 28-56) or adulthood (PND 74-102) induces gene specific changes in levels of expression within the mammary glands that persist for at least 4 weeks after the period of supplementation. Mice supplemented during the juvenile period demonstrate far greater numbers of differentially expressed genes compared to those supplemented during adulthood. Furthermore, gene expression shows minimal overlap between timepoints, identifying novel gene sets modulated by folic acid intake are critically dependent upon the timing of supplementation. Juvenile supplemented mice showed highly significant differential expression in enzymes linked with immune function as opposed to adult mice that show differential expression in genes that regulate cell differentiation, cell proliferation, and apoptosis. In the juvenile supplemented mice differentially regulated genes were shown to be clustered within the mitochondrion function as opposed to adults that show a large gene set component that function in the regulation of ECM, a critical component of mammary gland structure. Furthermore, juveniles showed high levels of gene enrichment in G2/M checkpoint, suggesting a role in cell cycle regulation as opposed to adult gene sets that cluster within EMT, suggesting a stem cell regulated change in cellular characteristics. Finally, juvenile supplemented mice showed distinct changes in pathways associated with mitochondrial dysfunction and networks of genes that regulate disease states centred on glucose metabolism. Conversely, adult supplemented mice showed changes in pathways associated with inhibition of MMPs which regulate ECM and gene networks based on tissue morphology and disease states centred on cancer, specifically metastasis and mammary cancer. Overall these findings show that the effects of folic acid supplementation on gene expression differ depending on timing. Furthermore, that supplementation during adulthood as opposed to during the juvenile period modulates levels of gene expression towards a breast cancer phenotype.

Chapter 7: The Effects of Folic Acid Deficiency during the Juvenile Period or Adulthood on *Brca1*, *Oct-4* Polycomb genes and Mammary Gland Morphology in the Female C57BL/6 Mouse Mammary Gland.

7.1 Introduction

Micronutrients are critical components in the maintenance of normal physiological development and health (Shenkin, 2006). Mandatory folic acid fortification of staple foods and supplementation has led to increased folic acid intake in many countries, however, globally, folate deficiency still occurs in women of childbearing age, pregnant females, young children and the elderly, all considered to be most vulnerable (Miller et al., 2016). In addition, research has shown that individuals who consume high levels of alcohol or have poor levels of nutritional intake are considered to be at high risk of folate deficiency (Bailey et al., 2010b).

Folate deficiency has been linked to a myriad of disease states and associated conditions that include megaloblastic macrocytic anaemia, atherosclerotic cardiovascular disease, neuropsychiatric disorders, diabetes-associated birth defects and the development of pre-neoplastic conditions and existing neoplasms (Varela-Moreiras et al., 2009, Suren et al., 2013). Folate deficiency is linked to the aetiology of cancer development, notably of the breast, colorectum, cervix and brain (Eichholzer et al., 2001). Current research suggests two principle mechanisms through which low folate status increases cancer risk, which include a reduction in intracellular levels of SAM, resulting in changes in cytosine methylation within DNA, leading to aberrant regulation of proto-oncogenes and subsequent malignant transformation; or dysregulated DNA synthesis resulting in uracil misincorporation, chromosomal breakage and DNA damage (Blount et al., 1997, Duthie and Hawdon, 1998). Folic acid intake in early life can affect the epigenome with long lasting effects that persist throughout the life-course. Folate deficiency during pregnancy has been shown to alter the methylation profiles of the offspring resulting in hypomethylation of DNA that persists into adulthood (McKay and Mathers, 2016). Furthermore, a folate and methyl deficient diet has been shown to decrease methyl-CpG-binding protein and Dnmts which results in hypomethylation of genomic DNA (Esfandiari et al 2003). Changes in DNA methylation have been linked to folate deficiency, more specifically through impaired methionine synthesis and decreased levels of SAM which lead to global DNA hypomethylation (James et al 2003). Levels of DNA methylation have been directly linked to changes in gene expression. Therefore; the timing of folic acid deficiency plays a major role on the epigenetic regulation of genes expression.

Although studies have linked folate deficiency to malignant transformation in cancer and suggested mechanisms for these effects, it currently remains unclear how changes in folate status during different periods of the life-course affect breast cancer risk. Furthermore, it is not

clear how periods of folate deficiency followed by adequate levels of folic acid intake at different time points affect the long term risk of breast cancer development.

To address this question the expression of *Brca1*, *Oct-4* and polycomb genes *Ezh2*, *Bmi1* and *Suz12* was quantified using qRT-PCR in the mammary glands of female C57BL/6 mice and mammary gland morphology examined in mice supplemented with a modified A193M folic acid deficient (0.2 mg/kg) diet during either the juvenile period or adulthood followed by a four or twelve week maintenance diet (2.1 mg/kg). This will inform on the effect of timing of dietary folic acid deficiency on the expression of key genes implicated in breast cancer risk.

7.1.1 Aims

1. To investigate if folic acid deficiency (0.2 mg/kg), for 28 days during either the juvenile period or adulthood, induces changes in the levels of *Brca1*, *Oct-4*, *Ezh2*, *Bmi1* and *Suz12* gene expression in the mammary glands of female virgin C57BL/6 mice that persists for 28 or 84 days after the period of deficiency.
2. To investigate if folic acid deficiency for 28 days during the juvenile period or adulthood leads to changes in epithelial density, the number of epithelial branches, epithelial junctions or TEBs that persist for 28 or 84 days after deficiency.

7.2 Methods

Female C57BL/6 mice (10 animals in each experimental dietary group) were fed modified AI93M folic acid diets containing basal (1 mg/kg), or low (0.2 mg/kg) folic acid in line with the detailed experimental design outlined in chapter 2. Total RNA was extracted from the right thoracic mammary glands and was reverse transcribed to cDNA. qRT-PCR was performed and mRNA expression was normalised to two reference genes selected using gNorm analysis. Gene expression levels were compared to the control group (1mg/kg folic acid). Mammary morphology was assessed in the same mice outlined above. The second and third thoracic mammary glands were excised, whole mounted onto glass slides, fixed in Carnoy's solution and stained in Carmine Alum solution. Mammary epithelial density, number of epithelial branches, number of junctions and the number of TEBs were calculated. Data was used to compare markers of mammary development and remodelling associated with breast cancer risk. Statistical analysis was performed using an independent t-test with the P value set to $P < 0.05$.

7.3 Results

7.3.1 Weight and Energy Intake Measurements

7.3.1.1 Juvenile Deficient Mouse Weights

Juvenile weights (g) were measured from PND 28 to PND 129 (Figure 7.1). Weight rapidly increased in both groups (0.2 mg/kg = 4.254 g, 1 mg/kg = 4.905g) between day 28 (weaning onto folic acid modified diets) and day 38 (Figure 7.1 A). Weight steadily increased from day 38 to day 129 (Figure 7.1 B). No significant differences in weight were identified between control and folic acid deficient groups at each time point apart from

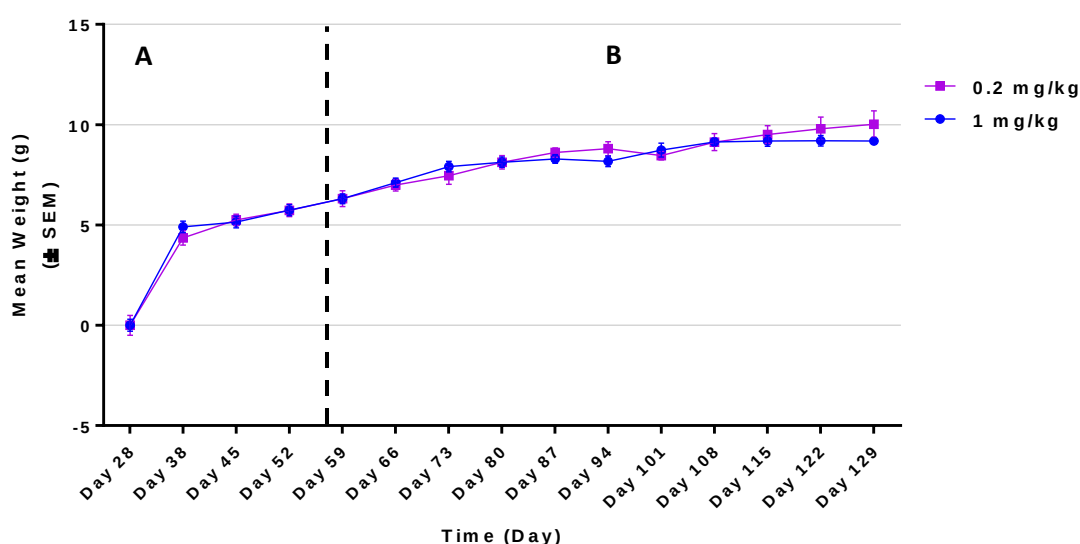


Figure 7.1 Mean Weights of Mice Fed a Folic Acid Deficient Diet during the Juvenile period.

Female C57BL/6 mice were fed either a control (1 mg/kg) or folic acid deficient (0.2 mg/kg) diet from PND 28 to PND 56 (A). Diets were modified to A193M maintenance diets (2.1 mg/kg folic acid) from PND 56 to PND 140. (B) Weights were recorded from PND 28 to 129. Data was baseline adjusted and displayed as mean weight (g) $\pm$ standard error of the mean (SEM).

7.3.1.2 Juvenile Deficient Mice Energy Intakes

Juvenile energy intakes (kJ per 10 g of mouse) were measured from PND 28 to PND 129 (Figure 7.2). Energy intakes rapidly decreased in both groups (1 mg/kg = 6.933 kJ, 0.2 mg/kg = 8.895 kJ) between day 28 (weaning onto folic acid modified diets) and day 38 before stabilising at day 45. Energy intake was maintained (between 19.105 kJ and 24.867 kJ per 10 g of mouse) between day 38 and day 129. Significant differences were observed at day 80 and day 101 (Figure 3.2, B).

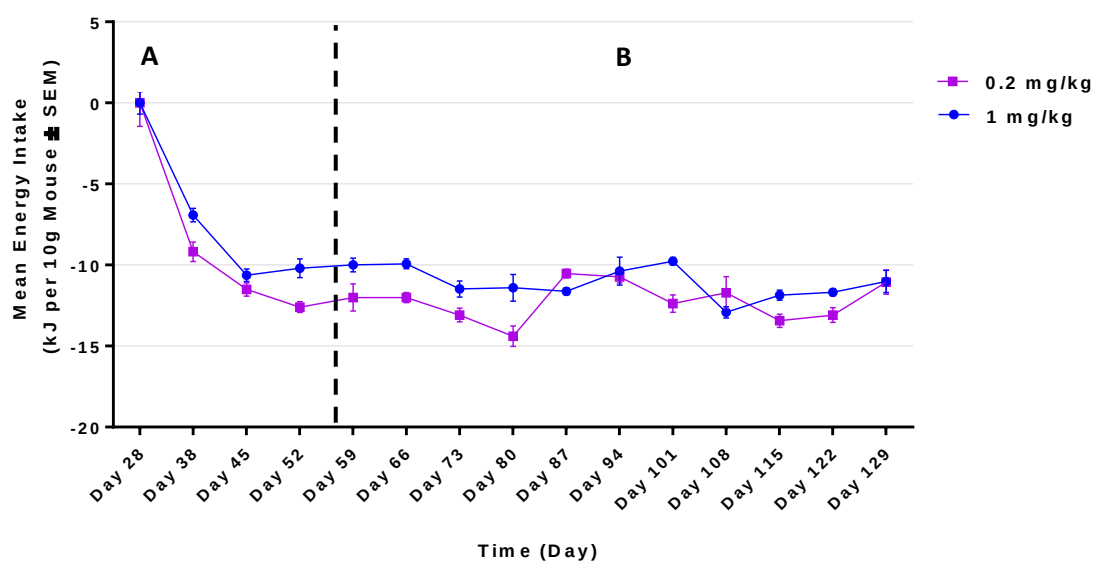


Figure 7.2. Mean Energy Intakes of Mice Fed a Folic Acid Deficient Diet during the Juvenile Period.

Female C57BL/6 mice were fed a control (1 mg/kg) or folic acid deficient diet (0.2 mg/kg) from PND 28 to PND 56 (**A**). Diets were modified to AI93M maintenance diets (2.1 mg/kg folic acid) from PND 56 to PND 140 (**B**). Energy intakes were recorded from PND 28 to 129. Energy intakes were baseline adjusted and displayed as mean energy intake (kJ per 10 g of mouse) ± standard error of the mean (SEM).

7.3.1.3 Adult Deficient Mouse Weights

Adult weights were measured from PND 74 to PND 181 (Figure 7.3). Weight increased in all groups from day 74 to day 102 (modified folic acid diets) (Figure 7.3 A) and day 102 to day 181 (maintenance diet) (Figure 7.3 B). No significant differences were observed between groups (folic acid concentrations) at each time point.

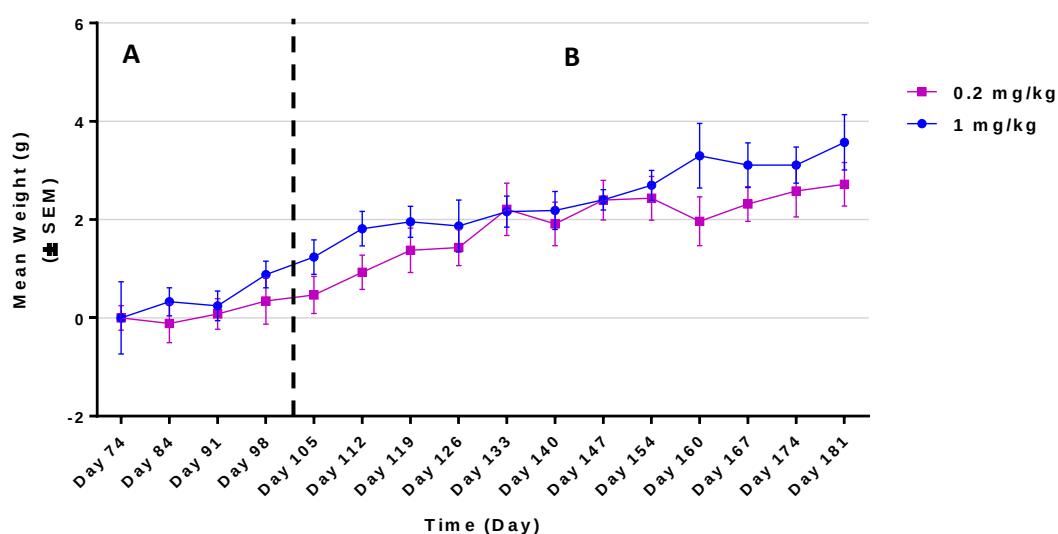


Figure 7.3. Mean Weights of Mice Fed a Folic Acid Deficient Diet during Adulthood.

Female C57BL/6 mice were fed a control (1 mg/kg) or folic acid deficient diet (0.2 mg/kg) from PND 74 to PND 102 (A). Diets were modified to AI93M maintenance diets (2.1 mg/kg folic acid) from PND 102 to PND 181 (B). Weights were recorded from PND 74 to 181. Weights were baseline adjusted and displayed as mean weight (g) ± standard error of the mean (SEM).

7.3.1.4 Adulthood Supplementation Energy Intakes

Adult energy intakes (kJ per 10 g of mouse) were measured from PND 74 to PND 181 (Figure 7.4). Energy intakes showed initial variance between groups during modified folic acid dietary supplementation (Figure 7.4, PND 74 to 102, A). Variance between groups was maintained from day 102 (start of maintenance diet) (Figure 7.4, B) to day 181. Statistical significance was observed between 1 mg/kg and 0.2 mg/kg folic acid at all time points apart from day 74, 91 and 98 (Figure 7.4).

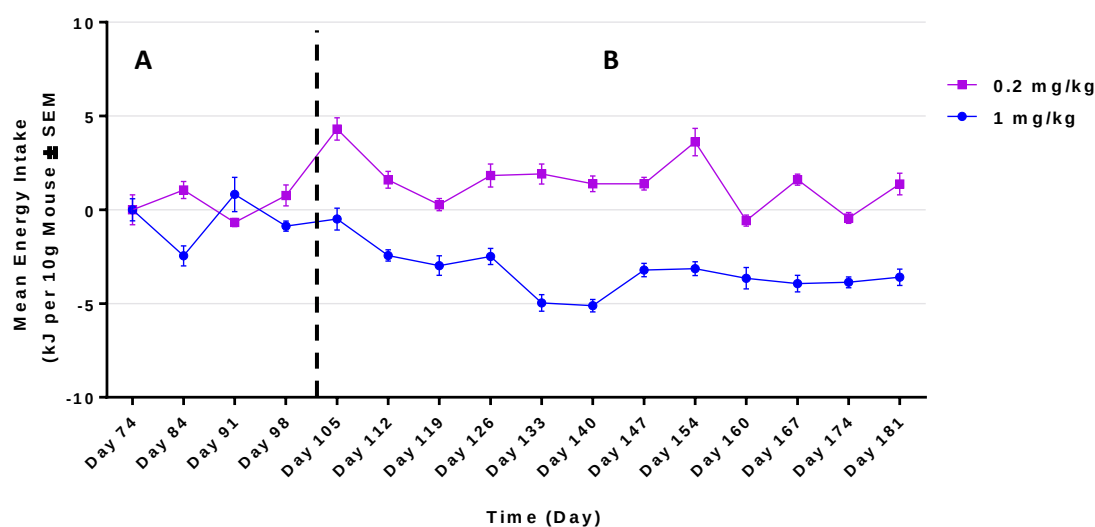


Figure 7.4 Mean Energy Intakes of Mice fed a Folic Acid Deficient Diet during Adulthood.

Female C57BL/6 mice were fed a control (1 mg/kg) or folic acid deficient (0.2 mg/kg) diet from PND 74 to PND 102 (A). Diets were modified to A193M maintenance diets (2.1 mg/kg folic acid) from PND 102 to PND 186 (B). Energy intakes were recorded from PND 28 to 129. Energy intakes were baseline adjusted and displayed as mean energy intake (kJ per 10 g of Mouse) $\pm$ standard error of the mean (SEM).

Table 7.1 Example of Average Food Consumption and Estimated Total Folic Acid Intake in Mice Fed a Folic Acid Deficient Diet during the Juvenile Period or Adulthood.

Folic acid intake was calculated in mice fed 0.2 mg/kg or 1 mg/kg, at (A) the end of the juvenile deficiency period (PND 56); (B) 4 weeks after the juvenile deficiency period (PND 84); (C) the end of the adult deficiency period (PND 102); (D) 4 weeks after the juvenile deficiency period (PND 130).

	Dietary Folic Acid Concentration	
	0.2 mg/kg	1 mg/kg
A End of Juvenile Supplementation		
Average food consumed per mouse mg/24 hours	2.35 (SD: 0.01)	2.39 (SD: 0.14)
Total folic acid intake per mouse (µg of FA per Mouse)	0.47	2.74
B 4 weeks after Juvenile Supplementation		
	2.1 mg/kg	2.1 mg/kg
Average food consumed per mouse mg/24 hours	2.39 (SD: 0.25)	2.57 (SD: 0.52)
Total folic acid intake per mouse (µg of FA per Mouse)	5.82	5.19
C End of Adult Supplementation		
	0.2 mg/kg	1 mg/kg
Average food consumed per mouse mg/24 hours	2.74 (SD: 0.00)	2.66 (SD: 0.00)
Total folic acid intake per mouse (µg of FA per Mouse)	0.54	2.93
D 4 weeks after Adult Supplementation		
	2.1 mg/kg	2.1 mg/kg
Average food consumed per mouse mg/24 hours	2.89 (SD: 0.34)	2.57 (SD: 0.00)
Total folic acid intake per mouse (µg of FA per Mouse)	6.06	5.39

SD = Standard Deviation. Maintenance diet contained 2.1 mg/kg FA for all post supplementation diets

7.3.2 Blood Folate Metabolites

Plasma 5-methyl tetrahydrofolate (5-meTHF) levels were measured in the mice directly after 4 weeks of folic acid deficiency (0.2 mg/kg) during the juvenile period or adulthood (Table 7.2). Average folate concentrations (nM) decreased in a dose response manner with a decrease in the level of folic acid in the juvenile mice but there was no significant difference in adult mice (Table 7.2) (Figure 7.5).

Table 7.2. Blood Plasma Concentrations of 5-meTHF in Mice Fed a Folic Acid Deficient Diet for 4 Weeks during the Juvenile Period or Adulthood.

	Supplemented Folic Acid Concentrations			
	0.2 mg/kg		1 mg/kg	
Period of supplementation	Average Blood Plasma Folate Concentration (nM)			
	Mean	Standard Deviation	Mean	Standard Deviation
Juvenile (PND 28-56)	76.2	19.41	104.8	6.952
Adulthood (PND 74-102)	135.0	34.1	140.2	60.51
Statistical Analysis (t-test)				
Juvenile 1 mg/kg vs 0.2 mg/kg	p<0.05, p = 0.01			
Statistical Analysis (t-test)				
Adult 1 mg/kg vs 0.2 mg/kg	P>0.05, p = 0.86			

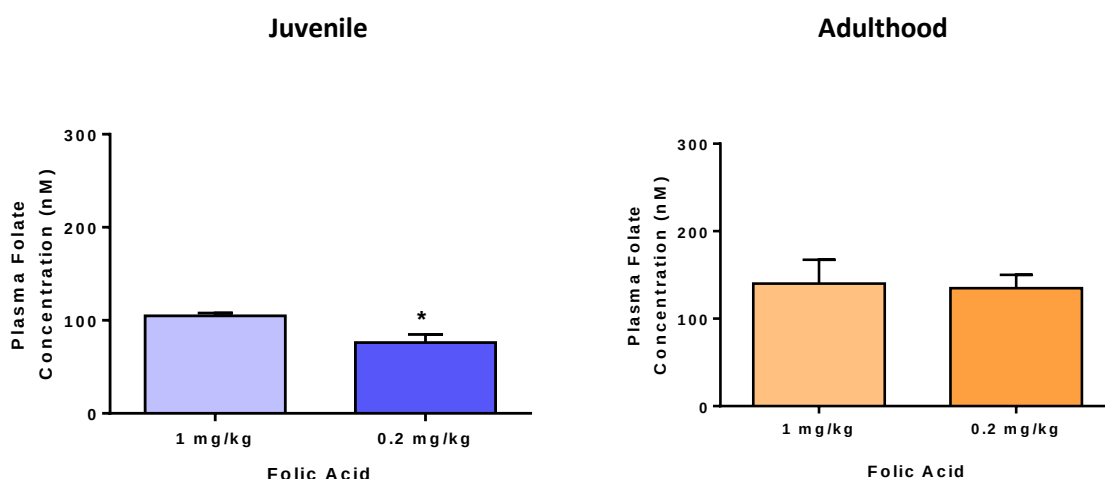


Figure 7.5 Plasma Folate Concentration of Mice Fed Different Levels of Folic Acid during the Pubertal Period or Adulthood.

Female C57BL/6 mice were fed 0.2 mg/kg of folic acid for 4 weeks (PND 28 to PND 56) during the juvenile period from (A) or during adulthood (PND 74-102) (B). Plasma folate levels were measured at the end of the supplementation periods (PND 56 for juvenile and PND 102 for adults). Plasma folate concentrations are displayed as Mean $\pm$ SEM. $n = 5-6$. * $P < 0.05$, (t-test).

7.3.3 Plasma Hormone Concentration

In juvenile mice plasma progesterone concentrations were widely distributed and representative of both proestrus and diestrus (Figure 7.6A). Estradiol concentrations were mainly representative of proestrus/metestrus, with a single sample in the 1mg/kg folic acid group having higher levels of estradiol consistent with estrus. However, there were no significant differences in the estradiol or progesterone concentrations between the groups. Within adults the majority of mice demonstrated progesterone concentrations consistent with proestrus or diestrus and estradiol-17 β concentrations consistent with proestrus/metestrus; however, in adult mice fed a folic acid deficient diet the majority of mice correlated with diestrus. There was no significant difference in estradiol concentration between the control and folic acid deficient group but there was a significant difference in levels of progesterone (Figure 3.7A). A significant difference in hormone concentration (reflective of diestrus) in the present study may reflect a folic acid mediated change in estrus cycle or simply the stage when mice

were culled. Further research is required to ascertain if folic acid deficiency affects hormone status in mice after the period of deficiency and the implications on disease risk (Figure 7.6B).

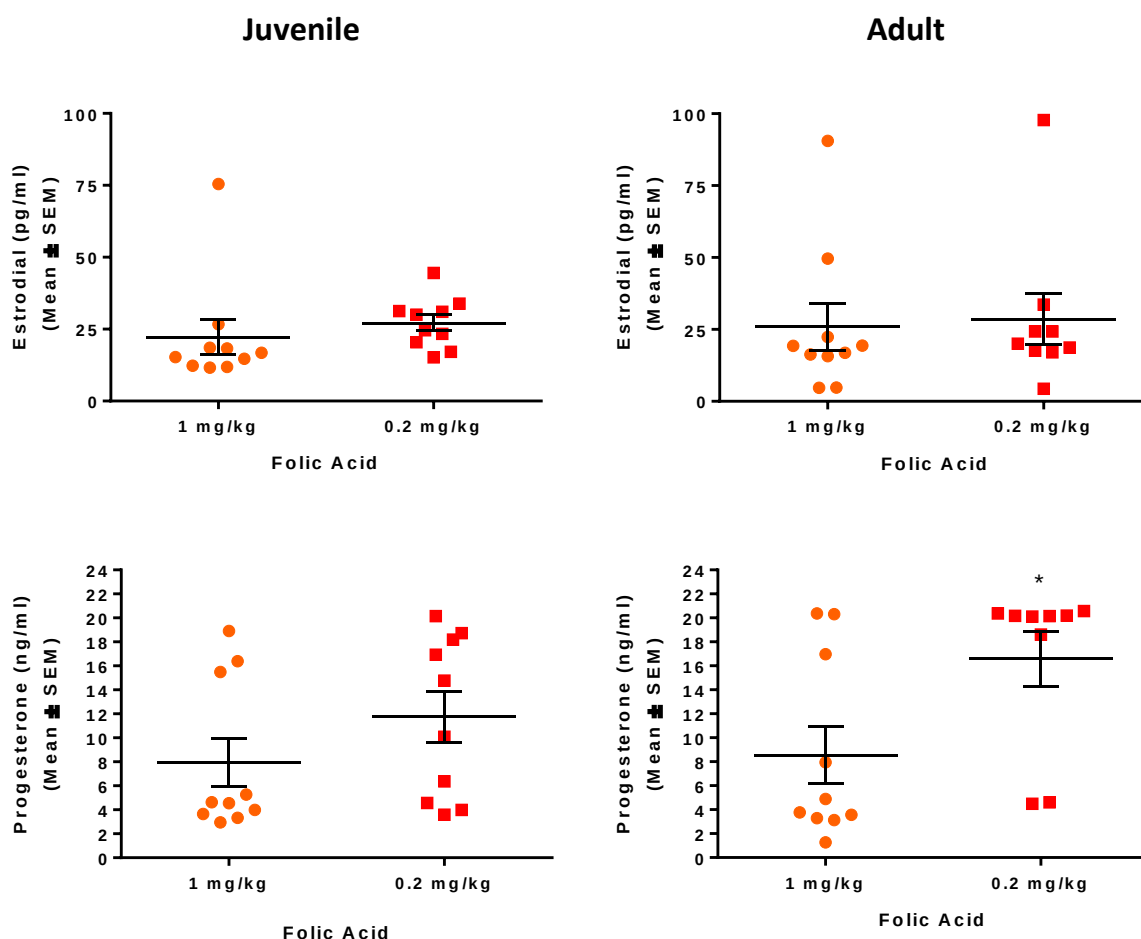


Figure 7.6. Plasma Hormone Concentrations in Female Mice fed Folic Acid during the Juvenile Period or Adulthood.

(A) Estradiol-17 β and progesterone concentrations were measured 4 weeks after folic acid supplementation. Original non-log transformed graphs shown for comparison of concentrations.

(B) Reference concentrations of Estradiol-17 β and progesterone with corresponding estrus stages in female mice adapted from Fata et al., (2001). * P < 0.05.

7.3.4 Folic Acid Deficiency during the Juvenile Period Induces Dose Specific Changes in Levels of *Brca1*, *Oct-4* and Polycomb genes

7.3.4.1 *Brca1* and *Oct-4* Expression

Brca1 expression significantly decreased in mice fed a folic acid deficient diet of 0.2 mg/kg at PND 84 ($P = 0.0001$) and PND 140 ($P = 0.0025$) (Figure 7.1). *Oct-4* expression significantly decreased at PND 84; however, this effect did not persist at PND 140 (Figure 7.7).

7.3.4.2 Polycomb Gene Expression

Ezh2, *Bmi1* and *Suz12* expression did not significantly differ ($P > 0.05$) in mice fed 0.2 mg/kg folic acid either 4 weeks (PND 86) or 12 weeks (PND 140) after a folic acid deficient diet (Figure 7.8).

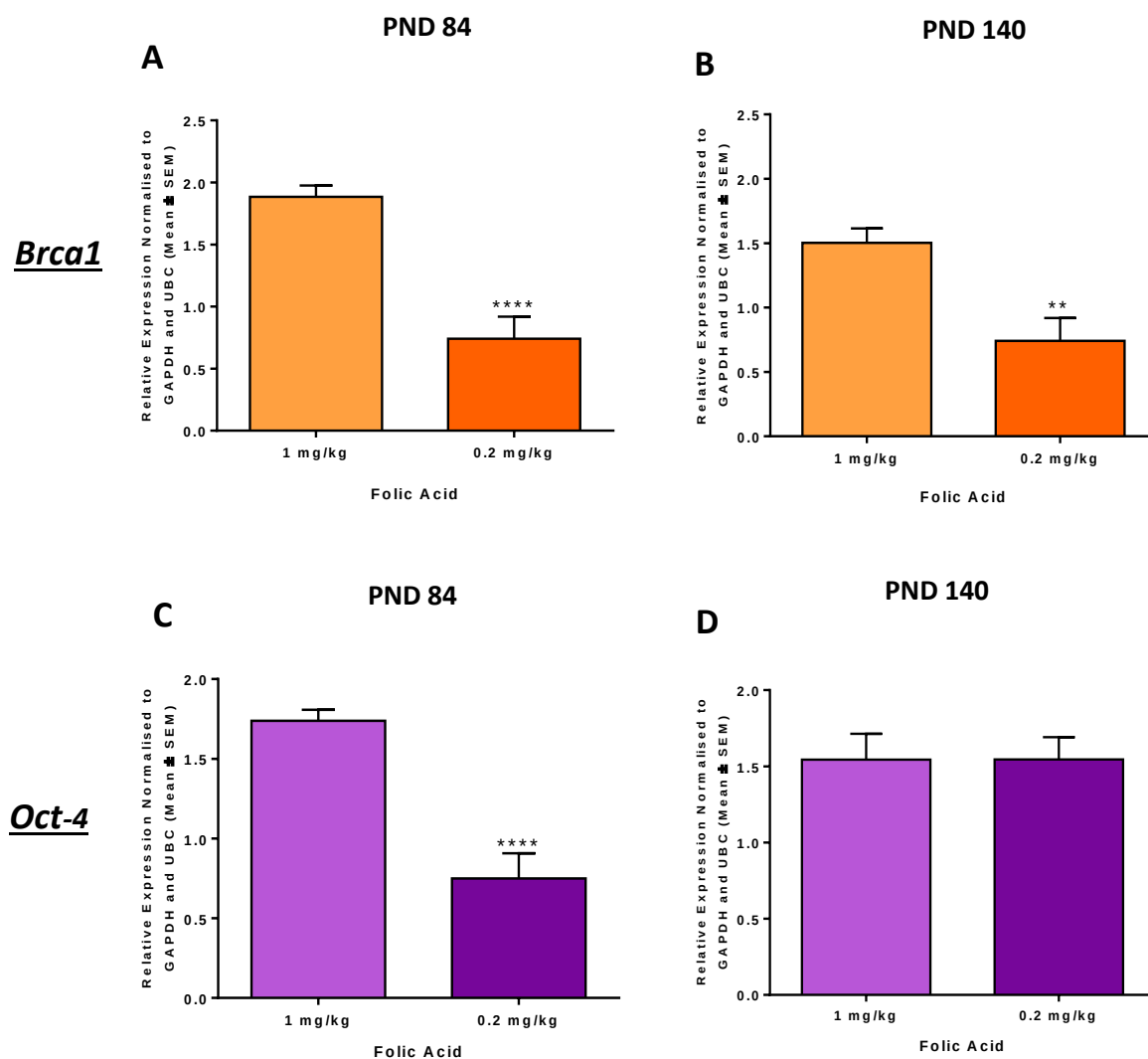


Figure 7.7. Relative mRNA Expression of *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Supplemented with FA during Juvenile Development.

Relative expression of *Brca1* (A, B) and *Oct-4* (C, D) in mice fed 1 mg/kg (1 x BDR - control), or 0.2 mg/kg (0.2 x BDR) for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56 – 84) (A – *Brca1*, C – *Oct-4*) or 12 weeks maintenance diet (PND 56 – 140) (B – *Brca1*, D – *Oct-4*). Data presented as mean ± SEM. n = 8-10 per group. ** P < 0.01, **** p < 0.0001.

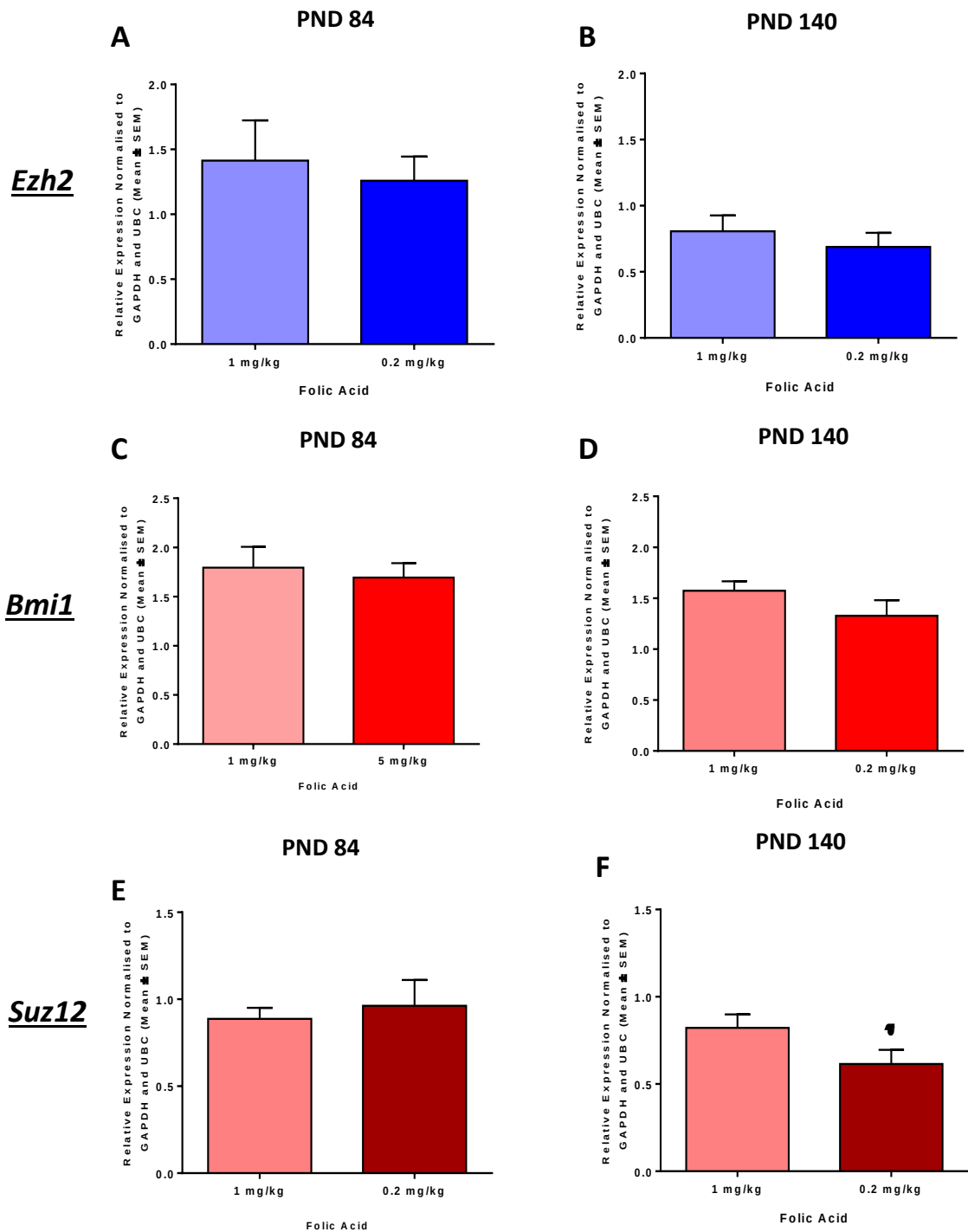


Figure 7.8. Relative mRNA expression of *Ezh2*, *Bmi1* and *Suz12* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Fed a Folic Acid Deficient Diet during the Juvenile Period.

Relative expression of *Ezh2* (A, B), *Bmi1* (B, C) and *Suz12* (E, F) in mice fed 1 mg/kg (1 x BDR) or 0.2 mg/kg (0.2 x BDR) folic acid for 4 weeks (PND 28 – 56) followed by a 4 week (A, C, E) (PND 56-84) or 12 week (B, D, F) maintenance diet (PND 56-140). Data presented as mean $\pm$ SEM. n = 8-10. τ = trend ($P < 0.01$).

7.3.5 Folic Acid Deficiency during the Juvenile Period induces Changes in Levels of Stem Cell Regulator genes with Ageing.

7.3.6 *Brca1* and *Oct4* Expression

Decreased *Brca1* expression seen with ageing ($P = 0.018$) was not observed in mice fed 0.2 mg/kg. However, levels of *Brca1* expression in mice fed 0.2 mg/kg folic acid were notably lower ($> 50\%$) when compared with animals fed 1 mg/kg both 4 weeks and 12 weeks post diet (Figure 7.9). Levels of *Oct-4* expression did not change in mice fed 1 mg/kg folic acid in response to ageing, however, in mice fed 0.2 mg/kg *Oct-4* expression significantly increased ($P = 0.003$) between PND 130 and PND 186. This suggests that folic acid deficiency at 0.2 mg/kg modulates *Oct-4* expression with ageing (Figure 7.9).

7.3.7 Polycomb Gene Expression

Decreased *Ezh2* expression seen with ageing (non- significant trend $P = 0.074$) was also observed in the presence of 0.2 mg/kg folic acid. There was no effect of ageing on *Bmi1* and *Suz12* expression in mice fed 1 mg/kg or 0.2 mg/kg folic acid (Figure 7.3).

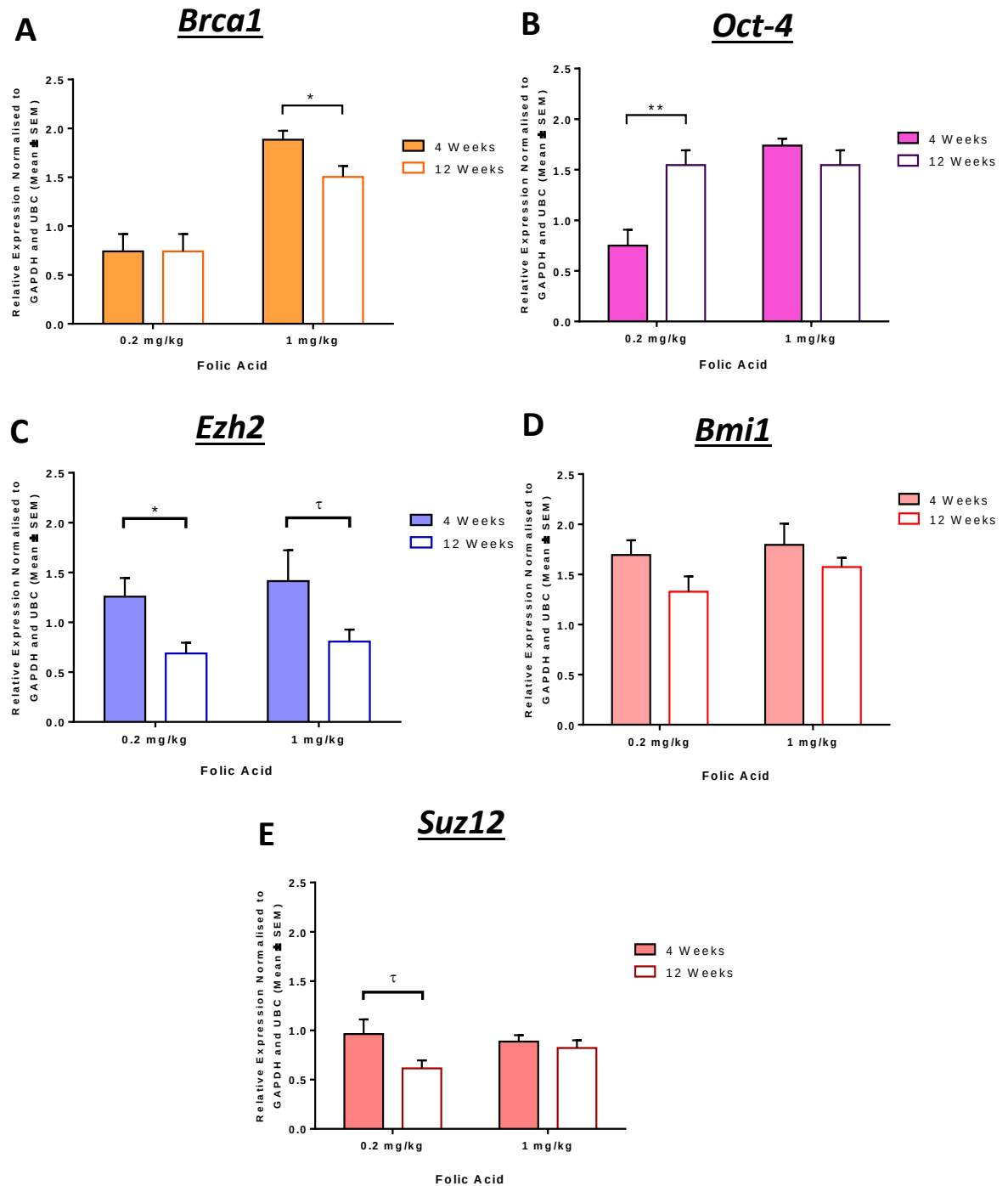


Figure 7.9. Comparison between Relative mRNA Expression of Polycomb Genes *Ezh2*, *Bmi1*, *Suz12* and *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Fed 0.2 mg/kg Folic Acid during the Juvenile Period.

Relative expression of *Brca1* (A), *Oct-4* (B), *Ezh2* (C), *Bmi1* (D) and *Suz12* (E) in mice fed 1 mg/kg (1 x BDR - control) or 0.2 mg/kg (0.2 x BDR) folic acid for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) or 12 week maintenance diet (PND 56-140). Data presented as mean $\pm$ SEM. n = 8-10 per group. * p < 0.05, ** p < 0.01).

7.3.8 Folic Acid Deficiency during Adulthood Induces Dose Specific Changes in Levels of Expression of Stem Cell Regulator Genes

7.3.8.1 *Brca1* and *Oct-4* Expression

Brca1 expression showed a non-significant trend towards an increase in expression ($P = 0.077$) in mice fed 0.2 mg/kg for 4 weeks at PND 130. No differences in levels of *Brca1* expression were observed at PND 186. *Oct-4* expression significantly increased in mice fed 0.2 mg/kg at PND 130 but this effect did not persist at PND 186 (Figure 7.10).

7.3.9 Polycomb Gene Expression

Ezh2 expression did not significantly differ in mice fed 0.2 mg/kg folic acid either 4 weeks (PND 130) or 12 weeks (PND 186) after supplementation (Figure 7.11). *Bmi1* and *Suz12* expression significantly increased in mice fed 0.2 mg/kg at PND 130 but these effects did not persist at PND 186 (Figure 7.11).

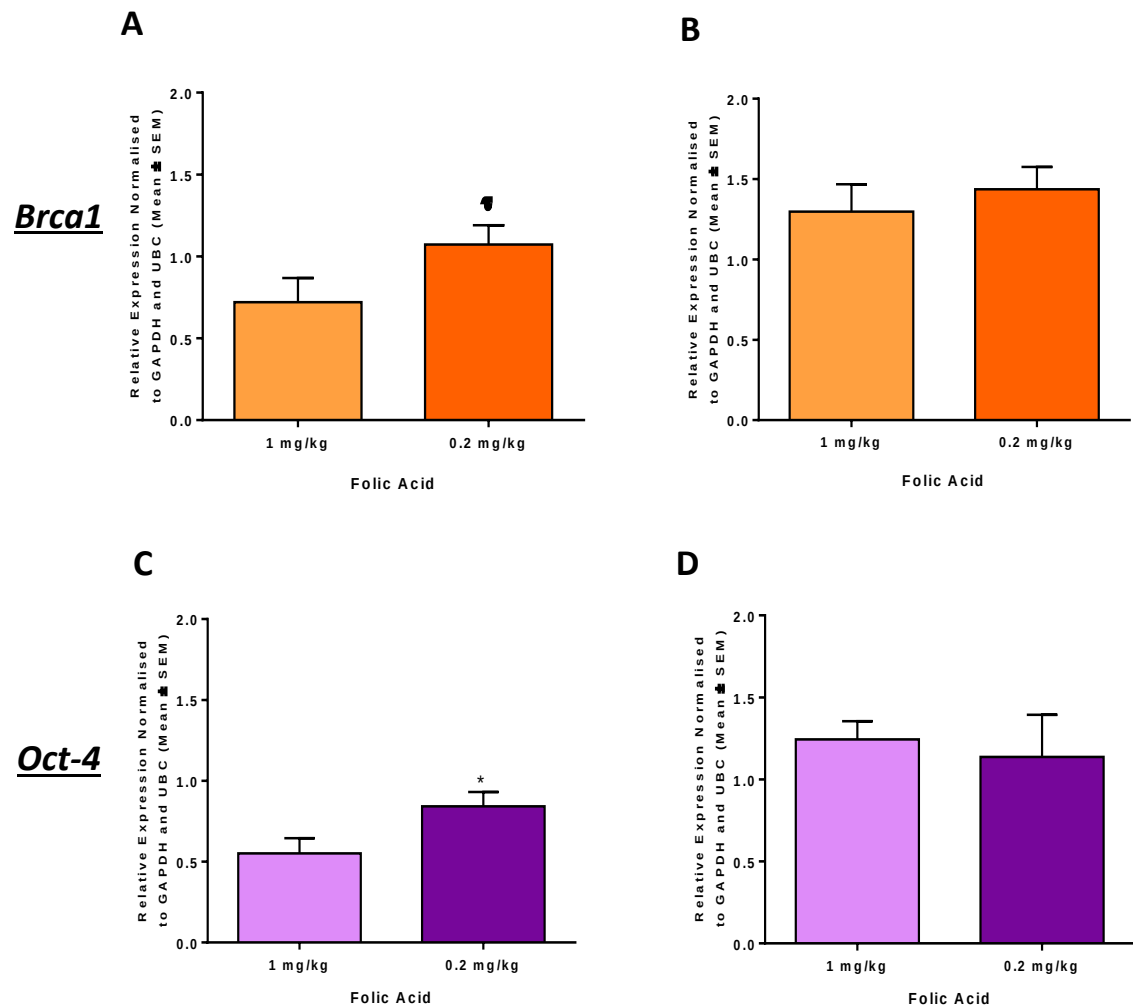


Figure 7.10. Relative mRNA Expression of *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Fed 0.2 mg/kg FA during Adulthood.

Relative expression of *Brca1* (A, B) and *Oct-4* (C, D) in mice fed 1 mg/kg (1 x BDR - control), or 0.2 mg/kg (0.2 x BDR) for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56 – 84) (A – *Brca1*, C – *Oct-4*) or 12 weeks maintenance diet (PND 56 – 140) (B – *Brca1*, D – *Oct-4*). Data presented as mean ± SEM. n = 8-10 per group. * P < 0.05, τ = trend (P = < 0.1).

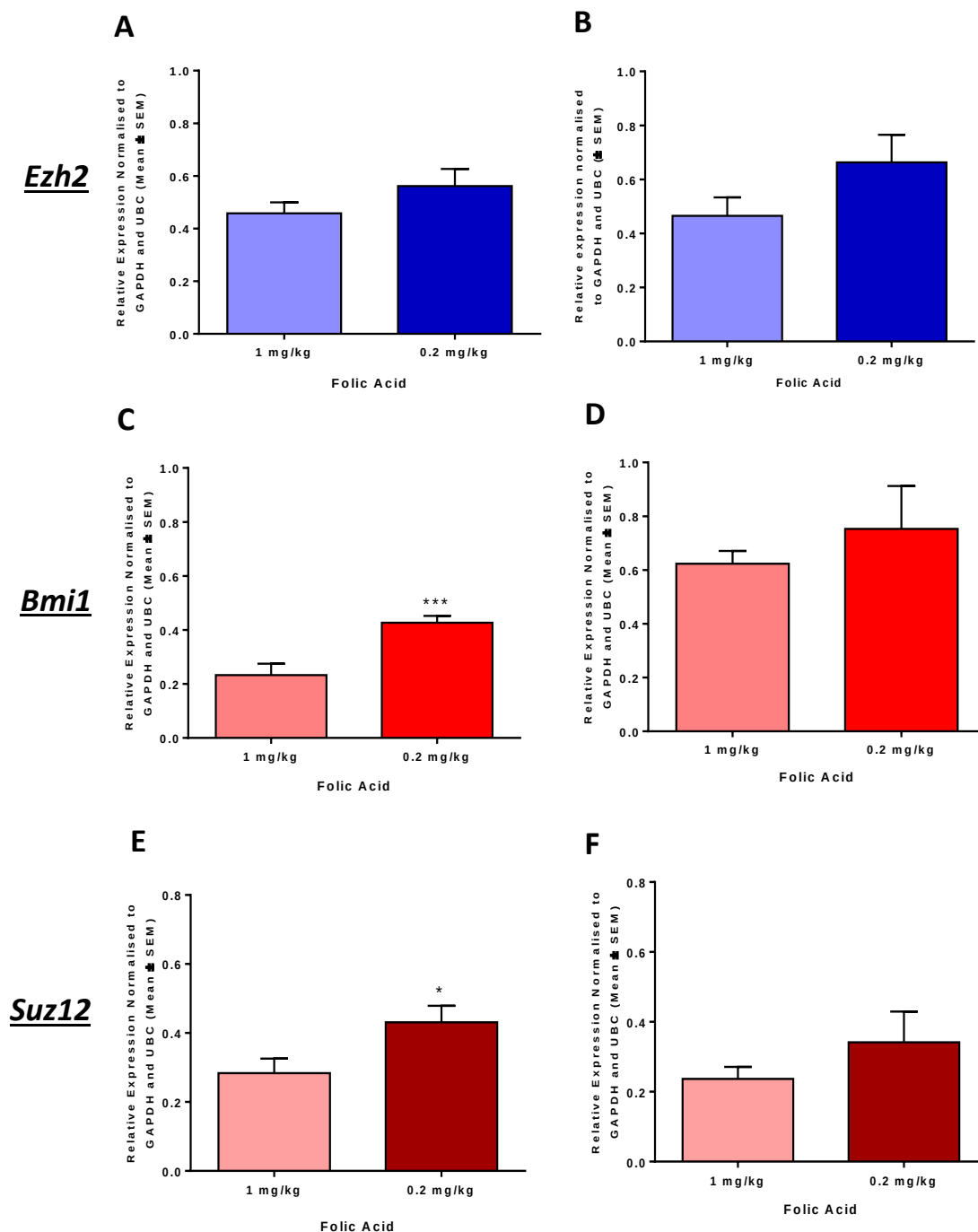


Figure 7.11. Relative mRNA Expression of *Ezh2*, *Bmi1* and *Suz12* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) Fed 0.2 mg/kg Folic Acid during Adulthood.

Relative expression of *Ezh2* (A, B), *Bmi1* (C, D) and *Suz12* (E, F) in mice fed 1 mg/kg (1 x BDR - control), or 0.2 mg/kg (0.2 x BDR) for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56 – 84) (A – *Ezh2*, C – *Bmi1*, E – *Suz12*) or 12 weeks maintenance diet (PND 56 – 140) (B – *Ezh2*, D – *Bmi1*, F – *Suz12*). Data presented as mean ± SEM. n = 8-10 per group. * P < 0.05, *** P < 0.001.

7.3.10 Folic Acid Deficiency during Adulthood induces Changes in Levels of Stem Cell Regulator genes with Ageing.

7.3.11 *Brca1* and *Oct4* Expression

Increased *Brca1* ($P = 0.021$) expression with ageing was not observed in mice fed 0.2 mg/kg (non-significant trend) between PND 130 and PND 186 suggesting *Brca1* deficiency may prematurely increase levels of *Brac1* expression (Figure 7.12). Increased *Oct-4* expression with ageing was not observed in mice fed 0.2 mg/kg suggesting folic acid deficiency increases *Oct-4* gene expression 4 weeks after intake to near levels associated with aging after 12 weeks (Figure 7.12).

7.3.12 Polycomb Gene Expression

Decreased *Ezh2* expression seen with ageing ($P = 0.025$) between 4 (PND 130) and 12 (PND 186) weeks was not observed in mice fed 0.2 mg/kg. There was no effect of ageing on *Bmi1* and *Suz12* expression in mice fed 0.2 mg/kg folic acid (Figure 7.12).

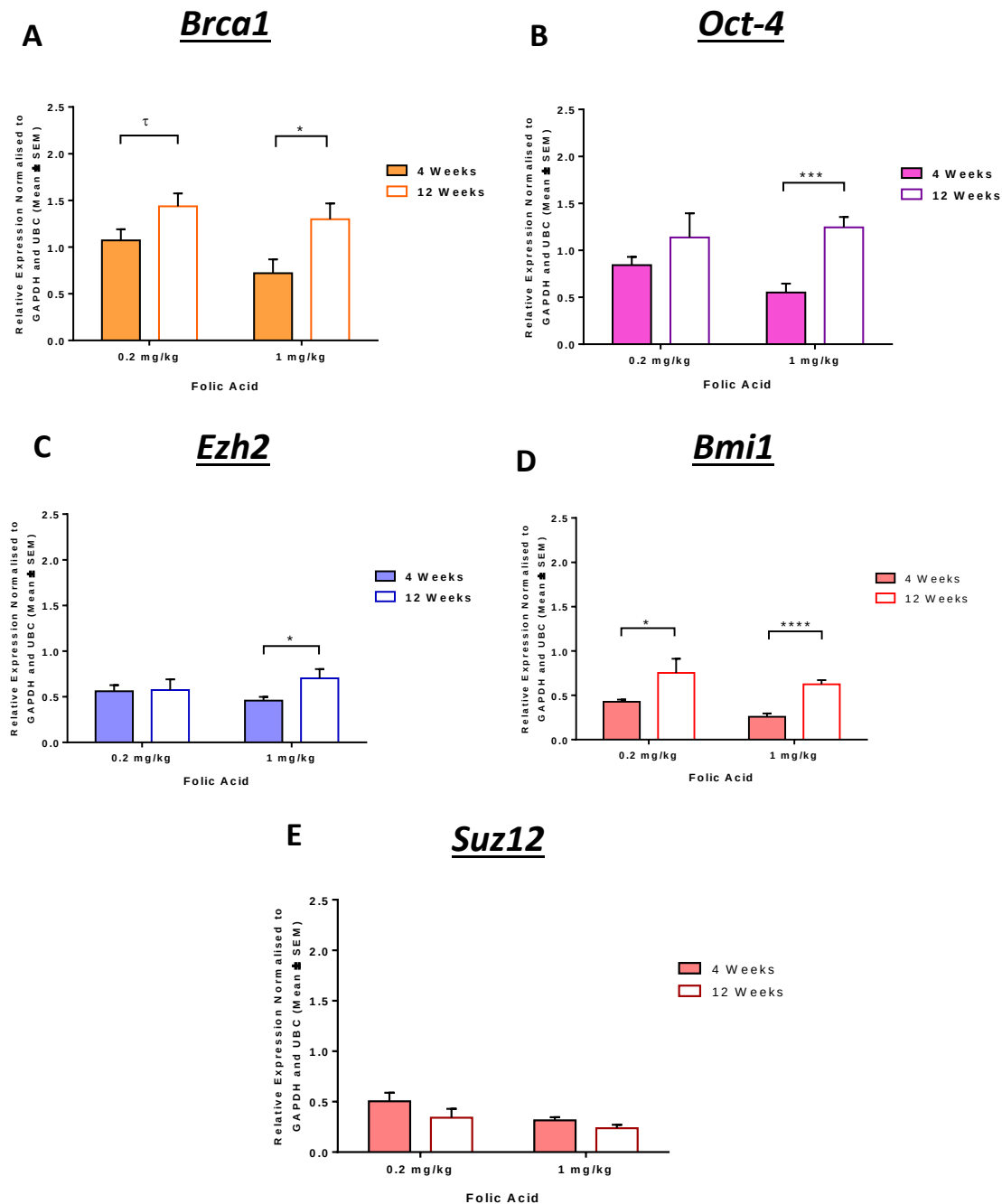


Figure 7.12. Comparison between Relative mRNA Expression of Polycomb Genes *Ezh2*, *Bmi1*, *Suz12* and *Brca1* and *Oct-4* in Female C57BL/6 Mammary Glands (2nd and 3rd thoracic) fed 0.2 mg/kg Folic Acid during Adulthood.

Relative expression of *Brca1* (A), *Oct-4* (B), *Ezh2* (C), *Bmi1* (D) and *Suz12* (E) in mice supplemented with a folic acid deficient diet (0.2 mg/kg) for 4 weeks (PND 28 – 56) followed by a 4 week maintenance diet (PND 56-84) or 12 week maintenance diet (PND 56-140) Data presented as mean $\pm$ SEM. n = 8-10 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. τ = Trend.

7.3.13 Folic Acid Deficiency during the Juvenile Period Does Not Induce Changes in Mammary Gland Morphology

No significant changes were observed in epithelial density, the number of epithelial branches, epithelial junctions or terminal end buds in mice fed 0.2 mg/kg folic acid. These results show that folic acid deficiency for 4 weeks during the juvenile period or adulthood does not induce persistent changes in morphology of the mammary gland 4 or 12 weeks after dietary intervention (Table 7.3).

7.3.14 Folic Acid Deficiency during Adulthood Induces Changes in Mammary Gland Morphology

Epithelial density was significantly increased ($P = 0.023$) in mice fed 0.2 mg/kg of folic acid during adulthood (PND 74 – 102) within the distal region of the mammary gland 4 weeks after the period of deficiency (PND 130) (Figure 7.13). This effect did not persist, with no change in epithelial density observed at 12 weeks. The number of epithelial branches and junctions showed no significant changes 4 weeks or 12 weeks after supplementation, however, a non-significant trend towards an increase in the number of epithelial branches and junctions was observed in the distal region of the mammary glands 4 weeks after folic acid supplementation (Figure 7.14 and 7.15). There were no significant differences in the numbers of TEBs in folic acid deficient groups.

Table 7.3. Effects of Folic Acid Deprivation on Mammary Gland Morphology in Mice Supplemented during the Juvenile Period

Ns – Non significant

4 Weeks							
Density		Branches		Junctions		TEBs	
Medial	Distal	Medial	Distal	Medial	Distal	Medial	Distal
Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
12 Weeks							
Density		Branches		Junctions		TEBs	
Density	Branches	Junctions	End buds	Medial	Distal	Medial	Distal
Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns

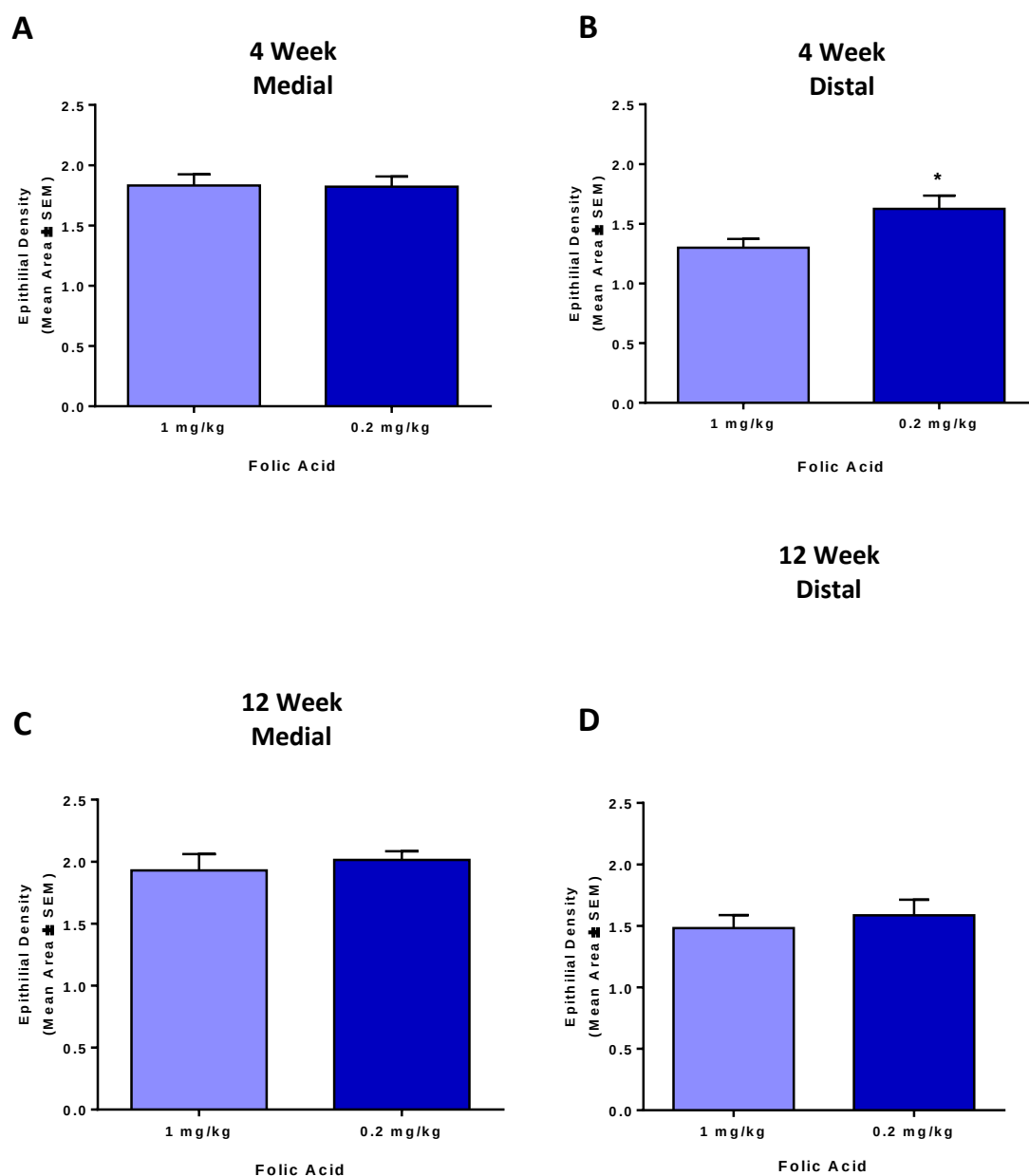


Figure 7.13. Epithelial Density of Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) fed 0.2 mg/kg Folic Acid during the Adult Period.

Mammary epithelial density in mice fed 1 mg/kg (1 x BDR) or 0.2 mg/kg (0.2 x BDR) folic acid for 4 weeks (PND 74 – 102) followed by a 4 week (A, B) (PND 102-130) or 12 week (C, D) (PND 102-186) maintenance diet. Data presented as mean ± SEM. n = 8-10 per group.

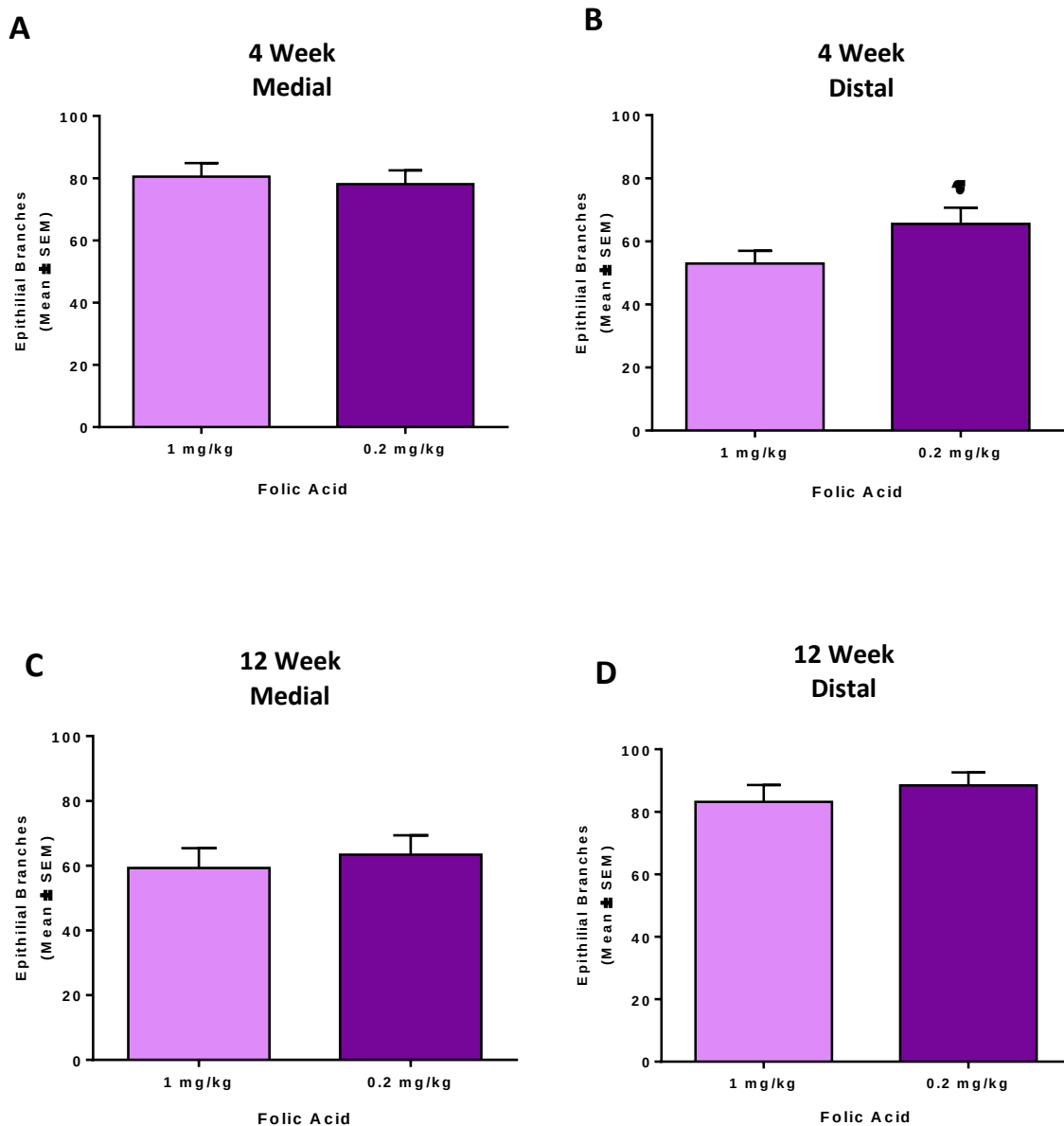


Figure 7.14. Epithelial Density of Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) fed 0.2 mg/kg of Folic Acid during the Adult Period.

Mammary epithelial density in mice fed 1 mg/kg (1 x BDR) or 0.2 mg/kg (0.2 x BDR) folic acid for 4 weeks (PND 74 – 102) followed by a 4 week (**A, B**) (PND 102-130) or (**C, D**) 12 week (PND 102-186) maintenance diet. Data presented as mean ± SEM. n = 8-10 per group.

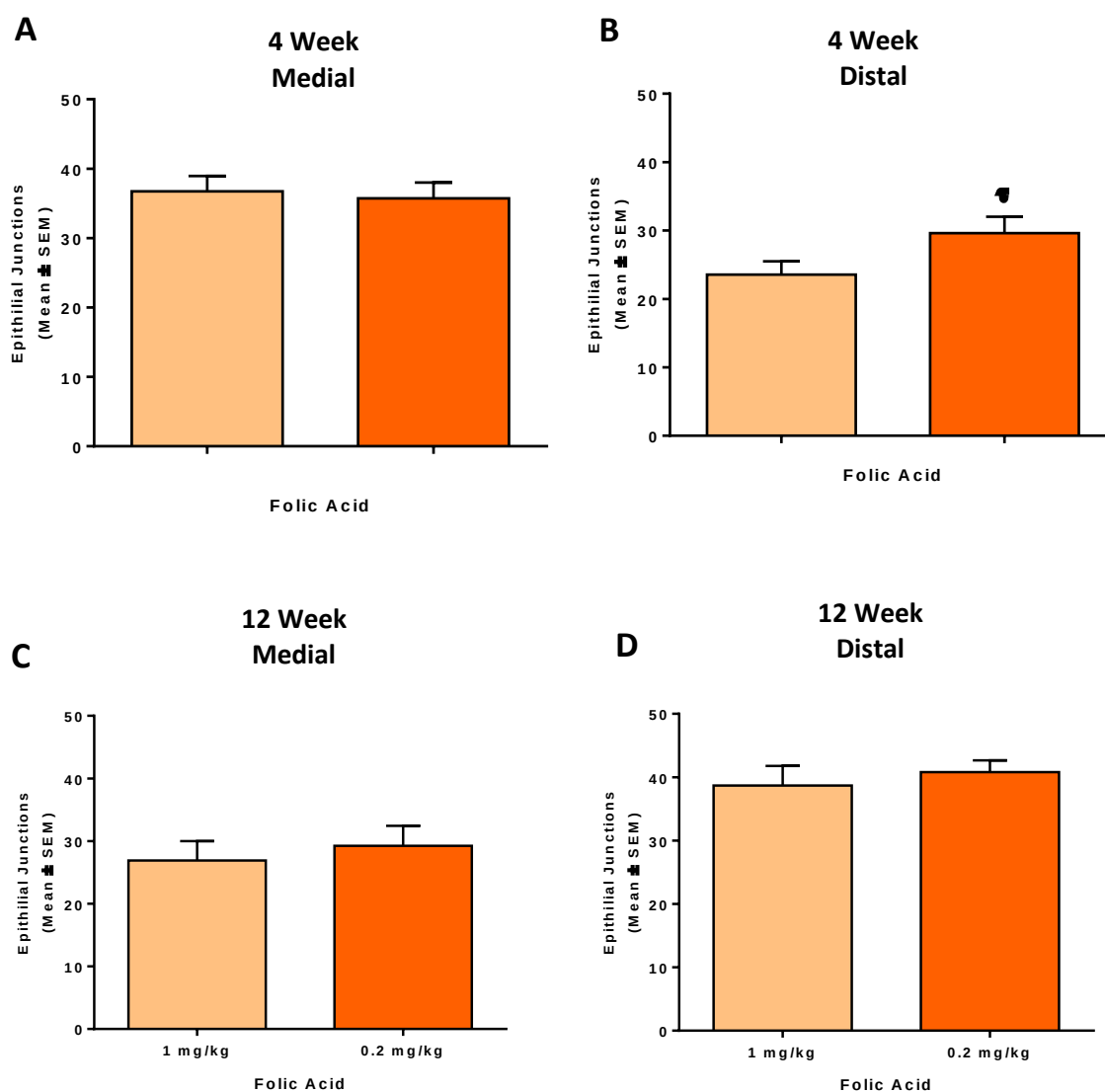


Figure 7.15. Epithelial Density of Female C57BL/6 Mammary Glands (2nd and 3rd left thoracic) Fed 0.2 mg/kg of Folic Acid during the Adult Period.

Mammary epithelial density in mice fed 1 mg/kg (1 x BDR) or 0.2 mg/kg (0.2 x BDR) folic acid for 4 weeks (PND 74 – 102) followed by a 4 week (A, B) (PND 102-130) or (C, D) 12 week (PND 102-186) maintenance diet. Data presented as mean ± SEM. n = 8-10 per group.

7.4 Discussion

Results from the present study demonstrate that folic acid deprivation in female C57/BL6 mice, during either the juvenile period (PND 28-56) or adulthood (PND 74-102) induces concentration, timing and duration specific changes in gene expression within mammary glands. Gene expression and changes in mammary gland morphology are summarised in table 7.4 and 7.5, respectively.

Table 7.4. Summary of Gene Expression Changes in Mice Supplemented with Folic Acid for 28 days during the Juvenile Period or Adulthood followed by a 4 week Maintenance Diet.

	Folic Acid Supplementation during the Juvenile Period		Folic Acid Supplementation during Adulthood	
	4 weeks after supplementation (PND 84)	12 weeks after supplementation (PND 140)	4 weeks after supplementation (PND 140)	12 weeks after supplementation (PND 140)
Gene	0.2 mg/kg	0.2mg/kg	0.2 mg/kg	0.2 mg/kg
<i>Brca1</i>	↓	↓	↑	----
<i>Oct-4</i>	↓	----	↑	----
<i>Ezh2</i>	----	----	----	----
<i>Bmi1</i>	----	----	↑	----
<i>Suz12</i>	----	----	↑	----

Energy intake did not differ significantly between the groups fed a folic acid deficient diet during the juvenile period, consistent with no differences in body weight between groups. However, in adult supplemented mice, energy intake differed significantly between control and folic acid deficient groups (Table 7.4). Mice fed a deficient concentration of folic acid demonstrated increased energy intake compared to baseline (1 mg/kg) (Figure 7.4). The increase in energy intake in mice supplemented during adulthood (PND 74 to PND 102) was not accompanied by an overall increase in body weight between groups and in some cases was accompanied by a

lower body weights at individual time points, suggesting energy is expended at an increased rate. As discussed in chapter 3, in response to supramaximal folic acid supplementation in which the same effects were observed, increased energy intake could be attributed to increased levels of physical or metabolic activity. These changes in energy intake in adult mice were also observed throughout the duration of the supplemental period and persisted until 12 weeks after the end of supplementation, which further reinforces a concentration specific, persistent effect that reflects changes in metabolism only within adult mice. These changes may, in part, reflect compensation behaviour in which mice consume an increased amount of diet to increase the levels of folic acid intake in an attempt to counteract folate deficiency.

Average plasma folate (5me-THF) decreased in a dose response manner at the end of juvenile deficiency between basal (1 mg/kg) and low (0.2 mg/kg) levels of folic acid intake. However, in adults there was no significant difference in plasma folate concentration. Despite adult mice consuming more diet than mice on the control diet, folic acid consumption was calculated to be approximately 5 fold lower than the control animals (0.47 ug/24 hours in deficient mice compared to 2.74 ug/24 hours in control). Therefore, levels of 5-meTHF would be predicted to be lower in the folic acid deficient mice. The liver does provide a store of THF which may be mobilised in response to a low folate diet, however, in the juvenile mice 5-meTHF levels were found to be reduced suggesting that an increase in mobilisation of stored folate may not account for these differences or that there are differences in the regulation of THF mobilisation between adult and juvenile mice. Further research is necessary to measure folate turnover, mobilisation and levels of stored folate within the liver.

Folic acid deficiency during the juvenile period was shown to have no effect on the expression of polycomb genes *Ezh2*, *Bmi1* or *Suz12*, either 4 weeks (PND 84) or 12 weeks (PND 140) after the period of supplementation. *Brca1* gene expression decreased in mice fed 0.2 mg/kg of folic acid 4 weeks after supplementation and this effect persisted at 12 weeks. Reduced levels of folic acid intake therefore affect the levels of *Brca1* expression within the mammary gland long after the period of supplementation. *Brca1* plays a critical role in the DNA damage response and in pathways that are involved in maintaining genome stability; therefore, its expression is tightly regulated (Mueller and Roskelley, 2003). Research has shown that reduced levels of *Brca1* expression is a characteristic feature of aggressive sporadic breast carcinomas and inherited breast cancer (Mueller and Roskelley, 2003). Furthermore, the magnitude of the decrease in

gene expression correlates with disease progression (Mueller and Roskelley, 2003). Thus, a folic acid deficiency-induced decrease in *Brca1* gene expression may be linked to breast cancer risk.

Mechanisms associated with these findings may be directly related to epigenetic regulation through changes in levels of DNA methylation. Research by Rice et al, (2000) has shown that methylation of the BRCA1 promoter is associated with decreased mRNA levels in clinical breast cancer specimens (Rice et al., 2000). Furthermore, BRCA1 promoter hypermethylation has been linked with down-regulated mRNA and protein levels in both tumours and cell lines (Hsu et al., 2013, Matros et al., 2005). Research by Pirouzpanah et al., (2015) has shown that dietary folate intake is inversely associated with methylated BRCA1. Furthermore, hypermethylation of the BRCA1 promoter is associated with reduced transcript levels in primary breast cancer samples (Pirouzpanah et al., 2015). The persistent decrease in *Brca1* expression up to 12 weeks post supplementation in the present study suggests a stable alteration in gene regulation, characteristic of an epigenetic mark, such as DNA methylation. Therefore, low folate intake may increase *Brca1* methylation which in turn may decrease levels of *Brca1* expression. Furthermore, low levels of *Brca1* gene expression may affect levels of DNA repair, cell cycle regulation, stem cell function and genome stability. Additional research could be conducted to ascertain if a decreased level of *Brca1* mRNA expression is associated with a decrease in *Brca1* protein, and if reduced levels of functional protein reduce DNA repair capacity which is an essential component of cell homeostasis and is dysregulated in breast cancer.

Oct-4 expression decreased in mice fed 0.2 mg/kg folic acid but this effect did not persist after 12 weeks. *Oct-4* has been shown to be lowly expressed in normal mammary glands from non-pregnant, non-lactating women and silenced in the majority of somatic cells; however, adult stem cell populations maintain expression of *Oct-4* which functions as a critical regulator of differentiation and proliferation (Tai et al., 2005). Decreased *Oct-4* expression and *Oct-4* silencing are associated with cellular differentiation and have been linked with the metastatic potential of breast cancer (Shen 2014). The effects of changes in the levels of *Oct-4* transcription on cancer risk appear to be tissue specific. Reduced levels of *Oct-4* expression are associated with reduced stem cell pool and decreased self-renewal (Shenghui et al., 2009). Therefore, a decrease in *Oct-4* expression in response to folic acid deficiency in the mammary gland may function to increase stem cell differentiation.

DNA methylation and chromatin remodelling have been shown to regulate *Oct-4* gene expression (Hattori et al., 2004). Methylation of the *Oct-4* promoter is considered a critical

switch which forces ESCs to differentiate as a result of decreased *Oct-4* transcription, confirming that *Oct-4* promoter methylation plays a direct role in cellular differentiation and proliferation (Gu et al., 2011). Modulation in levels of DNA methylation in response to folic acid deprivation may regulate *Oct-4* expression within adult stem cell populations of the mammary gland.

Mammary morphology showed no changes in response to low levels of folic acid intake during the juvenile period, either 4 weeks or 12 weeks after supplementation. Epithelial density, epithelial branches, epithelial junctions and number of TEBs were unchanged when compared to control groups. Therefore, during the juvenile period, an age associated with intense mammary development and plasticity, low folic acid intake does not induce mammary morphological changes that persist after the period of supplementation despite changes in levels of *Oct-4* expression, which suggests an increase in the levels of mammary stem cell differentiation. It may therefore be the case that changes in gene expression reflect changes in cellular differentiation that affect other aspects of morphology that have not been investigated. Increased mammary differentiation has been associated with changes in ductal and lobular structures, commonly classified as ductal or lobular hyperplasia (Mehta et al., 2001). Ductal hyperplasia occurs when increased cellular differentiation and proliferation results in an increase in the number of cells within the mammary ducts. Therefore, folic acid mediated changes in genes that control cellular differentiation such as *Oct-4* may pre-dispose or enhance the development of conditions such as ductal hyperplasia, which modulates breast cancer risk; however, this requires further investigation.

Interestingly, changes in gene expression were observed in adult folic acid deficient mice, despite no change in levels of 5-meTHF and increased energy intake. Therefore changes in expression may relate to an initial folate deficiency prior to the period of measurement, the resultant increase in energy intake or increased levels of folate mobilisation from liver stores in response to folate deficiency. These changes could also be related to the increased levels of progesterone that were significantly different from control groups, which may be effected by folate deficiency or simply reflect the stage of estrus cycle.

Folic acid deficiency during adulthood induced an increase in the expression of polycomb genes *Bmi1* and *Suz12*, 4 weeks after (PND 130) supplementation which did not persist after 12 weeks (PND 186). Interestingly, although the direction of *Ezh2* expression appeared to increase in response to low folic acid intake this did not reach significance. This provides a contrast to data from juvenile folic acid deficient mice where *Bmi1* and *Suz12* gene expression was unchanged;

suggesting the timing of folic acid deprivation is a critical component of polycomb gene regulation. Bmi1 mediates gene silencing through the regulation of chromatin structure, is critical for self-renewal in both normal and cancer stem cells and has been shown to be overexpressed in breast cancer (Bhattacharya et al., 2015). Research by Pietersen et al., (2008) suggests that mammary progenitor cell proliferation is hampered by the absence of Bmi1 and loss of Bmi1 prevents premature initiation of lobulo-alveolar differentiation (Pietersen et al., 2008). Furthermore, Bmi1 has been shown to promote EMT which induces stem-cell like properties in epithelial cells and is considered a key player in the development of cancer metastasis (Yang et al., 2010). Folic acid mediated increase in Bmi expression may therefore regulate progenitor proliferation and differentiation of cells down the lobulo-alveolar lineage. Increased Suz12 expression is associated with changes in transcription of genes that maintain pluripotency and self-renewal of stem/progenitor cells (Lee et al., 2006). Suz12 functions in the recruitment of DNA methyltransferases to target genes leading to de novo promoter methylation which has been linked to transcriptional repression (Reynolds et al., 2006). Upregulation of Suz12 and Bmi1 has been linked with dysregulated signalling related to epithelial differentiation, which results in dysregulated silencing of genes associated with tumour suppressor functions (Dimri et al., 2002). Changes in the levels of methyl donors such as folic acid may therefore play a role in the regulation of epithelial differentiation through polycomb mediated changes in methylation that lead to decreased levels of gene expression. Decreased levels of gene expression specifically in tumour suppressor genes have been linked to increased breast cancer risk. Overexpression of Bmi1 has been shown to decrease the levels of p16 and p19^{ARF}, tumour suppressor genes critically implicated in cancer development (Jacobs et al., 1999).

Brca1 gene expression was not significantly changed in response to folic acid deprivation in adult mice; however, *Oct-4* expression was significantly upregulated 4 weeks after supplementation. This effect is the opposite of what was observed in juvenile supplemented mice, with *Oct-4* expression shown to decrease at the same time point. Similar to the changes observed in polycomb regulators, this shows that the expression of *Oct-4* in response to folic acid intake is dependent on the timing of supplementation. As previously discussed *Oct-4* expression is reflective of changes in the regulation of stem cell differentiation and proliferation. Controlled upregulation of *Oct-4* and its associated gene network are implicated in normal remodelling of the breast during pregnancy and lactation towards a milk secretory pathway (Hassiotou et al., 2013b). Dysregulation of *Oct-4* expression outside of pregnancy or lactation is thought to affect

levels of cell proliferation and malignant cell transformation (Hassiotou et al., 2013b). Therefore, downregulation of *Oct-4* in juvenile supplemented mice in response to folic acid suggests the promotion of cell differentiation, and upregulation of *Oct-4* in adult mice (outside of pregnancy or lactation) suggests increased levels of cellular proliferation and malignant cell transformation, both of which are linked to increased breast cancer risk. Interestingly, changes in expression of *Brca1* in juveniles showed differences between folic acid deficiency and folic acid supplementation (Chapter 3), however, in adults, *Brca1*, *Oct-4* and polycomb genes showed distinct similarities in direction of expression. Both deficient and high levels of folic acid intake induced increased gene expression in these genes 4 weeks after supplementation. This suggests that changes in levels of folic acid intake are a key modulator of these genes. These findings also correlate with the U shaped relationship between folic acid intake and breast cancer risk.

Mammary morphology showed minimal changes in response to low levels of folic acid intake in adult mice. Epithelial density was significantly increased 12 weeks after folic acid supplementation, however, this effect was not observed 4 weeks after supplementation. This shows that folic acid deprivation induces changes in mammary morphology that only become evident long after the period of supplementation once folic acid intake has returned to baseline levels. Epithelial density is considered parallel to mammographic density analysis in humans (McGinley and Thompson, 2011). Increased epithelial density is associated with an increase in components of the epithelial tree. Although not significantly different, non-significant trends towards increased levels of epithelial branches and the number of epithelial junctions were observed 4 weeks after folic acid supplementation; however, the number of TEBS was unchanged. This suggests that folic acid may modify morphology, specifically epithelial density, by changing existing epithelial structures through mechanisms such as lateral side branching. This provides contrast with the lack of changes in morphology observed in juvenile supplemented mice, suggesting that the effects of folic acid deprivation on morphology are timing specific and may in part reflect differences in levels of plasticity and mammary development that occur between juvenile and adult mice. One pathway that is involved in the regulation of mammary morphogenesis, along with differentiation and proliferation, is controlled by Wnt proteins which regulate the ECM and cellular surface (Robinson et al., 2000).

Polycomb genes *Ezh2*, *Bmi1* and *Suz12* along with *Brca1* and *Oct-4* expression levels were compared for the effect of aging in mice fed a low dose of folic acid during the juvenile period. *Ezh2*, *Bmi1* and *Suz12* expression did not significantly differ from the pattern observed in control

mice between 4 and 12 weeks post folic acid supplementation. Therefore, folic acid deprivation does not alter levels of polycomb gene expression from that of baseline levels during ageing. Conversely, *Brca1* showed a significant decrease in expression with ageing between 4 and 12 weeks in control groups but in mice fed 0.2 mg/kg folic acid this loss of expression was not observed. As previously discussed in chapter 3, levels of *Brca1* expression have been shown to decrease with ageing. 0.2 mg/kg supplemented mice showed a reduction in the levels of *Brca1* expression 4 weeks after supplementation to below levels associated with normal ageing. Therefore, it appears that reduced folic acid intake dramatically prematurely reduces *Brca1* expression and that this accelerated decrease in *Brca1* expression occurs specifically during juvenile development.

Levels of *Oct-4* expression did not significantly change with ageing in mice fed 1 mg/kg folic acid, however, levels were significantly increased between 4 and 12 weeks post supplementation in mice fed 0.2 mg/kg, due to a reduction in *Oct-4* expression at 4 weeks. In line with observations in *Brca1* expression, this suggests that at 4 weeks post supplementation folic acid deprivation reduces *Oct-4* expression to levels below that which occur naturally with ageing, suggesting modulation of stem cell differentiation and proliferation that would otherwise not occur.

In terms of the effect of folic acid deprivation on gene expression with ageing in adults, an increase in *Ezh2* expression between 4 and 12 weeks in control groups was not mirrored in mice fed 0.2 mg/kg folic acid. Folic acid deprivation therefore increases *Ezh2* expression after 4 weeks to levels normally observed after 12 weeks. *Bmi1*, *Suz12* and *Brca1* expression did not significantly differ from the pattern observed in control mice between 4 and 12 weeks post folic acid supplementation, suggesting that levels of *Oct-4* expression significantly increased in mice fed 1 mg/kg folic acid; however, this effect was lost in mice fed 0.2 mg/kg. This was the opposite effect of what was observed in juveniles, showing an accelerated increase in expression after 4 weeks to near levels normally observed after 12 weeks.

Differences in gene expression with ageing between the juvenile period and adulthood may relate to the expansive remodelling and morphological changes associated with juvenile mammary development. This increased plasticity along with a much larger number of differentially regulated genes, as seen with RNA-seq analysis, may modulate downstream functions associated with BRCA1 and Oct-4, such as DNA repair and stem cell regulation, critical processes implicated in breast cancer risk.

Changes in levels of gene expression with ageing between folic acid deficiency and supplementation (chapter 3), in both juvenile and adult mice, showed no distinct patterns in gene expression of *Brca1*, *Oct-4* or polycomb genes. This suggests that persistent folic acid mediated changes in gene expression over time are specific to the timing and concentration of folic acid intake and reflect differences in the stages of mammary development.

7.5 Conclusion

In conclusion, this study has investigated the effects of folic acid deprivation at two different time points of the life-course on the expression of key genes implicated in breast cancer aetiology, namely polycomb genes *Ezh2*, *Bmi1* and *Suz12*, tumour suppressor *Brca1* and pluripotency regulator *Oct-4*. Key findings from this study have identified that a low dose of folic acid during the juvenile period does not affect levels of polycomb regulator genes; however, folic acid deprivation does cause a persistent decrease in the tumour suppressor gene *Brca1* and pluripotency gene *Oct-4*, but does not modify mammary morphology. In contrast, mice fed a low dose of folic acid during adulthood show persistent changes in expression in polycomb genes *Bmi1* and *Suz12* as well as *Brca1* and *Oct-4* and a persistent increase in levels of mammary epithelial density; however it is unclear how differences in progesterone concentrations between groups and the increase in energy intake contribute to these changes. Thus, the timing of folic acid deficiency plays a key role in both gene expression and mammary morphology. Persistent changes in gene expression that occur after low folic acid intake display characteristics consistent with epigenetic programming, which may suggest folic acid mediated changes in DNA methylation or polycomb mediated histone modifications that function to silence gene expression. Therefore, investigating levels of methylation within these genes would be a valuable next step to fully understand the role of folic acid deficiency on epigenetic programming. Furthermore, changes in gene expression and morphology may increase breast cancer risk through modulation of mammary stem cell regulation. Folic acid deficiency during the juvenile period may modulate mammary stem cells towards differentiation and during adulthood towards proliferation and cancer metastasis through mechanisms such as polycomb mediated EMT.

Chapter 8: Discussion

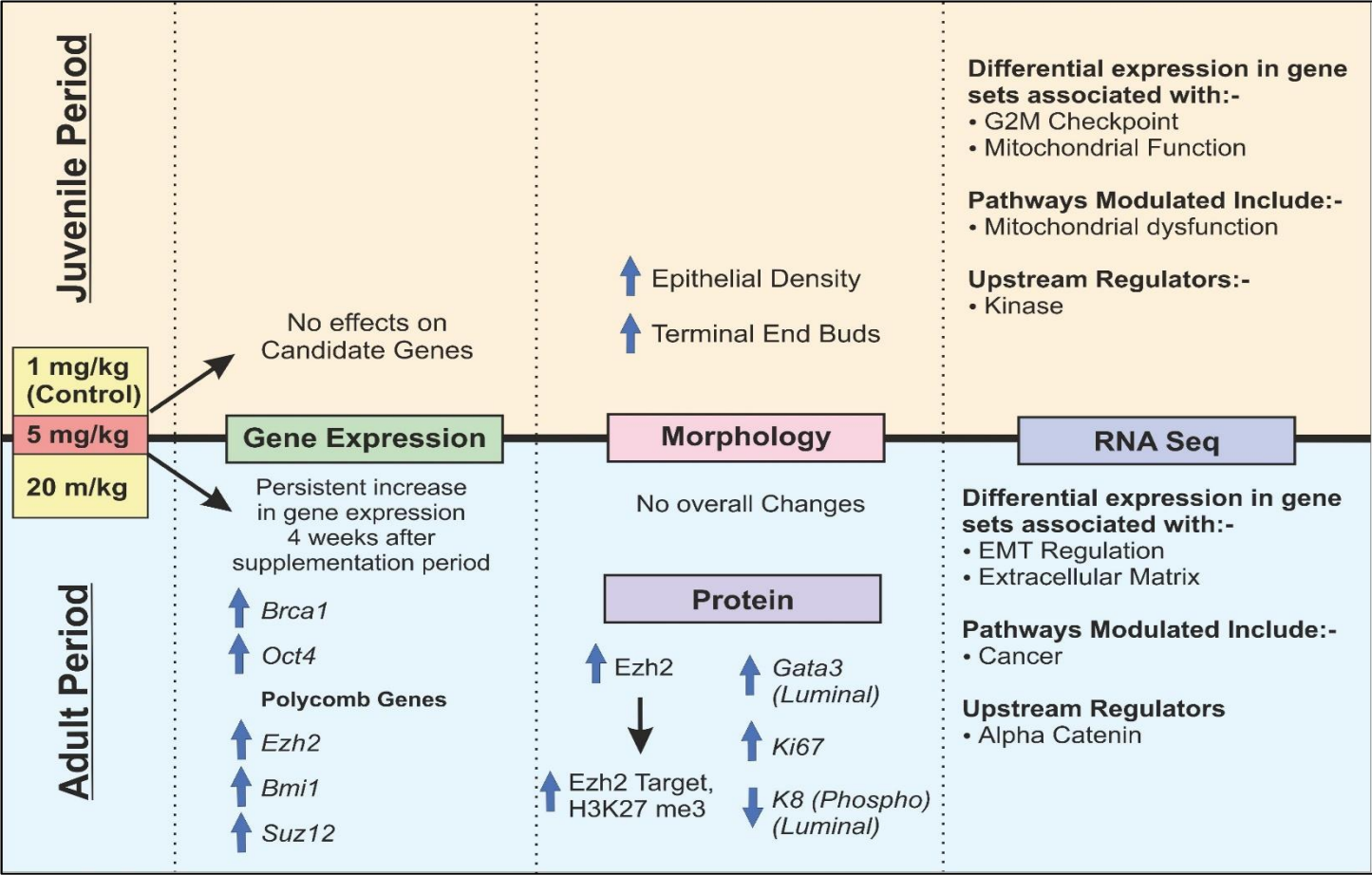


Figure 8.1. Summary Diagram of Key Findings. High folic acid intake (5 mg/kg) induced persistent changes in differential expression in key cancer determining genes and protein regulators of histone methylation specifically in adult mice. Furthermore changes in protein associated with epithelial differentiation and proliferation and enrichment of genes that regulate EMT were observed.

8.1 Discussion

There is substantial evidence that the environment, particularly nutritional intake, can have a large effect on disease risk. More specifically, modulation of methyl donors have been shown to influence disease risk later in life through changes in the epigenome (Vickers, 2014). Folic acid is a synthetic alternative to folate that can be used to supplement the diet and modulate folate status (Pietrzik et al., 2010). Evidence suggests that folic acid taken prior to and up to 12 weeks of gestation helps to prevent the occurrence of neural tube defects in new-borns (Pitkin, 2007a). However, there is a distinct lack of clarity within the literature with regards to correct folic acid dose, levels of folic acid consumption within the population and the effects of folic acid on long term health. Since the increase in folic acid intake due to mandatory food fortification combined with supplementation, an increasing number of studies have emerged providing putative links between high folic acid intake and breast cancer risk. Animal studies suggest a U shaped relationship between folic acid intake and breast cancer risk; furthermore, that folic acid increases existing mammary neoplasm development and also affects the disease risk of offspring, suggesting transgenerational effects conferred through epigenetic programming (Ly et al., 2011, Deghan Manshadi et al., 2014). This has raised many questions as to the safety of high doses of folic acid and prompted the requirement for further research to critically understand the effects of folic acid intake on long term breast cancer risk.

Using female C57BL/6 mice, the present study examined folic acid supplementation and deficiency during the juvenile period or adulthood with the aims of determining the persistent effects on gene expression, changes in mammary morphology and proteins associated with mammary cancer risk. Initial investigation was performed to ascertain how body weight, energy intake and levels of plasma folate and hormonal status were affected by dietary folic acid intake. Both weight and energy intake are important parameters to consider during dietary interventions to fully understand the influence of diet on growth, metabolism and development of disease states (Young and Kirkland, 2007). Results from the present study showed increased energy intake in mice fed a supramaximal level of folic acid acid (20 mg/kg) (Figure 3.2.4.1). Folic acid, along with all B vitamins, assists in the conversion of carbohydrates from food into fuel (glucose), which is used to produce energy (Mahmood, 2014). High levels of folic acid intake may induce changes in resting metabolic rate and increase levels of energy expenditure, which in turn result in a requirement to increase energy intake whilst maintaining body weight. Increased energy intake was also observed in mice fed a folic acid deficient diet (0.2 mg/kg). This may be,

in part, explained by a compensation effect, with mice consuming an increased amount of diet in an attempt to acquire baseline levels of folic acid. Research by Hambly et al., (2005) has shown that MF1 mice possess the ability to completely compensate for a 20% reduction in available energy by reducing components of their energy budget (Hambly and Speakman, 2005). Interestingly, individual mice demonstrate variability in their ability to compensate, for reasons unknown (Hambly and Speakman, 2005). Compensatory mechanisms have further been shown to slow down weight loss by directly reducing components of energy expenditure (Hambly and Speakman, 2005). The absence of changes in weight between these groups may be attributed to changes in resting metabolic rate, changes in energy expended in the form of increased levels of physical activity, or changes in thermoregulation (Hambly and Speakman, 2005).

Analysis of food intake at the end of the supplementation/deficiency period showed that adult mice fed 0.2 mg/kg did consume more diet and levels of plasma 5-meTHF in these mice were close to the folate levels measured in the mice fed the control diet (1 mg/kg) when measured directly after the period of supplementation (Chapter 3.4.2). Folic acid deficient mice also consumed increased levels of maintenance diet compared to controls 4 weeks after supplementation, suggesting that after a period of deficiency when dietary folic acid is returned to baseline, levels of food intake persistently remain high, the mechanisms of which are currently unknown. However, for mice to acquire baseline levels of folic acid (1 mg/kg) through a folic acid deficient (0.2 mg/kg) diet this would require dietary consumption 5 times greater than that of mice on a control diet which was not the case. The lack of difference between control and deficient plasma 5-meTHF may be related to an increase in efficiency of folic acid conversion into active 5-meTHF, with less lost as unmetabolised folic acid. This could also be combined with an increase in the release of stored folate from the liver elevating plasma 5-meTHF. In addition, during folate deficiency the demand for folate to supply one carbon groups to methylation reactions or nucleotide synthesis may be modified, preserving circulating level of 5-meTHF which therefore may not be reflective of the levels of these processes within tissues.

The increase in energy intake in mice supplemented with a restricted folic acid diet may, in part, be explained by the compensation effect, to attempt to acquire adequate intake of folic acid, but may also be attributed to dysregulated metabolism induced by reduced folate status. Interestingly, levels of plasma 5-meTHF measured directly after the period of supplementation in these studies only partly reflect levels of folic acid intake, with significant differences only observed in juvenile mice fed a supramaximal or folic acid deficient diet but no significant change

observed in adults. However, linear regression analysis showed that folic acid diet accounted for approximately 37% and 26% of changes in plasma folic acid in juvenile and adult mice, respectively. As discussed in Chapter 3, changes in levels of plasma 5-meTHF represent circulating folate and may be affected by levels of recent dietary intake, levels of unmetabolised folic acid and levels of folate uptake by the tissue; factors that would require additional measurement to fully understand folate status. Having identified changes in the expression of a number of key regulatory genes it will next be essential to determine the mechanisms behind how changes in gene expression occur and how folic acid metabolism and turnover within the plasma and tissues is affected by age and diet.

Although it is only possible to postulate as to the mechanisms involved, it is clear that specific concentrations of folic acid effect energy intake in adult mice and not juveniles, suggesting a novel, age-specific effect of folic acid on energy metabolism. Furthermore, the concentrations of folic acid intake affected are specific (both very high and very low). This shows that both supramaximal doses of folic acid and folic acid deficiency alter energy intake in adults, suggesting a novel concentration-specific role of folic acid intake on metabolism or feeding behaviours. Although compensation may in part explain increased energy intake associated with folic acid deficiency, this does not fully explain these findings and is not the case in mice supplemented with supramaximal levels of folic acid, suggesting other mechanisms are involved. One such mechanism that may relate to changes in energy intake in mice fed high and low levels of folic acid could result from effects of stress on behaviour and metabolism. Research by Moles et al., (2006) has identified that stress is associated with changes in behaviour and eating preferences in mice (Moles et al., 2006). Nutritional stress from both supramaximal intake of folic acid or folic acid deficiency could be associated with dysfunction of important biological functions such as the immune or neuroendocrine system which could directly affect metabolism and behaviour (Andrews and Abizaid, 2014). In order to investigate these changes further it would be necessary to monitor levels of mouse activity and behaviour to see if mice fed high or low levels of folic acid do in fact expend more energy than mice consuming basal levels. One way to achieve this would be to utilise metabolic cages which non-invasively measure oxygen (O₂) consumption, carbon dioxide (CO₂) production and calculate the respiratory exchange ratio (RER) of individual mice to reflect energy expenditure. Furthermore, levels of physical activity can be quantified as a measure of horizontal and vertical movement and combined with food/water intake over a given time period (Tschöp et al., 2011).

Folic acid intake has also been linked to cognitive function suggesting changes in intake can cause cognitive impairment which may lead to increased levels of feeding behaviour (Herbert, 1962). Previous research has shown tumour incidence in rodents is dependent upon daily caloric intake and bodyweight (Tannenbaum, 1940, Bocchinfuso et al., 2000). High caloric intake is sufficient to significantly increase development of mammary tumours in rats and mice (Lapidus et al., 1996). A plethora of research has implicated calorie restriction as protective against breast cancer (Abbott et al., 1999, Phelps et al., 2003, Marquis et al., 1995b). Furthermore, breast cancer mortality and incidence have been shown to increase with ageing (Zhang et al., 2005b). A specific folic acid mediated (very high 20 mg/kg, or very low 0.2 mg/kg) increase in energy intake observed in the present study, which persists for up to 84 days after modified folic acid supplementation, could therefore contribute towards a higher calorie intake and increased risk of mammary tumour development in susceptible animals.

Despite the significant effects of different levels of folic acid supplementation on energy intake, differences in the concentrations of supplemented folic acid were not consistently reflective of changes in the levels of 5-meTHF. However, although levels of 5-meTHF reflect circulating active folate, this is considered a proxy for folic acid intake. Thus, a non-significant change in levels of 5-meTHF in response to folic acid intake may not be representative of tissue folate status or levels of one carbon metabolism and needs to be combined with additional measurements to fully understand folate turnover.

Changes in the levels of folic acid intake were next investigated for effects on expression of key genes implicated in breast cancer risk, namely *Brca1*, *Oct-4* and polycomb regulators *Ezh2*, *Bmi1* and *Suz12*. Despite associations between levels of methyl donors, polycomb regulators and breast cancer risk, previous studies have not investigated the long term effects of folic acid supplementation on these genes in the mammary gland. The present study has shown that the effects of folic acid on gene expression are both dose and timing specific and persist beyond the period of supplementation. *Brca1* and polycomb gene expression was not affected by high or supramaximal folic acid intake during the juvenile period but *Oct-4* expression was decreased. Conversely, after adult supplementation, expression of all genes increased in the high folic acid (5 mg/kg) group, 4 weeks after folic acid intake. Moreover, unlike the decrease in *Oct-4* expression in juvenile mice, the direction of change in gene expression in adults increased and is therefore dependent on the timing of folic acid intake.

Previous research has shown that EZH2 transcript and protein are consistently elevated in invasive breast cancer (Kleer et al., 2003). Furthermore, changes in *Oct-4* and *Brca1* expression have been linked with breast cancer risk. Increased *Brca1* expression in some studies is considered protective against mammary tumour development, however, in vitro studies have shown *BRCA1* expression is increased in proliferating cells undergoing differentiation (Thompson et al., 1995). Furthermore, EZH2 has been shown to control the intracellular localisation of BRCA1 protein, and upregulation of EZH2 in benign mammary epithelial cells has been shown to induce nuclear export of BRCA1 protein, aberrant mitosis and genomic instability (Gonzalez et al., 2011). Thus, increased *Brca1* expression may not function as protective if elevated levels of *Ezh2* expression modulate nuclear localisation of *Brca1* in the present study.

Changes in levels of *Oct-4* expression is indicative of modulation of mammary stem cells, along with increased levels of *Ezh2* expression, suggesting high folic acid intake during adulthood may modulate stem cell regulation and levels of differentiation, processes that are implicated in cancer risk. Thus, folic acid intake during adulthood may demonstrate a greater period of risk compared to during juvenile development. Furthermore, folic acid mediated changes in gene expression at either time point persisted after 4 weeks but not after 12 weeks, showing changes in gene expression are also duration specific and effects can be lost after a sustained period on a basal concentration. The persistent change in gene expression that lasts for four weeks after supplementation suggests changes in gene regulation may be representative of modified epigenetic regulation. Epigenetic regulation of genes is generally associated with increased repression of gene expression based on methylation of DNA within promoter regions, which modulates transcription factor binding or methylation of residues within histones. Decreased levels of *Oct-4* expression in juveniles may well represent an epigenetic modification within the *Oct-4* gene that reduces the levels of gene expression. However in adult supplemented mice, persistent increases in gene expression do not suggest repressive DNA marks. These changes could be related to modulation of epigenetic marks in genes associated with the regulation of upregulated genes conferring changes through feedback loops and modified expression. For example, research by Di et al., (2010) suggests that *BRCA1* gene expression is tightly controlled by a dynamic equilibrium between transcriptional co-activators and co-repressors that regulate DNA accessibility, histone acetylation and the BRCA1 promoter (Di et al., 2010). Folic acid mediated changes in the epigenetic regulation of transcriptional co-activators or co-repressors

may function to modulate levels of gene expression that results in persistent upregulation of genes.

The dose specific effects on gene expression observed in adults may in part reflect the availability of folic acid in the mammary gland. Folic acid modulates gene expression at a physiological concentration (5 mg/kg); however, no dose response effect was observed in mice fed very high folic acid (20 mg/kg). Plasma 5-meTHF concentrations showed increased levels (mean folate concentration which did not reach significance) in mice fed 5 mg/kg and mice fed 20 mg/kg, suggestive of a dose response increase in plasma folate. Therefore, the lack of changes in gene expression at 20 mg/kg suggest very high levels of folic acid do not function in the same way as high concentrations (5 mg/kg). This may be explained by enzymatic constraints, specifically, limited folate accumulation in mammary tissues by folypolyglutamate synthase activity at high folic acid concentrations (Shane, 2010). Furthermore, this effect may be related to high levels of circulating unmetabolised folic acid which has been shown to inhibit dihydrofolate reductase (DHFR), critically involved in the reduction and methylation of folic acid, resulting in a decrease in the intracellular folate pool and reduced levels of cell proliferation, which is likely reflective of differences in gene expression (Deghan Manshadi et al., 2014). Furthermore, high folic acid induced DHFR inhibition has been shown to reduce thymidylate which regulates DNA synthesis and replication (Deghan Manshadi et al., 2014). Interestingly, research by Mansahdi et al (2014) draws parallels with findings from the present study showing absence of a dose-dependent effect of folic acid supplementation on mammary tumor progression in rat. Furthermore, mammary gland folate concentrations appeared to reach a plateau beyond a 5 mg/kg diet and were not significantly different among the three supplemental levels of folic acid (Deghan Manshadi et al., 2014). Further research is required to measure unmetabolised folic acid and levels of folate within mammary tissue to ascertain the exact mechanisms that are affected by supramaximal folic acid intake.

Timing specific gene expression changes may reflect the dynamic state of mammary development; with juvenile glands, which demonstrate a high level of plasticity, undergoing increased levels of epithelial development and remodelling when compared with adult glands (Conneely et al., 2003). During the juvenile period the mammary gland fully develops, undergoing extensive epithelial proliferation and growth which is regulated by hormones and growth factors, as opposed to the adult mammary gland (excluding pregnancy) which is relatively quiescent, demonstrates reduced plasticity and proliferation, and is modulated

predominantly by the spatial and functional coordination of hormonal and ECM regulatory factors (Macias and Hinck, 2012). Thus, the mammary epithelial microenvironment differs significantly between juvenile mammary development and adulthood. Decreased levels of *Oct-4* in juveniles suggest increased levels of stem cell differentiation which fits with the requirement for increased epithelial plasticity and remodelling during this period. However, increased *Oct-4*, *Brca1* and polycomb gene expression observed during adulthood suggests alternative mechanisms (Fata et al., 2001). Interestingly, no effects of either 17- β estradiol or progesterone, key hormones that represent stages of estrus, were observed 4 weeks after supplementation in juvenile or adult supplemented mice, suggesting folic acid-induced changes in gene expression are independent of hormone status. However, adult folic acid deficient mice (0.2 mg/kg) demonstrated a significant difference in progesterone which was reflective of diestrus phase. It is currently unknown if this change is caused by folate deficiency or if it is simply reflective of the estrus stage that the group of mice were in at the time of sacrifice. Furthermore, linear regression analysis comparing all concentrations of either progesterone or estradiol-17 β with expression data showed no significant correlation, suggesting hormone levels do not effect folic acid mediated changes in expression.

Increased levels of *Brca1*, *Oct-4* and polycomb gene expression suggest changes in the regulation and differentiation of mammary stem cell populations and an increased requirement for DNA repair or cell cycle regulation, which may in part be controlled through polycomb mediated changes in the epigenetic regulation of histones through PRC1 and PRC2 complexes. This provides a testable hypothesis to explain the persistent changes in expression observed through epigenetic regulation of chromatin, modulated by levels of folic acid. Persistent changes in gene expression at 4 weeks after supplementation show that the effects of folic acid intake extend beyond the period of supplementation. This key finding combined with the dose and timing specific effects of high folic acid intake shows that effects on the long term regulation of the genome are not limited to the dose or period of intake. However, findings clearly show that differential expression does not persist beyond a 12 week period post supplementation. The comparative age of humans to mice estimates that changes in expression persist for approximately 5 years after supplementation but are not present after approximately 15 years (Flurkey et al., 2007). Persistent changes in differential gene expression suggest that epigenetic regulation of DNA or histone proteins may play a role. Further investigation is necessary to determine levels of DNA methylation within these genes or genes that play a regulatory role.

To determine if a folic acid mediated increase in *Ezh2* expression was translated into functional protein and the effects on downstream targets, gene expression analysis was followed up with Western Blotting in adult mice fed 5 mg/kg 4 weeks after supplementation. Quantification of *Ezh2* revealed increased levels of protein and also increased levels of *Ezh2* target H3K27 (tri-methyl). High folic acid intake therefore modulates histone methylation, specifically H3K27 (tri-methyl), through modulation in levels of *Ezh2* expression. Methylation of H3K27 is considered a repressive chromatin mark which is tightly associated with inactive gene promoters (Barski et al., 2007). PRC2 mediated changes in levels of H3K27 (tri-methyl) plays a critical role in remodelling chromatin structure of genes required for multi-lineage differentiation of mammary stem/progenitor cells; therefore, increased methylation of H3K27 may function in the repression of genes that regulate mammary stem cell function (Huang and Esteller, 2010). Interestingly, an increase in the expression of *Oct-4*, a major regulator of stem cell function, shows this gene is not directly repressed; however, many other genes such as *Sox2*, *Lif* and *Wnt*, of which expression is implicated in mammary stem cell regulation and regulation of *Oct-4*, warrant further investigation. Increased expression of *EZH2* is strongly associated with breast cancer risk. Elevated levels of *EZH2* protein have been shown to indicate a precancerous state in morphologically normal mammary epithelium and have been shown to increase with breast cancer development (Ding et al., 2006b). Therefore, novel findings from this study suggest that high folic acid intake during adulthood may increase breast cancer risk through polycomb mediated regulation of chromatin. The next step to further understand the relationship between folic acid and *Ezh2* is to identify downstream targets and investigate how changes in levels of *Ezh2* expression affect these targets. Increased levels of *Ezh2* have been linked to H3K27 (tri-methyl) in the promoter region of a number of genes that include *RAD51*, *RUNX3*, *CDKN1C*, *FOXC1* and *CDH1* (Yoo and Hennighausen, 2012). In addition, epigenetic events in the *Ezh2* promoter could be studied using chromatin immunoprecipitation analysis (chIP), a technique which is currently underway.

It was hypothesised that changes in levels of gene and protein expression may be reflective of changes in gross mammary morphology. Gene expression analysis was therefore combined with direct morphological assessment of mammary epithelial structure using mammary whole mounts to fully understand morphological response to folic acid. Changes in mammary morphology were minimal in both juvenile and adult supplemented mice; however, persistent effects were observed in epithelial density and the number of TEBs, both established markers of mammary differentiation and epithelial development (Visvader and Stingl, 2014). Interestingly,

changes in density and TEBs in juvenile supplemented mice were observed in mice fed high (5 mg/kg) folic acid 12 weeks after the period of intake, showing long term changes in morphology. Long term changes in morphology were also observed in adult supplemented mice but only in TEBs. Collectively, increased TEBs alone or combined with increased density suggest epithelial expansion. Interestingly, expansion of TEBs is associated with distinct populations of mammary stem cells located within TEBs (Williams and Daniel, 1983b). Changes in levels of stem cell differentiation and cell proliferation have been shown to give rise to mammary ductal morphogenesis (Visvader and Stingl, 2014). Increased development of existing ductal structures could therefore give rise to an increase in mammary epithelial density. Interestingly, both TEB and epithelial density are considered markers of breast cancer risk, suggesting high folic acid modifies these markers which may in part modulate breast cancer risk.

In order to establish if high folic acid intake modifies mammary ducts and alveoli, protein markers within epithelial cell populations were investigated. Both Brca1 and Oct-4 protein expression was localised in ducts and alveoli and showed changes in immunopositive cells consistent with changes in gene expression. Of particular interest was the localisation of Brca1 protein which displayed high levels of cytoplasmic staining, inconsistent with the nuclear localisation required to regulate DNA repair and cell cycle. This could suggest Brca1 re-targeting to the cytoplasm, dysregulation of Brca1 transport into the nucleus or changes in the interactions with BARD1, which result in exportation to the cytoplasm. However, further research is necessary to establish the exact mechanism that may be affected and how folic acid may modulate this mechanism. A point of consideration if this is in fact the case would be that even if levels of Brca1 were increased, unless Brca1 is actively present within the nucleus the effects on DNA repair, cell cycle regulation and stem cell function would likely not increase and may actually be compromised. Therefore, levels of *Brca1* gene and protein expression may not necessarily be representative of Brca1 function.

Investigation of protein markers associated with the basal/myoepithelial and luminal components of ducts and alveoli in adult supplemented mice in response to high folic acid showed no significant changes in levels of basal marker α -SMA, however, luminal marker K8 Ser431 appeared to decrease with increasing folic acid intake, suggesting a change within luminal populations. Furthermore, these findings were combined with increased levels of cell proliferation observed through Ki-67 positive cells and an increase in Gata3 luminal stem cell marker. Collectively, these findings suggest that high folic acid persistently modulates luminal

K8 Ser431 expression, increases cell proliferation and luminal epithelial cell differentiation and commitment (Figure 8.1). In terms of the overall effects of changes in these markers on disease risk, modulation of levels of Keratin 8 increased cellular proliferation and Gata3 are associated with ductal morphological change and implicated in luminal breast cancer risk. Changes in levels of K8 are considered prognostic markers in epithelial tumours. Reduced expression of K8 has been associated with epithelial-to-mesenchymal cancer cell transition in colorectal cancer (Knösel et al., 2006). Increased K8 Ser431 phosphorylation is associated with cellular stress and increased mitosis. Interestingly, decreased expression of K8 Ser438 in the present study may therefore suggest reduced cellular stress and mitosis, however, it would be necessary to clarify if reduced levels of K8 Ser438 are a result of folic acid induced K8 de-phosphorylation or overall decreased levels of K8 (Karantza, 2011).

Gata-3 is strongly associated with luminal type breast cancer risk and has been shown to be frequently mutated and expression levels significantly elevated in luminal cancer (Chou et al., 2010). Research suggests that Gata-3 effects can switch between tumour suppression and tumour promotion during tumorigenesis, with the deregulation of three genes, mainly BCL2, DACH1 and THSD4, that represent Gata-3 control processes (Cohen et al., 2014). Interestingly, RNA-seq analysis conducted in the present study identified decreased differential expression of *Bcl2* (the second most differentially downregulated gene), suggesting a link between high folic acid and modulation of expression of Gata-3 control genes. Gata3 has also been linked to *Ezh2* which is predominantly expressed in luminal cells showing overexpression of EZH2 in MMTV-EZH2 virgin mice is accompanied by up-regulation of Gata-3 and premature differentiation of ductal epithelial cells (Li et al., 2009a, Michalak et al., 2013). Therefore, changes in K8 expression and Gata-3 expression may suggest an increased risk towards luminal breast cancer.

Collectively, high folic acid-mediated changes in *Ezh2* appear to modulate changes in H3K27 (tri-methyl) levels which can function as a repressive mark in histone H3. In addition, increased *Ezh2* and *Suz12* expression (PRC2 complex), and *Bmi1* expression (PRC1 complex) may regulate changes in G to M phase transition, which may affect levels of cellular proliferation and promote mammary progenitor cell expansion, in combination with Gata-3 and differentiation towards a luminal fate. Modulation of progenitor cells may either increase levels of stem cells which is reflective of increased Oct-4 expression or may promote stem cell differentiation, decreasing Oct-4. In addition, changes in levels of *Brca1* expression may function in the regulation of *Ezh2* (Figure 8.1).

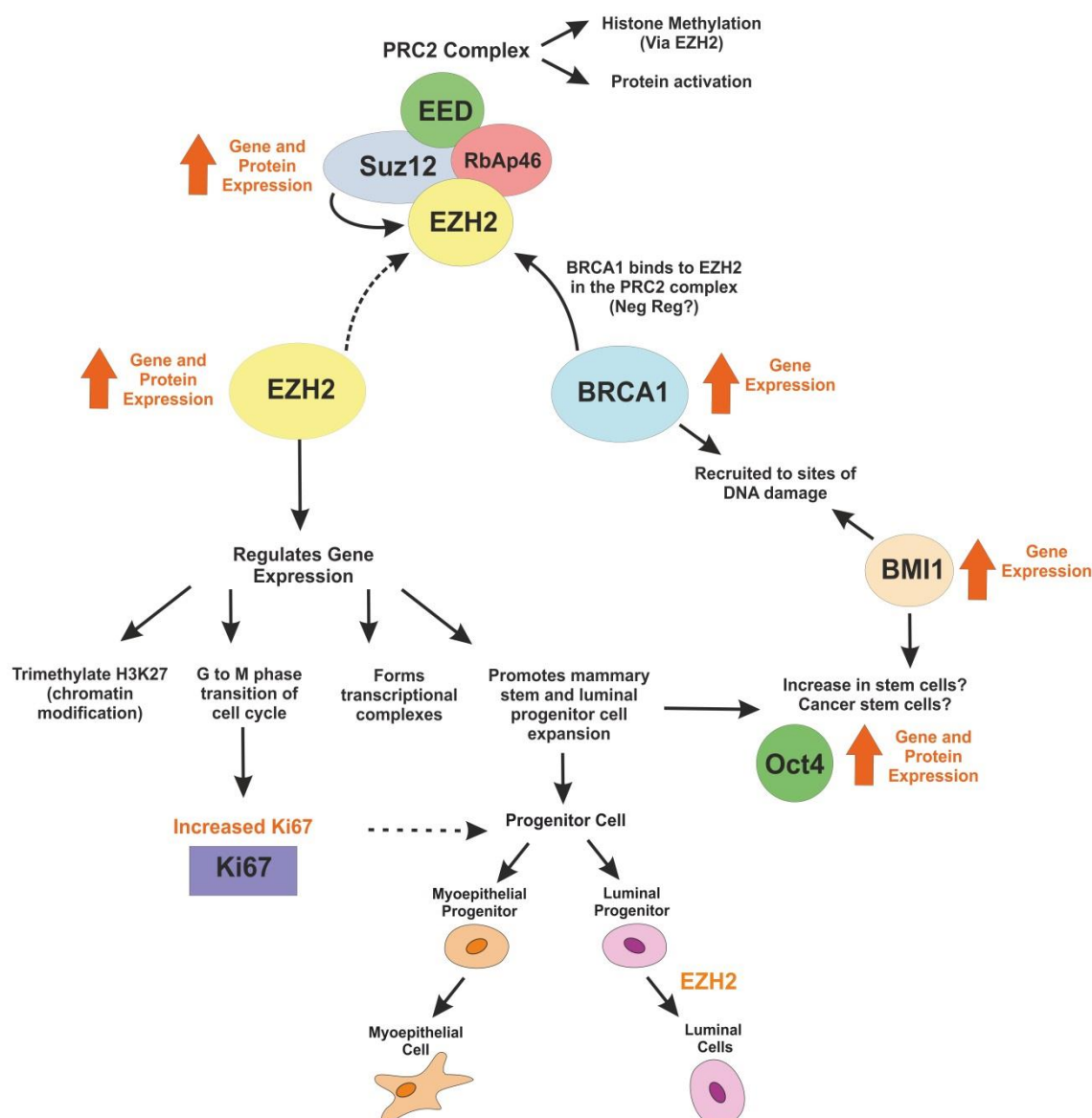


Figure 8.2. Putative Pathway Showing Possible Effects of High Folic Acid Supplementation on Candidate Gene and Protein Expression in Adult C57BL/6 Mice.

Changes in candidate gene expression and molecular markers were linked to provide a visual representation of possible folic acid mediated molecular interactions based on findings from the present study. *Brca1*, *Oct-4* and polycomb genes *Ezh2*, *Bmi1* and *Suz12* function together in the regulation of mammary stem cells. Increased expression of these genes may promote mammary progenitor stem cell expansion towards a luminal pathway.

In order to acquire a deeper understanding as to the effects of folic acid intake on gene expression and to investigate whether the timing of folic acid intake effects differential gene expression, gene enrichment and gene regulatory networks and pathways, candidate gene analysis was followed up using genome wide RNA-seq whole transcriptome analysis from mice supplemented with high folic acid (5 mg/kg) during the juvenile period or adulthood. This analysis identified a large difference in the number of differentially regulated genes between juvenile and adult time points with a much smaller gene set altered in adults. Of these gene sets, only 9 genes showed overlap (0.49% in juvenile and 4.05% in adults) with differences in gene ontology, GSEA and the pathways and networks affected. This data, along with the timing specific expression changes observed in the candidate gene approach (chapter 3), reinforce the key finding that the timing of folic acid supplementation is critical in the regulation of gene expression and in the case of the RNA-seq data, represents distinct gene sets that are relatively unrelated, differentially expressed at different time points and regulate different gene pathways and networks. These findings further support the major differences between the juvenile and adult mammary gland microenvironment previously discussed and the effect of folic acid during these different phases of mammary development. Furthermore, and more critically, the disease pathways implicated affect different processes. Highly differentially expressed genes in juvenile supplemented mice were focused on immunological function and lipid metabolism compared with adults that focused on genes that regulate cellular proliferation and apoptosis. More interestingly GSEA showed clear differences between juvenile and adult supplemented mice, with juvenile gene sets enriched on G2M checkpoints and adults on EMT. Also key to note is the direction of enrichment, with juvenile supplemented mice showing enriched gene sets upregulated in G2M and E2F targets and adults showing downregulated G2M and E2F within the top three enrichment sets. Therefore it appears that within GSEA there is some crossover in gene enrichment in response to high folic acid, however, the direction of expression is the opposite, further reinforcing the timing specific effects of folic acid supplementation.

Differences in gene expression between juveniles and adults further confer differences in gene regulatory pathways and disease risk. Within juvenile supplemented mice, changes in gene sets within GO focusing on mitochondrion were combined with mitochondrial dysfunction disease pathways and networks associated with glucose metabolism disorder, disglycemia and hypoglycaemia. Conversely, within mice fed during adulthood, changes in gene sets within GO focusing on ECM and cell migration was combined with inhibition of MMP disease pathways and networks associated with cancer tumour growth, cellular morphology and cellular assembly.

Thus, the timing of high folic acid intake critically determines the gene sets and disease pathways that are activated. Of particular interest in these findings is the response observed in mice supplemented during adulthood. Persistent changes in *ECM*, *MMP* and *Timp* expression suggest changes in regulation of mammary epithelial components. Changes in MMPs and TIMPs, components of the ECM, are consistent with previous reports identifying regulated tissue remodelling of the adult mammary gland epithelium (Fata et al., 2001). This combined with top disease networks regulating cancer; mammary tumour growth and metastasis provide a direct link between high folic acid intake during adulthood and breast cancer risk long after the period of supplementation. Furthermore, the links with gene sets enriched for processes that include upregulation of EMT and downregulation of G2M checkpoint (as well as many others) and changes observed in polycomb regulation identified using the candidate gene approach suggest these changes may be facilitated through the regulation of mammary stem cells. Enrichment of EMT genes within adults can also be linked to *Ezh2* which is critically implicated as a major regulator of EMT and epithelial stem cells.

Analysis of the effects of high folic acid intake on mammary function was followed up by investigating folic acid deficiency on candidate gene expression and gross morphology. Consistent with observations from folic acid supplemented mice, folic acid deficiency induced distinct differences in expression of polycomb genes, *Brca1* and *Oct-4* that were both dose, timing and duration specific. The direction of gene expression changes also differed between each time point, with down regulation of genes in juvenile deficient mice and upregulation of genes in adults, following the same expression pattern observed in mice supplemented with high folic acid. Gene changes also persisted for 4 weeks after deficiency for the majority of genes in similar fashion to supplemented mice; however, interestingly *Brca1* expression was persistently decreased both 4 and 12 weeks after the deficient diet, showing that folic acid deficiency can repress tumour suppressor gene expression as long as 15 years (human equivalent) after the period of supplementation. The similarity in direction of differential gene expression and genes affected suggest that folic acid deficiency and high levels of folic acid intake may control transcriptional regulation through similar mechanisms. Changes in cytosine methylation in DNA are a known mechanism associated with folate deficiency which leads to inappropriate activation of proto-oncogenes and induction of malignant transformation (Duthie, 1999). Consistent with these findings, in juvenile supplemented mice, *Brca1* and *Oct-4* expression was persistently repressed; suggesting folic acid deficiency-induced CpG methylation within these genes may be critically involved. Furthermore, increased expression of polycomb

genes in adults suggests chromatin modifications may also play a role. Both *Brca1* and *Oct-4* repression in juvenile mice and increased polycomb gene expression in adults, especially *Ezh2*, are characteristic of breast cancer risk. Therefore, in line with current literature, folic acid deficiency seems to modulate genes associated with increased breast cancer risk. However, novel data from the present study has shown that these changes persist after the period of supplementation for up to 12 weeks and, in line with data investigating high levels of folic acid supplementation using candidate gene and RNA-seq approaches, are dependent on the timing of intake. Consistent with mice fed a high folic acid diet, minimal changes were observed in mammary morphology in folic acid deficient mice.

8.1.1 Study Limitations

8.1.1.1 Plasma 5mTHF

Plasma 5-mTHF levels measured in folic acid supplemented and deficient groups showed significant changes in juvenile mice supplemented with a supramaximal and deficient concentration of folic acid; nonetheless, no differences in other groups were observed. Firstly, these findings were limited by the small size of the sample groups. Retrospective power calculations suggest the number of samples in juveniles (5) were adequate, however, in adults calculations showed that an increased number of mice (28) would be required to demonstrate significance. Secondly, 5-mTHF is a proxy measure of which a number of other factors have been shown to influence including, enzyme function and metabolism, tissue folate status and folate liver stores which can be mobilised during folate deficiency.

To fully understand how folic acid intake may affect folate metabolism and subsequent gene expression a number of additional measurements could be conducted to further understand the effects of folic acid intake on folate status. Measurement of folic acid in plasma would allow quantification of unmetabolised folic acid which would provide a deeper insight into folic acid absorption, conversion and the effects of circulating unmetabolised folic acid on folate turnover. In addition, the measurement of folate in red blood cells could be performed as this is considered more reflective of long term folate status compared to plasma folate which can be influenced by recent diet (Piyathilake et al., 2007). Levels of folate within mammary tissue could be measured as this would provide detailed information with regards to levels of tissue uptake

in response to changes in dietary folic acid, and also if changes in levels of tissue uptake effect levels of circulating 5-mTHF. Furthermore, the SAM/SAH ratio could be measured which would provide an indication of levels of methyl group donation into methylation reactions in response to changes in folic acid intake. Additionally, all of the above parameters would be measured immediately after supplementation and at both 4 weeks and 12 weeks after the modified folic acid diet to understand long term changes in folate status once an animal is returned to a maintenance diet.

Findings from the present study have wide reaching implications for informing on folic acid intake and mammary cancer risk. Key findings have shown that high folic acid intake modulates genes and proteins associated with breast cancer risk mainly in adult mice compared to juveniles. This suggests that high levels of mammary plasticity during juvenile development may function as protective and that reduced plasticity in the adult gland may increase susceptibility to modulation of cancer genes. With breast cancer considered one of the most common cancers during adulthood this highlights the importance for consideration of levels of folic acid intake in adult women. Furthermore, this raises questions as to the safety of high levels of folic acid intake on long term mammary health during pregnancy, during which women are often prescribed or advised to consume high doses of folic acid as a preventative measure for neural tube defect occurrence. In addition effects of high folic acid intake on the long term regulation of genes during ageing raises questions as to recommendations for folic acid intake in an increasingly aging population given that the timing of intake has been highlighted as important and has been shown to affect different genes and pathways. Collectively, these findings suggest that both the dose and timing of folic acid intake needs to be carefully considered, especially in adults with a previous history of mammary cancer or those at high risk who may be unknowingly consuming high levels. Furthermore, the results from this study highlight the need to determine whether the short term benefits of folic acid consumption to reduce neural tube defects outweighs the long term implications on mammary health in susceptible individuals.

8.2 Summary

The results from this study show that folic acid intake at 5 mg/kg, which is equivalent to doses recommended to women during pregnancy, women who have a history of neural tube defects and which is easily obtainable through fortified food intake combined with supplementation, appears to induce the most persistent effects on gene and protein expression in adult supplemented mice. High levels of folic acid intake during either the juvenile and adult periods induce dose, timing and duration specific changes in expression of genes, morphology and proteins implicated in breast cancer risk. Furthermore, these changes may be mediated by epigenetic regulation of histone modifications. Moreover, supplementation during adulthood is associated with pathways and networks implicated in tumour growth and mammary cancer as opposed to energy metabolism pathways in juveniles. This collectively suggests that high levels of folic acid supplementation during the juvenile period do not increase breast cancer risk, however, high intake during adulthood (5 mg/kg) may increase breast cancer risk long after the period of folic acid supplementation and levels of folic acid intake should therefore be carefully considered in adult populations, especially individuals at a higher risk of breast cancer through family history of the disease or breast cancer survivors and during old age with respect to minimising risks of breast cancer development and progression. Furthermore, considering the complexity of pathways involved in folate metabolism and gene regulation implicated in the present study, research should be conducted to critically assess the effects of high folic acid intake during pregnancy on the long term breast cancer risk of both the mother and offspring.

8.3 Future Directions

Research from the present study has identified a number of novel findings. Further work is required to fully understand the mechanisms behind observed folic acid induced changes in gene and protein expression and mammary gland morphology, along with how these changes affect mammary function and future disease risk.

8.3.1 Epigenetic Regulation of Gene Expression

Persistent changes in gene expression are suggestive of epigenetic regulation of DNA; therefore, it is necessary to investigate changes in levels of CpG methylation within the promoters of differentially expressed genes, such as *Brca1*, *Oct-4* or *Ezh2* to ascertain if changes in levels of folic acid effect CpG methylation within these genes. Currently, levels of *Oct-4* and *Brca1* CpG methylation are being investigated in mammary tissue from the experimental study outlined above (Chapter 2). DNA from mice fed folic acid during the juvenile or adult periods 4 weeks after the period of supplementation has been bisulfite converted and analysed using pyrosequencing with primers designed within the *Oct-4* and *Brca1* promoter regions. This will inform on the levels of CpG methylation within the sequenced regions in response to high (5 mg/kg) and supramaximal (20 mg/kg) levels of folic acid which may influence transcription factor binding and levels of gene transcription. Currently, preliminary investigations of *Oct-4* have shown significant increases and decreases in CpG methylation in mice fed 5 mg/kg and 20 mg/kg folic acid during the juvenile period and a significant decrease in methylation in mice fed 5 mg/kg during adulthood (Figure 8.2). Due to low levels of methylation identified in the *Brca1* promoter, further research is required to identify putative regions with higher levels of methylation which may be modulated by changes in levels of folic acid intake. Furthermore, as well as investigating methylation levels in mice 4 weeks after supplementation it is also necessary to assess levels after 12 weeks to see if changes in methylation persist.

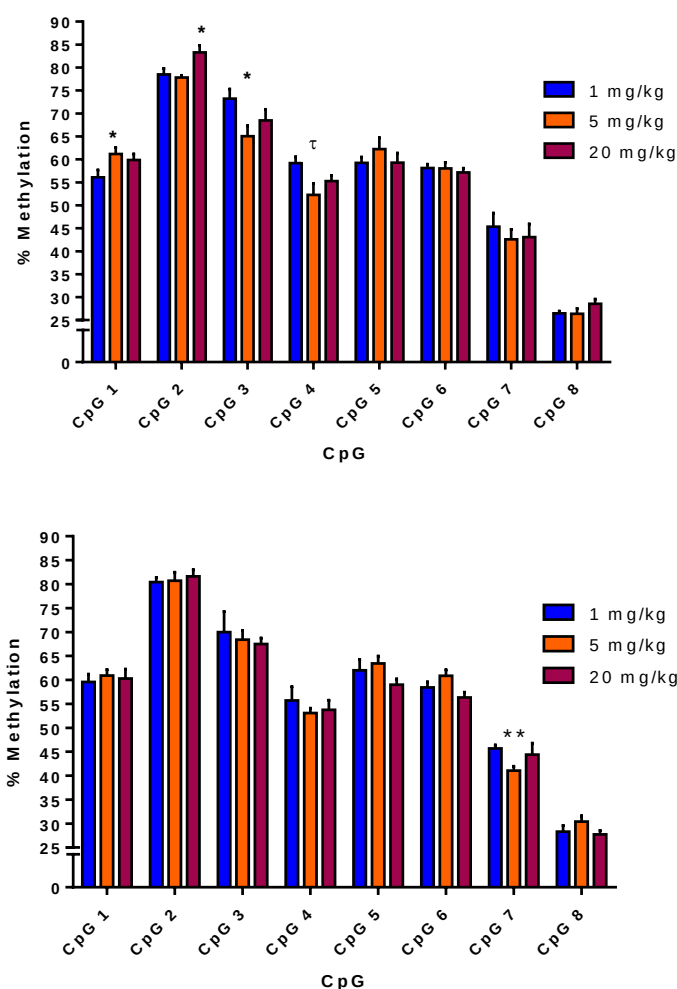


Figure 8.3 Preliminary Data showing Changes in *Oct-4* Promoter CpG Methylation in Response to Folic Acid Supplementation

(A) CpG methylation in mice supplemented for 4 weeks during the juvenile period (PND 28-56) or (B) adulthood (PND 74-102) followed by a 4 week maintenance diet.

8.3.2 Identification of Protein Binding Sites in DNA

In order to further understand the role of folic acid on Ezh2 function, it is necessary to understand if folic acid intake affects DNA/protein interactions. One way to do this is to use chromatin immunoprecipitation (ChIP) analysis to analyse interactions between Ezh2 protein and chromatin to establish which genomic regions of DNA Ezh2 is binding to and associations between these regions, such as transcription factors or DNA binding sites within the mammary

gland. In addition, H3K27me3 target of Ezh2 could be interrogated to investigate genomic regions associated with Ezh2 binding in response to folic acid intake. Currently chromatin extraction from mammary tissue is being optimised in preparation for ChIP analysis.

8.3.3 Immunohistochemistry

Further analysis could be performed on existing immunohistochemistry data in order to numerically quantify staining patterns for some markers including Brca1 and Oct-4. Due to the nature of cytoplasmic and low levels of nuclear staining methods could be developed in line with an imaging expert to generate quantitative data from fluorescent images. This would be particularly useful for publication of data within journals and is currently underway.

Immunohistochemistry analysis could be conducted to further understand the effects of folic acid on basal/myoepithelial and luminal cell populations. Dual staining for basal and luminal markers could be performed to assess if high levels of folic acid induce cellular disorganisation within mammary ducts. Furthermore, additional staining for mammary stem cell markers such as Sox4 which colocalises with Oct-4 and is involved in Ezh2 regulation in EMT.

In addition histological staining of mammary gland sections (4 µm) has been performed using hematoxylin and eosin (H&E) in mice fed 1 mg/kg and 5 mg/kg of folic acid during adulthood (Figure 8.3). Analysis suggests that high folic acid intake (5 mg/kg) may increase levels of mammary ductal hyperplasia, a condition in which the ductal wall thickens and in some cases obstructs the ductal lumen. Quantitative analysis is currently underway.

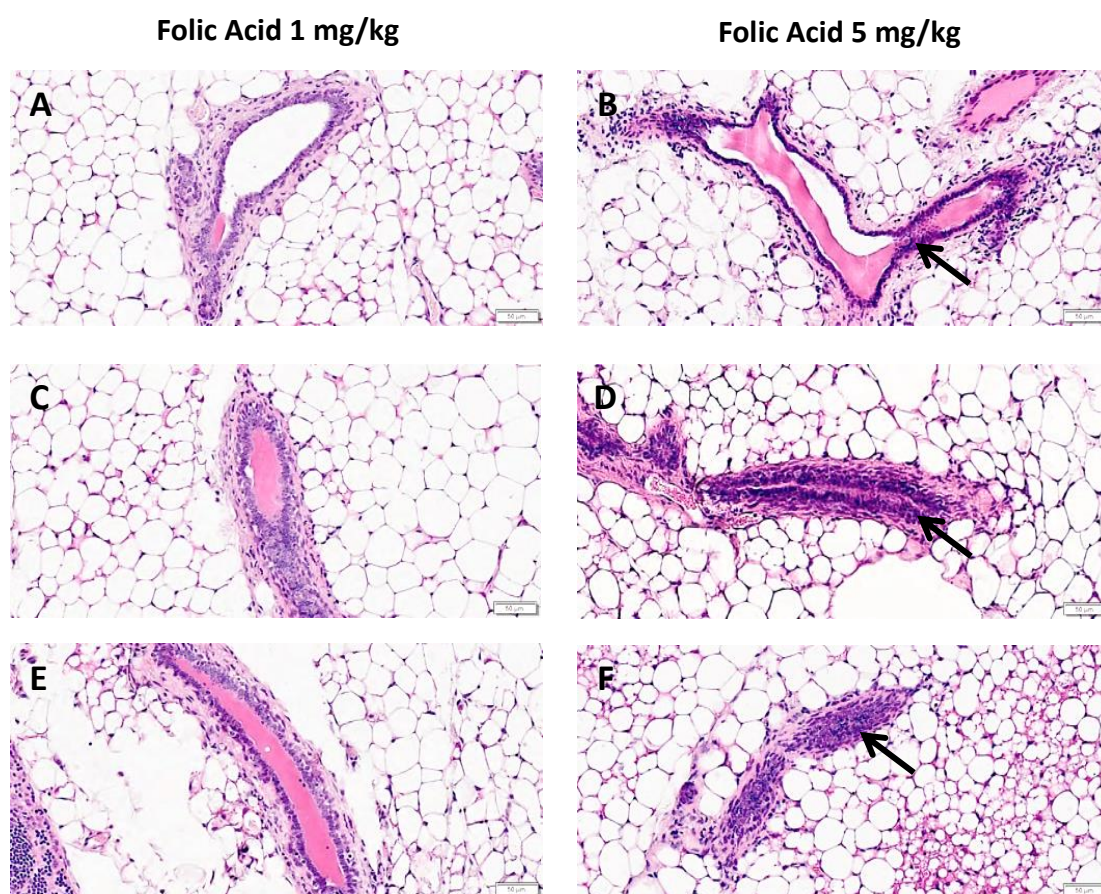


Figure 8.4 H&E Staining of Mammary Gland Sections from Mice Supplemented with Folic Acid during Adulthood.

Mammary gland sections (4 µm) showing ducts in mice fed 1 mg/kg (A, C, E) or 5 mg/kg (B, D, F) of folic acid. Arrow show possible ductal hyperplasia.

8.3.4 Next Generation Sequencing

With the changes in differential gene expression, gene enrichment and information from putative pathways changed in response to high (5 mg/kg) folic acid supplemented during the juvenile period or adulthood, this provides a large number of novel targets for further research. The strong association with EMT within adult mammary glands is particularly interesting as a marker of mammary cancer risk. In order to investigate this further, in vitro studies could be performed by treating a mammary cancer cell line such as MCF7 with folic acid and developing an assay to directly measure EMT. In addition, further research could be conducted in mammary tissue from the present study using immunohistochemistry to stain for protein markers of EMT between control and folic acid supplemented mice.

In addition to EMT, further analysis to understand the links between folic acid and ECM components, specifically Mmps and β -catenin, would prove useful. Immunohistochemistry analysis or flow cytometry (FACS) could be used to investigate markers of ECM, luminal and basal/myoepithelial components and to understand if mammary epithelial cell populations change or are remodelled in response to folic acid. This could be combined with Drop-seq technology which enables transcriptome analysis of single cells from populations of interest. From this it would be possible to establish differential gene expression within individual basal/myoepithelial, luminal cells and mammary stem cells within ducts in response to folic acid treatment. This may provide putative gene targets that could be explored to further understand the effects of folic acid intake on gene regulation.

8.3.5 Statistical Analysis

Due to the large amount of data and potential for a number of additional comparisons, further statistical analysis would provide a way to explore the data. This may further aid publication of thesis results in high impact journals and is currently being investigated.

Appendices

Appendix 1. Differentially Expressed Genes Upregulated in Mice Fed High Folic Acid during the Juvenile Period

ID	Gene	LogFC	LogCPM	LR	P Value	FDR	Mgi-symbol	Description
10405	ENSMUSG00000076609	6.564681	7.390155	14.8357	0.000117	0.004422	Igkc	immunoglobulin kappa constant [Source:MGI Symbol;Acc:MGI:96495]
2498	ENSMUSG00000022491	6.560779	7.897264	9.715238	0.001827	0.022728	Glycam1	glycosylation dependent cell adhesion molecule 1 [Source:MGI Symbol;Acc:MGI:95759]
10827	ENSMUSG000000104213	6.221723	7.053458	9.674148	0.001869	0.022999	Ighd	immunoglobulin heavy constant delta [Source:MGI Symbol;Acc:MGI:96447]
7031	ENSMUSG00000037922	4.543279	6.257205	10.76026	0.001037	0.017043	Bank1	B cell scaffold protein with ankyrin repeats 1 [Source:MGI Symbol;Acc:MGI:2442120]
								T cell immunoglobulin and mucin domain containing 4 [Source:MGI Symbol;Acc:MGI:2445125]
9263	ENSMUSG00000055546	3.98126	5.614558	11.08348	0.000871	0.015901	Timd4	RAS-related C3 botulinum substrate 2 [Source:MGI Symbol;Acc:MGI:97846]
6086	ENSMUSG00000033220	3.932237	6.122812	9.361136	0.002216	0.024747	Rac2	CD52 antigen [Source:MGI Symbol;Acc:MGI:1346088]
61	ENSMUSG00000000682	3.930667	5.838942	10.19144	0.001411	0.020034	Cd52	protein tyrosine phosphatase, non-receptor type 22 (lymphoid) [Source:MGI Symbol;Acc:MGI:107170]
4197	ENSMUSG00000027843	3.83613	4.354799	15.17232	9.81E-05	0.003921	Ptpn22	protein tyrosine phosphatase, receptor type, C [Source:MGI Symbol;Acc:MGI:97810]
3669	ENSMUSG00000026395	3.832467	8.455376	10.96042	0.000931	0.016244	Ptpnc	immunoglobulin heavy constant mu [Source:MGI Symbol;Acc:MGI:96448]
10406	ENSMUSG00000076617	3.800129	8.122198	10.01838	0.00155	0.020805	Ighm	SAM and SH3 domain containing 3 [Source:MGI Symbol;Acc:MGI:1921381]
5357	ENSMUSG00000031101	3.747423	5.112843	9.388847	0.002183	0.024615	Sash3	chemokine (C-X-C motif) receptor 4 [Source:MGI Symbol;Acc:MGI:109563]
8367	ENSMUSG00000045382	3.719055	4.758329	13.2515	0.000272	0.007728	Cxcr4	Rho GTPase activating protein 4 [Source:MGI Symbol;Acc:MGI:2159577]
5446	ENSMUSG00000031389	3.639534	5.054845	10.83855	0.000994	0.016715	Arhgap4	epithelial stromal interaction 1 (breast) [Source:MGI Symbol;Acc:MGI:1915168]
2306	ENSMUSG00000022014	3.630092	5.814857	10.62463	0.001116	0.01776	Epsti1	IKAROS family zinc finger 1 [Source:MGI Symbol;Acc:MGI:1342540]
1323	ENSMUSG00000018654	3.603533	6.14033	9.989091	0.001575	0.020924	Ikzf1	interferon regulatory factor 8 [Source:MGI Symbol;Acc:MGI:96395]
7881	ENSMUSG00000041515	3.569023	5.436267	11.32776	0.000764	0.014673	Irf8	kinesin family member 21B [Source:MGI Symbol;Acc:MGI:109234]
7901	ENSMUSG00000041642	3.524872	5.633802	9.544635	0.002005	0.023642	Kif21b	coronin, actin binding protein 1A [Source:MGI Symbol;Acc:MGI:1345961]
5228	ENSMUSG00000030707	3.522455	6.349805	7.557805	0.005975	0.041627	Coro1a	class II transactivator [Source:MGI Symbol;Acc:MGI:108445]
2502	ENSMUSG00000022504	3.506785	5.33122	12.35888	0.000439	0.010374	Ciita	pyrin and HIN domain family, member 1 [Source:MGI Symbol;Acc:MGI:2138243]
8180	ENSMUSG00000043263	3.472511	5.56765	10.87631	0.000974	0.016559	Pyhin1	pyrin domain containing 4 [Source:MGI Symbol;Acc:MGI:3695276]
10274	ENSMUSG00000073491	3.468141	4.896002	13.0403	0.000305	0.008202	Pydc4	pyrin domain containing 3 [Source:MGI Symbol;Acc:MGI:2442822]
9965	ENSMUSG00000066677	3.466844	4.392339	10.81814	0.001005	0.016771	Pydc3	Ena-vasodilator stimulated phosphoprotein [Source:MGI Symbol;Acc:MGI:1194884]
2026	ENSMUSG00000021262	3.459344	5.057383	12.71671	0.000362	0.009226	Evl	AT-hook transcription factor [Source:MGI Symbol;Acc:MGI:2140340]
7371	ENSMUSG00000039158	3.350024	5.737514	10.09728	0.001485	0.020485	Akna	protein tyrosine phosphatase, non-receptor type 6 [Source:MGI Symbol;Acc:MGI:96055]
484	ENSMUSG00000004266	3.335411	6.088851	8.579717	0.003399	0.031017	Ptpn6	kelch-like 6 [Source:MGI Symbol;Acc:MGI:2686922]
8156	ENSMUSG00000043008	3.325912	4.16496	10.93532	0.000943	0.016338	Klhl6	LIM domain containing 2 [Source:MGI Symbol;Acc:MGI:1915053]
7734	ENSMUSG00000040699	3.322533	5.424423	8.887504	0.002871	0.028467	Limd2	coagulation factor VIII [Source:MGI Symbol;Acc:MGI:88383]
5382	ENSMUSG00000031196	3.311886	5.410871	18.94959	1.34E-05	0.000903	F8	CD209b antigen [Source:MGI Symbol;Acc:MGI:1916415]
9930	ENSMUSG00000065987	3.302548	5.92836	14.18873	0.000165	0.005617	Cd209b	calcium/calmodulin-dependent protein kinase IV [Source:MGI Symbol;Acc:MGI:88258]
7088	ENSMUSG00000038128	3.295002	3.659874	14.46046	0.000143	0.005181	Camk4	pleckstrin and Sec7 domain containing 4 [Source:MGI Symbol;Acc:MGI:2674093]
3905	ENSMUSG00000026979	3.293261	4.967328	9.219477	0.002395	0.025731	Psd4	predicted gene 1966 [Source:MGI Symbol;Acc:MGI:3584360]
10297	ENSMUSG00000073902	3.288639	6.770475	8.333282	0.003893	0.033187	Gm1966	histocompatibility (minor) HA-1 [Source:MGI Symbol;Acc:MGI:1917969]
6581	ENSMUSG00000035697	3.2507	6.329973	10.08325	0.001496	0.020549	Hmha1	family with sequence similarity 65, member B [Source:MGI Symbol;Acc:MGI:2444879]
6636	ENSMUSG00000036006	3.212187	5.846872	8.675511	0.003225	0.030216	Fam65b	

3906	ENSMUSG000000026980	3.16763	3.957607	10.07002	0.001507	0.020577	Ly75	lymphocyte antigen 75 [Source:MGI Symbol;Acc:MGI:106662]
8796	ENSMUSG000000049744	3.156719	5.142861	8.971083	0.002743	0.027879	Arhgap15	Rho GTPase activating protein 15 [Source:MGI Symbol;Acc:MGI:1923367]
6257	ENSMUSG000000034116	3.130023	4.374957	8.528727	0.003496	0.031381	Vav1	vav 1 oncogene [Source:MGI Symbol;Acc:MGI:98923]
9052	ENSMUSG000000052889	3.127427	5.334356	8.066186	0.00451	0.035895	Prkcb	protein kinase C, beta [Source:MGI Symbol;Acc:MGI:97596]
7634	ENSMUSG000000040270	3.091473	4.671122	11.10924	0.000859	0.015837	Bach2	BTB and CNC homology 2 [Source:MGI Symbol;Acc:MGI:894679]
8718	ENSMUSG000000048865	3.072801	5.669586	10.07287	0.001505	0.020571	Arhgap30	Rho GTPase activating protein 30 [Source:MGI
8945	ENSMUSG000000051506	3.056728	5.938965	8.14938	0.004308	0.035283	Wdfy4	;Acc:MGI:2684948]
5989	ENSMUSG000000032776	3.047033	3.371808	16.57085	4.69E-05	0.00224	Mctp2	WD repeat and FYVE domain containing 4 [Source:MGI Symbol;Acc:MGI:3584510]
4794	ENSMUSG000000029333	3.016566	3.651994	17.40842	3.01E-05	0.001634	Rasgef1b	multiple C2 domains, transmembrane 2 [Source:MGI Symbol;Acc:MGI:2685335]
9902	ENSMUSG000000064262	3.013177	5.322063	9.360869	0.002217	0.024747	Gimap8	RasGEF domain family, member 1B [Source:MGI Symbol;Acc:MGI:2443755]
5074	ENSMUSG000000030220	3.011748	6.577629	8.810312	0.002995	0.028992	Arhgdib	GTPase, IMAP family member 8 [Source:MGI Symbol;Acc:MGI:2685303]
3681	ENSMUSG000000026430	3.007855	4.541824	10.33701	0.001304	0.019277	Rassf5	Rho, GDP dissociation inhibitor (GDI) beta [Source:MGI Symbol;Acc:MGI:101940]
137	ENSMUSG000000001281	3.001397	4.493219	7.146138	0.007513	0.04718	Itgb7	Ras association (RalGDS/AF-6) domain family member 5 [Source:MGI
5017	ENSMUSG000000030047	3.000995	4.51511	9.40858	0.00216	0.024496	Arhgap25	Symbol;Acc:MGI:1926375]
9179	ENSMUSG000000054435	2.977943	5.906841	11.7669	0.000603	0.012675	Gimap4	integrin beta 7 [Source:MGI Symbol;Acc:MGI:96616]
9676	ENSMUSG000000061132	2.963812	4.173854	9.862302	0.001687	0.021779	Blnk	Rho GTPase activating protein 25 [Source:MGI Symbol;Acc:MGI:2443687]
3742	ENSMUSG000000026594	2.95745	5.510756	8.073278	0.004492	0.035895	Ralgs2	GTPase, IMAP family member 4 [Source:MGI Symbol;Acc:MGI:1349656]
3073	ENSMUSG000000024677	2.949747	5.497812	11.74972	0.000609	0.012693	Ms4a6b	B cell linker [Source:MGI Symbol;Acc:MGI:96878]
8656	ENSMUSG000000048163	2.938883	5.409758	10.39607	0.001263	0.018979	Selplg	Ral GEF with PH domain and SH3 binding motif 2 [Source:MGI Symbol;Acc:MGI:1925505]
8309	ENSMUSG000000044770	2.924337	3.707238	9.740699	0.001802	0.022573	Scml4	membrane-spanning 4-domains, subfamily A, member 6B [Source:MGI
7510	ENSMUSG000000039747	2.899662	3.892651	9.07954	0.002585	0.026952	Orai2	Symbol;Acc:MGI:1917024]
4782	ENSMUSG000000029299	2.889664	3.901559	14.50985	0.000139	0.005098	Abcg3	selectin, platelet (p-selectin) ligand [Source:MGI Symbol;Acc:MGI:106689]
10589	ENSMUSG000000086825	2.856442	4.201013	12.75389	0.000355	0.009087	Gm15675	sex comb on midleg-like 4 (Drosophila) [Source:MGI Symbol;Acc:MGI:2446140]
8144	ENSMUSG000000042842	2.851164	3.722876	9.264105	0.002337	0.025361	Serpinb6b	ORAI calcium release-activated calcium modulator 2 [Source:MGI Symbol;Acc:MGI:2443195]
5375	ENSMUSG000000031165	2.837888	4.106125	10.15286	0.001441	0.020294	Was	ATP-binding cassette, sub-family G (WHITE), member 3 [Source:MGI
3917	ENSMUSG000000027009	2.837849	6.575209	10.62362	0.001117	0.01776	Itga4	Symbol;Acc:MGI:1351624]
2447	ENSMUSG000000022372	2.837653	4.738827	8.109923	0.004402	0.035611	Sla	predicted gene 15675 [Source:MGI Symbol;Acc:MGI:3783117]
9280	ENSMUSG000000055805	2.806728	4.851736	8.832791	0.002959	0.028861	Fmn1l	serine (or cysteine) peptidase inhibitor, clade B, member 6b [Source:MGI
8960	ENSMUSG000000051735	2.804073	3.550265	9.888968	0.001663	0.021645	Rinl	Symbol;Acc:MGI:894688]
2357	ENSMUSG000000022148	2.79408	5.113065	13.99083	0.000184	0.005966	Fyb	Wiskott-Aldrich syndrome homolog (human) [Source:MGI Symbol;Acc:MGI:105059]
3557	ENSMUSG000000026094	2.793001	6.597358	9.590107	0.001956	0.023296	Stk17b	integrin alpha 4 [Source:MGI Symbol;Acc:MGI:96603]
7104	ENSMUSG000000038179	2.763121	3.96119	8.583136	0.003393	0.031014	Slamf7	src-like adaptor [Source:MGI Symbol;Acc:MGI:104295]
7593	ENSMUSG000000040061	2.752916	3.978231	8.964378	0.002753	0.027929	Plcb2	formin-like 1 [Source:MGI Symbol;Acc:MGI:1888994]
8859	ENSMUSG000000050592	2.73843	4.283498	9.005127	0.002692	0.027583	Fam78a	Ras and Rab interactor-like [Source:MGI Symbol;Acc:MGI:2444024]
7753	ENSMUSG000000040747	2.721403	6.183861	7.563055	0.005958	0.041586	Cd53	FYN binding protein [Source:MGI Symbol;Acc:MGI:1346327]
								serine/threonine kinase 17b (apoptosis-inducing) [Source:MGI Symbol;Acc:MGI:2138162]
								SLAM family member 7 [Source:MGI Symbol;Acc:MGI:1922595]
								phospholipase C, beta 2 [Source:MGI Symbol;Acc:MGI:107465]
								family with sequence similarity 78, member A [Source:MGI Symbol;Acc:MGI:2443569]
								CD53 antigen [Source:MGI Symbol;Acc:MGI:88341]

1073	ENSMUSG00000015396	2.699521	4.05761	10.01446	0.001553	0.020805	Cd83	CD83 antigen [Source:MGI Symbol;Acc:MGI:1328316]
1578	ENSMUSG00000020143	2.673338	7.014353	9.823864	0.001723	0.02196	Dock2	dedicator of cyto-kinesis 2 [Source:MGI Symbol;Acc:MGI:2149010]
8944	ENSMUSG00000051504	2.668703	3.701348	8.598743	0.003364	0.030879	Siglech	sialic acid binding Ig-like lectin H [Source:MGI Symbol;Acc:MGI:2443256]
8347	ENSMUSG00000045165	2.664998	3.968137	7.682518	0.005576	0.040208	AI467606	expressed sequence AI467606 [Source:MGI Symbol;Acc:MGI:2141979]
7018	ENSMUSG00000037860	2.659393	3.592416	10.36968	0.001281	0.019135	Aim2	absent in melanoma 2 [Source:MGI Symbol;Acc:MGI:2686159]
7555	ENSMUSG00000039936	2.654854	5.381552	10.00013	0.001565	0.020876	Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide [Source:MGI Symbol;Acc:MGI:1098211]
10348	ENSMUSG00000074785	2.651428	4.702961	8.898751	0.002854	0.028431	Plxnc1	plexin C1 [Source:MGI Symbol;Acc:MGI:1890127]
10113	ENSMUSG00000070034	2.649934	4.60131	11.75954	0.000605	0.012684	Sp110	Sp110 nuclear body protein [Source:MGI Symbol;Acc:MGI:1923364]
1632	ENSMUSG00000020275	2.648745	4.679726	9.113565	0.002537	0.026608	Rel	reticuloendotheliosis oncogene [Source:MGI Symbol;Acc:MGI:97897]
1466	ENSMUSG00000019850	2.637739	4.462383	8.93837	0.002792	0.02812	Tnfaip3	tumor necrosis factor, alpha-induced protein 3 [Source:MGI Symbol;Acc:MGI:1196377]
5091	ENSMUSG00000030263	2.632259	4.409393	7.505908	0.00615	0.042356	Lrmp	lymphoid-restricted membrane protein [Source:MGI Symbol;Acc:MGI:108424]
2794	ENSMUSG00000023927	2.62364	6.206317	9.622386	0.001922	0.02312	Satb1	special AT-rich sequence binding protein 1 [Source:MGI Symbol;Acc:MGI:105084]
10594	ENSMUSG00000087107	2.622555	3.657667	8.756115	0.003086	0.029415	AI662270	expressed sequence AI662270 [Source:MGI Symbol;Acc:MGI:2144254]
2299	ENSMUSG00000021998	2.618723	7.790018	9.027802	0.002659	0.027385	Lcp1	lymphocyte cytosolic protein 1 [Source:MGI Symbol;Acc:MGI:104808]
7520	ENSMUSG00000039783	2.591989	3.644675	8.416238	0.003719	0.032678	Kmo	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase) [Source:MGI Symbol;Acc:MGI:2138151]
1630	ENSMUSG00000020272	2.580977	5.522552	9.781767	0.001763	0.022305	Stk10	serine/threonine kinase 10 [Source:MGI Symbol;Acc:MGI:1099439]
3549	ENSMUSG00000026070	2.573688	3.428488	9.342547	0.002239	0.024872	Il18r1	interleukin 18 receptor 1 [Source:MGI Symbol;Acc:MGI:105383]
722	ENSMUSG00000006731	2.570925	5.426139	9.058013	0.002615	0.027115	B4galnt1	beta-1,4-N-acetyl-galactosaminyl transferase 1 [Source:MGI Symbol;Acc:MGI:1342057]
2739	ENSMUSG00000023169	2.570453	5.032842	10.73183	0.001053	0.017203	Slc38a1	solute carrier family 38, member 1 [Source:MGI Symbol;Acc:MGI:2145895]
6724	ENSMUSG00000036469	2.556101	5.210196	8.697435	0.003187	0.029984	Mar-01	membrane-associated ring finger (C3HC4) 1 [Source:MGI Symbol;Acc:MGI:1920175]
196	ENSMUSG00000001741	2.549591	5.658154	8.556284	0.003443	0.031187	Il16	interleukin 16 [Source:MGI Symbol;Acc:MGI:1270855]
2087	ENSMUSG00000021457	2.535487	6.202888	11.65191	0.000641	0.013087	Syk	spleen tyrosine kinase [Source:MGI Symbol;Acc:MGI:99515]
3415	ENSMUSG00000025647	2.529446	7.375939	9.338487	0.002244	0.024872	Shisa5	shisa family member 5 [Source:MGI Symbol;Acc:MGI:1915044]
313	ENSMUSG00000002699	2.528576	4.697769	8.013075	0.004644	0.036412	Lcp2	lymphocyte cytosolic protein 2 [Source:MGI Symbol;Acc:MGI:1321402]
1477	ENSMUSG00000019876	2.525073	3.014114	12.00756	0.00053	0.011806	Pkib	protein kinase inhibitor beta, cAMP dependent, testis specific [Source:MGI Symbol;Acc:MGI:101937]
7825	ENSMUSG00000041202	2.522819	4.624408	11.88537	0.000566	0.012231	Pla2g2d	phospholipase A2, group IID [Source:MGI Symbol;Acc:MGI:1341796]
9835	ENSMUSG00000063286	2.508643	7.09426	7.936505	0.004845	0.037453	Gm8995	predicted gene 8995 [Source:MGI Symbol;Acc:MGI:3644223]
3181	ENSMUSG00000024965	2.493342	4.496902	9.238736	0.002369	0.025584	Fermt3	fermitin family homolog 3 (Drosophila) [Source:MGI Symbol;Acc:MGI:2147790]
4484	ENSMUSG00000028581	2.48966	6.850406	10.15182	0.001442	0.020294	Laptn5	lysosomal-associated protein transmembrane 5 [Source:MGI Symbol;Acc:MGI:108046]
1893	ENSMUSG00000020901	2.485365	3.722051	12.7666	0.000353	0.009047	Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101 [Source:MGI Symbol;Acc:MGI:2443588]
9631	ENSMUSG00000060550	2.46796	5.331744	10.25685	0.001362	0.019679	H2-Q7	histocompatibility 2, Q region locus 7 [Source:MGI Symbol;Acc:MGI:95936]
9602	ENSMUSG00000060131	2.465415	3.402437	11.57071	0.00067	0.013544	Atp8b4	ATPase, class I, type 8B, member 4 [Source:MGI Symbol;Acc:MGI:1859664]
9444	ENSMUSG00000057948	2.463344	3.44055	7.134163	0.007563	0.04744	Unc13d	unc-13 homolog D (C. elegans) [Source:MGI Symbol;Acc:MGI:1917700]
10220	ENSMUSG00000071714	2.461835	4.397366	13.73422	0.000211	0.006458	Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage) [Source:MGI Symbol;Acc:MGI:1339760]
5716	ENSMUSG00000032035	2.456768	7.330745	8.876309	0.002889	0.02859	Ets1	E26 avian leukemia oncogene 1, 5' domain [Source:MGI Symbol;Acc:MGI:95455]

7649	ENSMUSG00000040345	2.448768	3.716942	8.79389	0.003022	0.029169	Arhgap9	Rho GTPase activating protein 9 [Source:MGI Symbol;Acc:MGI:2143764]
3328	ENSMUSG00000025357	2.444665	5.923086	10.27598	0.001348	0.019612	Dgka	diacylglycerol kinase, alpha [Source:MGI Symbol;Acc:MGI:102952]
522	ENSMUSG00000004665	2.420322	5.709555	10.02317	0.001546	0.020805	Cnn2	calponin 2 [Source:MGI Symbol;Acc:MGI:105093]
8622	ENSMUSG00000047867	2.4101	6.124094	9.05207	0.002624	0.027152	Gimap6	GTPase, IMAP family member 6 [Source:MGI Symbol;Acc:MGI:1918876]
10264	ENSMUSG00000073409	2.404383	4.828265	11.35613	0.000752	0.014554	H2-Q6	histocompatibility 2, Q region locus 6 [Source:MGI Symbol;Acc:MGI:95935]
1568	ENSMUSG00000020120	2.379274	5.351449	8.361056	0.003833	0.033046	Plek	pleckstrin [Source:MGI Symbol;Acc:MGI:1860485]
8808	ENSMUSG00000049866	2.376926	4.909629	9.670535	0.001872	0.022999	Arl4c	ADP-ribosylation factor-like 4C [Source:MGI Symbol;Acc:MGI:2445172]
5266	ENSMUSG00000030789	2.37601	3.265698	12.55903	0.000394	0.009775	Itgax	integrin alpha X [Source:MGI Symbol;Acc:MGI:96609]
5360	ENSMUSG00000031112	2.372412	3.822191	12.64714	0.000376	0.009465	Stk26	serine/threonine kinase 26 [Source:MGI Symbol;Acc:MGI:1917665]
10235	ENSMUSG00000072620	2.342745	4.988062	8.326316	0.003907	0.033262	Slfn2	schlafen 2 [Source:MGI Symbol;Acc:MGI:1313258]
7226	ENSMUSG00000038608	2.342681	6.982856	7.472223	0.006266	0.042886	Dock10	dedicator of cytokinesis 10 [Source:MGI Symbol;Acc:MGI:2146320]
2635	ENSMUSG00000022831	2.338596	4.840224	8.302524	0.003959	0.033544	Hcls1	hematopoietic cell specific Lyn substrate 1 [Source:MGI Symbol;Acc:MGI:104568]
2495	ENSMUSG00000022488	2.333747	5.995417	8.013361	0.004643	0.036412	Nckap1l	NCK associated protein 1 like [Source:MGI Symbol;Acc:MGI:1926063]
10105	ENSMUSG00000069892	2.330357	6.625246	10.8436	0.000991	0.016696	993011J21Rik2	RIKEN cDNA 993011J21 gene 2 [Source:MGI Symbol;Acc:MGI:3711310]
3204	ENSMUSG00000025017	2.320532	4.15925	9.612201	0.001933	0.023195	Pik3ap1	phosphoinositide-3-kinase adaptor protein 1 [Source:MGI Symbol;Acc:MGI:1933177]
8848	ENSMUSG00000050379	2.304202	5.095845	8.143816	0.004321	0.035292	Sep-06	septin 6 [Source:MGI Symbol;Acc:MGI:1888939]
6494	ENSMUSG00000035208	2.289966	4.98438	10.18993	0.001412	0.020034	Slfn8	schlafen 8 [Source:MGI Symbol;Acc:MGI:2672859]
10106	ENSMUSG00000069893	2.284418	3.260533	8.46824	0.003614	0.032041	993011J21Rik1	RIKEN cDNA 993011J21 gene 1 [Source:MGI Symbol;Acc:MGI:3041173]
5222	ENSMUSG00000030691	2.283688	5.64672	7.241596	0.007123	0.045764	Fchsd2	FCH and double SH3 domains 2 [Source:MGI Symbol;Acc:MGI:2448475]
8039	ENSMUSG00000042349	2.26283	3.998252	10.42941	0.00124	0.018821	Ikbke	inhibitor of kappaB kinase epsilon [Source:MGI Symbol;Acc:MGI:1929612]
404	ENSMUSG00000003418	2.262268	3.567893	11.02579	0.000899	0.016032	St8sia6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6 [Source:MGI Symbol;Acc:MGI:2386797]
4240	ENSMUSG00000027962	2.260979	5.554771	14.30643	0.000155	0.005407	Vcam1	vascular cell adhesion molecule 1 [Source:MGI Symbol;Acc:MGI:98926]
9298	ENSMUSG00000056025	2.24354	3.360527	33.59304	6.79E-09	3.22E-06	Clca3a1	chloride channel accessory 3A1 [Source:MGI Symbol;Acc:MGI:1316732]
3635	ENSMUSG00000026288	2.241838	5.373055	8.491578	0.003568	0.031814	Inpp5d	inositol polyphosphate-5-phosphatase D [Source:MGI Symbol;Acc:MGI:107357]
9658	ENSMUSG00000060924	2.236645	3.061803	10.15882	0.001436	0.020269	Csmd1	CUB and Sushi multiple domains 1 [Source:MGI Symbol;Acc:MGI:2137383]
8512	ENSMUSG00000046805	2.229212	6.220277	14.91519	0.000112	0.004284	Mpeg1	macrophage expressed gene 1 [Source:MGI Symbol;Acc:MGI:1333743]
8550	ENSMUSG00000047180	2.218276	3.17979	9.84172	0.001706	0.021933	Neurl3	neuralized E3 ubiquitin protein ligase 3 [Source:MGI Symbol;Acc:MGI:2429944]
5737	ENSMUSG00000032089	2.211209	4.391416	8.80127	0.00301	0.029077	Il10ra	interleukin 10 receptor, alpha [Source:MGI Symbol;Acc:MGI:96538]
5038	ENSMUSG00000030091	2.20731	5.656402	9.716641	0.001826	0.022728	Nup210	nucleoporin 210 [Source:MGI Symbol;Acc:MGI:1859555]
856	ENSMUSG00000009687	2.204305	5.138416	9.625875	0.001919	0.02312	Fxyd5	FXD domain-containing ion transport regulator 5 [Source:MGI Symbol;Acc:MGI:1201785]
23	ENSMUSG00000000290	2.18862	5.302872	9.632735	0.001911	0.02312	Itgb2	integrin beta 2 [Source:MGI Symbol;Acc:MGI:96611]
6312	ENSMUSG00000034330	2.183775	5.254762	8.38149	0.003791	0.032963	Plcg2	phospholipase C, gamma 2 [Source:MGI Symbol;Acc:MGI:97616]
8443	ENSMUSG00000046169	2.179351	2.862804	8.085731	0.004462	0.03585	Adamts6	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 6 [Source:MGI Symbol;Acc:MGI:1347348]
1215	ENSMUSG00000017561	2.178927	4.729908	8.345612	0.003866	0.033071	Crlf3	cytokine receptor-like factor 3 [Source:MGI Symbol;Acc:MGI:1860086]
5635	ENSMUSG00000031838	2.135634	5.213424	7.807097	0.005204	0.038871	Ifi30	interferon gamma inducible protein 30 [Source:MGI Symbol;Acc:MGI:2137648]
10759	ENSMUSG00000097415	2.133324	3.71143	8.848013	0.002934	0.028802	AU020206	expressed sequence AU020206 [Source:MGI Symbol;Acc:MGI:2142134]
6996	ENSMUSG00000037754	2.13238	4.522282	7.315213	0.006837	0.044988	Ppp1r16b	protein phosphatase 1, regulatory (inhibitor) subunit 16B [Source:MGI Symbol;Acc:MGI:2151841]

9300	ENSMUSG00000056069	2.12912	3.241883	9.016214	0.002676	0.027533	Fam105a	family with sequence similarity 105, member A [Source:MGI Symbol;Acc:MGI:2687281]
5954	ENSMUSG00000032624	2.128415	5.210938	8.813705	0.00299	0.028983	Eml4	echinoderm microtubule associated protein like 4 [Source:MGI Symbol;Acc:MGI:1926048]
5358	ENSMUSG00000031103	2.113596	4.771016	9.630087	0.001914	0.02312	Elf4	E74-like factor 4 (ets domain transcription factor) [Source:MGI Symbol;Acc:MGI:1928377]
10845	ENSMUSG000000105353	2.113595	3.583997	13.0867	0.000297	0.008061	Gm42428	predicted gene 42428 [Source:MGI Symbol;Acc:MGI:5662565]
263	ENSMUSG00000002257	2.106498	3.131372	7.398597	0.006527	0.044038	Def6	differentially expressed in FDCP 6 [Source:MGI Symbol;Acc:MGI:1346328]
7947	ENSMUSG000000041849	2.105681	4.106532	9.873758	0.001677	0.021725	Card6	caspase recruitment domain family, member 6 [Source:MGI Symbol;Acc:MGI:3032959]
8732	ENSMUSG000000049044	2.10374	3.00741	6.971101	0.008284	0.049784	Rapgef4	Rap guanine nucleotide exchange factor (GEF) 4 [Source:MGI Symbol;Acc:MGI:1917723]
9005	ENSMUSG000000052397	2.103727	5.953817	10.12245	0.001465	0.020357	Ezr	ezrin [Source:MGI Symbol;Acc:MGI:98931]
1170	ENSMUSG000000016756	2.103361	6.436929	10.8891	0.000967	0.016494	Cmah	cytidine monophospho-N-acetylneuraminic acid hydroxylase [Source:MGI Symbol;Acc:MGI:103227]
4806	ENSMUSG000000029378	2.095892	3.189208	7.704306	0.005509	0.03999	Areg	amphiregulin [Source:MGI Symbol;Acc:MGI:88068]
10528	ENSMUSG000000079523	2.09342	6.328691	10.09158	0.001489	0.020518	Tmsb10	thymosin, beta 10 [Source:MGI Symbol;Acc:MGI:109146]
6679	ENSMUSG000000036246	2.091777	4.211586	7.189072	0.007335	0.046493	Gmip	Gem-interacting protein [Source:MGI Symbol;Acc:MGI:1926066]
9307	ENSMUSG000000056144	2.090909	4.323518	9.094336	0.002564	0.026837	Trim34a	tripartite motif-containing 34A [Source:MGI Symbol;Acc:MGI:2137359]
3051	ENSMUSG000000024610	2.07736	9.203497	7.922338	0.004883	0.037626	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) [Source:MGI Symbol;Acc:MGI:96534]
3819	ENSMUSG000000026786	2.068859	4.123587	10.71854	0.001061	0.017269	Apbb1ip	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein [Source:MGI Symbol;Acc:MGI:1861354]
5555	ENSMUSG000000031659	2.058453	5.42786	9.361795	0.002216	0.024747	Adcy7	adenylate cyclase 7 [Source:MGI Symbol;Acc:MGI:102891]
5701	ENSMUSG000000032000	2.057993	5.281493	8.878137	0.002886	0.028588	Birc3	baculoviral IAP repeat-containing 3 [Source:MGI Symbol;Acc:MGI:1197007]
4108	ENSMUSG000000027580	2.057289	6.230707	7.616772	0.005783	0.040942	Helz2	helicase with zinc finger 2, transcriptional coactivator [Source:MGI Symbol;Acc:MGI:2385169]
521	ENSMUSG000000004661	2.043408	3.228	7.627377	0.005749	0.040861	Arid3b	AT rich interactive domain 3B (BRIGHT-like) [Source:MGI Symbol;Acc:MGI:1930768]
6971	ENSMUSG000000037649	2.041813	4.734489	7.69548	0.005536	0.040079	H2-DMa	histocompatibility 2, class II, locus DMa [Source:MGI Symbol;Acc:MGI:95921]
5333	ENSMUSG000000031004	2.037062	5.193121	9.270489	0.002329	0.025334	Mki67	antigen identified by monoclonal antibody Ki 67 [Source:MGI Symbol;Acc:MGI:106035]
7823	ENSMUSG000000041187	2.036684	4.513762	10.05776	0.001517	0.020663	Prkd2	protein kinase D2 [Source:MGI Symbol;Acc:MGI:2141917]
7554	ENSMUSG000000039934	2.033941	3.326391	10.90394	0.00096	0.016466	Gsap	gamma-secretase activating protein [Source:MGI Symbol;Acc:MGI:2442259]
3077	ENSMUSG000000024691	2.031587	5.349408	7.507224	0.006145	0.042352	Fam111a	family with sequence similarity 111, member A [Source:MGI Symbol;Acc:MGI:1915508]
6820	ENSMUSG000000036944	2.02996	3.615236	6.962363	0.008324	0.04989	Tmem71	transmembrane protein 71 [Source:MGI Symbol;Acc:MGI:2146049]
3611	ENSMUSG000000026222	2.026907	6.418888	10.54	0.001168	0.018341	Sp100	nuclear antigen Sp100 [Source:MGI Symbol;Acc:MGI:109561]
848	ENSMUSG000000009585	2.025708	5.918802	9.832594	0.001714	0.021952	Apobec3	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 [Source:MGI Symbol;Acc:MGI:1933111]
10766	ENSMUSG000000097705	2.020037	3.008088	9.963297	0.001597	0.021142	Gm26740	predicted gene, 26740 [Source:MGI Symbol;Acc:MGI:5477234]
9262	ENSMUSG000000055541	2.019874	3.063327	9.49125	0.002065	0.024085	Lair1	leukocyte-associated Ig-like receptor 1 [Source:MGI Symbol;Acc:MGI:105492]
1127	ENSMUSG000000015950	2.018812	4.805178	7.732049	0.005425	0.039727	Ncf1	neutrophil cytosolic factor 1 [Source:MGI Symbol;Acc:MGI:97283]
6318	ENSMUSG000000034349	2.018359	6.083489	9.524871	0.002027	0.023775	Smc4	structural maintenance of chromosomes 4 [Source:MGI Symbol;Acc:MGI:1917349]
10648	ENSMUSG000000090272	2.017507	6.284391	12.16914	0.000486	0.011122	Mndal	myeloid nuclear differentiation antigen like [Source:MGI Symbol;Acc:MGI:3780953]
10807	ENSMUSG000000102850	2.01622	2.373683	10.93449	0.000944	0.016338	Gm37082	predicted gene, 37082 [Source:MGI Symbol;Acc:MGI:5610310]
860	ENSMUSG000000009772	2.013128	2.805925	7.634644	0.005726	0.040814	Nuak2	NUAK family, SNF1-like kinase, 2 [Source:MGI Symbol;Acc:MGI:1921387]
10859	ENSMUSG000000106734	2.009806	5.348476	8.384116	0.003785	0.032942	Gm20559	predicted gene, 20559 [Source:MGI Symbol;Acc:MGI:5295666]
3324	ENSMUSG000000025352	2.009655	2.971356	11.03357	0.000895	0.016032	Gdf11	growth differentiation factor 11 [Source:MGI Symbol;Acc:MGI:1338027]

9649	ENSMUSG00000060791	1.997578	3.95436	7.554613	0.005986	0.041674	Gmfg	glia maturation factor, gamma [Source:MGI Symbol;Acc:MGI:1927135]
2084	ENSMUSG00000021451	1.994874	4.530034	9.0663	0.002604	0.027044	Sema4d	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D [Source:MGI Symbol;Acc:MGI:109244]
4162	ENSMUSG00000027715	1.991883	2.306896	9.81895	0.001727	0.02196	Ccna2	cyclin A2 [Source:MGI Symbol;Acc:MGI:108069]
6460	ENSMUSG00000035021	1.987572	5.405513	9.125472	0.002521	0.026562	Baz1a	bromodomain adjacent to zinc finger domain 1A [Source:MGI Symbol;Acc:MGI:1309478]
10471	ENSMUSG00000078920	1.987411	3.901346	8.022046	0.004621	0.036355	Ifi47	interferon gamma inducible protein 47 [Source:MGI Symbol;Acc:MGI:99448]
2844	ENSMUSG00000024063	1.985372	5.325298	7.025228	0.008037	0.048867	Lbh	limb-bud and heart [Source:MGI Symbol;Acc:MGI:1925139]
4590	ENSMUSG00000028843	1.979431	5.321891	7.416083	0.006464	0.043694	Sh3bgrl3	SH3 domain binding glutamic acid-rich protein-like 3 [Source:MGI Symbol;Acc:MGI:1920973]
5771	ENSMUSG00000032202	1.977694	3.347113	9.638411	0.001906	0.02312	Rab27a	RAB27A, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:1861441]
7291	ENSMUSG00000038843	1.975296	4.118508	8.660813	0.003251	0.030338	Gcnt1	glucosaminyl (N-acetyl) transferase 1, core 2 [Source:MGI Symbol;Acc:MGI:95676]
6867	ENSMUSG00000037138	1.975295	4.729935	7.014632	0.008085	0.049075	Aff3	AF4/FMR2 family, member 3 [Source:MGI Symbol;Acc:MGI:106927]
7094	ENSMUSG00000038147	1.965479	4.829764	8.581041	0.003397	0.031017	Cd84	CD84 antigen [Source:MGI Symbol;Acc:MGI:1336885]
10849	ENSMUSG000000105703	1.964205	7.292047	8.069533	0.004502	0.035895	Gm43305	predicted gene 43305 [Source:MGI Symbol;Acc:MGI:5663442]
3109	ENSMUSG00000024795	1.963214	2.769991	8.000913	0.004675	0.036597	Kif20b	kinesin family member 20B [Source:MGI Symbol;Acc:MGI:2444576]
1286	ENSMUSG00000018381	1.959261	2.929718	9.254871	0.002349	0.025464	Abi3	ABI gene family, member 3 [Source:MGI Symbol;Acc:MGI:1913860]
9168	ENSMUSG00000054342	1.957348	2.772252	7.650829	0.005675	0.040567	Kcnn4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 [Source:MGI Symbol;Acc:MGI:1277957]
344	ENSMUSG00000002897	1.948148	4.803527	9.080716	0.002583	0.026952	Il17ra	interleukin 17 receptor A [Source:MGI Symbol;Acc:MGI:107399]
269	ENSMUSG00000002297	1.941751	2.620276	11.78021	0.000599	0.012617	Dbf4	DBF4 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1351328]
1269	ENSMUSG00000018209	1.930081	6.432612	7.570659	0.005933	0.041454	Stk4	serine/threonine kinase 4 [Source:MGI Symbol;Acc:MGI:1929004]
2824	ENSMUSG00000024013	1.926248	3.658538	10.07712	0.001501	0.020549	Fgd2	FYVE, RhoGEF and PH domain containing 2 [Source:MGI Symbol;Acc:MGI:1347084]
9476	ENSMUSG00000058396	1.923629	2.894326	20.19763	6.98E-06	0.000568	Gpr182	G protein-coupled receptor 182 [Source:MGI Symbol;Acc:MGI:109545]
8632	ENSMUSG00000047945	1.921218	4.128633	9.059186	0.002614	0.027115	Marcks1	MARCKS-like 1 [Source:MGI Symbol;Acc:MGI:97143]
3766	ENSMUSG00000026655	1.919118	5.869503	7.057547	0.007893	0.048489	Fam107b	family with sequence similarity 107, member B [Source:MGI Symbol;Acc:MGI:1913790]
2317	ENSMUSG00000022037	1.918308	7.23969	10.01836	0.00155	0.020805	Clu	clusterin [Source:MGI Symbol;Acc:MGI:88423]
8260	ENSMUSG00000044250	1.910597	2.96722	9.835588	0.001712	0.021952	Pced1b	PC-esterase domain containing 1B [Source:MGI Symbol;Acc:MGI:2446270]
10311	ENSMUSG00000074151	1.907899	5.271708	8.102906	0.004419	0.035643	Nlrc5	NLR family, CARD domain containing 5 [Source:MGI Symbol;Acc:MGI:3612191]
6955	ENSMUSG00000037533	1.904216	6.624941	7.908878	0.004919	0.037705	Rapgef6	Rap guanine nucleotide exchange factor (GEF) 6 [Source:MGI Symbol;Acc:MGI:2384761]
7806	ENSMUSG00000041112	1.89678	4.987061	7.653026	0.005668	0.040567	Elmo1	engulfment and cell motility 1 [Source:MGI Symbol;Acc:MGI:2153044]
4021	ENSMUSG00000027322	1.888279	6.048764	14.03185	0.00018	0.005917	Siglec1	sialic acid binding Ig-like lectin 1, sialoadhesin [Source:MGI Symbol;Acc:MGI:99668]
7235	ENSMUSG00000038642	1.886312	6.56768	9.310582	0.002278	0.025152	Ctss	cathepsin S [Source:MGI Symbol;Acc:MGI:107341]
6937	ENSMUSG00000037447	1.884689	3.913152	12.5362	0.000399	0.009817	Arid5a	AT rich interactive domain 5A (MRF1-like) [Source:MGI Symbol;Acc:MGI:2443039]
6012	ENSMUSG00000032870	1.861951	5.566811	8.533493	0.003487	0.031372	Smap2	small ArfGAP 2 [Source:MGI Symbol;Acc:MGI:1917030]
9202	ENSMUSG00000054702	1.855224	2.662735	8.199548	0.00419	0.034561	Ap1s3	adaptor-related protein complex AP-1, sigma 3 [Source:MGI Symbol;Acc:MGI:1891304]
10699	ENSMUSG00000094030	1.853019	2.576479	21.68028	3.22E-06	0.000337	Gm21833	predicted gene, 21833 [Source:MGI Symbol;Acc:MGI:5433997]
8277	ENSMUSG00000044456	1.847978	4.053383	12.33599	0.000444	0.010456	Rin3	Ras and Rab interactor 3 [Source:MGI Symbol;Acc:MGI:2385708]
304	ENSMUSG00000002603	1.839869	3.443968	10.03659	0.001535	0.020765	Tgfb1	transforming growth factor, beta 1 [Source:MGI Symbol;Acc:MGI:98725]
4394	ENSMUSG00000028381	1.83831	5.729668	9.166513	0.002465	0.026151	Ugcg	UDP-glucose ceramide glucosyltransferase [Source:MGI Symbol;Acc:MGI:1332243]
10551	ENSMUSG00000082292	1.834556	2.57093	9.767154	0.001777	0.022379	Gm12250	predicted gene 12250 [Source:MGI Symbol;Acc:MGI:3649299]
10681	ENSMUSG00000091575	1.831542	3.468092	7.39657	0.006535	0.044038	201001618Rik	RIKEN cDNA 201001618 gene [Source:MGI Symbol;Acc:MGI:1916456]

5630	ENSMUSG000000031827	1.827099	5.184072	8.644866	0.00328	0.030441	Cotl1	coactosin-like 1 (Dictyostelium) [Source:MGI Symbol;Acc:MGI:1919292]
6726	ENSMUSG000000036478	1.821564	6.300013	7.913364	0.004907	0.037705	Btg1	B cell translocation gene 1, anti-proliferative [Source:MGI Symbol;Acc:MGI:88215]
6409	ENSMUSG000000034786	1.814415	3.005921	9.925121	0.00163	0.021386	Gpsm3	G-protein signalling modulator 3 (AGS3-like, C. elegans) [Source:MGI Symbol;Acc:MGI:2146785]
2053	ENSMUSG000000021356	1.806045	6.164352	6.978526	0.008249	0.04966	Irf4	interferon regulatory factor 4 [Source:MGI Symbol;Acc:MGI:1096873]
1757	ENSMUSG000000020573	1.797049	4.644562	7.93542	0.004848	0.037453	Pik3cg	phosphoinositide-3-kinase, catalytic, gamma polypeptide [Source:MGI Symbol;Acc:MGI:1353576]
1576	ENSMUSG000000020134	1.792215	5.065884	9.577007	0.00197	0.023335	Peli1	pellino 1 [Source:MGI Symbol;Acc:MGI:1914495]
3671	ENSMUSG000000026399	1.79027	6.529283	13.30237	0.000265	0.007561	Cd55	CD55 molecule, decay accelerating factor for complement [Source:MGI Symbol;Acc:MGI:104850]
10287	ENSMUSG000000073678	1.789271	5.177457	7.900898	0.004941	0.037781	Pgap1	post-GPI attachment to proteins 1 [Source:MGI Symbol;Acc:MGI:2443342]
10826	ENSMUSG000000104125	1.787652	3.059985	9.251723	0.002353	0.025483	Gm37488	predicted gene, 37488 [Source:MGI Symbol;Acc:MGI:5610716]
7476	ENSMUSG000000039621	1.782055	5.344574	7.979972	0.00473	0.03687	Prex1	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1 [Source:MGI Symbol;Acc:MGI:3040696]
1993	ENSMUSG000000021182	1.780106	5.413803	10.35775	0.001289	0.019192	Ccdc88c	coiled-coil domain containing 88C [Source:MGI Symbol;Acc:MGI:1915589]
7368	ENSMUSG000000039153	1.773245	2.36631	8.860475	0.002914	0.028753	Runx2	runt related transcription factor 2 [Source:MGI Symbol;Acc:MGI:99829]
5790	ENSMUSG000000032254	1.770028	2.322265	14.1029	0.000173	0.005803	Kif23	kinesin family member 23 [Source:MGI Symbol;Acc:MGI:1919069]
7729	ENSMUSG000000040687	1.766017	4.572183	9.34863	0.002232	0.024836	Madd	MAP-kinase activating death domain [Source:MGI Symbol;Acc:MGI:2444672]
7575	ENSMUSG000000039997	1.762819	6.712501	13.23697	0.000274	0.007752	Ifi203	interferon activated gene 203 [Source:MGI Symbol;Acc:MGI:96428]
6372	ENSMUSG000000034613	1.759449	3.958091	8.775332	0.003053	0.029277	Ppm1h	protein phosphatase 1H (PP2C domain containing) [Source:MGI Symbol;Acc:MGI:2442087]
9189	ENSMUSG000000054520	1.753533	3.659135	8.446244	0.003658	0.032273	Sh3bp2	SH3-domain binding protein 2 [Source:MGI Symbol;Acc:MGI:1346349]
10751	ENSMUSG000000097312	1.751925	8.940151	7.470821	0.006271	0.042886	Gm26870	predicted gene, 26870 [Source:MGI Symbol;Acc:MGI:5477364]
9632	ENSMUSG000000060586	1.751228	7.310391	7.09988	0.007709	0.047915	H2-Eb1	histocompatibility 2, class II antigen E beta [Source:MGI Symbol;Acc:MGI:95901]
2057	ENSMUSG000000021365	1.748313	5.578696	10.4047	0.001257	0.018917	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9 [Source:MGI Symbol;Acc:MGI:97302]
10843	ENSMUSG000000105270	1.728751	2.031732	12.55281	0.000396	0.009775	Gm42863	predicted gene 42863 [Source:MGI Symbol;Acc:MGI:5663000]
10472	ENSMUSG000000078921	1.720892	3.631963	13.7672	0.000207	0.006423	Tgtp2	T cell specific GTPase 2 [Source:MGI Symbol;Acc:MGI:3710083]
10267	ENSMUSG000000073421	1.714041	7.723995	8.365413	0.003824	0.032993	H2-Ab1	histocompatibility 2, class II antigen A, beta 1 [Source:MGI Symbol;Acc:MGI:103070]
1908	ENSMUSG000000020941	1.712137	3.439258	8.855763	0.002922	0.028781	Map3k14	mitogen-activated protein kinase kinase kinase 14 [Source:MGI Symbol;Acc:MGI:1858204]
9377	ENSMUSG000000057110	1.711329	5.770443	8.29394	0.003978	0.033624	Cntrl	centriolin [Source:MGI Symbol;Acc:MGI:1889576]
4391	ENSMUSG000000028364	1.705805	5.061868	17.69488	2.59E-05	0.001427	Tnc	tenascin C [Source:MGI Symbol;Acc:MGI:101922]
8999	ENSMUSG000000052331	1.703974	6.122097	8.482984	0.003585	0.031875	Ankrd44	ankyrin repeat domain 44 [Source:MGI Symbol;Acc:MGI:3045243]
8608	ENSMUSG000000047735	1.695813	6.868994	7.030691	0.008012	0.048773	Samd9l	sterile alpha motif domain containing 9-like [Source:MGI Symbol;Acc:MGI:1343184]
5706	ENSMUSG000000032011	1.690046	5.280723	11.16965	0.000831	0.01554	Thy1	thymus cell antigen 1, theta [Source:MGI Symbol;Acc:MGI:98747]
6831	ENSMUSG000000036986	1.685489	5.014444	9.785164	0.001759	0.022289	Pml	promyelocytic leukemia [Source:MGI Symbol;Acc:MGI:104662]
5632	ENSMUSG000000031833	1.684043	3.587362	7.699188	0.005525	0.040048	Mast3	microtubule associated serine/threonine kinase 3 [Source:MGI Symbol;Acc:MGI:2683541]
2973	ENSMUSG000000024397	1.680301	2.782064	10.44024	0.001233	0.01879	Aif1	allograft inflammatory factor 1 [Source:MGI Symbol;Acc:MGI:1343098]
340	ENSMUSG000000002870	1.677884	3.17644	8.209849	0.004166	0.034417	Mcm2	minichromosome maintenance deficient 2 mitotin (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:105380]
6927	ENSMUSG000000037405	1.674441	4.634244	7.676899	0.005593	0.040227	Icam1	intercellular adhesion molecule 1 [Source:MGI Symbol;Acc:MGI:96392]

9003	ENSMUSG000000052384	1.666498	4.565736	7.959167	0.004784	0.037184	Nrros	negative regulator of reactive oxygen species [Source:MGI Symbol;Acc:MGI:2445095]
2194	ENSMUSG000000021728	1.660236	4.147811	10.90397	0.00096	0.016466	Emb	embigin [Source:MGI Symbol;Acc:MGI:95321]
9609	ENSMUSG000000060216	1.659188	4.375777	10.72289	0.001058	0.01726	Arrb2	arrestin, beta 2 [Source:MGI Symbol;Acc:MGI:99474]
8019	ENSMUSG000000042228	1.6589	5.381781	7.937809	0.004841	0.037453	Lyn	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog [Source:MGI Symbol;Acc:MGI:96892]
4175	ENSMUSG000000027778	1.652776	4.153804	10.47475	0.00121	0.018651	Ift80	intraflagellar transport 80 [Source:MGI Symbol;Acc:MGI:1915509]
7949	ENSMUSG000000041859	1.650512	3.521072	8.234128	0.004111	0.034145	Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:101845]
229	ENSMUSG000000001986	1.648166	2.443795	9.270375	0.002329	0.025334	Gria3	glutamate receptor, ionotropic, AMPA3 (alpha 3) [Source:MGI Symbol;Acc:MGI:95810]
10053	ENSMUSG000000068735	1.644252	4.262251	7.835587	0.005123	0.038556	Trp53i11	transformation related protein 53 inducible protein 11 [Source:MGI Symbol;Acc:MGI:2670995]
10734	ENSMUSG000000096768	1.631335	2.403168	9.433613	0.00213	0.024384	Erd1	erythroid differentiation regulator 1 [Source:MGI Symbol;Acc:MGI:2384747]
6388	ENSMUSG000000034663	1.624753	5.943223	7.231697	0.007163	0.045855	Bmp2k	BMP2 inducible kinase [Source:MGI Symbol;Acc:MGI:2155456]
9944	ENSMUSG000000066258	1.606311	4.748938	9.967318	0.001593	0.021122	Trim12a	tripartite motif-containing 12A [Source:MGI Symbol;Acc:MGI:1923931]
252	ENSMUSG000000002111	1.602784	3.301722	7.234052	0.007153	0.045855	Spi1	spleen focus forming virus (SFFV) proviral integration oncogene [Source:MGI Symbol;Acc:MGI:98282]
1562	ENSMUSG000000020108	1.59664	3.169441	7.818361	0.005172	0.038771	Ddit4	DNA-damage-inducible transcript 4 [Source:MGI Symbol;Acc:MGI:1921997]
6370	ENSMUSG000000034610	1.589733	5.677787	9.627775	0.001917	0.02312	Zcchc11	zinc finger, CCHC domain containing 11 [Source:MGI Symbol;Acc:MGI:2445126]
7960	ENSMUSG000000041920	1.586123	3.034725	13.75126	0.000209	0.006428	Slc16a6	solute carrier family 16 (monocarboxylic acid transporters), member 6 [Source:MGI Symbol;Acc:MGI:2144585]
10370	ENSMUSG000000075028	1.58541	2.776907	8.24445	0.004088	0.034056	Prdm11	PR domain containing 11 [Source:MGI Symbol;Acc:MGI:2685553]
10829	ENSMUSG0000000104235	1.583675	3.898762	7.423838	0.006437	0.043561	Gm37589	predicted gene, 37589 [Source:MGI Symbol;Acc:MGI:5610817]
3797	ENSMUSG000000026737	1.578106	4.800808	9.219719	0.002394	0.025731	Pip4k2a	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha [Source:MGI Symbol;Acc:MGI:1298206]
10857	ENSMUSG0000000106631	1.575864	3.011427	7.371965	0.006625	0.044268	Gm42669	predicted gene 42669 [Source:MGI Symbol;Acc:MGI:5662806]
5309	ENSMUSG000000030921	1.574226	5.490986	8.632967	0.003301	0.030536	Trim30a	tripartite motif-containing 30A [Source:MGI Symbol;Acc:MGI:98178]
1065	ENSMUSG000000015340	1.560854	5.643861	8.089224	0.004453	0.035828	Cybb	cytochrome b-245, beta polypeptide [Source:MGI Symbol;Acc:MGI:88574]
6557	ENSMUSG000000035566	1.558087	2.862863	10.73976	0.001049	0.017181	Pcdh17	protocadherin 17 [Source:MGI Symbol;Acc:MGI:2684924]
8364	ENSMUSG000000045328	1.556265	2.405439	7.793166	0.005244	0.039059	Cenpe	centromere protein E [Source:MGI Symbol;Acc:MGI:1098230]
3314	ENSMUSG000000025316	1.555224	3.407688	14.14529	0.000169	0.005708	Banp	BTG3 associated nuclear protein [Source:MGI Symbol;Acc:MGI:1889023]
513	ENSMUSG000000004609	1.541869	3.892145	11.20425	0.000816	0.015358	Cd33	CD33 antigen [Source:MGI Symbol;Acc:MGI:99440]
4781	ENSMUSG000000029298	1.530733	5.745278	10.95852	0.000932	0.016244	Gbp9	guanylate-binding protein 9 [Source:MGI Symbol;Acc:MGI:3605620]
10475	ENSMUSG000000078945	1.528875	3.371518	9.44725	0.002115	0.024357	Naip2	NLR family, apoptosis inhibitory protein 2 [Source:MGI Symbol;Acc:MGI:1298226]
10652	ENSMUSG000000090394	1.526993	5.663386	8.394485	0.003764	0.032833	4930523C07Rik	RIKEN cDNA 4930523C07 gene [Source:MGI Symbol;Acc:MGI:1914897]
4882	ENSMUSG000000029552	1.525196	4.301678	7.50383	0.006157	0.042378	Tes	testis derived transcript [Source:MGI Symbol;Acc:MGI:105081]
5027	ENSMUSG000000030067	1.497771	6.484554	8.830367	0.002963	0.028873	Foxp1	forkhead box P1 [Source:MGI Symbol;Acc:MGI:1914004]
7004	ENSMUSG000000037795	1.49241	4.893921	10.43392	0.001237	0.018821	N4bp2	NEDD4 binding protein 2 [Source:MGI Symbol;Acc:MGI:2684414]
1047	ENSMUSG000000015143	1.490774	5.056365	10.30361	0.001328	0.019523	Actn1	actinin, alpha 1 [Source:MGI Symbol;Acc:MGI:2137706]
5353	ENSMUSG000000031093	1.488455	6.33742	7.753788	0.00536	0.03956	Dock11	dedicator of cytokinesis 11 [Source:MGI Symbol;Acc:MGI:1923224]
8293	ENSMUSG000000044583	1.48552	3.968628	10.0334	0.001537	0.020765	Tlr7	toll-like receptor 7 [Source:MGI Symbol;Acc:MGI:2176882]
10830	ENSMUSG0000000104377	1.483643	4.434259	9.237195	0.002371	0.025584	Gm37515	predicted gene, 37515 [Source:MGI Symbol;Acc:MGI:5610743]

9432	ENSMUSG00000057722	1.479521	4.671124	12.36868	0.000437	0.01036	Lepr	leptin receptor [Source:MGI Symbol;Acc:MGI:104993]
1254	ENSMUSG00000018008	1.477501	4.835404	7.899275	0.004945	0.037788	Cyth4	cytohesin 4 [Source:MGI Symbol;Acc:MGI:2441702]
5004	ENSMUSG00000029999	1.4773	2.468533	10.70324	0.001069	0.017315	Tgfa	transforming growth factor alpha [Source:MGI Symbol;Acc:MGI:98724]
7220	ENSMUSG00000038578	1.475379	3.21776	9.513393	0.00204	0.023873	Susd1	sushi domain containing 1 [Source:MGI Symbol;Acc:MGI:3651543]
10583	ENSMUSG00000086370	1.469416	3.138055	7.737785	0.005408	0.039699	Ftx	Ftx transcript, Xist regulator (non-protein coding) [Source:MGI Symbol;Acc:MGI:1926128]
6821	ENSMUSG00000036948	1.465785	3.027737	9.294858	0.002298	0.025249	BC037034	cDNA sequence BC037034 [Source:MGI Symbol;Acc:MGI:2385896]
10478	ENSMUSG00000078995	1.464548	2.444272	9.605545	0.00194	0.023228	Zfp456	zinc finger protein 456 [Source:MGI Symbol;Acc:MGI:3040694]
10836	ENSMUSG000000104563	1.46265	2.315428	11.5225	0.000688	0.013693	Gm43041	predicted gene 43041 [Source:MGI Symbol;Acc:MGI:5663178]
10710	ENSMUSG00000095123	1.452838	2.495465	7.463457	0.006296	0.042932	Gm21781	predicted gene, 21781 [Source:MGI Symbol;Acc:MGI:5433945]
5285	ENSMUSG00000030844	1.451527	3.701221	8.144117	0.00432	0.035292	Rgs10	regulator of G-protein signalling 10 [Source:MGI Symbol;Acc:MGI:1915115]
2193	ENSMUSG00000021725	1.443536	4.422697	21.94324	2.81E-06	0.000309	Parp8	poly (ADP-ribose) polymerase family, member 8 [Source:MGI Symbol;Acc:MGI:1098713]
								tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator) [Source:MGI Symbol;Acc:MGI:2675303]
8037	ENSMUSG00000042333	1.439411	2.428884	13.67261	0.000218	0.006605	Tnfrsf14	myelocytomatosis oncogene [Source:MGI Symbol;Acc:MGI:97250]
2433	ENSMUSG00000022346	1.439239	3.301048	16.34731	5.27E-05	0.002445	Myc	RNA binding motif protein 12 B1 [Source:MGI Symbol;Acc:MGI:1919647]
8487	ENSMUSG00000046667	1.432355	1.935166	14.06492	0.000177	0.005849	Rbm12b1	predicted gene, 37954 [Source:MGI Symbol;Acc:MGI:5611182]
10824	ENSMUSG000000104060	1.42446	3.676965	8.147023	0.004313	0.035283	Gm37954	sphingosine-1-phosphate receptor 1 [Source:MGI Symbol;Acc:MGI:1096355]
8340	ENSMUSG00000045092	1.420541	6.186468	10.66079	0.001094	0.017586	S1pr1	family with sequence similarity 46, member C [Source:MGI Symbol;Acc:MGI:1921895]
8280	ENSMUSG00000044468	1.41756	3.604807	12.95582	0.000319	0.008496	Fam46c	coiled coil domain containing 88A [Source:MGI Symbol;Acc:MGI:1925177]
5981	ENSMUSG00000032740	1.413194	5.602334	11.18458	0.000825	0.015442	Ccdc88a	predicted gene, 37903 [Source:MGI Symbol;Acc:MGI:5611131]
10813	ENSMUSG000000103070	1.410679	2.751832	8.353463	0.00385	0.033062	Gm37903	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 [Source:MGI Symbol;Acc:MGI:1333853]
								predicted gene, 36989 [Source:MGI Symbol;Acc:MGI:5610217]
10127	ENSMUSG00000070407	1.40577	2.816463	19.34866	1.09E-05	0.000781	Hs3st3b1	SAM domain and HD domain, 1 [Source:MGI Symbol;Acc:MGI:1927468]
10801	ENSMUSG000000102496	1.403399	2.601081	7.55853	0.005973	0.041627	Gm36989	solute carrier family 15, member 4 [Source:MGI Symbol;Acc:MGI:2140796]
4130	ENSMUSG00000027639	1.402944	6.462403	9.46113	0.002099	0.024276	Samhd1	predicted gene 29055 [Source:MGI Symbol;Acc:MGI:5579761]
4825	ENSMUSG00000029416	1.398073	3.751737	9.833137	0.001714	0.021952	Slc15a4	Kruppel-like factor 2 (lung) [Source:MGI Symbol;Acc:MGI:1342772]
10787	ENSMUSG000000100768	1.397186	3.787951	7.290537	0.006932	0.045282	Gm29055	interleukin 18 binding protein [Source:MGI Symbol;Acc:MGI:1333800]
9237	ENSMUSG00000055148	1.395252	4.081549	9.711236	0.001831	0.022728	Klf2	
10130	ENSMUSG00000070427	1.391432	2.589708	8.733678	0.003124	0.029649	Il18bp	
10879	ENSMUSG000000108173	1.388396	3.720714	7.355351	0.006686	0.044478		
10075	ENSMUSG00000069094	1.387681	5.485915	13.29573	0.000266	0.007568	Pde7a	phosphodiesterase 7A [Source:MGI Symbol;Acc:MGI:1202402]
3216	ENSMUSG00000025044	1.383431	3.315705	7.709135	0.005494	0.039937	Msr1	macrophage scavenger receptor 1 [Source:MGI Symbol;Acc:MGI:98257]
239	ENSMUSG00000002028	1.382937	6.431044	8.095606	0.004437	0.035761	Kmt2a	lysine (K)-specific methyltransferase 2A [Source:MGI Symbol;Acc:MGI:96995]
6614	ENSMUSG00000035891	1.380238	4.602262	9.687811	0.001855	0.022942	Cerk	ceramide kinase [Source:MGI Symbol;Acc:MGI:2386052]
1526	ENSMUSG00000020027	1.379781	3.976128	13.15334	0.000287	0.007917	Socs2	suppressor of cytokine signaling 2 [Source:MGI Symbol;Acc:MGI:1201787]
6082	ENSMUSG00000033192	1.376777	2.489251	10.41563	0.00125	0.01885	Lpcat2	lysophosphatidylcholine acyltransferase 2 [Source:MGI Symbol;Acc:MGI:3606214]
5502	ENSMUSG00000031537	1.375208	5.621392	7.297966	0.006903	0.045154	Ikbkb	inhibitor of kappaB kinase beta [Source:MGI Symbol;Acc:MGI:1338071]
2441	ENSMUSG00000022360	1.375035	4.134649	7.892773	0.004963	0.037848	Atad2	ATPase family, AAA domain containing 2 [Source:MGI Symbol;Acc:MGI:1917722]
3479	ENSMUSG00000025880	1.373584	3.310803	9.450141	0.002111	0.024344	Smad7	SMAD family member 7 [Source:MGI Symbol;Acc:MGI:1100518]
1675	ENSMUSG00000020387	1.373349	4.603261	8.425467	0.0037	0.032539	Jade2	jade family PHD finger 2 [Source:MGI Symbol;Acc:MGI:1924151]
10156	ENSMUSG00000070808	1.371017	3.031135	7.871925	0.005021	0.038123	Gltscr1	glioma tumor suppressor candidate region gene 1 [Source:MGI Symbol;Acc:MGI:2154263]

3575	ENSMUSG000000026126	1.369746	3.386869	8.618211	0.003328	0.03068	Ptpn18	protein tyrosine phosphatase, non-receptor type 18 [Source:MGI Symbol;Acc:MGI:108410]
134	ENSMUSG000000001248	1.367232	5.472529	7.211198	0.007245	0.046142	Gramd1a	GRAM domain containing 1A [Source:MGI Symbol;Acc:MGI:105490]
2841	ENSMUSG000000024054	1.362159	6.514935	8.549916	0.003455	0.031246	Smchd1	SMC hinge domain containing 1 [Source:MGI Symbol;Acc:MGI:1921605]
8028	ENSMUSG000000042292	1.361175	4.529223	6.968451	0.008296	0.049804	Mkl1	MKL (megakaryoblastic leukemia)/myocardin-like 1 [Source:MGI Symbol;Acc:MGI:2384495]
2943	ENSMUSG000000024300	1.359596	3.949001	8.700677	0.003181	0.029956	Myo1f	myosin IF [Source:MGI Symbol;Acc:MGI:107711]
1824	ENSMUSG000000020733	1.35951	3.95832	8.583158	0.003393	0.031014	Slc9a3r1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1 [Source:MGI Symbol;Acc:MGI:1349482]
3744	ENSMUSG000000026600	1.358182	4.764288	9.761698	0.001782	0.022416	Soat1	sterol O-acyltransferase 1 [Source:MGI Symbol;Acc:MGI:104665]
7846	ENSMUSG000000041313	1.355601	3.5342	7.360738	0.006666	0.044372	Slc7a1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 [Source:MGI Symbol;Acc:MGI:88117]
4071	ENSMUSG000000027469	1.354205	2.364681	7.298287	0.006902	0.045154	Tpx2	TPX2, microtubule-associated protein homolog (Xenopus laevis) [Source:MGI Symbol;Acc:MGI:1919369]
7886	ENSMUSG000000041559	1.353465	2.632508	8.859359	0.002916	0.028753	Fmod	fibromodulin [Source:MGI Symbol;Acc:MGI:1328364]
7086	ENSMUSG000000038126	1.351065	3.342962	8.918376	0.002823	0.028299	Mphosph9	M-phase phosphoprotein 9 [Source:MGI Symbol;Acc:MGI:2443138]
9685	ENSMUSG000000061288	1.345756	4.620988	8.765374	0.00307	0.029381	Taok3	TAO kinase 3 [Source:MGI Symbol;Acc:MGI:3041177]
1100	ENSMUSG000000015745	1.340576	3.936313	7.678794	0.005587	0.040227	Plekho1	pleckstrin homology domain containing, family O member 1 [Source:MGI Symbol;Acc:MGI:1914470]
3052	ENSMUSG000000024613	1.337675	4.741612	8.112221	0.004397	0.035592	Tcof1	Treacher Collins Franceschetti syndrome 1, homolog [Source:MGI Symbol;Acc:MGI:892003]
8413	ENSMUSG000000045868	1.334202	2.521503	10.218	0.001391	0.019861	Gvin1	GTPase, very large interferon inducible 1 [Source:MGI Symbol;Acc:MGI:1921808]
1135	ENSMUSG000000016087	1.333852	5.558735	9.652797	0.001891	0.023095	Fli1	Friend leukemia integration 1 [Source:MGI Symbol;Acc:MGI:95554]
6957	ENSMUSG000000037552	1.333525	4.238863	7.776338	0.005293	0.039264	Plekhg2	pleckstrin homology domain containing, family G (with RhoGef domain) member 2 [Source:MGI Symbol;Acc:MGI:2141874]
777	ENSMUSG000000007880	1.326467	6.301564	7.587837	0.005876	0.041282	Arid1a	AT rich interactive domain 1A (SWI-like) [Source:MGI Symbol;Acc:MGI:1935147]
7370	ENSMUSG000000039157	1.326286	4.657337	7.669966	0.005615	0.040355	Fam102a	family with sequence similarity 102, member A [Source:MGI Symbol;Acc:MGI:2138935]
9422	ENSMUSG000000057596	1.319153	3.926727	7.852803	0.005074	0.038368	Trim30d	tripartite motif-containing 30D [Source:MGI Symbol;Acc:MGI:3035181]
6040	ENSMUSG000000033016	1.313482	5.363549	8.205387	0.004177	0.034476	Nfatc1	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1 [Source:MGI Symbol;Acc:MGI:102469]
6145	ENSMUSG000000033538	1.307929	3.037219	10.01315	0.001554	0.020805	Casp4	caspase 4, apoptosis-related cysteine peptidase [Source:MGI Symbol;Acc:MGI:107700]
10377	ENSMUSG000000075284	1.307492	5.3995	7.905525	0.004928	0.037737	Wipf1	WAS/WASL interacting protein family, member 1 [Source:MGI Symbol;Acc:MGI:2178801]
4736	ENSMUSG000000029174	1.29844	4.787364	7.468757	0.006278	0.042886	Tbc1d1	TBC1 domain family, member 1 [Source:MGI Symbol;Acc:MGI:1889508]
6707	ENSMUSG000000036381	1.297788	2.585218	11.04532	0.000889	0.015986	P2ry14	purinergic receptor P2Y, G-protein coupled, 14 [Source:MGI Symbol;Acc:MGI:2155705]
7742	ENSMUSG000000040721	1.291331	2.783316	10.44992	0.001227	0.018738	Zfhx2	zinc finger homeobox 2 [Source:MGI Symbol;Acc:MGI:2686934]
9558	ENSMUSG000000059552	1.286205	4.454519	9.581017	0.001966	0.023335	Trp53	transformation related protein 53 [Source:MGI Symbol;Acc:MGI:98834]
10556	ENSMUSG000000083594	1.283645	3.536537	8.340211	0.003878	0.033138	Gm13722	predicted gene 13722 [Source:MGI Symbol;Acc:MGI:3651668]
1716	ENSMUSG000000020474	1.283325	2.097686	7.640823	0.005706	0.040717	Polm	polymerase (DNA directed), mu [Source:MGI Symbol;Acc:MGI:1860191]
10797	ENSMUSG0000000101970	1.27883	4.720902	8.403173	0.003746	0.032822	1810026B05Rik	RIKEN cDNA 1810026B05 gene [Source:MGI Symbol;Acc:MGI:1916420]
6536	ENSMUSG000000035439	1.277407	2.449887	7.538081	0.006041	0.041951	Haus8	4HAUS augmin-like complex, subunit 8 [Source:MGI Symbol;Acc:MGI:1923728]
10092	ENSMUSG000000069682	1.267243	3.331695	7.756739	0.005351	0.039557	Gm10275	predicted pseudogene 10275 [Source:MGI Symbol;Acc:MGI:3704354]
5089	ENSMUSG000000030257	1.265529	2.928209	7.041119	0.007966	0.048641	Srgap3	SLIT-ROBO Rho GTPase activating protein 3 [Source:MGI Symbol;Acc:MGI:2152938]
2791	ENSMUSG000000023919	1.265456	2.368529	8.895635	0.002859	0.028431	Cenpq	centromere protein Q [Source:MGI Symbol;Acc:MGI:1933744]

5458	ENSMUSG000000031431	1.264366	6.140826	9.635884	0.001908	0.02312	Tsc22d3	TSC22 domain family, member 3 [Source:MGI Symbol;Acc:MGI:1196284]
10513	ENSMUSG000000079419	1.262013	3.774519	9.724189	0.001819	0.022698	Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C [Source:MGI Symbol;Acc:MGI:2385644]
7506	ENSMUSG000000039738	1.256525	3.50529	7.0281	0.008024	0.048816	Slx4	SLX4 structure-specific endonuclease subunit homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:106299]
6723	ENSMUSG000000036461	1.254914	6.047352	8.60513	0.003352	0.030823	Elf1	E74-like factor 1 [Source:MGI Symbol;Acc:MGI:107180]
10860	ENSMUSG000000106819	1.250967	2.855965	7.087276	0.007763	0.048089	Gm43498	predicted gene 43498 [Source:MGI Symbol;Acc:MGI:5663635]
5972	ENSMUSG000000032712	1.250804	6.188719	19.66902	9.21E-06	0.000697	2810474O19Rik	RIKEN cDNA 2810474O19 gene [Source:MGI Symbol;Acc:MGI:1914496]
10769	ENSMUSG000000097815	1.250697	2.263614	7.05523	0.007903	0.048489	Gm26809	predicted gene, 26809 [Source:MGI Symbol;Acc:MGI:5477303]
10633	ENSMUSG000000089989	1.242964	2.845861	7.678	0.00559	0.040227	Flt3l	FMS-like tyrosine kinase 3 ligand [Source:MGI Symbol;Acc:MGI:95560]
4514	ENSMUSG000000028654	1.236304	3.180538	8.22896	0.004123	0.034187	Myl	v-myc myelocytomatosis viral oncogene homolog, lung carcinoma derived (avian) [Source:MGI Symbol;Acc:MGI:96799]
3430	ENSMUSG000000025742	1.235639	4.231119	7.690397	0.005552	0.040144	Prps2	phosphoribosyl pyrophosphate synthetase 2 [Source:MGI Symbol;Acc:MGI:97776]
9187	ENSMUSG000000054517	1.222728	3.613642	8.368938	0.003817	0.032993	Trim65	tripartite motif-containing 65 [Source:MGI Symbol;Acc:MGI:2442815]
10613	ENSMUSG000000089417	1.221622	7.328004	11.52091	0.000688	0.013693	Gm22009	predicted gene, 22009 [Source:MGI Symbol;Acc:MGI:5451786]
3958	ENSMUSG000000027176	1.220941	3.040846	8.915827	0.002827	0.028313	Cstf3	cleavage stimulation factor, 3' pre-RNA, subunit 3 [Source:MGI Symbol;Acc:MGI:1351825]
10865	ENSMUSG000000106948	1.21985	4.264455	7.055951	0.0079	0.048489	Gm42785	predicted gene 42785 [Source:MGI Symbol;Acc:MGI:5662922]
10431	ENSMUSG000000078502	1.219254	2.381257	8.70995	0.003165	0.029856	Gm13212	predicted gene 13212 [Source:MGI Symbol;Acc:MGI:3651014]
1250	ENSMUSG000000017929	1.218492	3.695768	8.046052	0.00456	0.036111	B4galt5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 [Source:MGI Symbol;Acc:MGI:1927169]
4605	ENSMUSG000000028883	1.218083	2.780098	8.894215	0.002861	0.028431	Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A [Source:MGI Symbol;Acc:MGI:107558]
8401	ENSMUSG000000045730	1.217381	3.374311	13.17485	0.000284	0.007847	Adrb2	adrenergic receptor, beta 2 [Source:MGI Symbol;Acc:MGI:87938]
10799	ENSMUSG000000102307	1.217003	4.288749	16.67755	4.43E-05	0.002145	Gm38194	predicted gene, 38194 [Source:MGI Symbol;Acc:MGI:5611422]
6657	ENSMUSG000000036106	1.215784	2.420904	7.26421	0.007034	0.045598	Prr5	proline rich 5 (renal) [Source:MGI Symbol;Acc:MGI:1924714]
3026	ENSMUSG000000024542	1.210441	4.851446	7.58382	0.005889	0.041337	Cep192	centrosomal protein 192 [Source:MGI Symbol;Acc:MGI:1918049]
10578	ENSMUSG000000085811	1.203723	2.114781	10.50087	0.001193	0.01852	Cep112it	centrosomal protein 112, intronic transcript [Source:MGI Symbol;Acc:MGI:3649867]
1935	ENSMUSG000000021025	1.202706	5.398861	12.02279	0.000526	0.011758	Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha [Source:MGI Symbol;Acc:MGI:104741]
2559	ENSMUSG000000022637	1.197956	5.32268	8.52696	0.003499	0.031381	Cblb	Casitas B-lineage lymphoma b [Source:MGI Symbol;Acc:MGI:2146430]
6329	ENSMUSG000000034422	1.197502	6.401163	11.95712	0.000544	0.011939	Parp14	poly (ADP-ribose) polymerase family, member 14 [Source:MGI Symbol;Acc:MGI:1919489]
4267	ENSMUSG000000028034	1.190885	6.300276	9.666933	0.001876	0.022999	Fubp1	far upstream element (FUSE) binding protein 1 [Source:MGI Symbol;Acc:MGI:1196294]
10121	ENSMUSG000000070327	1.1895	7.893995	9.125204	0.002521	0.026562	Rnf213	ring finger protein 213 [Source:MGI Symbol;Acc:MGI:1289196]
4461	ENSMUSG000000028525	1.188282	5.373164	37.16698	1.08E-09	6.95E-07	Pde4b	phosphodiesterase 4B, cAMP specific [Source:MGI Symbol;Acc:MGI:99557]
8311	ENSMUSG000000044786	1.186687	4.779263	8.889149	0.002869	0.028467	Zfp36	zinc finger protein 36 [Source:MGI Symbol;Acc:MGI:99180]
4970	ENSMUSG000000029798	1.186497	3.609857	7.526332	0.00608	0.042145	Herc6	hect domain and RLD 6 [Source:MGI Symbol;Acc:MGI:1914388]
8287	ENSMUSG000000044519	1.184959	2.312048	8.032073	0.004596	0.036236	Zfp488	zinc finger protein 488 [Source:MGI Symbol;Acc:MGI:2686052]
4339	ENSMUSG000000028211	1.18344	6.093245	8.366188	0.003823	0.032993	Trp53inp1	transformation related protein 53 inducible nuclear protein 1 [Source:MGI Symbol;Acc:MGI:1926609]

9319	ENSMUSG00000056260	1.182329	4.562229	12.93521	0.000322	0.00857	Lrif1	ligand dependent nuclear receptor interacting factor 1 [Source:MGI Symbol;Acc:MGI:2445214]
5575	ENSMUSG00000031709	1.182225	3.234625	10.13381	0.001456	0.020342	Tbc1d9	TBC1 domain family, member 9 [Source:MGI Symbol;Acc:MGI:1918560]
2818	ENSMUSG00000023994	1.17844	3.933497	8.3575	0.003841	0.033062	Nfya	nuclear transcription factor-Y alpha [Source:MGI Symbol;Acc:MGI:97316]
6574	ENSMUSG00000035649	1.178087	5.381812	9.694382	0.001848	0.022912	Zcchc7	zinc finger, CCHC domain containing 7 [Source:MGI Symbol;Acc:MGI:2442912]
1782	ENSMUSG00000020640	1.177446	6.347917	7.439726	0.00638	0.043264	Itsn2	intersectin 2 [Source:MGI Symbol;Acc:MGI:1338049]
7443	ENSMUSG00000039456	1.174723	5.315774	9.096488	0.002561	0.026831	Morc3	microrchidia 3 [Source:MGI Symbol;Acc:MGI:2136841]
2769	ENSMUSG00000023764	1.17049	3.736538	8.626751	0.003313	0.030608	Sfi1	Sfi1 homolog, spindle assembly associated (yeast) [Source:MGI Symbol;Acc:MGI:1926137]
8977	ENSMUSG00000052005	1.168836	2.361177	21.95493	2.79E-06	0.000309	Gm9864	predicted gene 9864 [Source:MGI Symbol;Acc:MGI:3708663]
10798	ENSMUSG000000102151	1.166851	2.581392	7.223127	0.007197	0.045974	Gm37472	predicted gene, 37472 [Source:MGI Symbol;Acc:MGI:5610700]
6786	ENSMUSG00000036815	1.163767	2.50951	9.045263	0.002634	0.027227	Dpp10	dipeptidylpeptidase 10 [Source:MGI Symbol;Acc:MGI:2442409]
9383	ENSMUSG00000057143	1.160397	4.308597	7.000448	0.008149	0.049302	Trim12c	tripartite motif-containing 12C [Source:MGI Symbol;Acc:MGI:4821183]
6381	ENSMUSG00000034641	1.149984	2.93451	12.48878	0.000409	0.009961	Cd300ld	CD300 molecule-like family member d [Source:MGI Symbol;Acc:MGI:2442358]
1682	ENSMUSG00000020400	1.149814	4.690286	9.355728	0.002223	0.024783	Tnip1	TNFAIP3 interacting protein 1 [Source:MGI Symbol;Acc:MGI:1926194]
8409	ENSMUSG00000045827	1.148641	4.640465	7.279074	0.006976	0.045408	Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9 [Source:MGI Symbol;Acc:MGI:106603]
6170	ENSMUSG00000033685	1.14685	6.775169	12.25401	0.000464	0.010769	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier) [Source:MGI Symbol;Acc:MGI:109354]
1522	ENSMUSG00000020021	1.146749	3.479701	10.46191	0.001219	0.018703	Fgd6	FYVE, RhoGEF and PH domain containing 6 [Source:MGI Symbol;Acc:MGI:1261419]
7312	ENSMUSG00000038910	1.142723	5.215157	11.64571	0.000644	0.013106	Plcl2	phospholipase C-like 2 [Source:MGI Symbol;Acc:MGI:1352756]
2819	ENSMUSG00000024002	1.141088	5.151866	7.211019	0.007246	0.046142	Brd4	bromodomain containing 4 [Source:MGI Symbol;Acc:MGI:1888520]
5776	ENSMUSG00000032220	1.137397	5.122042	7.197405	0.007301	0.046372	Myo1e	myosin IE [Source:MGI Symbol;Acc:MGI:106621]
3431	ENSMUSG00000025743	1.129653	5.657948	11.55004	0.000677	0.01362	Sdc3	syndecan 3 [Source:MGI Symbol;Acc:MGI:1349163]
4856	ENSMUSG00000029475	1.126551	3.767123	9.543081	0.002007	0.023642	Kdm2b	lysine (K)-specific demethylase 2B [Source:MGI Symbol;Acc:MGI:1354737]
3316	ENSMUSG00000025323	1.126399	4.86856	7.282503	0.006963	0.045391	Sp4	trans-acting transcription factor 4 [Source:MGI Symbol;Acc:MGI:107595]
3875	ENSMUSG00000026915	1.125474	5.271939	10.98133	0.00092	0.016148	Strbp	spermatid perinuclear RNA binding protein [Source:MGI Symbol;Acc:MGI:104626]
251	ENSMUSG00000002109	1.124991	3.269572	7.043219	0.007957	0.048641	Ddb2	damage specific DNA binding protein 2 [Source:MGI Symbol;Acc:MGI:1355314]
6977	ENSMUSG00000037674	1.123557	5.634418	7.109414	0.007668	0.047858	Rfx7	regulatory factor X, 7 [Source:MGI Symbol;Acc:MGI:2442675]
9206	ENSMUSG00000054720	1.122126	4.528396	9.876968	0.001674	0.021725	Lrrc8c	leucine rich repeat containing 8 family, member C [Source:MGI Symbol;Acc:MGI:2140839]
10582	ENSMUSG00000086320	1.11969	4.180972	10.75681	0.001039	0.017049	Gm12840	predicted gene 12840 [Source:MGI Symbol;Acc:MGI:3650917]
10855	ENSMUSG000000106411	1.116018	2.703987	6.982478	0.008231	0.049578	Gm42445	predicted gene 42445 [Source:MGI Symbol;Acc:MGI:5662582]
6728	ENSMUSG00000036501	1.114315	4.467094	7.179851	0.007373	0.046659	Fam13b	family with sequence similarity 13, member B [Source:MGI Symbol;Acc:MGI:2447834]
6457	ENSMUSG00000035004	1.114041	2.758206	12.59748	0.000386	0.009653	Igsf6	immunoglobulin superfamily, member 6 [Source:MGI Symbol;Acc:MGI:1891393]
10842	ENSMUSG000000105263	1.106127	6.087296	9.622064	0.001923	0.02312	Gm42427	predicted gene 42427 [Source:MGI Symbol;Acc:MGI:5662564]
9019	ENSMUSG000000052544	1.103401	2.293138	9.921523	0.001634	0.021386	St6galnac3	ST6 (alpha-N-acetyl-neuraminy-2,3-beta-galactosyl-1,3)-N-acetylglactosaminide alpha-2,6-sialyltransferase 3 [Source:MGI Symbol;Acc:MGI:1341828]
7445	ENSMUSG00000039458	1.103323	4.318367	7.908312	0.004921	0.037705	Mtmr12	myotubularin related protein 12 [Source:MGI Symbol;Acc:MGI:2443034]
3060	ENSMUSG00000024642	1.103123	4.472848	13.5398	0.000234	0.006915	Tle4	transducin-like enhancer of split 4, homolog of Drosophila E(spl) [Source:MGI Symbol;Acc:MGI:104633]
10410	ENSMUSG00000077394	1.097165	2.195566	16.2126	5.66E-05	0.002574	Gm24339	predicted gene, 24339 [Source:MGI Symbol;Acc:MGI:5454116]
1462	ENSMUSG00000019843	1.09612	5.137834	8.634111	0.003299	0.030536	Fyn	Fyn proto-oncogene [Source:MGI Symbol;Acc:MGI:95602]

10729	ENSMUSG000000096463	1.095996	2.309783	9.854	0.001695	0.021826	Gm21750	predicted gene, 21750 [Source:MGI Symbol;Acc:MGI:5433914]
2477	ENSMUSG000000022443	1.095503	8.530498	7.071818	0.007831	0.048286	Myh9	myosin, heavy polypeptide 9, non-muscle [Source:MGI Symbol;Acc:MGI:107717]
8710	ENSMUSG000000048779	1.094568	2.149121	14.02269	0.000181	0.005928	P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6 [Source:MGI Symbol;Acc:MGI:2673874]
1506	ENSMUSG000000019979	1.089131	4.108583	8.250436	0.004074	0.034044	Apaf1	apoptotic peptidase activating factor 1 [Source:MGI Symbol;Acc:MGI:1306796]
6283	ENSMUSG000000034218	1.082284	5.658974	7.489973	0.006204	0.042604	Atm	ataxia telangiectasia mutated [Source:MGI Symbol;Acc:MGI:107202]
10489	ENSMUSG000000079084	1.074917	4.094151	7.833035	0.00513	0.038556	Ccdc82	coiled-coil domain containing 82 [Source:MGI Symbol;Acc:MGI:1913646]
8650	ENSMUSG000000048118	1.074553	5.389438	10.47938	0.001207	0.018651	Arid4a	AT rich interactive domain 4A (RBP1-like) [Source:MGI Symbol;Acc:MGI:2444354]
9509	ENSMUSG000000058818	1.073995	4.197014	8.311448	0.00394	0.033431	Pirb	paired Ig-like receptor B [Source:MGI Symbol;Acc:MGI:894311]
743	ENSMUSG000000007041	1.07016	5.151029	7.079491	0.007797	0.048164	Clic1	chloride intracellular channel 1 [Source:MGI Symbol;Acc:MGI:2148924]
1767	ENSMUSG000000020601	1.067399	4.197875	9.080731	0.002583	0.026952	Trib2	tribbles homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:2145021]
10669	ENSMUSG000000091183	1.067387	2.170917	9.138365	0.002503	0.026453	Gm5141	predicted gene 5141 [Source:MGI Symbol;Acc:MGI:3779466]
10444	ENSMUSG000000078671	1.060419	6.578961	10.23727	0.001376	0.01981	Chd2	chromodomain helicase DNA binding protein 2 [Source:MGI Symbol;Acc:MGI:2448567]
9007	ENSMUSG000000052407	1.060396	2.593785	12.87486	0.000333	0.008765	Ccdc171	coiled-coil domain containing 171 [Source:MGI Symbol;Acc:MGI:1922152]
								regulatory factor X, 3 (influences HLA class II expression) [Source:MGI Symbol;Acc:MGI:106582]
7784	ENSMUSG000000040929	1.058187	3.285377	7.130073	0.00758	0.04744	Rfx3	interferon regulatory factor 7 [Source:MGI Symbol;Acc:MGI:1859212]
3374	ENSMUSG000000025498	1.057344	4.397188	8.764187	0.003072	0.029381	Irf7	G patch domain containing 4 [Source:MGI Symbol;Acc:MGI:1913864]
4284	ENSMUSG000000028069	1.054581	2.498525	14.98775	0.000108	0.004166	Gpatch4	peter pan homolog (Drosophila) [Source:MGI Symbol;Acc:MGI:2178445]
476	ENSMUSG000000004100	1.050061	3.128634	7.837505	0.005117	0.038556	Ppan	guanylate binding protein 5 [Source:MGI Symbol;Acc:MGI:2429943]
10847	ENSMUSG000000010504	1.049109	3.907246	7.008902	0.008111	0.049188	Gbp5	zinc finger CCCH type, antiviral 1 [Source:MGI Symbol;Acc:MGI:1926031]
4979	ENSMUSG000000029826	1.049098	6.359494	6.996436	0.008167	0.049302	Zc3hav1	RIKEN cDNA 4930503L19 gene [Source:MGI Symbol;Acc:MGI:1922045]
8323	ENSMUSG000000044906	1.048769	2.647129	9.894498	0.001658	0.021606	4930503L19Rik	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) [Source:MGI Symbol;Acc:MGI:1918764]
								zinc finger protein 292 [Source:MGI Symbol;Acc:MGI:1353423]
4578	ENSMUSG000000028820	1.042088	6.13166	9.084113	0.002578	0.026952	Sfpq	poly (ADP-ribose) polymerase family, member 11 [Source:MGI Symbol;Acc:MGI:2141505]
7564	ENSMUSG000000039967	1.04184	6.469009	8.079444	0.004477	0.035895	Zfp292	nucleolar protein 9 [Source:MGI Symbol;Acc:MGI:1921285]
7050	ENSMUSG000000037997	1.041349	3.647948	7.32191	0.006812	0.044956	Parp11	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor [Source:MGI Symbol;Acc:MGI:1917689]
4633	ENSMUSG000000028948	1.039465	3.628463	11.27861	0.000784	0.014883	Nol9	RCSD domain containing 1 [Source:MGI Symbol;Acc:MGI:2676394]
								K(lysine) acetyltransferase 6A [Source:MGI Symbol;Acc:MGI:2442415]
1801	ENSMUSG000000020680	1.038791	3.782636	7.14876	0.007502	0.04718	Taf15	mediator of DNA damage checkpoint 1 [Source:MGI Symbol;Acc:MGI:3525201]
7744	ENSMUSG000000040723	1.038552	5.891315	8.088191	0.004455	0.035828	Rcsd1	zinc finger protein 800 [Source:MGI Symbol;Acc:MGI:1889334]
5505	ENSMUSG000000031540	1.03417	6.488024	8.019992	0.004626	0.03637	Kat6a	G protein-coupled receptor kinase-interactor 2 [Source:MGI Symbol;Acc:MGI:1347053]
9713	ENSMUSG000000061607	1.032869	4.682607	7.684328	0.00557	0.040194	Mdc1	PIN2/TERF1 interacting, telomerase inhibitor 1 [Source:MGI Symbol;Acc:MGI:1919650]
7533	ENSMUSG000000039841	1.02706	4.458822	9.464341	0.002095	0.024276	Zfp800	Hspb associated protein 1 [Source:MGI Symbol;Acc:MGI:1913917]
7955	ENSMUSG000000041890	1.026919	5.617079	7.623382	0.005762	0.040898	Git2	zinc finger, DHHC domain containing 20 [Source:MGI Symbol;Acc:MGI:1923215]
2282	ENSMUSG000000021958	1.024267	1.996538	8.106468	0.004411	0.035626	Pinx1	interferon gamma induced GTPase [Source:MGI Symbol;Acc:MGI:107729]
2641	ENSMUSG000000022849	1.018496	1.832152	9.587721	0.001959	0.023301	Hspbap1	interleukin 6 receptor, alpha [Source:MGI Symbol;Acc:MGI:105304]
2287	ENSMUSG000000021969	1.016469	5.199899	8.397871	0.003757	0.032824	Zdhhc20	predicted gene 17167 [Source:MGI Symbol;Acc:MGI:4937994]
10460	ENSMUSG000000078853	1.016091	3.874008	8.714031	0.003158	0.029815	Igtp	
4231	ENSMUSG000000027947	1.013934	4.66549	11.51806	0.000689	0.013693	Il6ra	
10678	ENSMUSG000000091542	1.013315	2.197513	9.665775	0.001877	0.022999	Gm17167	

913	ENSMUSG00000011256	1.010876	4.501535	11.85786	0.000574	0.012389	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta) [Source:MGI Symbol;Acc:MGI:105377]
10850	ENSMUSG000000105987	1.003879	4.775954	12.1614	0.000488	0.011131	AI506816	expressed sequence AI506816 [Source:MGI Symbol;Acc:MGI:2140929]
6197	ENSMUSG000000033799	1.002544	5.859263	7.102157	0.007699	0.047915	Fam208b	family with sequence similarity 208, member B [Source:MGI Symbol;Acc:MGI:2145274]
6673	ENSMUSG000000036202	1.001268	5.799346	10.26194	0.001358	0.019651	Rif1	Rap1 interacting factor 1 homolog (yeast) [Source:MGI Symbol;Acc:MGI:1098622]
3561	ENSMUSG000000026098	1.001069	2.659486	7.900889	0.004941	0.037781	Pms1	postmeiotic segregation increased 1 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1202302]
7640	ENSMUSG000000040296	0.997558	5.421274	8.899732	0.002852	0.028431	Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 [Source:MGI Symbol;Acc:MGI:2442858]
10474	ENSMUSG000000078942	0.995813	2.557009	10.79001	0.00102	0.016915	Naip6	NLR family, apoptosis inhibitory protein 6 [Source:MGI Symbol;Acc:MGI:1298222]
7685	ENSMUSG000000040481	0.989651	6.675838	7.58313	0.005892	0.041337	Bptf	bromodomain PHD finger transcription factor [Source:MGI Symbol;Acc:MGI:2444008]
10116	ENSMUSG000000070056	0.988939	3.622228	7.244288	0.007113	0.04575	Mfhas1	malignant fibrous histiocytoma amplified sequence 1 [Source:MGI Symbol;Acc:MGI:1098644]
8205	ENSMUSG000000043671	0.988911	2.810448	7.063219	0.007868	0.048409	Dpy19l3	dpy-19-like 3 (C. elegans) [Source:MGI Symbol;Acc:MGI:2443952]
8254	ENSMUSG000000044167	0.988281	5.739663	8.12703	0.004361	0.035485	Foxo1	forkhead box O1 [Source:MGI Symbol;Acc:MGI:1890077]
2168	ENSMUSG000000021676	0.986246	4.891459	13.92938	0.00019	0.006065	Iqgap2	IQ motif containing GTPase activating protein 2 [Source:MGI Symbol;Acc:MGI:2449975]
5070	ENSMUSG000000030213	0.983666	6.458544	8.191643	0.004208	0.034673	Atf7ip	activating transcription factor 7 interacting protein [Source:MGI Symbol;Acc:MGI:1858965]
2817	ENSMUSG000000023991	0.983526	3.634469	7.217205	0.007221	0.046064	Foxp4	forkhead box P4 [Source:MGI Symbol;Acc:MGI:1921373]
5542	ENSMUSG000000031627	0.982845	4.700055	10.03265	0.001538	0.020765	Irf2	interferon regulatory factor 2 [Source:MGI Symbol;Acc:MGI:96591]
2421	ENSMUSG000000022313	0.977703	2.384654	8.037551	0.004582	0.036228	Utp23	UTP23, small subunit (SSU) processome component, homolog (yeast) [Source:MGI Symbol;Acc:MGI:1925831]
6620	ENSMUSG000000035929	0.975539	5.495231	8.281299	0.004006	0.033738	H2-Q4	histocompatibility 2, Q region locus 4 [Source:MGI Symbol;Acc:MGI:95933]
7192	ENSMUSG000000038500	0.974449	1.863636	10.03474	0.001536	0.020765	Prr3	proline-rich polypeptide 3 [Source:MGI Symbol;Acc:MGI:1922460]
8937	ENSMUSG000000051413	0.973844	3.606794	7.193476	0.007317	0.046406	Plagl2	pleiomorphic adenoma gene-like 2 [Source:MGI Symbol;Acc:MGI:1933165]
274	ENSMUSG000000002325	0.973176	4.831543	7.524573	0.006086	0.042159	Irf9	interferon regulatory factor 9 [Source:MGI Symbol;Acc:MGI:107587]
7975	ENSMUSG000000041992	0.971559	3.345032	10.41378	0.001251	0.01885	Rapgef5	Rap guanine nucleotide exchange factor (GEF) 5 [Source:MGI Symbol;Acc:MGI:2444365]
4051	ENSMUSG000000027405	0.968341	4.674488	7.041758	0.007963	0.048641	Nop56	NOP56 ribonucleoprotein [Source:MGI Symbol;Acc:MGI:1914384]
3536	ENSMUSG000000026029	0.966295	4.739969	7.698103	0.005528	0.040048	Casp8	caspase 8 [Source:MGI Symbol;Acc:MGI:1261423]
10382	ENSMUSG000000075327	0.965298	3.364298	8.788222	0.003032	0.029234	Zbtb2	zinc finger and BTB domain containing 2 [Source:MGI Symbol;Acc:MGI:2685949]
5789	ENSMUSG000000032253	0.964026	7.114001	7.535488	0.00605	0.041985	Phip	pleckstrin homology domain interacting protein [Source:MGI Symbol;Acc:MGI:1932404]
9047	ENSMUSG000000052812	0.963403	4.572464	8.528373	0.003497	0.031381	Atad2b	ATPase family, AAA domain containing 2B [Source:MGI Symbol;Acc:MGI:2444798]
3565	ENSMUSG000000026104	0.960085	4.894007	7.162087	0.007446	0.046897	Stat1	signal transducer and activator of transcription 1 [Source:MGI Symbol;Acc:MGI:103063]
5741	ENSMUSG000000032103	0.953474	3.093348	8.990185	0.002714	0.027666	Pus3	pseudouridine synthase 3 [Source:MGI Symbol;Acc:MGI:1914299]
5008	ENSMUSG000000030019	0.953107	3.836998	7.391342	0.006554	0.044051	Fbxl14	F-box and leucine-rich repeat protein 14 [Source:MGI Symbol;Acc:MGI:2141676]
3507	ENSMUSG000000025958	0.95252	6.1183	9.303055	0.002288	0.02523	Creb1	cAMP responsive element binding protein 1 [Source:MGI Symbol;Acc:MGI:88494]
9952	ENSMUSG000000066415	0.951658	5.208311	7.737133	0.00541	0.039699	Msl2	male-specific lethal 2 homolog (Drosophila) [Source:MGI Symbol;Acc:MGI:1925103]
10487	ENSMUSG000000079065	0.949213	3.61716	10.98598	0.000918	0.016148	BC005561	cDNA sequence BC005561 [Source:MGI Symbol;Acc:MGI:3040669]
10492	ENSMUSG000000079109	0.948391	2.647808	8.782587	0.003041	0.029246	Pms2	postmeiotic segregation increased 2 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:104288]
610	ENSMUSG000000005621	0.946371	5.222527	8.660627	0.003252	0.030338	Zfp592	zinc finger protein 592 [Source:MGI Symbol;Acc:MGI:2443541]
7228	ENSMUSG000000038612	0.945967	7.316571	9.60076	0.001945	0.023263	Mcl1	myeloid cell leukemia sequence 1 [Source:MGI Symbol;Acc:MGI:101769]
4976	ENSMUSG000000029817	0.945231	5.16433	9.442419	0.00212	0.024369	Tra2a	transformer 2 alpha homolog (Drosophila) [Source:MGI Symbol;Acc:MGI:1933972]
3768	ENSMUSG000000026657	0.944238	4.175411	11.09854	0.000864	0.015878	Frmd4a	FERM domain containing 4A [Source:MGI Symbol;Acc:MGI:1919850]
5522	ENSMUSG000000031578	0.942831	4.322908	9.775138	0.001769	0.02234	Mak16	MAK16 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1915170]

10756	ENSMUSG00000097354	0.942002	2.541782	8.859564	0.002916	0.028753	Z310001H17Rik	RIKEN cDNA 2310001H17 gene [Source:MGI Symbol;Acc:MGI:1923682]
9321	ENSMUSG00000056268	0.939881	5.65819	8.509234	0.003533	0.031584	Dennd1b	DENN/MADD domain containing 1B [Source:MGI Symbol;Acc:MGI:2447812]
6313	ENSMUSG00000034333	0.874214	2.931967	8.964537	0.002753	0.027929	Zbed4	zinc finger, BED type containing 4 [Source:MGI Symbol;Acc:MGI:2682302]
4452	ENSMUSG00000028495	0.87314	7.849468	7.912878	0.004908	0.037705	Rps6	ribosomal protein S6 [Source:MGI Symbol;Acc:MGI:98159]
7548	ENSMUSG00000039908	0.872625	1.994746	11.97975	0.000538	0.011887	Slc26a11	solute carrier family 26, member 11 [Source:MGI Symbol;Acc:MGI:2444589]
373	ENSMUSG00000003134	0.870514	3.96092	7.403473	0.00651	0.043974	Tbc1d8	TBC1 domain family, member 8 [Source:MGI Symbol;Acc:MGI:1927225]
8497	ENSMUSG000000046721	0.869925	2.948487	9.494304	0.002061	0.024071	Rpl14-ps1	ribosomal protein L14, pseudogene 1 [Source:MGI Symbol;Acc:MGI:3710579]
7019	ENSMUSG00000037876	0.869878	7.016032	8.27291	0.004024	0.033806	Jmjd1c	jumonji domain containing 1C [Source:MGI Symbol;Acc:MGI:1918614]
2346	ENSMUSG00000022120	0.869609	2.415579	9.948742	0.00161	0.021258	Rnf219	ring finger protein 219 [Source:MGI Symbol;Acc:MGI:1919736]
370	ENSMUSG00000003119	0.866525	5.868629	8.333339	0.003892	0.033187	Cdk12	cyclin-dependent kinase 12 [Source:MGI Symbol;Acc:MGI:1098802]
6384	ENSMUSG00000034652	0.865145	2.872488	9.659613	0.001884	0.023035	Cd300a	CD300A antigen [Source:MGI Symbol;Acc:MGI:2443411]
6819	ENSMUSG00000036943	0.86481	5.575457	7.610159	0.005804	0.041025	Rab8b	RAB8B, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:2442982]
5259	ENSMUSG00000030779	0.863746	6.234035	7.836632	0.00512	0.038556	Rbbp6	retinoblastoma binding protein 6 [Source:MGI Symbol;Acc:MGI:894835]
								glutathione S-transferase, C-terminal domain containing [Source:MGI Symbol;Acc:MGI:1914803]
4259	ENSMUSG00000028018	0.862558	2.089297	7.614382	0.00579	0.040969	Gstcd	SDA1 domain containing 1 [Source:MGI Symbol;Acc:MGI:2140779]
4824	ENSMUSG00000029415	0.856506	4.478002	8.459289	0.003632	0.032147	Sdad1	synaptotagmin-like 2 [Source:MGI Symbol;Acc:MGI:1933366]
5198	ENSMUSG00000030616	0.854201	3.474072	11.45827	0.000712	0.013974	Syt12	interferon-induced protein 44 [Source:MGI Symbol;Acc:MGI:2443016]
4270	ENSMUSG00000028037	0.853966	2.811322	11.5615	0.000673	0.013586	Ifi44	nucleoporin 153 [Source:MGI Symbol;Acc:MGI:2385621]
2062	ENSMUSG00000021374	0.85268	6.216513	9.593286	0.001953	0.023296	Nup153	enhancer of polycomb homolog 1 (Drosophila) [Source:MGI Symbol;Acc:MGI:1278322]
2920	ENSMUSG00000024240	0.851574	4.752652	9.142815	0.002497	0.026415	Epc1	RNA binding motif protein 5 [Source:MGI Symbol;Acc:MGI:1933204]
5938	ENSMUSG00000032580	0.848286	6.654375	11.33744	0.00076	0.014649	Rbm5	rosbin, round spermatid basic protein 1 [Source:MGI Symbol;Acc:MGI:2444993]
8247	ENSMUSG00000044098	0.847808	5.004876	8.744935	0.003105	0.029544	Rsbn1	RCC1 domain containing 1 [Source:MGI Symbol;Acc:MGI:2444156]
7314	ENSMUSG00000038930	0.844143	2.136227	7.418922	0.006454	0.043653	Rccd1	polymerase (DNA-directed), delta 3, accessory subunit [Source:MGI Symbol;Acc:MGI:1915217]
5234	ENSMUSG00000030726	0.84336	3.858555	10.38863	0.001268	0.018979	Pold3	SKI-like [Source:MGI Symbol;Acc:MGI:106203]
4140	ENSMUSG00000027660	0.841536	5.592291	7.483126	0.006228	0.042706	Skil	E1A binding protein p300 [Source:MGI Symbol;Acc:MGI:1276116]
9228	ENSMUSG00000055024	0.83889	6.424894	7.042876	0.007958	0.048641	Ep300	WD repeat domain 31 [Source:MGI Symbol;Acc:MGI:1918604]
4398	ENSMUSG00000028391	0.838268	1.931752	7.078291	0.007802	0.048167	Wdr31	putative homeodomain transcription factor 1 [Source:MGI Symbol;Acc:MGI:1332671]
9474	ENSMUSG00000058388	0.835548	3.667399	10.29215	0.001336	0.019575	Phtf1	zinc finger (CCCH type), RNA binding motif and serine/arginine rich 2 [Source:MGI Symbol;Acc:MGI:103287]
5436	ENSMUSG00000031370	0.835381	3.096436	8.369417	0.003816	0.032993	Zrsr2	cyclin L1 [Source:MGI Symbol;Acc:MGI:1922664]
4194	ENSMUSG00000027829	0.834069	5.456439	7.449961	0.006344	0.04312	Ccnl1	PR domain containing 2, with ZNF domain [Source:MGI Symbol;Acc:MGI:107628]
9425	ENSMUSG00000057637	0.827786	5.557006	10.12964	0.001459	0.020342	Prdm2	centrosomal protein 162 [Source:MGI Symbol;Acc:MGI:1925343]
9364	ENSMUSG00000056919	0.827285	4.193887	8.836612	0.002952	0.028861	Cep162	E2F transcription factor 4 [Source:MGI Symbol;Acc:MGI:103012]
1023	ENSMUSG00000014859	0.824226	4.192869	9.913862	0.00164	0.021431	E2f4	predicted gene, 20186 [Source:MGI Symbol;Acc:MGI:5012371]
10863	ENSMUSG000000106874	0.822573	5.55086	7.619715	0.005773	0.040928	Gm20186	zinc finger protein 281 [Source:MGI Symbol;Acc:MGI:3029290]
7877	ENSMUSG00000041483	0.821473	4.696098	11.76443	0.000604	0.012675	Zfp281	cryptochrome 1 (photolyase-like) [Source:MGI Symbol;Acc:MGI:1270841]
1530	ENSMUSG00000020038	0.819085	2.356982	7.45223	0.006336	0.04312	Cry1	polypyrimidine tract binding protein 3 [Source:MGI Symbol;Acc:MGI:1923334]
4395	ENSMUSG00000028382	0.817136	7.71518	7.379845	0.006596	0.044214	Ptbp3	RNA binding motif protein 41 [Source:MGI Symbol;Acc:MGI:2444923]
5460	ENSMUSG00000031433	0.814424	3.386978	10.63595	0.001109	0.01776	Rbm41	

3531	ENSMUSG00000026020	0.812256	4.527924	10.64369	0.001104	0.017724	Nop58	NOP58 ribonucleoprotein [Source:MGI Symbol;Acc:MGI:1933184] B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB [Source:MGI Symbol;Acc:MGI:1347077]
8788	ENSMUSG00000049658	0.811574	5.908635	11.79053	0.000595	0.012617	Bdp1	DAZ interacting protein 1 [Source:MGI Symbol;Acc:MGI:1914311]
8004	ENSMUSG00000042156	0.8097	3.143701	13.09408	0.000296	0.008049	Dzip1	WD repeat domain 44 [Source:MGI Symbol;Acc:MGI:1919654]
6778	ENSMUSG00000036769	0.808097	4.392561	22.10919	2.58E-06	0.000294	Wdr44	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B [Source:MGI Symbol;Acc:MGI:1914878]
4382	ENSMUSG00000028333	0.805369	3.95931	11.54504	0.000679	0.013631	Anp32b	SAFB-like, transcription modulator [Source:MGI Symbol;Acc:MGI:1913910]
5772	ENSMUSG00000032212	0.803707	5.071068	7.632114	0.005734	0.040814	Sltm	DNA cross-link repair 1C, PSO2 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:2441769]
3765	ENSMUSG00000026648	0.803365	3.893365	8.824946	0.002971	0.028896	Dclre1c	digestive organ expansion factor homolog (zebrafish) [Source:MGI Symbol;Acc:MGI:2138080]
1137	ENSMUSG00000016181	0.802851	2.923801	8.908699	0.002838	0.028397	Diexf	tripartite motif-containing 26 [Source:MGI Symbol;Acc:MGI:1337056]
2997	ENSMUSG00000024457	0.800225	4.641245	7.470707	0.006271	0.042886	Trim26	C-type lectin domain family 1, member a [Source:MGI Symbol;Acc:MGI:2444151]
6057	ENSMUSG00000033082	0.800039	3.575059	9.032765	0.002652	0.027336	Clec1a	solute carrier family 8 (sodium/calcium exchanger), member 1 [Source:MGI Symbol;Acc:MGI:107956]
9195	ENSMUSG00000054640	0.799919	3.255722	12.46888	0.000414	0.009997	Slc8a1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1 [Source:MGI Symbol;Acc:MGI:1926953]
6104	ENSMUSG00000033323	0.798651	3.727393	8.271843	0.004026	0.033806	Ctdp1	additional sex combs like 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1922552]
6944	ENSMUSG00000037486	0.79799	5.511522	7.311018	0.006853	0.045037	Asxl2	tumor necrosis factor receptor superfamily, member 11a, NFkB activator [Source:MGI Symbol;Acc:MGI:1314891]
3647	ENSMUSG00000026321	0.794004	3.139912	10.82353	0.001002	0.016748	Tnfrsf11a	RIKEN cDNA 2210018M11 gene [Source:MGI Symbol;Acc:MGI:1924203]
6533	ENSMUSG00000035401	0.79201	5.36402	9.144	0.002495	0.026415	2210018M11Rik	integrator complex subunit 8 [Source:MGI Symbol;Acc:MGI:1919906]
7750	ENSMUSG00000040738	0.790558	4.611361	10.50086	0.001193	0.01852	Ints8	pre B cell leukemia homeobox 3 [Source:MGI Symbol;Acc:MGI:97496]
7258	ENSMUSG00000038718	0.789083	2.293357	11.05352	0.000885	0.015945	Pbx3	RNA binding motif protein 25 [Source:MGI Symbol;Acc:MGI:1914289]
896	ENSMUSG00000010608	0.788914	6.574069	8.120447	0.004377	0.035485	Rbm25	zinc finger and SCAN domain containing 22 [Source:MGI Symbol;Acc:MGI:2443312]
9204	ENSMUSG00000054715	0.786192	2.355502	7.874716	0.005013	0.038091	Zscan22	CREB regulated transcription coactivator 2 [Source:MGI Symbol;Acc:MGI:1921593]
4226	ENSMUSG00000027936	0.786144	4.02158	9.714782	0.001828	0.022728	Crtc2	PRP39 pre-mRNA processing factor 39 homolog (yeast) [Source:MGI Symbol;Acc:MGI:104602]
6563	ENSMUSG00000035597	0.785663	5.097385	7.774929	0.005298	0.039267	Prpf39	receptor (TNFRSF)-interacting serine-threonine kinase 1 [Source:MGI Symbol;Acc:MGI:108212]
2072	ENSMUSG00000021408	0.785644	4.580764	11.32556	0.000764	0.014673	Ripk1	dynein light chain LC8-type 1 [Source:MGI Symbol;Acc:MGI:1861457]
822	ENSMUSG00000009013	0.785482	5.11369	11.11047	0.000858	0.015837	Dynll1	CREB binding protein [Source:MGI Symbol;Acc:MGI:1098280]
2513	ENSMUSG00000022521	0.783346	6.704137	7.056883	0.007896	0.048489	Crebbp	zinc finger protein 26 [Source:MGI Symbol;Acc:MGI:99173]
9819	ENSMUSG000000063108	0.78294	5.104377	7.052109	0.007917	0.04854	Zfp26	teneurin transmembrane protein 3 [Source:MGI Symbol;Acc:MGI:1345183]
5515	ENSMUSG000000031561	0.782873	3.223059	8.525067	0.003503	0.031388	Tenm3	RIKEN cDNA 4933421O10 gene [Source:MGI Symbol;Acc:MGI:1918323]
10565	ENSMUSG000000085037	0.782436	2.007222	7.853783	0.005071	0.038368	4933421O10Rik	protein arginine N-methyltransferase 3 [Source:MGI Symbol;Acc:MGI:1919224]
5152	ENSMUSG000000030505	0.778891	3.698422	7.348813	0.006711	0.044555	Prmt3	formin-like 2 [Source:MGI Symbol;Acc:MGI:1918659]
6644	ENSMUSG000000036053	0.777808	4.533479	10.52953	0.001175	0.018377	Fmn12	zinc finger protein 1 [Source:MGI Symbol;Acc:MGI:99154]
9282	ENSMUSG000000055835	0.777237	2.863048	9.9304	0.001626	0.021386	Zfp1	caspase activity and apoptosis inhibitor 1 [Source:MGI Symbol;Acc:MGI:1915020]
4482	ENSMUSG000000028578	0.777154	2.444604	7.829985	0.005139	0.038588	Caap1	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D [Source:MGI Symbol;Acc:MGI:1922566]
5675	ENSMUSG000000031939	0.773576	4.191769	7.74143	0.005397	0.039679	Taf1d	

10598	ENSMUSG000000087259	0.770782	1.961749	7.601503	0.005832	0.041103	2610035D17Rik	RIKEN cDNA 2610035D17 gene [Source:MGI Symbol;Acc:MGI:1919636] TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor [Source:MGI Symbol;Acc:MGI:2388097]
3444	ENSMUSG000000025782	0.769693	3.066752	8.583801	0.003392	0.031014	Taf3	N(alpha)-acetyltransferase 16, NatA auxiliary subunit [Source:MGI Symbol;Acc:MGI:1914147]
2310	ENSMUSG000000022020	0.768362	3.410154	7.647928	0.005684	0.040583	Naa16	AT hook containing transcription factor 1 [Source:MGI Symbol;Acc:MGI:1915033]
3705	ENSMUSG000000026491	0.76443	6.21014	10.44791	0.001228	0.018738	Ahctf1	heat shock 105kDa/110kDa protein 1 [Source:MGI Symbol;Acc:MGI:105053]
4923	ENSMUSG000000029657	0.763666	4.433354	10.2322	0.00138	0.019811	Hsph1	leucine rich repeat (in FLII) interacting protein 1 [Source:MGI Symbol;Acc:MGI:1342770]
3638	ENSMUSG000000026305	0.763067	5.850207	8.025231	0.004613	0.036317	Lrrfip1	URB2 ribosome biogenesis 2 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:2681124]
5688	ENSMUSG000000031976	0.762457	3.143028	9.269751	0.00233	0.025334	Urb2	Janus kinase 2 [Source:MGI Symbol;Acc:MGI:96629]
3107	ENSMUSG000000024789	0.760948	6.159203	10.41717	0.001248	0.01885	Jak2	protein kinase C, theta [Source:MGI Symbol;Acc:MGI:97601]
3815	ENSMUSG000000026778	0.760834	4.891354	6.967877	0.008299	0.049804	Prkcq	aryl hydrocarbon receptor nuclear translocator-like [Source:MGI Symbol;Acc:MGI:1096381]
9235	ENSMUSG000000055116	0.759989	3.071512	9.840645	0.001707	0.021933	Arntl	RIKEN cDNA 5830417110 gene [Source:MGI Symbol;Acc:MGI:1923272]
10448	ENSMUSG000000078684	0.759564	3.67564	11.74056	0.000612	0.012693	5830417110Rik	transgelin 2 [Source:MGI Symbol;Acc:MGI:1312985]
3724	ENSMUSG0000000026547	0.759303	5.7989	11.43397	0.000721	0.014107	Tagln2	apoptotic chromatin condensation inducer 1 [Source:MGI Symbol;Acc:MGI:1891824]
2369	ENSMUSG000000022185	0.758796	6.117274	10.12771	0.001461	0.020342	Acin1	chemokine (C-X-C motif) ligand 12 [Source:MGI Symbol;Acc:MGI:103556]
9690	ENSMUSG000000061353	0.748555	6.531952	12.63587	0.000378	0.0095	Cxcl12	REX4, RNA exonuclease 4 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:2684957]
9006	ENSMUSG000000052406	0.746585	4.006708	7.130495	0.007578	0.04744	Rexo4	RNA binding motif protein 26 [Source:MGI Symbol;Acc:MGI:1921463]
2345	ENSMUSG000000022119	0.744619	5.523066	7.089189	0.007755	0.048065	Rbm26	filamin A interacting protein 1-like [Source:MGI Symbol;Acc:MGI:1925999]
8186	ENSMUSG000000043336	0.739757	6.516347	8.477045	0.003597	0.031923	Filip1l	transcription termination factor, RNA polymerase I [Source:MGI Symbol;Acc:MGI:105044]
3828	ENSMUSG000000026803	0.739143	3.806875	7.306058	0.006872	0.045108	Ttf1	zinc finger protein 638 [Source:MGI Symbol;Acc:MGI:1203484]
5007	ENSMUSG000000030016	0.738696	6.662095	9.385849	0.002187	0.024615	Zfp638	RING1 and YY1 binding protein [Source:MGI Symbol;Acc:MGI:1929059]
10247	ENSMUSG000000072872	0.737387	3.802833	9.291945	0.002302	0.025249	Rybp	ArfGAP with dual PH domains 2 [Source:MGI Symbol;Acc:MGI:2663075]
1815	ENSMUSG000000020709	0.732446	3.274665	8.599673	0.003362	0.030879	Adap2	LUC7-like 3 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1914934]
1876	ENSMUSG000000020863	0.729111	5.822992	8.717392	0.003152	0.029786	Luc7l3	DEAH (Asp-Glu-Ala-His) box polypeptide 15 [Source:MGI Symbol;Acc:MGI:1099786]
4733	ENSMUSG000000029169	0.728405	6.401583	7.72419	0.005449	0.039792	Dhx15	zinc finger protein 747 [Source:MGI Symbol;Acc:MGI:2443581]
9170	ENSMUSG000000054381	0.724874	2.044782	9.004264	0.002694	0.027583	Zfp747	rearranged L-myc fusion sequence [Source:MGI Symbol;Acc:MGI:1924705]
8810	ENSMUSG000000049878	0.724373	5.084909	7.311447	0.006852	0.045037	Rlf	zinc finger and BTB domain containing 11 [Source:MGI Symbol;Acc:MGI:2443876]
2547	ENSMUSG000000022601	0.72326	4.391751	7.379247	0.006598	0.044214	Zbtb11	metal response element binding transcription factor 2 [Source:MGI Symbol;Acc:MGI:105050]
4775	ENSMUSG000000029267	0.721455	4.581084	7.816502	0.005177	0.038771	Mtf2	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 [Source:MGI Symbol;Acc:MGI:1920591]
5363	ENSMUSG000000031133	0.720366	6.095266	10.12615	0.001462	0.020342	Arhgef6	SUMO-interacting motifs containing 1 [Source:MGI Symbol;Acc:MGI:2442599]
8172	ENSMUSG000000043183	0.719448	3.010843	9.921392	0.001634	0.021386	Simc1	tripartite motif-containing 56 [Source:MGI Symbol;Acc:MGI:2685298]
8181	ENSMUSG000000043279	0.717093	5.88417	7.680639	0.005582	0.040223	Trim56	RIO kinase 1 (yeast) [Source:MGI Symbol;Acc:MGI:1918590]
2081	ENSMUSG000000021428	0.716275	3.955045	9.066326	0.002604	0.027044	Riok1	cyclin K [Source:MGI Symbol;Acc:MGI:1276106]
2025	ENSMUSG000000021258	0.711282	3.977865	12.52817	0.000401	0.009837	Ccnk	pogo transposable element with ZNF domain [Source:MGI Symbol;Acc:MGI:2442117]
7310	ENSMUSG000000038902	0.70982	4.46527	7.081681	0.007788	0.048158	Pogz	CDC-like kinase 1 [Source:MGI Symbol;Acc:MGI:107403]
3539	ENSMUSG000000026034	0.709665	6.868293	8.131027	0.004351	0.035462	Clk1	SET domain, bifurcated 1 [Source:MGI Symbol;Acc:MGI:1934229]
1096	ENSMUSG000000015697	0.70901	4.483177	8.134545	0.004343	0.035446	Setdb1	trinucleotide repeat containing 6C [Source:MGI Symbol;Acc:MGI:2443265]
3396	ENSMUSG000000025571	0.708714	5.605828	7.147131	0.007508	0.04718	Tnrc6c	ELK3, member of ETS oncogene family [Source:MGI Symbol;Acc:MGI:101762]
799	ENSMUSG000000008398	0.706048	4.431983	7.049207	0.00793	0.04857	Elk3	cytochrome P450, family 1, subfamily b, polypeptide 1 [Source:MGI Symbol;Acc:MGI:88590]
2862	ENSMUSG000000024087	0.70445	5.429261	8.897782	0.002855	0.028431	Cyp1b1	

5652	ENSMUSG000000031885	0.702044	5.906237	10.5512	0.001161	0.018256	Cbfb	core binding factor beta [Source:MGI Symbol;Acc:MGI:99851]
1679	ENSMUSG000000020392	0.69868	4.278238	8.366311	0.003822	0.032993	Cdkn2aipnl	CDKN2A interacting protein N-terminal like [Source:MGI Symbol;Acc:MGI:1261797]
2082	ENSMUSG000000021431	0.698316	3.378153	8.073432	0.004492	0.035895	Snrnp48	small nuclear ribonucleoprotein 48 (U11/U12) [Source:MGI Symbol;Acc:MGI:1915047]
9193	ENSMUSG000000054611	0.696096	6.620127	8.647695	0.003275	0.030436	Kdm2a	lysine (K)-specific demethylase 2A [Source:MGI Symbol;Acc:MGI:1354736]
7848	ENSMUSG000000041328	0.693332	6.199374	7.727397	0.005439	0.039773	Pcf11	cleavage and polyadenylation factor subunit homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1919579]
3007	ENSMUSG000000024491	0.692851	5.258565	7.350255	0.006705	0.04455	Rbm27	RNA binding motif protein 27 [Source:MGI Symbol;Acc:MGI:2147194]
7497	ENSMUSG000000039697	0.692076	3.91571	9.412343	0.002155	0.024496	Ncoa7	nuclear receptor coactivator 7 [Source:MGI Symbol;Acc:MGI:2444847]
10499	ENSMUSG000000079184	0.690677	3.925005	8.588352	0.003383	0.031014	Mphosph8	M-phase phosphoprotein 8 [Source:MGI Symbol;Acc:MGI:1922589]
3684	ENSMUSG000000026436	0.687242	6.423075	7.347545	0.006715	0.044555	Elk4	ELK4, member of ETS oncogene family [Source:MGI Symbol;Acc:MGI:102853]
1044	ENSMUSG000000015127	0.684773	3.612851	8.351484	0.003854	0.033062	Unkl	unkempt-like (Drosophila) [Source:MGI Symbol;Acc:MGI:1921404]
7675	ENSMUSG000000040447	0.682933	4.766505	12.31803	0.000449	0.010512	Spns2	spinster homolog 2 [Source:MGI Symbol;Acc:MGI:2384936]
7209	ENSMUSG000000038535	0.682807	5.278929	7.374334	0.006616	0.044268	Zfp280d	zinc finger protein 280D [Source:MGI Symbol;Acc:MGI:2384583]
4734	ENSMUSG000000029171	0.682344	3.175011	11.94466	0.000548	0.011971	Pgm1	phosphoglucomutase 1 [Source:MGI Symbol;Acc:MGI:97564]
6303	ENSMUSG000000034297	0.680622	7.272168	7.270217	0.007011	0.045524	Med13	mediator complex subunit 13 [Source:MGI Symbol;Acc:MGI:3029632]
2452	ENSMUSG000000022387	0.678242	5.53903	10.50954	0.001188	0.018486	Brd1	bromodomain containing 1 [Source:MGI Symbol;Acc:MGI:1924161]
10083	ENSMUSG000000069495	0.677277	5.112109	9.19848	0.002422	0.02589	Epc2	enhancer of polycomb homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1278321]
2428	ENSMUSG000000022335	0.676403	2.274211	7.806269	0.005207	0.038871	Zfat	zinc finger and AT hook domain containing [Source:MGI Symbol;Acc:MGI:2681865]
1292	ENSMUSG000000018412	0.672152	5.6728	7.72306	0.005452	0.039792	Kansl1	KAT8 regulatory NSL complex subunit 1 [Source:MGI Symbol;Acc:MGI:1923969]
7801	ENSMUSG000000041057	0.66677	5.214644	10.78015	0.001026	0.016963	Wdr43	WD repeat domain 43 [Source:MGI Symbol;Acc:MGI:1919765]
1206	ENSMUSG000000017421	0.665948	6.824502	8.44689	0.003657	0.032273	Zfp207	zinc finger protein 207 [Source:MGI Symbol;Acc:MGI:1340045]
9506	ENSMUSG000000058761	0.662016	4.479012	8.234324	0.004111	0.034145	Rnf169	ring finger protein 169 [Source:MGI Symbol;Acc:MGI:1920257]
9925	ENSMUSG000000065265	0.660321	2.512839	10.47493	0.00121	0.018651	Gm23455	predicted gene, 23455 [Source:MGI Symbol;Acc:MGI:5453232]
1164	ENSMUSG000000016552	0.657301	3.13756	10.35909	0.001288	0.019192	Foxred2	FAD-dependent oxidoreductase domain containing 2 [Source:MGI Symbol;Acc:MGI:106315]
10276	ENSMUSG000000073555	0.656689	3.365425	8.257141	0.004059	0.03397	Gm4951	predicted gene 4951 [Source:MGI Symbol;Acc:MGI:3644953]
6382	ENSMUSG000000034647	0.656498	5.837189	7.262688	0.00704	0.045604	Ankrd12	ankyrin repeat domain 12 [Source:MGI Symbol;Acc:MGI:1914357]
4459	ENSMUSG000000028522	0.656418	5.956405	11.36105	0.00075	0.014541	Mier1	mesoderm induction early response 1 homolog (Xenopus laevis) [Source:MGI Symbol;Acc:MGI:1918398]
2780	ENSMUSG000000023852	0.655875	6.249351	7.131209	0.007575	0.04744	Chd1	chromodomain helicase DNA binding protein 1 [Source:MGI Symbol;Acc:MGI:88393]
7278	ENSMUSG000000038784	0.653917	5.19775	13.22129	0.000277	0.007752	Cnot4	CCR4-NOT transcription complex, subunit 4 [Source:MGI Symbol;Acc:MGI:1859026]
10203	ENSMUSG000000071645	0.653721	3.354495	7.711463	0.005487	0.039931	Tut1	terminal uridylyl transferase 1, U6 snRNA-specific [Source:MGI Symbol;Acc:MGI:1917294]
8177	ENSMUSG000000043257	0.653339	2.471803	7.109201	0.007669	0.047858	Pigv	phosphatidylinositol glycan anchor biosynthesis, class V [Source:MGI Symbol;Acc:MGI:2442480]
5564	ENSMUSG000000031681	0.650617	4.236287	7.165456	0.007432	0.046836	Smad1	SMAD family member 1 [Source:MGI Symbol;Acc:MGI:109452]
10194	ENSMUSG000000071456	0.645178	2.899374	7.318023	0.006827	0.044961	1110002L01Rik	RIKEN cDNA 1110002L01 gene [Source:MGI Symbol;Acc:MGI:1915821]
3634	ENSMUSG000000026283	0.642656	3.874305	7.059159	0.007886	0.048489	Ing5	inhibitor of growth family, member 5 [Source:MGI Symbol;Acc:MGI:1922816]
3199	ENSMUSG000000024999	0.640594	3.820825	9.176988	0.002451	0.026114	Noc3l	nucleolar complex associated 3 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1932610]
6168	ENSMUSG000000033671	0.640181	6.872132	7.176316	0.007387	0.046689	Cep350	centrosomal protein 350 [Source:MGI Symbol;Acc:MGI:1921331]

8418	ENSMUSG00000045932	0.639679	4.372898	7.084052	0.007777	0.048121	Ifit2	interferon-induced protein with tetratricopeptide repeats 2 [Source:MGI Symbol;Acc:MGI:99449]
927	ENSMUSG00000011960	0.63786	5.406496	10.63264	0.001111	0.01776	Ccnt1	cyclin T1 [Source:MGI Symbol;Acc:MGI:1328363]
1925	ENSMUSG00000020994	0.637148	4.960826	8.122726	0.004371	0.035485	Pnn	pinin [Source:MGI Symbol;Acc:MGI:1100514]
9599	ENSMUSG00000060090	0.632033	4.920697	7.539615	0.006036	0.041942	Rp2	retinitis pigmentosa 2 homolog (human) [Source:MGI Symbol;Acc:MGI:1277953]
10321	ENSMUSG00000074221	0.627118	3.557815	11.39375	0.000737	0.014364	Zfp568	zinc finger protein 568 [Source:MGI Symbol;Acc:MGI:2142347]
6788	ENSMUSG00000036819	0.626977	3.354538	12.48101	0.000411	0.009961	Jmjd4	jumonji domain containing 4 [Source:MGI Symbol;Acc:MGI:2144404]
4478	ENSMUSG00000028572	0.625311	4.05489	10.52801	0.001176	0.018377	Hook1	hook homolog 1 (Drosophila) [Source:MGI Symbol;Acc:MGI:1925213]
4668	ENSMUSG00000029014	0.622909	4.540596	7.796177	0.005236	0.039021	Dnajc2	Dnaj (Hsp40) homolog, subfamily C, member 2 [Source:MGI Symbol;Acc:MGI:99470]
5395	ENSMUSG00000031229	0.622002	7.580554	7.069687	0.00784	0.048289	Atrx	alpha thalassemia/mental retardation syndrome X-linked homolog (human) [Source:MGI Symbol;Acc:MGI:103067]
8094	ENSMUSG00000042599	0.621738	6.514813	8.171637	0.004255	0.034938	Kdm7a	lysine (K)-specific demethylase 7A [Source:MGI Symbol;Acc:MGI:2443388]
2726	ENSMUSG00000023087	0.618687	5.602472	9.185977	0.002439	0.026026	Noct	nocturnin [Source:MGI Symbol;Acc:MGI:109382]
9378	ENSMUSG00000057113	0.618585	7.337117	7.174039	0.007397	0.046694	Npm1	nucleophosmin 1 [Source:MGI Symbol;Acc:MGI:106184]
7149	ENSMUSG00000038335	0.61813	4.28139	8.271718	0.004027	0.033806	Tsr1	TSR1 20S rRNA accumulation [Source:MGI Symbol;Acc:MGI:2144566]
6103	ENSMUSG00000033319	0.617419	4.38421	7.782463	0.005276	0.039237	Fem1c	fem-1 homolog c (C.elegans) [Source:MGI Symbol;Acc:MGI:2444737]
7180	ENSMUSG00000038446	0.616993	4.788655	9.436091	0.002128	0.024379	Cdc40	cell division cycle 40 [Source:MGI Symbol;Acc:MGI:1918963]
4761	ENSMUSG00000029227	0.61571	5.514606	7.332331	0.006773	0.044778	Fip1l1	FIP1 like 1 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1914149]
5056	ENSMUSG00000030157	0.612614	7.005513	10.30929	0.001324	0.019489	Clec2d	C-type lectin domain family 2, member d [Source:MGI Symbol;Acc:MGI:2135589]
2290	ENSMUSG00000021975	0.606986	3.699641	8.27011	0.00403	0.033806	Ints9	integrator complex subunit 9 [Source:MGI Symbol;Acc:MGI:1098533]
6874	ENSMUSG00000037174	0.606525	5.548368	8.955603	0.002766	0.028038	Elf2	E74-like factor 2 [Source:MGI Symbol;Acc:MGI:1916507]
1975	ENSMUSG00000021127	0.605718	6.408605	7.018459	0.008067	0.049025	Zfp36l1	zinc finger protein 36, C3H type-like 1 [Source:MGI Symbol;Acc:MGI:107946]
3702	ENSMUSG00000026484	0.596696	4.849142	11.48619	0.000701	0.01384	Rnf2	ring finger protein 2 [Source:MGI Symbol;Acc:MGI:1101759]
6566	ENSMUSG00000035614	0.595694	5.106204	18.31475	1.87E-05	0.001153	Fam179b	family with sequence similarity 179, member B [Source:MGI Symbol;Acc:MGI:2684313]
7449	ENSMUSG00000039473	0.586763	5.602852	11.35141	0.000754	0.014565	Ubn1	ubinnuclein 1 [Source:MGI Symbol;Acc:MGI:1891307]
7627	ENSMUSG00000040253	0.584739	5.656186	12.79881	0.000347	0.008955	Gbp7	guanylate binding protein 7 [Source:MGI Symbol;Acc:MGI:2444421]
9893	ENSMUSG00000064127	0.584319	5.422847	6.959371	0.008338	0.049919	Med14	mediator complex subunit 14 [Source:MGI Symbol;Acc:MGI:1349442]
2482	ENSMUSG00000022462	0.583989	6.071649	7.261704	0.007044	0.045604	Slc38a2	solute carrier family 38, member 2 [Source:MGI Symbol;Acc:MGI:1915010]
8354	ENSMUSG00000045216	0.583205	4.052965	12.36536	0.000437	0.01036	Hs6st1	heparan sulfate 6-O-sulfotransferase 1 [Source:MGI Symbol;Acc:MGI:1354958]
7395	ENSMUSG00000039220	0.578415	5.13269	7.490523	0.006202	0.042604	Ppp1r10	protein phosphatase 1, regulatory subunit 10 [Source:MGI Symbol;Acc:MGI:1289273]
741	ENSMUSG00000007038	0.57748	3.901289	13.69887	0.000215	0.00654	Neu1	neuraminidase 1 [Source:MGI Symbol;Acc:MGI:97305]
6965	ENSMUSG00000037608	0.575013	6.959842	11.60838	0.000657	0.013322	Bclaf1	BCL2-associated transcription factor 1 [Source:MGI Symbol;Acc:MGI:1917580]
5734	ENSMUSG00000032078	0.568306	3.599326	8.345343	0.003867	0.033071	Zpr1	ZPR1 zinc finger [Source:MGI Symbol;Acc:MGI:1330262]
4332	ENSMUSG00000028187	0.568054	4.043618	7.754839	0.005357	0.03956	Rpf1	ribosome production factor 1 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1917535]
662	ENSMUSG00000006169	0.565003	5.576405	7.822388	0.00516	0.038724	Clint1	clathrin interactor 1 [Source:MGI Symbol;Acc:MGI:2144243]
10231	ENSMUSG00000072501	0.559279	6.184304	7.07348	0.007823	0.048269	Phf20l1	PHD finger protein 20-like 1 [Source:MGI Symbol;Acc:MGI:2444412]
6233	ENSMUSG00000033972	0.557856	3.420244	7.532813	0.006059	0.04202	Zfp944	zinc finger protein 944 [Source:MGI Symbol;Acc:MGI:2442394]
3614	ENSMUSG00000026234	0.554062	6.872496	8.245319	0.004086	0.034056	Ncl	nucleolin [Source:MGI Symbol;Acc:MGI:97286]
1261	ENSMUSG00000018160	0.551281	5.617747	6.962492	0.008324	0.04989	Med1	mediator complex subunit 1 [Source:MGI Symbol;Acc:MGI:1100846]

7017	ENSMUSG000000037857	0.549313	6.160105	9.926868	0.001629	0.021386	Nufip2	nuclear fragile X mental retardation protein interacting protein 2 [Source:MGI Symbol;Acc:MGI:1915814]
4555	ENSMUSG000000028760	0.545689	6.17616	7.608825	0.005808	0.041025	Eif4g3	eukaryotic translation initiation factor 4 gamma, 3 [Source:MGI Symbol;Acc:MGI:1923935]
7676	ENSMUSG000000040451	0.542946	5.182427	8.271075	0.004028	0.033806	Sgms1	sphingomyelin synthase 1 [Source:MGI Symbol;Acc:MGI:2444110]
5825	ENSMUSG000000032332	0.542565	4.996285	7.494056	0.00619	0.042582	Col12a1	collagen, type XII, alpha 1 [Source:MGI Symbol;Acc:MGI:88448]
4186	ENSMUSG000000027806	0.539433	5.0103	7.576932	0.005912	0.041399	Tsc22d2	TSC22 domain family, member 2 [Source:MGI Symbol;Acc:MGI:1919283]
9214	ENSMUSG000000054792	0.538748	3.526174	7.787298	0.005261	0.039159	Klhl18	kelch-like 18 [Source:MGI Symbol;Acc:MGI:2143315]
2795	ENSMUSG000000023932	0.537916	5.271382	8.280763	0.004007	0.033738	Cdc5l	cell division cycle 5-like (S. pombe) [Source:MGI Symbol;Acc:MGI:1918952]
6225	ENSMUSG000000033943	0.537161	6.859104	7.110616	0.007663	0.047858	Mga	MAX gene associated [Source:MGI Symbol;Acc:MGI:1352483]
5058	ENSMUSG000000030166	0.532138	3.190314	7.241939	0.007122	0.045764	Rad52	RAD52 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:101949]
7609	ENSMUSG000000040167	0.532089	4.139455	8.294303	0.003977	0.033624	Ikzf5	IKAROS family zinc finger 5 [Source:MGI Symbol;Acc:MGI:1914393]
6385	ENSMUSG000000034653	0.531442	4.089149	14.04408	0.000179	0.005896	Ythdc2	YTH domain containing 2 [Source:MGI Symbol;Acc:MGI:2448561]
2935	ENSMUSG000000024283	0.529321	6.102289	7.371544	0.006626	0.044268	Wac	WW domain containing adaptor with coiled-coil [Source:MGI Symbol;Acc:MGI:2387357]
6503	ENSMUSG000000035248	0.525735	6.250017	8.012665	0.004645	0.036412	Zcchc6	zinc finger, CCHC domain containing 6 [Source:MGI Symbol;Acc:MGI:2387179]
1240	ENSMUSG000000017781	0.524887	5.832083	8.482146	0.003586	0.031875	Pitpna	phosphatidylinositol transfer protein, alpha [Source:MGI Symbol;Acc:MGI:99887]
8943	ENSMUSG000000051495	0.524689	5.516908	9.171096	0.002459	0.026114	Irf2bp2	interferon regulatory factor 2 binding protein 2 [Source:MGI Symbol;Acc:MGI:2443921]
6717	ENSMUSG000000036437	0.523829	2.225661	7.833494	0.005129	0.038556	Npy1r	neuropeptide Y receptor Y1 [Source:MGI Symbol;Acc:MGI:104963]
3	ENSMUSG000000000078	0.521302	6.320868	7.594643	0.005854	0.041153	Klf6	Kruppel-like factor 6 [Source:MGI Symbol;Acc:MGI:1346318]
1079	ENSMUSG000000015501	0.515994	6.186779	9.115622	0.002534	0.026608	Hivep2	human immunodeficiency virus type I enhancer binding protein 2 [Source:MGI Symbol;Acc:MGI:1338076]
9718	ENSMUSG000000061689	0.511873	4.465798	9.410288	0.002158	0.024496	Dlgap4	discs, large homolog-associated protein 4 (Drosophila) [Source:MGI Symbol;Acc:MGI:2138865]
8079	ENSMUSG000000042520	0.510427	6.435064	8.402378	0.003747	0.032822	Ubp2l	ubiquitin-associated protein 2-like [Source:MGI Symbol;Acc:MGI:1921633]
1543	ENSMUSG000000020074	0.50874	5.527135	7.329383	0.006784	0.044797	Ccar1	cell division cycle and apoptosis regulator 1 [Source:MGI Symbol;Acc:MGI:1914750]
4449	ENSMUSG000000028484	0.508604	5.270456	11.42944	0.000723	0.014116	Psip1	PC4 and SFRS1 interacting protein 1 [Source:MGI Symbol;Acc:MGI:2142116]
2397	ENSMUSG000000022247	0.503556	3.493368	7.232344	0.00716	0.045855	Brix1	BRX1, biogenesis of ribosomes, homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1915082]
10543	ENSMUSG000000081049	0.503528	2.259199	7.859979	0.005054	0.038296	Rps24-ps3	ribosomal protein S24, pseudogene 3 [Source:MGI Symbol;Acc:MGI:3644407]
8249	ENSMUSG000000044147	0.500991	6.298384	7.350428	0.006705	0.04455	Arf6	ADP-ribosylation factor 6 [Source:MGI Symbol;Acc:MGI:99435]
9723	ENSMUSG000000061755	0.498382	6.495677	7.847618	0.005089	0.038452	Bod1l	bioorientation of chromosomes in cell division 1-like [Source:MGI Symbol;Acc:MGI:2444804]
47	ENSMUSG000000000532	0.497391	4.178649	7.688986	0.005556	0.040144	Acvr1b	activin A receptor, type 1B [Source:MGI Symbol;Acc:MGI:1338944]
7620	ENSMUSG000000040225	0.490841	7.715995	7.946385	0.004818	0.037394	Prrc2c	proline-rich coiled-coil 2C [Source:MGI Symbol;Acc:MGI:1913754]
1702	ENSMUSG000000020454	0.487841	4.885052	8.485894	0.003579	0.031861	Eif4enif1	eukaryotic translation initiation factor 4E nuclear import factor 1 [Source:MGI Symbol;Acc:MGI:1921453]
6833	ENSMUSG000000036990	0.487442	6.375353	13.75637	0.000208	0.006428	Otd4	OTU domain containing 4 [Source:MGI Symbol;Acc:MGI:1098801]
517	ENSMUSG000000004633	0.485765	1.901597	13.22509	0.000276	0.007752	Chn2	chimerin 2 [Source:MGI Symbol;Acc:MGI:1917243]
1793	ENSMUSG000000020659	0.485595	3.942043	7.766488	0.005322	0.039398	Cbl1l	Casitas B-lineage lymphoma-like 1 [Source:MGI Symbol;Acc:MGI:2144842]
3523	ENSMUSG000000025997	0.483228	4.41347	7.23867	0.007135	0.045812	Ikzf2	IKAROS family zinc finger 2 [Source:MGI Symbol;Acc:MGI:1342541]
4362	ENSMUSG000000028268	0.482409	4.8508	7.300439	0.006894	0.045154	Gbp3	guanylate binding protein 3 [Source:MGI Symbol;Acc:MGI:1926263]
7470	ENSMUSG000000039585	0.479759	6.17777	7.070502	0.007836	0.048289	Myo9a	myosin IXa [Source:MGI Symbol;Acc:MGI:107735]

942	ENSMUSG00000012640	0.477247	4.386889	8.10299	0.004419	0.035643	Zfp715	zinc finger protein 715 [Source:MGI Symbol;Acc:MGI:1917180]
1091	ENSMUSG00000015656	0.473557	8.766477	8.184719	0.004224	0.034766	Hspa8	heat shock protein 8 [Source:MGI Symbol;Acc:MGI:105384]
4551	ENSMUSG00000028745	0.47062	5.925194	11.05314	0.000885	0.015945	Capzb	capping protein (actin filament) muscle Z-line, beta [Source:MGI Symbol;Acc:MGI:104652]
5338	ENSMUSG00000031016	0.468002	1.935346	9.727228	0.001816	0.022686	Wee1	WEE 1 homolog 1 (S. pombe) [Source:MGI Symbol;Acc:MGI:103075]
10776	ENSMUSG00000098557	0.465465	6.656272	7.199862	0.007291	0.046372	Kctd12	potassium channel tetramerisation domain containing 12 [Source:MGI Symbol;Acc:MGI:2145823]
7369	ENSMUSG00000039156	0.464609	4.57057	10.89277	0.000965	0.016494	Stim2	stromal interaction molecule 2 [Source:MGI Symbol;Acc:MGI:2151156]
6290	ENSMUSG00000034252	0.454576	6.136656	7.2587	0.007056	0.045635	Senp6	SUMO/sentrin specific peptidase 6 [Source:MGI Symbol;Acc:MGI:1922075]
650	ENSMUSG00000006005	0.44997	7.716168	7.869287	0.005028	0.038152	Tpr	translocated promoter region, nuclear basket protein [Source:MGI Symbol;Acc:MGI:1922066]
8175	ENSMUSG00000043241	0.449001	5.111385	8.465506	0.003619	0.032063	Upf2	UPF2 regulator of nonsense transcripts homolog (yeast) [Source:MGI Symbol;Acc:MGI:2449307]
7394	ENSMUSG00000039219	0.442627	5.443971	7.711733	0.005486	0.039931	Arid4b	AT rich interactive domain 4B (RBP1-like) [Source:MGI Symbol;Acc:MGI:2137512]
1105	ENSMUSG00000015755	0.442258	6.274195	8.567314	0.003423	0.031102	Tab2	TGF-beta activated kinase 1/MAP3K7 binding protein 2 [Source:MGI Symbol;Acc:MGI:1915902]
9766	ENSMUSG00000062328	0.440089	8.124714	8.429026	0.003693	0.032528	Rpl17	ribosomal protein L17 [Source:MGI Symbol;Acc:MGI:2448270]
8522	ENSMUSG00000046865	0.435014	3.08038	7.174952	0.007393	0.046694	Fbl	fibrillarin [Source:MGI Symbol;Acc:MGI:95486]
1519	ENSMUSG00000020015	0.432476	5.137066	7.736851	0.005411	0.039699	Cdk17	cyclin-dependent kinase 17 [Source:MGI Symbol;Acc:MGI:97517]
2857	ENSMUSG00000024081	0.432113	5.213431	8.24479	0.004087	0.034056	Cebpz	CCAAT/enhancer binding protein zeta [Source:MGI Symbol;Acc:MGI:109386]
5968	ENSMUSG00000032690	0.429939	3.026051	10.84715	0.000989	0.016689	Oas2	2'-5' oligoadenylate synthetase 2 [Source:MGI Symbol;Acc:MGI:2180852]
6875	ENSMUSG00000037197	0.427901	4.946476	7.932443	0.004856	0.037469	Rbm17	RNA binding motif protein 17 [Source:MGI Symbol;Acc:MGI:1924188]
6027	ENSMUSG00000032939	0.427401	3.078833	9.173595	0.002455	0.026114	Nup93	nucleoporin 93 [Source:MGI Symbol;Acc:MGI:1919055]
4184	ENSMUSG00000027804	0.425517	5.0136	8.12194	0.004373	0.035485	Ppid	peptidylprolyl isomerase D (cyclophilin D) [Source:MGI Symbol;Acc:MGI:1914988]
1012	ENSMUSG00000014763	0.421369	4.520276	7.464711	0.006292	0.042929	Fam120b	family with sequence similarity 120, member B [Source:MGI Symbol;Acc:MGI:1914794]
5047	ENSMUSG00000030107	0.412176	3.097262	7.718005	0.005467	0.039847	Usp18	ubiquitin specific peptidase 18 [Source:MGI Symbol;Acc:MGI:1344364]
5733	ENSMUSG00000032077	0.409638	2.840047	19.5232	9.94E-06	0.000737	Bud13	BUD13 homolog (yeast) [Source:MGI Symbol;Acc:MGI:2443443]
9150	ENSMUSG00000054072	0.405515	5.872792	7.741608	0.005396	0.039679	ligp1	interferon inducible GTPase 1 [Source:MGI Symbol;Acc:MGI:1926259]
8404	ENSMUSG00000045763	0.399488	3.552967	13.44952	0.000245	0.00714	Basp1	brain abundant, membrane attached signal protein 1 [Source:MGI Symbol;Acc:MGI:1917600]
757	ENSMUSG00000007613	0.39229	5.573821	8.848108	0.002934	0.028802	Tgfbr1	transforming growth factor, beta receptor I [Source:MGI Symbol;Acc:MGI:98728]
5977	ENSMUSG00000032727	0.391291	4.807628	7.015256	0.008082	0.049075	Mier3	mesoderm induction early response 1, family member 3 [Source:MGI Symbol;Acc:MGI:2442317]
1013	ENSMUSG00000014767	0.389337	3.990556	13.06517	0.000301	0.008114	Tbp	TATA box binding protein [Source:MGI Symbol;Acc:MGI:101838]
1953	ENSMUSG00000021068	0.3868	5.694764	9.463359	0.002096	0.024276	Nin	ninein [Source:MGI Symbol;Acc:MGI:105108]
3313	ENSMUSG00000025314	0.384262	6.216106	7.805187	0.00521	0.038871	Ptptrj	protein tyrosine phosphatase, receptor type, J [Source:MGI Symbol;Acc:MGI:104574]
8439	ENSMUSG00000046138	0.383287	5.161608	8.919251	0.002822	0.028299	9930021J03Rik	RIKEN cDNA 9930021J03 gene [Source:MGI Symbol;Acc:MGI:2444398]
3730	ENSMUSG00000026565	0.381725	5.197065	7.098879	0.007713	0.047915	Pou2f1	POU domain, class 2, transcription factor 1 [Source:MGI Symbol;Acc:MGI:101898]
5489	ENSMUSG00000031513	0.371574	4.147179	7.742327	0.005394	0.039679	Leprotl1	leptin receptor overlapping transcript-like 1 [Source:MGI Symbol;Acc:MGI:1915442]
8190	ENSMUSG00000043415	0.369037	2.406722	9.649298	0.001894	0.023113	Otd1	OTU domain containing 1 [Source:MGI Symbol;Acc:MGI:1918448]
10545	ENSMUSG00000081094	0.356349	2.897649	9.29054	0.002303	0.025249	Rpl19-ps11	ribosomal protein L19, pseudogene 11 [Source:MGI Symbol;Acc:MGI:3642193]
5535	ENSMUSG00000031608	0.341123	3.438939	8.823973	0.002973	0.028896	Galnt7	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7 [Source:MGI Symbol;Acc:MGI:1349449]

6770	ENSMUSG000000036712	0.338586	5.829333	8.003446	0.004669	0.036572	Cyld	cylindromatosis (turban tumor syndrome) [Source:MGI Symbol;Acc:MGI:1921506]
290	ENSMUSG000000002428	0.336048	5.019168	7.14624	0.007512	0.04718	Hltf	helicase-like transcription factor [Source:MGI Symbol;Acc:MGI:1196437]
1856	ENSMUSG000000020806	0.32336	3.401374	8.946126	0.002781	0.02812	Rhbdf2	rhomboid 5 homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:2442473]
8639	ENSMUSG000000048039	0.315748	3.863972	7.120013	0.007623	0.047652	Isg20l2	interferon stimulated exonuclease gene 20-like 2 [Source:MGI Symbol;Acc:MGI:2140076]
8345	ENSMUSG000000045128	0.31321	8.034678	9.131906	0.002512	0.026521	Rpl18a	ribosomal protein L18A [Source:MGI Symbol;Acc:MGI:1924058]
2548	ENSMUSG000000022604	0.285297	3.494464	8.304905	0.003954	0.033526	Cep97	centrosomal protein 97 [Source:MGI Symbol;Acc:MGI:1921451]
7048	ENSMUSG000000037992	0.282759	3.945003	9.234204	0.002375	0.0256	Rara	retinoic acid receptor, alpha [Source:MGI Symbol;Acc:MGI:97856]
3946	ENSMUSG000000027130	0.277684	5.122801	7.982658	0.004723	0.036862	Slc12a6	solute carrier family 12, member 6 [Source:MGI Symbol;Acc:MGI:2135960]
3022	ENSMUSG000000024534	0.270071	1.7465	9.409272	0.002159	0.024496	Sncap	synuclein, alpha interacting protein (synphilin) [Source:MGI Symbol;Acc:MGI:1915097]
716	ENSMUSG000000006678	0.267534	3.571527	8.567541	0.003422	0.031102	Pola1	polymerase (DNA directed), alpha 1 [Source:MGI Symbol;Acc:MGI:99660]

Appendix 2. Differentially Expressed Genes Downregulated in Mice Fed High Folic Acid during the Juvenile Period

1899	ENSMUSG00000020917	-3.1258	9.710161	13.52869	0.000235	0.006938	Acly	ATP citrate lyase [Source:MGI Symbol;Acc:MGI:103251]
6423	ENSMUSG00000034853	-2.99763	5.720653	7.928921	0.004865	0.037516	Acot11	acyl-CoA thioesterase 11 [Source:MGI Symbol;Acc:MGI:1913736]
8510	ENSMUSG00000046794	-2.8694	4.512623	14.66338	0.000129	0.004763	Ppp1r3b	protein phosphatase 1, regulatory (inhibitor) subunit 3B [Source:MGI Symbol;Acc:MGI:2177268]
6980	ENSMUSG00000037686	-2.7664	3.681234	7.992208	0.004698	0.036694	Aspg	asparaginase homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:2144822]
4118	ENSMUSG00000027605	-2.63932	6.844697	17.7994	2.45E-05	0.001379	Acss2	acyl-CoA synthetase short-chain family member 2 [Source:MGI Symbol;Acc:MGI:1890410]
7829	ENSMUSG00000041220	-2.61972	8.792145	10.94759	0.000937	0.016314	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast) [Source:MGI Symbol;Acc:MGI:2156528]
422	ENSMUSG00000003528	-2.52678	7.506797	19.64524	9.32E-06	0.000701	Slc25a1	solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 [Source:MGI Symbol;Acc:MGI:1345283]
3498	ENSMUSG00000025934	-2.47654	2.741168	60.85536	6.14E-15	1.67E-11	Gsta3	glutathione S-transferase, alpha 3 [Source:MGI Symbol;Acc:MGI:95856]
6579	ENSMUSG00000035686	-2.40495	8.685336	20.43801	6.16E-06	0.000516	Thrsp	thyroid hormone responsive [Source:MGI Symbol;Acc:MGI:109126]
7780	ENSMUSG00000040907	-2.37909	3.251368	14.00969	0.000182	0.005933	Atp1a3	ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide [Source:MGI Symbol;Acc:MGI:88107]
1742	ENSMUSG00000020532	-2.35886	10.40299	19.093	1.25E-05	0.00087	Acaca	acetyl-Coenzyme A carboxylase alpha [Source:MGI Symbol;Acc:MGI:108451]
5870	ENSMUSG00000032418	-2.35783	8.386963	11.08216	0.000872	0.015901	Me1	malic enzyme 1, NADP(+)-dependent, cytosolic [Source:MGI Symbol;Acc:MGI:97043]
4307	ENSMUSG00000028116	-2.34864	4.774428	20.73426	5.28E-06	0.000467	Myoz2	myozenin 2 [Source:MGI Symbol;Acc:MGI:1913063]
1954	ENSMUSG00000021069	-2.31461	7.298861	16.8955	3.95E-05	0.001992	Pygl	liver glycogen phosphorylase [Source:MGI Symbol;Acc:MGI:97829]
2281	ENSMUSG00000021957	-2.26003	9.258589	14.41473	0.000147	0.005256	Tkt	transketolase [Source:MGI Symbol;Acc:MGI:105992]
3251	ENSMUSG00000025153	-2.2585	12.18972	20.78749	5.13E-06	0.000458	Fasn	fatty acid synthase [Source:MGI Symbol;Acc:MGI:95485]
4849	ENSMUSG00000029467	-2.21382	7.586125	36.88156	1.26E-09	7.60E-07	Atp2a2	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2 [Source:MGI Symbol;Acc:MGI:88110]
4204	ENSMUSG00000027861	-2.20199	3.080572	40.56507	1.90E-10	1.59E-07	Casq2	calsequestrin 2 [Source:MGI Symbol;Acc:MGI:1309469]
7362	ENSMUSG00000039126	-2.20131	5.045289	23.3564	1.35E-06	0.000201	Prune2	prune homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1925004]
156	ENSMUSG00000001420	-2.18625	3.063979	23.11367	1.53E-06	0.000216	Tmem79	transmembrane protein 79 [Source:MGI Symbol;Acc:MGI:1919163]
5081	ENSMUSG00000030244	-2.16274	3.580783	17.3886	3.05E-05	0.001635	Gys2	glycogen synthase 2 [Source:MGI Symbol;Acc:MGI:2385254]
8167	ENSMUSG00000043122	-2.12447	5.745437	70.98479	3.60E-17	1.96E-13	A530016L24Rik	RIKEN cDNA A530016L24 gene [Source:MGI Symbol;Acc:MGI:2443020]
3879	ENSMUSG00000026922	-2.08116	7.446977	26.31996	2.89E-07	6.29E-05	Agpat2	1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) [Source:MGI Symbol;Acc:MGI:1914762]
787	ENSMUSG00000008153	-2.04421	3.808185	10.00799	0.001559	0.02082	Clstn3	calsyntenin 3 [Source:MGI Symbol;Acc:MGI:2178323]
29	ENSMUSG00000000326	-1.97605	6.409027	19.39231	1.06E-05	0.000773	Comt	catechol-O-methyltransferase [Source:MGI Symbol;Acc:MGI:88470]

7579	ENSMUSG00000040016	-1.94994	5.573683	44.18643	2.99E-11	4.07E-08	Ptger3	prostaglandin E receptor 3 (subtype EP3) [Source:MGI Symbol;Acc:MGI:97795]
5244	ENSMUSG00000030747	-1.94649	7.940379	31.46986	2.03E-08	7.34E-06	Dgat2	diacylglycerol O-acyltransferase 2 [Source:MGI Symbol;Acc:MGI:1915050]
2700	ENSMUSG00000023019	-1.90967	9.443283	7.999583	0.004679	0.036598	Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble) [Source:MGI Symbol;Acc:MGI:95679]
1633	ENSMUSG00000020277	-1.89965	5.81342	24.80403	6.35E-07	0.000115	Pfkl	phosphofructokinase, liver, B-type [Source:MGI Symbol;Acc:MGI:97547]
10419	ENSMUSG00000078234	-1.88474	5.715739	42.97631	5.54E-11	6.04E-08	Klhdc7a	kelch domain containing 7A [Source:MGI Symbol;Acc:MGI:2444612]
6868	ENSMUSG00000037139	-1.85725	4.608043	20.03559	7.60E-06	0.0006	Myom3	myomesin family, member 3 [Source:MGI Symbol;Acc:MGI:2685280]
9860	ENSMUSG00000063684	-1.84932	2.690598	17.81232	2.44E-05	0.001379	Gm13910	predicted gene 13910 [Source:MGI Symbol;Acc:MGI:3651143]
4023	ENSMUSG00000027327	-1.84123	4.916486	18.39728	1.79E-05	0.001116	1700037H04Rik	RIKEN cDNA 1700037H04 gene [Source:MGI Symbol;Acc:MGI:1914576]
9900	ENSMUSG00000064225	-1.81871	4.852182	21.0908	4.38E-06	0.000411	Paqr9	progesterin and adipoQ receptor family member IX [Source:MGI Symbol;Acc:MGI:1922802]
7904	ENSMUSG00000041653	-1.81844	5.595562	34.92487	3.43E-09	1.78E-06	Pnpla3	patatin-like phospholipase domain containing 3 [Source:MGI Symbol;Acc:MGI:2151796]
8360	ENSMUSG00000045294	-1.81206	6.839017	47.91911	4.44E-12	8.07E-09	Insig1	insulin induced gene 1 [Source:MGI Symbol;Acc:MGI:1916289]
915	ENSMUSG00000011305	-1.81063	3.637666	10.91349	0.000955	0.016433	Plin5	perilipin 5 [Source:MGI Symbol;Acc:MGI:1914218]
3985	ENSMUSG00000027223	-1.80092	2.987915	12.11332	0.000501	0.011294	Mapk8ip1	mitogen-activated protein kinase 8 interacting protein 1 [Source:MGI Symbol;Acc:MGI:1309464]
2778	ENSMUSG00000023832	-1.78804	4.286338	22.2289	2.42E-06	0.000287	Acat2	acetyl-Coenzyme A acetyltransferase 2 [Source:MGI Symbol;Acc:MGI:87871]
2524	ENSMUSG00000022546	-1.78135	4.294425	27.77971	1.36E-07	3.70E-05	Gpt	glutamic pyruvic transaminase, soluble [Source:MGI Symbol;Acc:MGI:95802]
4642	ENSMUSG00000028961	-1.77022	5.765046	12.73369	0.000359	0.009164	Pgd	phosphogluconate dehydrogenase [Source:MGI Symbol;Acc:MGI:97553]
8874	ENSMUSG00000050737	-1.7684	4.419058	24.21228	8.63E-07	0.000147	Ptges	prostaglandin E synthase [Source:MGI Symbol;Acc:MGI:1927593]
6913	ENSMUSG00000037353	-1.75904	5.432551	9.823059	0.001723	0.02196	Letmd1	LETM1 domain containing 1 [Source:MGI Symbol;Acc:MGI:1915864]
3147	ENSMUSG00000024892	-1.75473	8.708622	24.04427	9.41E-07	0.000155	Pcx	pyruvate carboxylase [Source:MGI Symbol;Acc:MGI:97520]
4261	ENSMUSG00000028024	-1.74584	5.947484	21.53424	3.48E-06	0.000354	Enpep	glutamyl aminopeptidase [Source:MGI Symbol;Acc:MGI:106645]
3274	ENSMUSG00000025203	-1.74221	7.012673	32.67855	1.09E-08	4.56E-06	Scd2	stearoyl-Coenzyme A desaturase 2 [Source:MGI Symbol;Acc:MGI:98240]
2450	ENSMUSG00000022383	-1.73762	4.000641	11.27306	0.000786	0.014902	Ppara	peroxisome proliferator activated receptor alpha [Source:MGI Symbol;Acc:MGI:104740]
3088	ENSMUSG00000024747	-1.72082	4.659946	15.1219	0.000101	0.00395	Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7 [Source:MGI Symbol;Acc:MGI:1347050]
6804	ENSMUSG00000036880	-1.7184	6.854046	9.531574	0.00202	0.023739	Acaa2	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) [Source:MGI Symbol;Acc:MGI:1098623]
4859	ENSMUSG00000029482	-1.71549	5.584742	26.22841	3.03E-07	6.36E-05	Aacs	acetoacetyl-CoA synthetase [Source:MGI Symbol;Acc:MGI:1926144]
1959	ENSMUSG00000021094	-1.71401	5.490061	14.72662	0.000124	0.004622	Dhrs7	dehydrogenase/reductase (SDR family) member 7 [Source:MGI Symbol;Acc:MGI:1913625]
9178	ENSMUSG00000054434	-1.70033	4.909533	23.23915	1.43E-06	0.000205	Tmem120b	transmembrane protein 120B [Source:MGI Symbol;Acc:MGI:3603158]

3426	ENSMUSG000000025732	-1.68892	3.089197	11.31596	0.000768	0.014689	Fam195a	family with sequence similarity 195, member A [Source:MGI Symbol;Acc:MGI:1915491]
8099	ENSMUSG000000042616	-1.68291	2.555097	16.84278	4.06E-05	0.002002	Oscp1	organic solute carrier partner 1 [Source:MGI Symbol;Acc:MGI:1916308]
2201	ENSMUSG000000021748	-1.67963	7.215709	11.14394	0.000843	0.015649	Pdhb	pyruvate dehydrogenase (lipoamide) beta [Source:MGI Symbol;Acc:MGI:1915513]
9335	ENSMUSG000000056486	-1.67877	3.247275	21.75384	3.10E-06	0.000331	Chn1	chimerin 1 [Source:MGI Symbol;Acc:MGI:1915674]
5897	ENSMUSG000000032492	-1.64778	3.247981	17.73664	2.54E-05	0.00141	Pth1r	parathyroid hormone 1 receptor [Source:MGI Symbol;Acc:MGI:97801]
9252	ENSMUSG000000055407	-1.64587	2.72691	15.6413	7.66E-05	0.003246	Map6	microtubule-associated protein 6 [Source:MGI Symbol;Acc:MGI:1201690]
6851	ENSMUSG000000037071	-1.64348	14.03915	25.45369	4.53E-07	8.66E-05	Scd1	stearyl-Coenzyme A desaturase 1 [Source:MGI Symbol;Acc:MGI:98239]
2380	ENSMUSG000000022206	-1.63405	5.762834	13.31593	0.000263	0.007546	Npr3	natriuretic peptide receptor 3 [Source:MGI Symbol;Acc:MGI:97373]
7117	ENSMUSG000000038217	-1.62826	2.229049	10.93304	0.000945	0.016338	Tlcd2	TLC domain containing 2 [Source:MGI Symbol;Acc:MGI:1917141]
10614	ENSMUSG000000089665	-1.61418	2.455749	23.76882	1.09E-06	0.000174	Fcor	Foxo1 corepressor [Source:MGI Symbol;Acc:MGI:1915484]
3731	ENSMUSG000000026568	-1.61288	5.672489	13.10095	0.000295	0.00804	Mpc2	mitochondrial pyruvate carrier 2 [Source:MGI Symbol;Acc:MGI:1917706]
4092	ENSMUSG000000027533	-1.61189	5.309571	15.29733	9.18E-05	0.003762	Fabp5	fatty acid binding protein 5, epidermal [Source:MGI Symbol;Acc:MGI:101790]
9851	ENSMUSG000000063524	-1.60963	7.13642	15.17027	9.82E-05	0.003921	Eno1	enolase 1, alpha non-neuron [Source:MGI Symbol;Acc:MGI:95393]
3194	ENSMUSG000000024990	-1.60671	4.417832	30.57335	3.21E-08	1.03E-05	Rbp4	retinol binding protein 4, plasma [Source:MGI Symbol;Acc:MGI:97879]
5860	ENSMUSG000000032401	-1.60062	3.533807	13.73064	0.000211	0.006458	Lct1	lactase-like [Source:MGI Symbol;Acc:MGI:2183549]
4233	ENSMUSG000000027952	-1.59818	3.813542	14.36536	0.000151	0.005335	Pmvk	phosphomevalonate kinase [Source:MGI Symbol;Acc:MGI:1915853]
5729	ENSMUSG000000032064	-1.58836	3.649152	31.41001	2.09E-08	7.34E-06	Dixdc1	DIX domain containing 1 [Source:MGI Symbol;Acc:MGI:2679721]
9302	ENSMUSG000000056091	-1.5773	3.11098	48.08998	4.07E-12	8.07E-09	St3gal5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5 [Source:MGI Symbol;Acc:MGI:1339963]
8654	ENSMUSG000000048142	-1.56744	5.182933	19.04677	1.28E-05	0.000879	Nat8l	N-acetyltransferase 8-like [Source:MGI Symbol;Acc:MGI:2447776]
10700	ENSMUSG000000094377	-1.56392	2.688212	11.99616	0.000533	0.01183	Gm24407	predicted gene, 24407 [Source:MGI Symbol;Acc:MGI:5454184]
1316	ENSMUSG000000018574	-1.55474	7.524388	7.934515	0.00485	0.037453	Acadv1	acyl-Coenzyme A dehydrogenase, very long chain [Source:MGI Symbol;Acc:MGI:895149]
8653	ENSMUSG000000048138	-1.55273	3.548617	31.92159	1.61E-08	6.25E-06	Dmrt2	doublesex and mab-3 related transcription factor 2 [Source:MGI Symbol;Acc:MGI:1330307]
9989	ENSMUSG000000067235	-1.55256	5.683686	24.28294	8.32E-07	0.000144	H2-Q10	histocompatibility 2, Q region locus 10 [Source:MGI Symbol;Acc:MGI:95929]
2781	ENSMUSG000000023861	-1.55177	7.724413	8.077198	0.004483	0.035895	Mpc1	mitochondrial pyruvate carrier 1 [Source:MGI Symbol;Acc:MGI:1915240]
5426	ENSMUSG000000031349	-1.54982	4.221263	18.41829	1.77E-05	0.001115	Nsdhl	NAD(P) dependent steroid dehydrogenase-like [Source:MGI Symbol;Acc:MGI:1099438]
5710	ENSMUSG000000032018	-1.54603	5.732056	79.12955	5.82E-19	6.34E-15	Sc5d	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1353611]
3841	ENSMUSG000000026827	-1.53536	7.474199	7.290811	0.006931	0.045282	Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial [Source:MGI Symbol;Acc:MGI:99778]

5599	ENSMUSG00000031767	-1.5343	4.860264	22.50209	2.10E-06	0.000263	Nudt7	nudix (nucleoside diphosphate linked moiety X)-type motif 7 [Source:MGI Symbol;Acc:MGI:1914778]
9548	ENSMUSG00000059447	-1.52544	8.116983	8.987029	0.002719	0.027688	Hadhb	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit [Source:MGI Symbol;Acc:MGI:2136381]
1841	ENSMUSG00000020774	-1.52182	3.599582	24.94449	5.90E-07	0.000109	Aspa	aspartoacylase [Source:MGI Symbol;Acc:MGI:87914]
7384	ENSMUSG00000039196	-1.52057	5.209172	15.13862	9.99E-05	0.00395	Orm1	orosomuroid 1 [Source:MGI Symbol;Acc:MGI:97443]
9899	ENSMUSG00000064215	-1.51844	6.013631	31.78857	1.72E-08	6.46E-06	Ifi27	interferon, alpha-inducible protein 27 [Source:MGI Symbol;Acc:MGI:1277180]
7778	ENSMUSG00000040883	-1.51373	3.12357	16.49559	4.88E-05	0.0023	Tmem205	transmembrane protein 205 [Source:MGI Symbol;Acc:MGI:3045495]
7911	ENSMUSG00000041696	-1.51025	3.677338	33.34778	7.71E-09	3.36E-06	Ras12	RAS-like, family 12 [Source:MGI Symbol;Acc:MGI:1918034]
3666	ENSMUSG00000026385	-1.4957	7.695657	24.69985	6.70E-07	0.00012	Dbi	diazepam binding inhibitor [Source:MGI Symbol;Acc:MGI:94865]
9525	ENSMUSG00000059146	-1.49248	4.358174	15.72989	7.31E-05	0.003141	Ntrk3	neurotrophic tyrosine kinase, receptor, type 3 [Source:MGI Symbol;Acc:MGI:97385]
644	ENSMUSG00000005950	-1.49136	2.643146	10.94452	0.000939	0.016315	P2rx5	purinergic receptor P2X, ligand-gated ion channel, 5 [Source:MGI Symbol;Acc:MGI:2137026]
1746	ENSMUSG00000020538	-1.49126	6.504828	41.36754	1.26E-10	1.25E-07	Srebf1	sterol regulatory element binding transcription factor 1 [Source:MGI Symbol;Acc:MGI:107606]
2586	ENSMUSG00000022707	-1.48676	6.395034	10.70556	0.001068	0.017315	Gbe1	glucan (1,4-alpha-), branching enzyme 1 [Source:MGI Symbol;Acc:MGI:1921435]
3190	ENSMUSG00000024981	-1.47451	6.098291	10.41572	0.001249	0.01885	Acs15	acyl-CoA synthetase long-chain family member 5 [Source:MGI Symbol;Acc:MGI:1919129]
4451	ENSMUSG00000028494	-1.45927	6.954562	14.44883	0.000144	0.005196	Plin2	perilipin 2 [Source:MGI Symbol;Acc:MGI:87920]
5036	ENSMUSG00000030088	-1.45884	5.40205	21.60451	3.35E-06	0.000348	Aldh1l1	aldehyde dehydrogenase 1 family, member L1 [Source:MGI Symbol;Acc:MGI:1340024]
920	ENSMUSG00000011752	-1.45594	6.023544	23.58227	1.20E-06	0.000186	Pgam1	phosphoglycerate mutase 1 [Source:MGI Symbol;Acc:MGI:97552]
6449	ENSMUSG00000034957	-1.45591	6.326963	62.98401	2.08E-15	7.57E-12	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha [Source:MGI Symbol;Acc:MGI:99480]
7425	ENSMUSG00000039349	-1.4545	5.603318	43.59342	4.04E-11	4.89E-08	C130074G19Rik	RIKEN cDNA C130074G19 gene [Source:MGI Symbol;Acc:MGI:2444831]
7560	ENSMUSG00000039956	-1.45356	5.161671	18.75526	1.49E-05	0.000975	Mrap	melanocortin 2 receptor accessory protein [Source:MGI Symbol;Acc:MGI:1924287]
6043	ENSMUSG00000033022	-1.45193	7.843306	19.91424	8.10E-06	0.00063	Cdo1	cysteine dioxygenase 1, cytosolic [Source:MGI Symbol;Acc:MGI:105925]
301	ENSMUSG00000002550	-1.44402	5.479373	26.89692	2.15E-07	4.87E-05	Uck1	uridine-cytidine kinase 1 [Source:MGI Symbol;Acc:MGI:98904]
7912	ENSMUSG00000041697	-1.44218	6.400148	14.32358	0.000154	0.005376	Cox6a1	cytochrome c oxidase subunit VIa polypeptide 1 [Source:MGI Symbol;Acc:MGI:103099]
6180	ENSMUSG00000033720	-1.44194	3.812446	21.58338	3.39E-06	0.000348	Sfxn5	sideroflexin 5 [Source:MGI Symbol;Acc:MGI:2137681]
10379	ENSMUSG00000075296	-1.44004	2.698443	10.6271	0.001114	0.01776	Aldh3b2	aldehyde dehydrogenase 3 family, member B2 [Source:MGI Symbol;Acc:MGI:2147613]

585	ENSMUSG00000005373	-1.43839	5.204694	15.63187	7.69E-05	0.00325	Mlxipl	MLX interacting protein-like [Source:MGI Symbol;Acc:MGI:1927999]
1034	ENSMUSG00000015016	-1.43683	3.414539	22.54539	2.05E-06	0.00026	Acsf3	acyl-CoA synthetase family member 3 [Source:MGI Symbol;Acc:MGI:2182591]
2489	ENSMUSG00000022474	-1.43653	4.192613	35.2757	2.86E-09	1.56E-06	Pmm1	phosphomannomutase 1 [Source:MGI Symbol;Acc:MGI:1353418]
4245	ENSMUSG00000027983	-1.43555	3.164405	13.74995	0.000209	0.006428	Cyp2u1	cytochrome P450, family 2, subfamily u, polypeptide 1 [Source:MGI Symbol;Acc:MGI:1918769]
6612	ENSMUSG00000035885	-1.43462	6.234074	19.31842	1.11E-05	0.000788	Cox8a	cytochrome c oxidase subunit VIIIa [Source:MGI Symbol;Acc:MGI:105959]
4748	ENSMUSG00000029195	-1.43403	4.712884	22.91713	1.69E-06	0.000226	Klb	klotho beta [Source:MGI Symbol;Acc:MGI:1932466]
6020	ENSMUSG00000032902	-1.43347	5.395118	10.82946	0.000999	0.016746	Slc16a1	solute carrier family 16 (monocarboxylic acid transporters), member 1 [Source:MGI Symbol;Acc:MGI:106013]
8551	ENSMUSG00000047182	-1.43308	3.109747	36.07032	1.90E-09	1.09E-06	Irs3	insulin receptor substrate 3 [Source:MGI Symbol;Acc:MGI:1194882]
7344	ENSMUSG00000039063	-1.41619	3.145099	16.20689	5.68E-05	0.002574	Echdc3	enoyl Coenzyme A hydratase domain containing 3 [Source:MGI Symbol;Acc:MGI:1915106]
7462	ENSMUSG00000039518	-1.41604	3.726034	22.12807	2.55E-06	0.000294	Cdsn	corneodesmosin [Source:MGI Symbol;Acc:MGI:3505689]
3376	ENSMUSG00000025503	-1.41398	6.864537	20.71462	5.33E-06	0.000468	Taldo1	transaldolase 1 [Source:MGI Symbol;Acc:MGI:1274789]
1803	ENSMUSG00000020682	-1.40666	2.917211	20.98666	4.62E-06	0.000423	Mmp28	matrix metalloproteinase 28 (epilysin) [Source:MGI Symbol;Acc:MGI:2153062]
1403	ENSMUSG00000019437	-1.39862	3.624548	23.72615	1.11E-06	0.000175	Tlcd1	TLC domain containing 1 [Source:MGI Symbol;Acc:MGI:1915572]
6063	ENSMUSG00000033105	-1.39567	4.066877	21.21948	4.10E-06	0.000388	Lss	lanosterol synthase [Source:MGI Symbol;Acc:MGI:1336155]
12	ENSMUSG00000000168	-1.39466	6.791867	8.868682	0.002901	0.028684	Dlat	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) [Source:MGI Symbol;Acc:MGI:2385311]
7938	ENSMUSG000000041801	-1.39359	4.872614	15.49613	8.27E-05	0.003451	Phlda3	pleckstrin homology-like domain, family A, member 3 [Source:MGI Symbol;Acc:MGI:1351485]
3068	ENSMUSG00000024664	-1.39257	5.212149	21.46032	3.61E-06	0.000355	Fads3	fatty acid desaturase 3 [Source:MGI Symbol;Acc:MGI:1928740]
10400	ENSMUSG00000075706	-1.38633	7.583109	15.42961	8.56E-05	0.003561	Gpx4	glutathione peroxidase 4 [Source:MGI Symbol;Acc:MGI:104767]
4521	ENSMUSG00000028671	-1.38569	2.670324	40.67489	1.80E-10	1.59E-07	Gale	galactose-4-epimerase, UDP [Source:MGI Symbol;Acc:MGI:1921496]
9137	ENSMUSG000000053898	-1.38346	6.875789	11.26202	0.000791	0.014965	Ech1	enoyl coenzyme A hydratase 1, peroxisomal [Source:MGI Symbol;Acc:MGI:1858208]
2914	ENSMUSG00000024228	-1.38133	4.38647	11.96731	0.000541	0.011918	Nudt12	nudix (nucleoside diphosphate linked moiety X)-type motif 12 [Source:MGI Symbol;Acc:MGI:1915243]
9873	ENSMUSG000000063873	-1.38119	4.109575	21.02803	4.53E-06	0.000419	Slc24a3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3 [Source:MGI Symbol;Acc:MGI:2137513]
6623	ENSMUSG00000035936	-1.37659	3.214519	18.80351	1.45E-05	0.000957	Aldh5a1	aldehyde dehydrogenase family 5, subfamily A1 [Source:MGI Symbol;Acc:MGI:2441982]
3016	ENSMUSG00000024525	-1.3727	2.250561	12.38737	0.000432	0.010306	Impa2	inositol (myo)-1(or 4)-monophosphatase 2 [Source:MGI Symbol;Acc:MGI:2149728]
3969	ENSMUSG00000027195	-1.36821	6.311018	19.35459	1.09E-05	0.000781	Hsd17b12	hydroxysteroid (17-beta) dehydrogenase 12 [Source:MGI Symbol;Acc:MGI:1926967]

7921	ENSMUSG00000041736	-1.36731	4.934778	45.15939	1.82E-11	2.83E-08	Tspo	translocator protein [Source:MGI Symbol;Acc:MGI:88222]
8856	ENSMUSG00000050556	-1.36471	4.377664	10.68191	0.001082	0.01749	Kcnb1	potassium voltage gated channel, Shab-related subfamily, member 1 [Source:MGI Symbol;Acc:MGI:96666]
3360	ENSMUSG00000025465	-1.36083	6.161147	10.60601	0.001127	0.017852	Echs1	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial [Source:MGI Symbol;Acc:MGI:2136460]
10166	ENSMUSG00000071014	-1.35925	4.339556	12.35268	0.00044	0.010385	Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6 [Source:MGI Symbol;Acc:MGI:2684983]
4246	ENSMUSG00000027984	-1.35854	7.067584	10.10965	0.001475	0.020395	Hadh	hydroxyacyl-Coenzyme A dehydrogenase [Source:MGI Symbol;Acc:MGI:96009]
9420	ENSMUSG00000057580	-1.3579	2.456437	7.254913	0.007071	0.045663	Gm10012	predicted pseudogene 10012 [Source:MGI Symbol;Acc:MGI:3642454]
10151	ENSMUSG00000070730	-1.35429	4.757482	17.90923	2.32E-05	0.001329	Rmdn3	regulator of microtubule dynamics 3 [Source:MGI Symbol;Acc:MGI:1915059]
2427	ENSMUSG00000022332	-1.35355	3.185322	9.991002	0.001573	0.020924	Khdrbs3	KH domain containing, RNA binding, signal transduction associated 3 [Source:MGI Symbol;Acc:MGI:1313312]
5480	ENSMUSG00000031489	-1.35316	5.54515	19.7275	8.93E-06	0.00068	Adrb3	adrenergic receptor, beta 3 [Source:MGI Symbol;Acc:MGI:87939]
7967	ENSMUSG00000041957	-1.35148	2.902205	20.78865	5.13E-06	0.000458	Pkp2	plakophilin 2 [Source:MGI Symbol;Acc:MGI:1914701]
5831	ENSMUSG00000032348	-1.34988	3.534991	8.28186	0.004004	0.033738	Gsta4	glutathione S-transferase, alpha 4 [Source:MGI Symbol;Acc:MGI:1309515]
9687	ENSMUSG00000061313	-1.34937	5.926565	13.69637	0.000215	0.00654	Ddhd2	DDHD domain containing 2 [Source:MGI Symbol;Acc:MGI:1919358]
1426	ENSMUSG00000019718	-1.34792	2.841349	14.81898	0.000118	0.004446	L3hypdh	L-3-hydroxyproline dehydratase (trans-) [Source:MGI Symbol;Acc:MGI:1914467]
7183	ENSMUSG00000038462	-1.3454	6.161854	10.42533	0.001243	0.018837	Uqcrcf1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 [Source:MGI Symbol;Acc:MGI:1913944]
2530	ENSMUSG00000022555	-1.34122	5.556142	30.38338	3.55E-08	1.10E-05	Dgat1	diacylglycerol O-acyltransferase 1 [Source:MGI Symbol;Acc:MGI:1333825]
3450	ENSMUSG00000025792	-1.34043	5.673272	20.35427	6.43E-06	0.000531	Slc25a10	solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter), member 10 [Source:MGI Symbol;Acc:MGI:1353497]
8888	ENSMUSG00000050953	-1.33931	5.286633	19.0383	1.28E-05	0.000879	Gja1	gap junction protein, alpha 1 [Source:MGI Symbol;Acc:MGI:95713]
7978	ENSMUSG00000042010	-1.3378	7.719106	7.466365	0.006286	0.042917	Acacb	acetyl-Coenzyme A carboxylase beta [Source:MGI Symbol;Acc:MGI:2140940]
5853	ENSMUSG00000032387	-1.33474	3.330093	10.66873	0.00109	0.017563	Rbpms2	RNA binding protein with multiple splicing 2 [Source:MGI Symbol;Acc:MGI:1919223]
5738	ENSMUSG00000032092	-1.33392	3.931683	13.52271	0.000236	0.006941	Mpzl2	myelin protein zero-like 2 [Source:MGI Symbol;Acc:MGI:1289160]
4139	ENSMUSG00000027656	-1.33161	1.823974	13.35732	0.000257	0.007401	Wisp2	WNT1 inducible signaling pathway protein 2 [Source:MGI Symbol;Acc:MGI:1328326]
2876	ENSMUSG00000024132	-1.33128	5.84942	7.394139	0.006544	0.044038	Eci1	enoyl-Coenzyme A delta isomerase 1 [Source:MGI Symbol;Acc:MGI:94871]
1335	ENSMUSG00000018740	-1.33092	3.917469	14.20962	0.000164	0.005586	Slc25a35	solute carrier family 25, member 35 [Source:MGI Symbol;Acc:MGI:1919248]
6547	ENSMUSG00000035504	-1.32898	2.755354	9.008383	0.002687	0.027583	Reep6	receptor accessory protein 6 [Source:MGI Symbol;Acc:MGI:1917585]
943	ENSMUSG00000012705	-1.32599	7.085426	18.31426	1.87E-05	0.001153	Retn	resistin [Source:MGI Symbol;Acc:MGI:1888506]
3003	ENSMUSG00000024479	-1.32559	2.605154	11.64047	0.000645	0.013118	Mal2	mal, T cell differentiation protein 2 [Source:MGI Symbol;Acc:MGI:2146021]
3984	ENSMUSG00000027222	-1.32553	4.360822	39.61555	3.09E-10	2.41E-07	Pex16	peroxisomal biogenesis factor 16 [Source:MGI Symbol;Acc:MGI:1338829]

5147	ENSMUSG00000030470	-1.32447	4.315938	9.385743	0.002187	0.024615	Csrp3	cysteine and glycine-rich protein 3 [Source:MGI Symbol;Acc:MGI:1330824]
5680	ENSMUSG00000031958	-1.32375	4.579619	10.27545	0.001348	0.019612	Ldhd	lactate dehydrogenase D [Source:MGI Symbol;Acc:MGI:106428]
4491	ENSMUSG00000028607	-1.3232	6.039983	9.502201	0.002052	0.023993	Cpt2	carnitine palmitoyltransferase 2 [Source:MGI Symbol;Acc:MGI:109176]
3361	ENSMUSG00000025466	-1.31747	2.794874	10.03975	0.001532	0.020765	Fuom	fucose mutarotase [Source:MGI Symbol;Acc:MGI:1916314]
5824	ENSMUSG00000032330	-1.31705	5.576929	9.584284	0.001962	0.023319	Cox7a2	cytochrome c oxidase subunit VIIa 2 [Source:MGI Symbol;Acc:MGI:1316715]
5171	ENSMUSG00000030546	-1.31499	7.950164	22.76365	1.83E-06	0.000238	Plin1	perilipin 1 [Source:MGI Symbol;Acc:MGI:1890505]
9369	ENSMUSG00000056973	-1.30775	8.085236	10.60679	0.001127	0.017852	Ces1d	carboxylesterase 1D [Source:MGI Symbol;Acc:MGI:2148202]
5169	ENSMUSG00000030541	-1.30768	6.232903	11.46561	0.000709	0.013944	Idh2	isocitrate dehydrogenase 2 (NADP+), mitochondrial [Source:MGI Symbol;Acc:MGI:96414]
4913	ENSMUSG00000029632	-1.30535	6.670111	8.449274	0.003652	0.032273	Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 [Source:MGI Symbol;Acc:MGI:107686]
8811	ENSMUSG00000049892	-1.30291	3.245634	24.00311	9.62E-07	0.000156	Rasd1	RAS, dexamethasone-induced 1 [Source:MGI Symbol;Acc:MGI:1270848]
5668	ENSMUSG00000031924	-1.30023	7.15578	25.78766	3.81E-07	7.69E-05	Cyb5b	cytochrome b5 type B [Source:MGI Symbol;Acc:MGI:1913677]
9424	ENSMUSG00000057614	-1.29738	5.570432	31.25522	2.26E-08	7.70E-06	Gnai1	guanine nucleotide binding protein (G protein), alpha inhibiting 1 [Source:MGI Symbol;Acc:MGI:95771]
3857	ENSMUSG00000026858	-1.29672	4.570371	32.13355	1.44E-08	5.81E-06	Fam73b	family with sequence similarity 73, member B [Source:MGI Symbol;Acc:MGI:1922035]
1549	ENSMUSG00000020085	-1.29341	5.695425	18.0019	2.21E-05	0.001272	Aifm2	apoptosis-inducing factor, mitochondrion-associated 2 [Source:MGI Symbol;Acc:MGI:1918611]
2048	ENSMUSG00000021319	-1.29099	2.560917	7.710598	0.00549	0.039931	Sfrp4	secreted frizzled-related protein 4 [Source:MGI Symbol;Acc:MGI:892010]
6022	ENSMUSG00000032911	-1.29073	4.982065	20.42187	6.21E-06	0.000517	Cspg4	chondroitin sulfate proteoglycan 4 [Source:MGI Symbol;Acc:MGI:2153093]
5452	ENSMUSG00000031400	-1.28679	6.396457	10.86627	0.000979	0.016592	G6pdx	glucose-6-phosphate dehydrogenase X-linked [Source:MGI Symbol;Acc:MGI:105979]
9619	ENSMUSG00000060376	-1.28593	6.059715	8.476441	0.003598	0.031923	Bckdha	branched chain ketoacid dehydrogenase E1, alpha polypeptide [Source:MGI Symbol;Acc:MGI:107701]
8272	ENSMUSG00000044405	-1.2815	4.571738	9.86703	0.001683	0.021749	Adig	adipogenin [Source:MGI Symbol;Acc:MGI:2675492]
13	ENSMUSG00000000171	-1.27513	6.230408	10.29324	0.001335	0.019575	Sdhd	succinate dehydrogenase complex, subunit D, integral membrane protein [Source:MGI Symbol;Acc:MGI:1914175]
9039	ENSMUSG00000052738	-1.27183	6.302585	7.034127	0.007997	0.048734	Suclg1	succinate-CoA ligase, GDP-forming, alpha subunit [Source:MGI Symbol;Acc:MGI:1927234]
1313	ENSMUSG00000018566	-1.27061	6.702899	8.660895	0.003251	0.030338	Slc2a4	solute carrier family 2 (facilitated glucose transporter), member 4 [Source:MGI Symbol;Acc:MGI:95758]
9520	ENSMUSG00000059027	-1.26966	2.549845	13.30319	0.000265	0.007561	9630013D21Rik	RIKEN cDNA 9630013D21 gene [Source:MGI Symbol;Acc:MGI:2442649]
6967	ENSMUSG00000037624	-1.26452	2.285112	9.621306	0.001923	0.02312	Kcnk2	potassium channel, subfamily K, member 2 [Source:MGI Symbol;Acc:MGI:109366]
5202	ENSMUSG00000030630	-1.25947	4.627673	24.1533	8.90E-07	0.000149	Fah	fumarylacetoacetate hydrolase [Source:MGI Symbol;Acc:MGI:95482]
5	ENSMUSG00000000088	-1.25741	5.252291	10.01972	0.001549	0.020805	Cox5a	cytochrome c oxidase subunit Va [Source:MGI Symbol;Acc:MGI:88474]

6127	ENSMUSG000000033429	-1.25654	3.973921	8.062041	0.00452	0.035916	Mcee	methylmalonyl CoA epimerase [Source:MGI Symbol;Acc:MGI:1920974]
7143	ENSMUSG000000038302	-1.25417	4.009451	15.19394	9.70E-05	0.003921	Lace1	lactation elevated 1 [Source:MGI Symbol;Acc:MGI:2148801]
6720	ENSMUSG000000036446	-1.24798	6.57412	10.76689	0.001033	0.017008	Lum	lumican [Source:MGI Symbol;Acc:MGI:109347]
1422	ENSMUSG000000019689	-1.24737	2.485424	9.066516	0.002603	0.027044	1110001J03Rik	RIKEN cDNA 1110001J03 gene [Source:MGI Symbol;Acc:MGI:1913367]
3939	ENSMUSG000000027099	-1.24582	4.422579	15.97053	6.43E-05	0.002827	Mtx2	metaxin 2 [Source:MGI Symbol;Acc:MGI:1859652]
2334	ENSMUSG000000022099	-1.24457	3.249446	16.232	5.60E-05	0.002566	Dmtn	dematin actin binding protein [Source:MGI Symbol;Acc:MGI:99670]
5725	ENSMUSG000000032051	-1.24409	2.854007	9.033995	0.00265	0.027336	Fdx1	ferredoxin 1 [Source:MGI Symbol;Acc:MGI:103224]
7249	ENSMUSG000000038690	-1.24311	6.074409	9.488808	0.002067	0.024086	Atp5j2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2 [Source:MGI Symbol;Acc:MGI:1927558]
371	ENSMUSG00000003123	-1.23635	8.133753	21.79965	3.03E-06	0.000327	Lipe	lipase, hormone sensitive [Source:MGI Symbol;Acc:MGI:96790]
1651	ENSMUSG000000020321	-1.23521	7.578051	12.20352	0.000477	0.011011	Mdh1	malate dehydrogenase 1, NAD (soluble) [Source:MGI Symbol;Acc:MGI:97051]
4337	ENSMUSG000000028199	-1.23342	2.874047	13.83427	0.0002	0.006289	Cryz	crystallin, zeta [Source:MGI Symbol;Acc:MGI:88527]
3865	ENSMUSG000000026879	-1.23322	10.45576	15.03405	0.000106	0.004097	Gsn	gelsolin [Source:MGI Symbol;Acc:MGI:95851]
10376	ENSMUSG000000075273	-1.23303	2.855843	10.56856	0.00115	0.018153	Ttc30b	tetratricopeptide repeat domain 30B [Source:MGI Symbol;Acc:MGI:1919671]
1938	ENSMUSG000000021033	-1.2326	6.875171	18.61918	1.60E-05	0.001029	Gstz1	glutathione transferase zeta 1 (maleylacetoacetate isomerase) [Source:MGI Symbol;Acc:MGI:1341859]
4344	ENSMUSG000000028223	-1.23228	6.784234	7.807029	0.005204	0.038871	Decr1	2,4-dienoyl CoA reductase 1, mitochondrial [Source:MGI Symbol;Acc:MGI:1914710]
2649	ENSMUSG000000022878	-1.23205	9.126323	15.65425	7.60E-05	0.003237	Adipoq	adiponectin, C1Q and collagen domain containing [Source:MGI Symbol;Acc:MGI:106675]
5766	ENSMUSG000000032192	-1.23156	2.600865	15.1272	0.000101	0.00395	Gnb5	guanine nucleotide binding protein (G protein), beta 5 [Source:MGI Symbol;Acc:MGI:101848]
244	ENSMUSG000000002059	-1.22732	3.564408	30.874	2.75E-08	9.09E-06	Rab34	RAB34, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:104606]
862	ENSMUSG000000009863	-1.22693	6.621318	7.198904	0.007295	0.046372	Sdhb	succinate dehydrogenase complex, subunit B, iron sulfur (lp) [Source:MGI Symbol;Acc:MGI:1914930]
3338	ENSMUSG000000025375	-1.22655	2.777731	10.01632	0.001552	0.020805	Aatk	apoptosis-associated tyrosine kinase [Source:MGI Symbol;Acc:MGI:1197518]
7657	ENSMUSG000000040370	-1.22446	5.046296	9.400562	0.002169	0.024544	Lym5	LYR motif containing 5 [Source:MGI Symbol;Acc:MGI:1914886]
2527	ENSMUSG000000022551	-1.22222	5.676843	8.605628	0.003351	0.030823	Cyc1	cytochrome c-1 [Source:MGI Symbol;Acc:MGI:1913695]
4311	ENSMUSG000000028127	-1.22215	6.272042	9.540938	0.002009	0.023644	Abcd3	ATP-binding cassette, sub-family D (ALD), member 3 [Source:MGI Symbol;Acc:MGI:1349216]
6452	ENSMUSG000000034981	-1.21971	5.857288	10.22239	0.001387	0.019861	Parm1	prostate androgen-regulated mucin-like protein 1 [Source:MGI Symbol;Acc:MGI:2443349]
199	ENSMUSG000000001755	-1.21958	4.498911	27.42608	1.63E-07	4.14E-05	Coasy	Coenzyme A synthase [Source:MGI Symbol;Acc:MGI:1918993]
2011	ENSMUSG000000021235	-1.21958	3.572623	12.42298	0.000424	0.010156	Coq6	coenzyme Q6 homolog (yeast) [Source:MGI Symbol;Acc:MGI:1924408]
221	ENSMUSG000000001918	-1.21902	7.52678	39.34634	3.55E-10	2.58E-07	Slc1a5	solute carrier family 1 (neutral amino acid transporter), member 5 [Source:MGI Symbol;Acc:MGI:105305]

7864	ENSMUSG000000041426	-1.21891	6.053048	13.81076	0.000202	0.006312	Hibch	3-hydroxyisobutyryl-Coenzyme A hydrolase [Source:MGI Symbol;Acc:MGI:1923792]
9592	ENSMUSG000000060002	-1.21744	9.008201	11.78274	0.000598	0.012617	Chpt1	choline phosphotransferase 1 [Source:MGI Symbol;Acc:MGI:2384841]
6077	ENSMUSG000000033174	-1.21518	7.79389	16.68115	4.42E-05	0.002145	Mgll	monoglyceride lipase [Source:MGI Symbol;Acc:MGI:1346042]
10698	ENSMUSG000000093930	-1.21444	5.941766	27.52216	1.55E-07	4.04E-05	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 [Source:MGI Symbol;Acc:MGI:107592]
1297	ENSMUSG000000018428	-1.21125	5.68145	8.10662	0.00441	0.035626	Akap1	A kinase (PRKA) anchor protein 1 [Source:MGI Symbol;Acc:MGI:104729]
3432	ENSMUSG000000025745	-1.21048	8.400399	7.367334	0.006642	0.044318	Hadha	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit [Source:MGI Symbol;Acc:MGI:2135593]
5549	ENSMUSG000000031641	-1.20892	3.462587	15.23059	9.51E-05	0.003883	Cbr4	carbonyl reductase 4 [Source:MGI Symbol;Acc:MGI:2384567]
6176	ENSMUSG000000033707	-1.20786	3.45957	12.70167	0.000365	0.009257	Lrrc24	leucine rich repeat containing 24 [Source:MGI Symbol;Acc:MGI:3605040]
1138	ENSMUSG000000016194	-1.20594	5.127912	20.67601	5.44E-06	0.00047	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1 [Source:MGI Symbol;Acc:MGI:103562]
1887	ENSMUSG000000020889	-1.20419	6.180699	13.58341	0.000228	0.00685	Nr1d1	nuclear receptor subfamily 1, group D, member 1 [Source:MGI Symbol;Acc:MGI:2444210]
4879	ENSMUSG000000029545	-1.20311	5.188294	8.410055	0.003732	0.03275	Acads	acyl-Coenzyme A dehydrogenase, short chain [Source:MGI Symbol;Acc:MGI:87868]
6485	ENSMUSG000000035172	-1.20134	3.08056	16.58766	4.65E-05	0.00223	Plekhh3	pleckstrin homology domain containing, family H (with MyTH4 domain) member 3 [Source:MGI Symbol;Acc:MGI:2384950]
2765	ENSMUSG000000023571	-1.1999	3.34101	15.97633	6.41E-05	0.002827	Fam132a	family with sequence similarity 132, member A [Source:MGI Symbol;Acc:MGI:1914639]
905	ENSMUSG000000010914	-1.19934	5.082854	8.3276	0.003905	0.033262	Pdhx	pyruvate dehydrogenase complex, component X [Source:MGI Symbol;Acc:MGI:1351627]
9855	ENSMUSG000000063568	-1.19758	2.348888	10.28821	0.001339	0.019581	Jazf1	JAZF zinc finger 1 [Source:MGI Symbol;Acc:MGI:2141450]
3275	ENSMUSG000000025204	-1.19642	5.722137	8.993697	0.002709	0.027639	Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 [Source:MGI Symbol;Acc:MGI:1914514]
527	ENSMUSG000000004748	-1.19439	2.836306	7.254065	0.007074	0.045663	Mtfp1	mitochondrial fission process 1 [Source:MGI Symbol;Acc:MGI:1916686]
4619	ENSMUSG000000028910	-1.19423	3.947049	18.10176	2.09E-05	0.001222	Mecr	mitochondrial trans-2-enoyl-CoA reductase [Source:MGI Symbol;Acc:MGI:1349441]
8384	ENSMUSG000000045569	-1.19218	2.287927	17.10874	3.53E-05	0.001828	Mc2r	melanocortin 2 receptor [Source:MGI Symbol;Acc:MGI:96928]
8449	ENSMUSG000000046275	-1.19212	6.463161	14.07627	0.000176	0.005849	Tusc5	tumor suppressor candidate 5 [Source:MGI Symbol;Acc:MGI:3029307]
7022	ENSMUSG000000037892	-1.19193	2.988531	8.941559	0.002788	0.02812	Pcdh18	protocadherin 18 [Source:MGI Symbol;Acc:MGI:1920423]
1832	ENSMUSG000000020744	-1.18996	4.985861	11.01524	0.000904	0.016039	Slc25a19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19 [Source:MGI Symbol;Acc:MGI:1914533]
3476	ENSMUSG000000025875	-1.18917	2.119548	12.82709	0.000342	0.008884	Tspan17	tetraspanin 17 [Source:MGI Symbol;Acc:MGI:1921507]
7439	ENSMUSG000000039428	-1.18886	5.931038	28.57529	9.01E-08	2.65E-05	Tmem135	transmembrane protein 135 [Source:MGI Symbol;Acc:MGI:1920009]
5412	ENSMUSG000000031299	-1.18203	8.656694	7.091789	0.007744	0.048023	Pdha1	pyruvate dehydrogenase E1 alpha 1 [Source:MGI Symbol;Acc:MGI:97532]

6060	ENSMUSG00000033096	-1.1794	5.959794	11.78088	0.000598	0.012617	Apmap	adipocyte plasma membrane associated protein [Source:MGI Symbol;Acc:MGI:1919131]
8709	ENSMUSG00000048772	-1.175	2.01949	11.072	0.000876	0.015901	Tmem53	transmembrane protein 53 [Source:MGI Symbol;Acc:MGI:1916027]
6626	ENSMUSG00000035948	-1.17298	5.037216	7.68931	0.005555	0.040144	Acss3	acyl-CoA synthetase short-chain family member 3 [Source:MGI Symbol;Acc:MGI:2685720]
6919	ENSMUSG00000037366	-1.17002	2.663214	10.87085	0.000977	0.01658	Pafah2	platelet-activating factor acetylhydrolase 2 [Source:MGI Symbol;Acc:MGI:2140321]
359	ENSMUSG00000002997	-1.17002	7.902476	9.38353	0.002189	0.024619	Prkar2b	protein kinase, cAMP dependent regulatory, type II beta [Source:MGI Symbol;Acc:MGI:97760]
804	ENSMUSG00000008540	-1.16796	7.35518	18.19599	1.99E-05	0.001193	Mgst1	microsomal glutathione S-transferase 1 [Source:MGI Symbol;Acc:MGI:1913850]
325	ENSMUSG00000002781	-1.16459	4.580747	8.354903	0.003846	0.033062	Tmem143	transmembrane protein 143 [Source:MGI Symbol;Acc:MGI:1917459]
5994	ENSMUSG00000032802	-1.16117	3.565529	9.759836	0.001784	0.022416	Srxn1	sulfiredoxin 1 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:104971]
4648	ENSMUSG000000028973	-1.15971	4.099935	18.42748	1.76E-05	0.001115	Abcb8	ATP-binding cassette, sub-family B (MDR/TAP), member 8 [Source:MGI Symbol;Acc:MGI:1351667]
8322	ENSMUSG00000044894	-1.15858	3.77491	7.13051	0.007578	0.04744	Uqcrcq	ubiquinol-cytochrome c reductase, complex III subunit VII [Source:MGI Symbol;Acc:MGI:107807]
8375	ENSMUSG00000045438	-1.15675	3.305306	25.01531	5.69E-07	0.000107	Cox19	cytochrome c oxidase assembly protein 19 [Source:MGI Symbol;Acc:MGI:1915283]
5913	ENSMUSG00000032527	-1.15352	5.830204	10.08036	0.001499	0.020549	Pccb	propionyl Coenzyme A carboxylase, beta polypeptide [Source:MGI Symbol;Acc:MGI:1914154]
1185	ENSMUSG00000017167	-1.1526	2.289596	22.38721	2.23E-06	0.000276	Cntnap1	contactin associated protein-like 1 [Source:MGI Symbol;Acc:MGI:1858201]
4442	ENSMUSG000000028470	-1.15079	2.408682	12.00211	0.000531	0.011817	Hint2	histidine triad nucleotide binding protein 2 [Source:MGI Symbol;Acc:MGI:1916167]
2424	ENSMUSG000000022324	-1.1496	3.876579	23.46026	1.28E-06	0.000196	Matn2	matrilin 2 [Source:MGI Symbol;Acc:MGI:109613]
1480	ENSMUSG00000019883	-1.14723	7.192953	8.738173	0.003116	0.029602	Echdc1	enoyl Coenzyme A hydratase domain containing 1 [Source:MGI Symbol;Acc:MGI:1277169]
1980	ENSMUSG000000021136	-1.14443	5.147308	16.58748	4.65E-05	0.00223	Smoc1	SPARC related modular calcium binding 1 [Source:MGI Symbol;Acc:MGI:1929878]
80	ENSMUSG00000000811	-1.14323	2.483438	18.80662	1.45E-05	0.000957	Txnrd3	thioredoxin reductase 3 [Source:MGI Symbol;Acc:MGI:2386711]
3241	ENSMUSG000000025137	-1.14201	4.21838	21.48759	3.56E-06	0.000354	Pcyt2	phosphate cytidyltransferase 2, ethanolamine [Source:MGI Symbol;Acc:MGI:1915921]
4269	ENSMUSG000000028036	-1.13973	2.693997	9.820387	0.001726	0.02196	Ptgfr	prostaglandin F receptor [Source:MGI Symbol;Acc:MGI:97796]
8445	ENSMUSG000000046186	-1.13814	2.064448	9.297544	0.002295	0.025249	Cd109	CD109 antigen [Source:MGI Symbol;Acc:MGI:2445221]
2537	ENSMUSG000000022562	-1.13759	5.245948	7.177413	0.007383	0.046687	Oplah	5-oxoprolinase (ATP-hydrolysing) [Source:MGI Symbol;Acc:MGI:1922725]
4926	ENSMUSG000000029669	-1.13645	5.377012	19.2035	1.17E-05	0.000831	Tspan12	tetraspanin 12 [Source:MGI Symbol;Acc:MGI:1889818]
3500	ENSMUSG000000025937	-1.13445	5.553461	16.1898	5.73E-05	0.00258	Lactb2	lactamase, beta 2 [Source:MGI Symbol;Acc:MGI:2442551]
5427	ENSMUSG000000031351	-1.13319	2.457806	7.607188	0.005814	0.041027	Zfp185	zinc finger protein 185 [Source:MGI Symbol;Acc:MGI:108095]

8813	ENSMUSG00000049929	-1.12825	2.028038	10.08904	0.001492	0.02052	Lpar4	lysophosphatidic acid receptor 4 [Source:MGI Symbol;Acc:MGI:1925384]
7173	ENSMUSG00000038412	-1.12612	3.622455	14.12662	0.000171	0.005748	Higd1a	HIG1 domain family, member 1A [Source:MGI Symbol;Acc:MGI:1930666]
3011	ENSMUSG000000024507	-1.12273	6.620563	17.39668	3.03E-05	0.001635	Hsd17b4	hydroxysteroid (17-beta) dehydrogenase 4 [Source:MGI Symbol;Acc:MGI:105089]
2263	ENSMUSG000000021906	-1.12138	3.92727	8.929856	0.002805	0.028208	Oxnad1	oxidoreductase NAD-binding domain containing 1 [Source:MGI Symbol;Acc:MGI:1916953]
8896	ENSMUSG000000051041	-1.12118	3.590438	16.52077	4.81E-05	0.00228	Olfml1	olfactomedin-like 1 [Source:MGI Symbol;Acc:MGI:2679264]
2381	ENSMUSG000000022210	-1.12021	4.009281	12.6527	0.000375	0.009464	Dhrs4	dehydrogenase/reductase (SDR family) member 4 [Source:MGI Symbol;Acc:MGI:90169]
5170	ENSMUSG000000030545	-1.11988	3.001902	11.74016	0.000612	0.012693	Pex11a	peroxisomal biogenesis factor 11 alpha [Source:MGI Symbol;Acc:MGI:1338788]
531	ENSMUSG000000004789	-1.11794	7.043227	8.222584	0.004137	0.034271	Dlst	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) [Source:MGI Symbol;Acc:MGI:1926170]
10137	ENSMUSG000000070493	-1.11561	5.884926	12.48438	0.00041	0.009961	Chchd2	coiled-coil-helix-coiled-coil-helix domain containing 2 [Source:MGI Symbol;Acc:MGI:1261428]
3837	ENSMUSG000000026820	-1.11393	3.552126	12.15619	0.000489	0.011131	Ptges2	prostaglandin E synthase 2 [Source:MGI Symbol;Acc:MGI:1917592]
3477	ENSMUSG000000025876	-1.11361	3.109977	13.45603	0.000244	0.00714	Unc5a	unc-5 homolog A (C. elegans) [Source:MGI Symbol;Acc:MGI:894682]
2040	ENSMUSG000000021286	-1.11345	2.296489	22.97879	1.64E-06	0.000223	Zfyve21	zinc finger, FYVE domain containing 21 [Source:MGI Symbol;Acc:MGI:1915770]
5461	ENSMUSG000000031434	-1.11092	3.537343	17.26263	3.26E-05	0.001734	Morc4	microorchidia 4 [Source:MGI Symbol;Acc:MGI:1922996]
9365	ENSMUSG000000056938	-1.10908	4.689855	27.82717	1.33E-07	3.70E-05	Acbd4	acyl-Coenzyme A binding domain containing 4 [Source:MGI Symbol;Acc:MGI:1914381]
3100	ENSMUSG000000024777	-1.10819	3.949564	21.4218	3.69E-06	0.000356	Ppp2r5b	protein phosphatase 2, regulatory subunit B', beta [Source:MGI Symbol;Acc:MGI:2388480]
7257	ENSMUSG000000038717	-1.10522	5.747374	7.222839	0.007198	0.045974	Atp5l	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G [Source:MGI Symbol;Acc:MGI:1351597]
10325	ENSMUSG000000074264	-1.10446	6.686817	12.1475	0.000492	0.011158	Amy1	amylase 1, salivary [Source:MGI Symbol;Acc:MGI:88019]
9774	ENSMUSG000000062515	-1.10146	10.80116	17.153	3.45E-05	0.001815	Fabp4	fatty acid binding protein 4, adipocyte [Source:MGI Symbol;Acc:MGI:88038]
8057	ENSMUSG000000042429	-1.09608	5.11829	13.02969	0.000307	0.008228	Adora1	adenosine A1 receptor [Source:MGI Symbol;Acc:MGI:99401]
5438	ENSMUSG000000031373	-1.09352	6.610176	11.78025	0.000599	0.012617	Car5b	carbonic anhydrase 5b, mitochondrial [Source:MGI Symbol;Acc:MGI:1926249]
4988	ENSMUSG000000029864	-1.09332	3.711049	8.530716	0.003492	0.031381	Gstk1	glutathione S-transferase kappa 1 [Source:MGI Symbol;Acc:MGI:1923513]
3547	ENSMUSG000000026064	-1.0927	7.224234	16.9412	3.86E-05	0.001956	Ptp4a1	protein tyrosine phosphatase 4a1 [Source:MGI Symbol;Acc:MGI:1277096]
5674	ENSMUSG000000031938	-1.09257	5.782153	7.883897	0.004988	0.03795	4931406C07Rik	RIKEN cDNA 4931406C07 gene [Source:MGI Symbol;Acc:MGI:1918234]
4252	ENSMUSG000000027999	-1.09257	4.042155	16.86921	4.00E-05	0.002002	Pla2g12a	phospholipase A2, group XIA [Source:MGI Symbol;Acc:MGI:1913600]
347	ENSMUSG000000002944	-1.09147	9.744671	17.12953	3.49E-05	0.00182	Cd36	CD36 antigen [Source:MGI Symbol;Acc:MGI:107899]
798	ENSMUSG000000008393	-1.08757	5.536124	23.4291	1.30E-06	0.000196	Carhsp1	calcium regulated heat stable protein 1 [Source:MGI Symbol;Acc:MGI:1196368]

5947	ENSMUSG000000032602	-1.08454	6.047837	8.676999	0.003223	0.030216	Slc25a20	solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20 [Source:MGI Symbol;Acc:MGI:1928738]
2157	ENSMUSG000000021646	-1.08347	4.93802	9.994238	0.00157	0.020917	Mccc2	methylcrotonoyl-Coenzyme A carboxylase 2 (beta) [Source:MGI Symbol;Acc:MGI:1925288]
7315	ENSMUSG000000038936	-1.08181	4.069065	12.86653	0.000335	0.008783	Sccpdh	saccharopine dehydrogenase (putative) [Source:MGI Symbol;Acc:MGI:1924486]
7482	ENSMUSG000000039640	-1.08016	4.744172	12.28853	0.000456	0.010634	Mrpl12	mitochondrial ribosomal protein L12 [Source:MGI Symbol;Acc:MGI:1926273]
2043	ENSMUSG000000021294	-1.079	2.199051	12.19309	0.00048	0.011045	Kif26a	kinesin family member 26A [Source:MGI Symbol;Acc:MGI:2447072]
560	ENSMUSG000000005057	-1.07809	4.001611	19.99743	7.75E-06	0.000608	Sh2b2	SH2B adaptor protein 2 [Source:MGI Symbol;Acc:MGI:1345171]
5622	ENSMUSG000000031818	-1.0775	7.457869	9.294263	0.002299	0.025249	Cox4i1	cytochrome c oxidase subunit IV isoform 1 [Source:MGI Symbol;Acc:MGI:88473]
8505	ENSMUSG000000046764	-1.07707	2.556783	14.32755	0.000154	0.005376	A530053G22Rik	RIKEN cDNA A530053G22 gene [Source:MGI Symbol;Acc:MGI:2443347]
6528	ENSMUSG000000035376	-1.07691	4.726309	12.51903	0.000403	0.009844	Hacd2	3-hydroxyacyl-CoA dehydratase 2 [Source:MGI Symbol;Acc:MGI:1918007]
6600	ENSMUSG000000035799	-1.07405	2.235728	15.55838	8.00E-05	0.003352	Twist1	twist basic helix-loop-helix transcription factor 1 [Source:MGI Symbol;Acc:MGI:98872]
5818	ENSMUSG000000032314	-1.07132	5.698432	6.997402	0.008163	0.049302	Etfa	electron transferring flavoprotein, alpha polypeptide [Source:MGI Symbol;Acc:MGI:106092]
457	ENSMUSG000000003948	-1.07098	7.495671	23.24814	1.42E-06	0.000205	Mmd	monocyte to macrophage differentiation-associated [Source:MGI Symbol;Acc:MGI:1914718]
872	ENSMUSG000000010051	-1.06808	2.143193	12.85392	0.000337	0.008821	Hyal1	hyaluronoglucosaminidase 1 [Source:MGI Symbol;Acc:MGI:96298]
7613	ENSMUSG000000040181	-1.0675	6.434633	10.89975	0.000962	0.016477	Fmo1	flavin containing monooxygenase 1 [Source:MGI Symbol;Acc:MGI:1310002]
4538	ENSMUSG000000028701	-1.06608	3.61437	8.897832	0.002855	0.028431	Lurap1	leucine rich adaptor protein 1 [Source:MGI Symbol;Acc:MGI:1915325]
10480	ENSMUSG000000079017	-1.06494	6.79921	7.225723	0.007187	0.045959	Ifi27l2a	interferon, alpha-inducible protein 27 like 2A [Source:MGI Symbol;Acc:MGI:1924183]
9463	ENSMUSG000000058258	-1.06487	4.064544	8.686782	0.003205	0.030107	Idi1	isopentenyl-diphosphate delta isomerase [Source:MGI Symbol;Acc:MGI:2442264]
4396	ENSMUSG000000028383	-1.0645	6.754345	7.651889	0.005671	0.040567	Hsd12	hydroxysteroid dehydrogenase like 2 [Source:MGI Symbol;Acc:MGI:1919729]
6740	ENSMUSG000000036560	-1.06359	2.147506	8.350108	0.003857	0.033062	Lgi4	leucine-rich repeat LGI family, member 4 [Source:MGI Symbol;Acc:MGI:2180197]
3368	ENSMUSG000000025486	-1.06332	2.538698	8.132444	0.004348	0.035461	Sirt3	sirtuin 3 [Source:MGI Symbol;Acc:MGI:1927665]
3174	ENSMUSG000000024953	-1.06246	5.324999	11.73684	0.000613	0.012693	Prdx5	peroxiredoxin 5 [Source:MGI Symbol;Acc:MGI:1859821]
9954	ENSMUSG000000066441	-1.06017	3.441983	9.013837	0.002679	0.027543	Rdh11	retinol dehydrogenase 11 [Source:MGI Symbol;Acc:MGI:102581]
7205	ENSMUSG000000038527	-1.05922	2.480167	15.29777	9.18E-05	0.003762	C1rl	complement component 1, r subcomponent-like [Source:MGI Symbol;Acc:MGI:2660692]
1626	ENSMUSG000000020264	-1.05911	6.383307	6.996766	0.008166	0.049302	Slc36a2	solute carrier family 36 (proton/amino acid symporter), member 2 [Source:MGI Symbol;Acc:MGI:1891430]
3457	ENSMUSG000000025813	-1.05856	3.597612	7.892618	0.004964	0.037848	Homer2	homer homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1347354]

9453	ENSMUSG00000058076	-1.05818	6.123552	7.051297	0.007921	0.04854	Sdhc	succinate dehydrogenase complex, subunit C, integral membrane protein [Source:MGI Symbol;Acc:MGI:1913302]
4750	ENSMUSG00000029198	-1.05696	4.95029	9.644769	0.001899	0.023118	Grpel1	GrpE-like 1, mitochondrial [Source:MGI Symbol;Acc:MGI:1334417]
10010	ENSMUSG00000067847	-1.05669	2.965469	10.57609	0.001146	0.018117	Romo1	reactive oxygen species modulator 1 [Source:MGI Symbol;Acc:MGI:1914317]
9853	ENSMUSG00000063558	-1.05554	5.402938	8.42681	0.003697	0.032539	Aox1	aldehyde oxidase 1 [Source:MGI Symbol;Acc:MGI:88035]
9278	ENSMUSG00000055782	-1.05384	7.287366	22.06481	2.64E-06	0.000296	Abcd2	ATP-binding cassette, sub-family D (ALD), member 2 [Source:MGI Symbol;Acc:MGI:1349467]
2788	ENSMUSG00000023909	-1.05322	2.722259	20.12744	7.24E-06	0.00058	Paqr4	progesterone and adiponectin receptor family member IV [Source:MGI Symbol;Acc:MGI:1923748]
6415	ENSMUSG00000034810	-1.052	6.977553	9.232179	0.002378	0.025603	Scn7a	sodium channel, voltage-gated, type VII, alpha [Source:MGI Symbol;Acc:MGI:102965]
1472	ENSMUSG00000019864	-1.05135	3.474401	11.01494	0.000904	0.016039	Rtn4ip1	reticulon 4 interacting protein 1 [Source:MGI Symbol;Acc:MGI:2178759]
7903	ENSMUSG00000041650	-1.04894	4.588284	13.58512	0.000228	0.00685	Pcca	propionyl-Coenzyme A carboxylase, alpha polypeptide [Source:MGI Symbol;Acc:MGI:97499]
2208	ENSMUSG000000021764	-1.0474	5.411426	7.367726	0.00664	0.044318	Ndufs4	NADH dehydrogenase (ubiquinone) Fe-S protein 4 [Source:MGI Symbol;Acc:MGI:1343135]
4467	ENSMUSG00000028551	-1.0471	4.712024	14.17794	0.000166	0.005628	Cdkn2c	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) [Source:MGI Symbol;Acc:MGI:105388]
5581	ENSMUSG000000031722	-1.04673	5.852777	7.608432	0.00581	0.041025	Hp	haptoglobin [Source:MGI Symbol;Acc:MGI:96211]
4793	ENSMUSG00000029330	-1.04515	2.124189	15.20925	9.62E-05	0.003912	Cds1	CDP-diacylglycerol synthase 1 [Source:MGI Symbol;Acc:MGI:1921846]
3200	ENSMUSG00000025006	-1.0451	7.052747	15.94721	6.51E-05	0.00285	Sorbs1	sorbin and SH3 domain containing 1 [Source:MGI Symbol;Acc:MGI:700014]
3981	ENSMUSG00000027217	-1.04418	4.713586	8.833372	0.002958	0.028861	Tspan18	tetraspanin 18 [Source:MGI Symbol;Acc:MGI:1917186]
2010	ENSMUSG00000021226	-1.04148	3.692157	7.728379	0.005436	0.039773	Acot2	acyl-CoA thioesterase 2 [Source:MGI Symbol;Acc:MGI:2159605]
9721	ENSMUSG000000061740	-1.04096	3.700523	9.407909	0.002161	0.024496	Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22 [Source:MGI Symbol;Acc:MGI:1929474]
4652	ENSMUSG00000028978	-1.03791	3.826568	15.18029	9.77E-05	0.003921	Nos3	nitric oxide synthase 3, endothelial cell [Source:MGI Symbol;Acc:MGI:97362]
2	ENSMUSG000000000058	-1.03781	6.12078	27.89894	1.28E-07	3.67E-05	Cav2	caveolin 2 [Source:MGI Symbol;Acc:MGI:107571]
113	ENSMUSG000000001100	-1.0363	5.276865	9.856482	0.001692	0.021822	Poldip2	polymerase (DNA-directed), delta interacting protein 2 [Source:MGI Symbol;Acc:MGI:1915061]
3380	ENSMUSG00000025509	-1.03576	8.406571	10.24297	0.001372	0.019775	Pnpla2	patatin-like phospholipase domain containing 2 [Source:MGI Symbol;Acc:MGI:1914103]
4003	ENSMUSG00000027282	-1.03529	6.485943	9.216688	0.002398	0.025745	Mtch2	mitochondrial carrier homolog 2 (C. elegans) [Source:MGI Symbol;Acc:MGI:1929260]
2015	ENSMUSG00000021241	-1.03468	3.989819	9.955998	0.001603	0.0212	Isca2	iron-sulfur cluster assembly 2 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1921566]
7405	ENSMUSG00000039246	-1.0345	2.610492	8.351159	0.003854	0.033062	Lyplal1	lysophospholipase-like 1 [Source:MGI Symbol;Acc:MGI:2385115]
271	ENSMUSG00000002308	-1.02956	2.321128	17.52012	2.84E-05	0.001557	Cd320	CD320 antigen [Source:MGI Symbol;Acc:MGI:1860083]
849	ENSMUSG000000009614	-1.0295	2.86118	26.45211	2.70E-07	6.01E-05	Sardh	sarcosine dehydrogenase [Source:MGI Symbol;Acc:MGI:2183102]

2419	ENSMUSG00000022309	-1.02775	4.159478	7.172425	0.007403	0.046709	Angpt1	angiopoietin 1 [Source:MGI Symbol;Acc:MGI:108448]
3175	ENSMUSG00000024955	-1.02767	4.097885	17.25838	3.26E-05	0.001734	Esrra	estrogen related receptor, alpha [Source:MGI Symbol;Acc:MGI:1346831]
645	ENSMUSG00000005951	-1.0274	2.571458	18.16436	2.03E-05	0.0012	Shpk	sedoheptulokinase [Source:MGI Symbol;Acc:MGI:1921887]
5295	ENSMUSG00000030869	-1.02671	5.044127	7.776315	0.005294	0.039264	Ndufab1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1 [Source:MGI Symbol;Acc:MGI:1917566]
7163	ENSMUSG00000038375	-1.02368	6.539649	8.190924	0.00421	0.034673	Trp53inp2	transformation related protein 53 inducible nuclear protein 2 [Source:MGI Symbol;Acc:MGI:1915978]
3104	ENSMUSG00000024782	-1.02338	6.423456	13.96567	0.000186	0.005966	Ak3	adenylate kinase 3 [Source:MGI Symbol;Acc:MGI:1860835]
5113	ENSMUSG00000030317	-1.02012	3.177427	7.815123	0.005181	0.038773	Timp4	tissue inhibitor of metalloproteinase 4 [Source:MGI Symbol;Acc:MGI:109125]
2444	ENSMUSG00000022365	-1.01915	6.157811	22.10094	2.59E-06	0.000294	Der1	Der1-like domain family, member 1 [Source:MGI Symbol;Acc:MGI:1915069]
3907	ENSMUSG00000026986	-1.0154	3.594589	10.70455	0.001069	0.017315	Hnmt	histamine N-methyltransferase [Source:MGI Symbol;Acc:MGI:2153181]
3505	ENSMUSG00000025950	-1.01483	6.835732	9.830385	0.001717	0.021952	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble [Source:MGI Symbol;Acc:MGI:96413]
2227	ENSMUSG00000021809	-1.01459	3.55921	13.5851	0.000228	0.00685	Nudt13	nudix (nucleoside diphosphate linked moiety X)-type motif 13 [Source:MGI Symbol;Acc:MGI:1914975]
7411	ENSMUSG00000039270	-1.01347	5.012669	18.97021	1.33E-05	0.000899	Megf9	multiple EGF-like-domains 9 [Source:MGI Symbol;Acc:MGI:1918264]
7852	ENSMUSG00000041351	-1.01229	2.609178	10.3426	0.0013	0.019245	Rap1gap	Rap1 GTPase-activating protein [Source:MGI Symbol;Acc:MGI:109338]
6747	ENSMUSG00000036585	-1.01219	4.414948	8.786086	0.003035	0.029243	Fgf1	fibroblast growth factor 1 [Source:MGI Symbol;Acc:MGI:95515]
4420	ENSMUSG00000028427	-1.01215	5.472618	7.380783	0.006592	0.044214	Aqp7	aquaporin 7 [Source:MGI Symbol;Acc:MGI:1314647]
7341	ENSMUSG00000039048	-1.01158	4.123565	12.21216	0.000475	0.010984	Foxred1	FAD-dependent oxidoreductase domain containing 1 [Source:MGI Symbol;Acc:MGI:2446262]
1129	ENSMUSG00000015970	-1.0073	4.651192	8.068595	0.004504	0.035895	Chdh	choline dehydrogenase [Source:MGI Symbol;Acc:MGI:1860776]
4399	ENSMUSG00000028393	-1.00621	3.720425	27.11473	1.92E-07	4.64E-05	Alad	aminolevulinate, delta-, dehydratase [Source:MGI Symbol;Acc:MGI:96853]
420	ENSMUSG00000003526	-1.00326	2.953753	13.82844	0.0002	0.006289	Prodh	proline dehydrogenase [Source:MGI Symbol;Acc:MGI:97770]
7137	ENSMUSG00000038286	-1.00276	2.927957	16.33314	5.31E-05	0.002453	Bphl	biphenyl hydrolase-like (serine hydrolase, breast epithelial mucin-associated antigen) [Source:MGI Symbol;Acc:MGI:1915271]
9183	ENSMUSG000000054474	-1.00127	2.345384	7.882704	0.004991	0.03795	Thnsl2	threonine synthase-like 2 (bacterial) [Source:MGI Symbol;Acc:MGI:3041254]
688	ENSMUSG00000006373	-1.00086	5.589273	25.48933	4.45E-07	8.66E-05	Pgrmc1	progesterone receptor membrane component 1 [Source:MGI Symbol;Acc:MGI:1858305]
6541	ENSMUSG00000035473	-0.99908	3.738505	11.51613	0.00069	0.013693	Galm	galactose mutarotase [Source:MGI Symbol;Acc:MGI:2442420]
10251	ENSMUSG00000072946	-0.99906	6.013443	11.52042	0.000688	0.013693	Ptgr2	prostaglandin reductase 2 [Source:MGI Symbol;Acc:MGI:1916372]
7509	ENSMUSG00000039745	-0.99841	2.38373	21.69213	3.20E-06	0.000337	Htatip2	HIV-1 tat interactive protein 2, homolog (human) [Source:MGI Symbol;Acc:MGI:1859271]
6096	ENSMUSG00000033287	-0.99649	3.239605	27.51812	1.56E-07	4.04E-05	Kctd17	potassium channel tetramerisation domain containing 17 [Source:MGI Symbol;Acc:MGI:1920094]
7797	ENSMUSG00000041028	-0.99604	7.44946	7.630784	0.005738	0.040814	Ghitm	growth hormone inducible transmembrane protein [Source:MGI Symbol;Acc:MGI:1913342]

4954	ENSMUSG00000029759	-0.99552	5.430019	7.522381	0.006094	0.042165	Pon3	paraoxonase 3 [Source:MGI Symbol;Acc:MGI:106686]
1375	ENSMUSG00000019082	-0.99495	4.413502	22.83369	1.77E-06	0.000232	Slc25a22	solute carrier family 25 (mitochondrial carrier, glutamate), member 22 [Source:MGI Symbol;Acc:MGI:1915517]
8799	ENSMUSG000000049760	-0.99434	4.425905	9.393831	0.002177	0.024583	2410015M20Rik	RIKEN cDNA 2410015M20 gene [Source:MGI Symbol;Acc:MGI:2442174]
4547	ENSMUSG000000028737	-0.99428	4.472636	7.816449	0.005177	0.038771	Aldh4a1	aldehyde dehydrogenase 4 family, member A1 [Source:MGI Symbol;Acc:MGI:2443883]
4147	ENSMUSG000000027673	-0.99372	5.577443	7.975458	0.004742	0.036903	Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 [Source:MGI Symbol;Acc:MGI:1913296]
8119	ENSMUSG000000042709	-0.99344	4.492098	12.12048	0.000499	0.011274	Atpaf2	ATP synthase mitochondrial F1 complex assembly factor 2 [Source:MGI Symbol;Acc:MGI:2180561]
5204	ENSMUSG000000030647	-0.99308	5.08426	7.092475	0.007741	0.048023	Ndufc2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2 [Source:MGI Symbol;Acc:MGI:1344370]
2730	ENSMUSG000000023094	-0.99301	2.541298	7.704982	0.005507	0.03999	Msrb2	methionine sulfoxide reductase B2 [Source:MGI Symbol;Acc:MGI:1923717]
8132	ENSMUSG000000042770	-0.99274	3.723324	22.12194	2.56E-06	0.000294	Hebp1	heme binding protein 1 [Source:MGI Symbol;Acc:MGI:1333880]
10187	ENSMUSG000000071347	-0.99203	3.614663	7.193606	0.007316	0.046406	C1qtnf9	C1q and tumor necrosis factor related protein 9 [Source:MGI Symbol;Acc:MGI:3045252]
4404	ENSMUSG000000028399	-0.99201	5.572496	9.197396	0.002424	0.02589	Ptprd	protein tyrosine phosphatase, receptor type, D [Source:MGI Symbol;Acc:MGI:97812]
759	ENSMUSG000000007655	-0.98986	8.050034	19.74904	8.83E-06	0.000678	Cav1	caveolin 1, caveolae protein [Source:MGI Symbol;Acc:MGI:102709]
785	ENSMUSG000000008090	-0.9894	4.855402	14.34898	0.000152	0.005355	Fgfr1	fibroblast growth factor receptor-like 1 [Source:MGI Symbol;Acc:MGI:2150920]
598	ENSMUSG000000005510	-0.98684	5.294899	6.961396	0.008329	0.04989	Ndufs3	NADH dehydrogenase (ubiquinone) Fe-S protein 3 [Source:MGI Symbol;Acc:MGI:1915599]
10030	ENSMUSG000000068220	-0.9866	7.113203	14.41833	0.000146	0.005256	Lgals1	lectin, galactose binding, soluble 1 [Source:MGI Symbol;Acc:MGI:96777]
474	ENSMUSG000000004098	-0.98633	6.083197	16.55591	4.72E-05	0.002248	Col5a3	collagen, type V, alpha 3 [Source:MGI Symbol;Acc:MGI:1858212]
2404	ENSMUSG000000022265	-0.98564	5.228829	7.151389	0.007491	0.04715	Ank	progressive ankylosis [Source:MGI Symbol;Acc:MGI:3045421]
4190	ENSMUSG000000027820	-0.98534	6.693253	16.06475	6.12E-05	0.002722	Mme	membrane metallo endopeptidase [Source:MGI Symbol;Acc:MGI:97004]
5389	ENSMUSG000000031209	-0.9852	4.563568	14.28342	0.000157	0.005456	Heph	hephaestin [Source:MGI Symbol;Acc:MGI:1332240]
9949	ENSMUSG000000066361	-0.98398	4.153107	8.040822	0.004573	0.036189	Serpina3c	serine (or cysteine) peptidase inhibitor, clade A, member 3C [Source:MGI Symbol;Acc:MGI:102848]
5034	ENSMUSG000000030086	-0.9839	2.541361	18.85965	1.41E-05	0.000941	Chchd6	coiled-coil-helix-coiled-coil-helix domain containing 6 [Source:MGI Symbol;Acc:MGI:1913348]
1329	ENSMUSG000000018677	-0.98338	5.544489	13.21466	0.000278	0.007752	Slc25a39	solute carrier family 25, member 39 [Source:MGI Symbol;Acc:MGI:1196386]
832	ENSMUSG000000009281	-0.98315	4.743845	11.50756	0.000693	0.013707	Rarres2	retinoic acid receptor responder (tazarotene induced) 2 [Source:MGI Symbol;Acc:MGI:1918910]
4840	ENSMUSG000000029455	-0.97874	6.876431	10.85297	0.000986	0.016663	Aldh2	aldehyde dehydrogenase 2, mitochondrial [Source:MGI Symbol;Acc:MGI:99600]
7383	ENSMUSG000000039195	-0.97843	3.25014	10.77322	0.00103	0.016989	1110008P14Rik	RIKEN cDNA 1110008P14 gene [Source:MGI Symbol;Acc:MGI:1920987]

8206	ENSMUSG00000043673	-0.97767	2.788639	11.08319	0.000871	0.015901	Kcns3	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3 [Source:MGI Symbol;Acc:MGI:1098804]
2148	ENSMUSG000000021619	-0.97735	2.742394	26.9272	2.11E-07	4.87E-05	Atg10	autophagy related 10 [Source:MGI Symbol;Acc:MGI:1914045]
6848	ENSMUSG000000037060	-0.9769	3.931266	22.68767	1.91E-06	0.000244	Prkcdbp	protein kinase C, delta binding protein [Source:MGI Symbol;Acc:MGI:1923422]
6114	ENSMUSG000000033377	-0.97607	5.092345	11.08011	0.000873	0.015901	Palmd	palmdelphin [Source:MGI Symbol;Acc:MGI:2148896]
561	ENSMUSG000000005069	-0.97583	4.583928	19.02976	1.29E-05	0.000879	Pex5	peroxisomal biogenesis factor 5 [Source:MGI Symbol;Acc:MGI:1098808]
3866	ENSMUSG000000026880	-0.97477	6.040764	7.318393	0.006825	0.044961	Stom	stomatin [Source:MGI Symbol;Acc:MGI:95403]
7095	ENSMUSG000000038150	-0.97463	6.242519	14.06661	0.000176	0.005849	Ormdl3	ORM1-like 3 (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1913862]
135	ENSMUSG000000001270	-0.97334	2.949132	21.85609	2.94E-06	0.00032	Ckb	creatine kinase, brain [Source:MGI Symbol;Acc:MGI:88407]
9243	ENSMUSG000000055254	-0.97195	6.211422	13.97005	0.000186	0.005966	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2 [Source:MGI Symbol;Acc:MGI:97384]
636	ENSMUSG000000005882	-0.97025	4.931569	7.961603	0.004778	0.03716	Uqcc1	ubiquinol-cytochrome c reductase complex assembly factor 1 [Source:MGI Symbol;Acc:MGI:1929472]
6556	ENSMUSG000000035559	-0.96951	2.47647	21.4182	3.69E-06	0.000356	Mpv17l2	MPV17 mitochondrial membrane protein-like 2 [Source:MGI Symbol;Acc:MGI:2681846]
2711	ENSMUSG000000023044	-0.96517	5.220316	17.71537	2.57E-05	0.001419	Csad	cysteine sulfinic acid decarboxylase [Source:MGI Symbol;Acc:MGI:2180098]
9446	ENSMUSG0000000057969	-0.96144	3.262475	11.97079	0.00054	0.011918	Sema3b	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B [Source:MGI Symbol;Acc:MGI:107561]
415	ENSMUSG000000003464	-0.96109	6.087983	14.85552	0.000116	0.004391	Pex19	peroxisomal biogenesis factor 19 [Source:MGI Symbol;Acc:MGI:1334458]
1395	ENSMUSG000000019326	-0.95912	6.888796	9.770209	0.001774	0.022367	Aoc3	amine oxidase, copper containing 3 [Source:MGI Symbol;Acc:MGI:1306797]
7952	ENSMUSG0000000041881	-0.95813	5.211895	10.31012	0.001323	0.019489	Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (B14.5a) [Source:MGI Symbol;Acc:MGI:1913666]
9248	ENSMUSG000000005322	-0.95562	8.25955	10.66386	0.001092	0.017583	Tns1	tensin 1 [Source:MGI Symbol;Acc:MGI:104552]
1341	ENSMUSG000000018796	-0.95521	9.805467	8.647257	0.003276	0.030436	Acsl1	acyl-CoA synthetase long-chain family member 1 [Source:MGI Symbol;Acc:MGI:102797]
7385	ENSMUSG0000000039197	-0.95499	4.723464	8.492218	0.003567	0.031814	Adk	adenosine kinase [Source:MGI Symbol;Acc:MGI:87930]
9361	ENSMUSG0000000056899	-0.95467	2.749319	9.04268	0.002637	0.02724	Imm2p2l	IMP2 inner mitochondrial membrane peptidase-like (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:2135611]
62	ENSMUSG000000000686	-0.95458	3.14811	12.03841	0.000521	0.011684	Abhd15	abhydrolase domain containing 15 [Source:MGI Symbol;Acc:MGI:1914727]
7587	ENSMUSG0000000040037	-0.95446	4.512689	8.062701	0.004519	0.035916	Negr1	neuronal growth regulator 1 [Source:MGI Symbol;Acc:MGI:2444846]
1125	ENSMUSG000000015943	-0.95291	1.81518	12.00769	0.00053	0.011806	Bola1	bolA-like 1 (E. coli) [Source:MGI Symbol;Acc:MGI:1916418]
4097	ENSMUSG0000000027546	-0.95271	5.268397	14.475	0.000142	0.005159	Atp9a	ATPase, class II, type 9A [Source:MGI Symbol;Acc:MGI:1330826]
9526	ENSMUSG0000000059173	-0.95229	4.207796	7.595546	0.005851	0.041153	Pde1a	phosphodiesterase 1A, calmodulin-dependent [Source:MGI Symbol;Acc:MGI:1201792]
6842	ENSMUSG0000000037022	-0.95217	3.341494	20.6796	5.43E-06	0.00047	Mmaa	methylmalonic aciduria (cobalamin deficiency) type A [Source:MGI Symbol;Acc:MGI:1923805]

3786	ENSMUSG00000026701	-0.95013	5.674716	33.48454	7.18E-09	3.26E-06	Prdx6	peroxiredoxin 6 [Source:MGI Symbol;Acc:MGI:894320]
9026	ENSMUSG000000052605	-0.95003	1.907605	17.74878	2.52E-05	0.001409	Isoc2b	isochorismatase domain containing 2b [Source:MGI Symbol;Acc:MGI:1914691]
983	ENSMUSG000000014294	-0.94928	4.163701	7.281738	0.006966	0.045391	Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2 [Source:MGI Symbol;Acc:MGI:1343103]
322	ENSMUSG000000002767	-0.94881	3.806962	9.419463	0.002147	0.024495	Mrpl2	mitochondrial ribosomal protein L2 [Source:MGI Symbol;Acc:MGI:1351622]
5343	ENSMUSG000000031059	-0.94876	5.388722	7.10637	0.007681	0.047907	Ndufb11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11 [Source:MGI Symbol;Acc:MGI:1349919]
1270	ENSMUSG000000018217	-0.94865	5.928034	8.37996	0.003794	0.032964	Pmp22	peripheral myelin protein 22 [Source:MGI Symbol;Acc:MGI:97631]
9874	ENSMUSG000000063882	-0.94857	6.703807	7.085265	0.007772	0.048116	Uqcrrh	ubiquinol-cytochrome c reductase hinge protein [Source:MGI Symbol;Acc:MGI:1913826]
9055	ENSMUSG000000052914	-0.94746	3.755164	9.461676	0.002098	0.024276	Cyp2j6	cytochrome P450, family 2, subfamily j, polypeptide 6 [Source:MGI Symbol;Acc:MGI:1270148]
8676	ENSMUSG000000048371	-0.94577	4.427462	7.469878	0.006274	0.042886	Pdp2	pyruvate dehydrogenase phosphatase catalytic subunit 2 [Source:MGI Symbol;Acc:MGI:1918878]
9891	ENSMUSG000000064105	-0.94467	3.503423	21.50085	3.54E-06	0.000354	Cnnm2	cyclin M2 [Source:MGI Symbol;Acc:MGI:2151054]
1765	ENSMUSG000000020593	-0.94349	7.259212	7.264085	0.007035	0.045598	Lpin1	lipin 1 [Source:MGI Symbol;Acc:MGI:1891340]
10430	ENSMUSG000000078496	-0.94256	2.847849	7.444859	0.006362	0.043189	Gm13152	predicted gene 13152 [Source:MGI Symbol;Acc:MGI:3701121]
6153	ENSMUSG000000033576	-0.94254	5.020572	9.275586	0.002322	0.025334	Apol6	apolipoprotein L 6 [Source:MGI Symbol;Acc:MGI:1919189]
3844	ENSMUSG000000026834	-0.94109	6.4224	8.99589	0.002706	0.027639	Acvr1c	activin A receptor, type IC [Source:MGI Symbol;Acc:MGI:2661081]
1339	ENSMUSG000000018770	-0.94079	5.429129	11.15031	0.00084	0.015622	Atp5g3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9) [Source:MGI Symbol;Acc:MGI:2442035]
9445	ENSMUSG000000057963	-0.94065	4.339894	9.280631	0.002316	0.025334	Itpk1	inositol 1,3,4-triphosphate 5/6 kinase [Source:MGI Symbol;Acc:MGI:2446159]
3929	ENSMUSG000000027076	-0.94002	2.882309	9.170838	0.002459	0.026114	Timm10	translocase of inner mitochondrial membrane 10 [Source:MGI Symbol;Acc:MGI:1353429]
3250	ENSMUSG000000025151	-0.93975	6.288362	23.06899	1.56E-06	0.000218	Maged1	melanoma antigen, family D, 1 [Source:MGI Symbol;Acc:MGI:1930187]
6522	ENSMUSG000000035342	-0.93964	4.122908	13.13835	0.000289	0.007957	Lzts2	leucine zipper, putative tumor suppressor 2 [Source:MGI Symbol;Acc:MGI:2385095]
2460	ENSMUSG000000022404	-0.93671	4.484579	21.48115	3.57E-06	0.000354	Slc25a17	solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17 [Source:MGI Symbol;Acc:MGI:1342248]
9510	ENSMUSG000000058833	-0.93603	3.179345	10.39191	0.001266	0.018979	2810428115Rik	RIKEN cDNA 2810428115 gene [Source:MGI Symbol;Acc:MGI:1913712]
4407	ENSMUSG000000028405	-0.93582	6.403901	18.09856	2.10E-05	0.001222	Aco1	aconitase 1 [Source:MGI Symbol;Acc:MGI:87879]
2972	ENSMUSG000000024395	-0.93558	3.917145	7.309981	0.006857	0.045037	Lims2	LIM and senescent cell antigen like domains 2 [Source:MGI Symbol;Acc:MGI:2385067]
7332	ENSMUSG000000039016	-0.93401	4.304817	7.0384	0.007978	0.048672	Timm8b	translocase of inner mitochondrial membrane 8B [Source:MGI Symbol;Acc:MGI:1353424]

4040	ENSMUSG00000027371	-0.93373	2.416823	10.56441	0.001153	0.018153	Fahd2a	fumarylacetoacetate hydrolase domain containing 2A [Source:MGI Symbol;Acc:MGI:1915376]
2435	ENSMUSG00000022351	-0.93259	3.05018	10.7965	0.001017	0.016915	Sqle	squalene epoxidase [Source:MGI Symbol;Acc:MGI:109296]
8033	ENSMUSG00000042312	-0.9301	3.946987	11.81767	0.000587	0.012553	S100a13	S100 calcium binding protein A13 [Source:MGI Symbol;Acc:MGI:109581]
7542	ENSMUSG00000039883	-0.92943	5.452818	7.522024	0.006095	0.042165	Lrrc17	leucine rich repeat containing 17 [Source:MGI Symbol;Acc:MGI:1921761]
1830	ENSMUSG00000020741	-0.92856	6.724193	11.15697	0.000837	0.015593	Cluh	clustered mitochondria (cluA/CLU1) homolog [Source:MGI Symbol;Acc:MGI:1921398]
9780	ENSMUSG000000062619	-0.92826	1.870828	7.170486	0.007411	0.046732	2310039H08Rik	RIKEN cDNA 2310039H08 gene [Source:MGI Symbol;Acc:MGI:1914351]
4025	ENSMUSG00000027332	-0.92751	6.193058	8.759847	0.003079	0.029381	Ivd	isovaleryl coenzyme A dehydrogenase [Source:MGI Symbol;Acc:MGI:1929242]
8823	ENSMUSG00000050043	-0.92735	4.209364	22.29259	2.34E-06	0.000283	Tmx2	thioredoxin-related transmembrane protein 2 [Source:MGI Symbol;Acc:MGI:1914208]
2903	ENSMUSG00000024197	-0.92649	4.565921	38.2396	6.26E-10	4.26E-07	Plin3	perilipin 3 [Source:MGI Symbol;Acc:MGI:1914155]
368	ENSMUSG00000003072	-0.92608	6.014788	6.998945	0.008156	0.049302	Atp5d	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit [Source:MGI Symbol;Acc:MGI:1913293]
9394	ENSMUSG00000057315	-0.92299	4.211987	22.98495	1.63E-06	0.000223	Arhgap24	Rho GTPase activating protein 24 [Source:MGI Symbol;Acc:MGI:1922647]
310	ENSMUSG00000002660	-0.92291	3.800626	15.39858	8.71E-05	0.003593	Clpp	caseinolytic mitochondrial matrix peptidase proteolytic subunit [Source:MGI Symbol;Acc:MGI:1858213]
5792	ENSMUSG00000032263	-0.9219	4.287427	11.55625	0.000675	0.013599	Bckdhd	branched chain ketoacid dehydrogenase E1, beta polypeptide [Source:MGI Symbol;Acc:MGI:88137]
2487	ENSMUSG00000022471	-0.92129	4.364898	11.69613	0.000626	0.0129	Xrcc6	X-ray repair complementing defective repair in Chinese hamster cells 6 [Source:MGI Symbol;Acc:MGI:95606]
10588	ENSMUSG000000086784	-0.92038	4.226995	11.02484	0.000899	0.016032	Isoc2a	isochorismatase domain containing 2a [Source:MGI Symbol;Acc:MGI:3609243]
3755	ENSMUSG00000026626	-0.91981	7.990289	21.02368	4.54E-06	0.000419	Ppp2r5a	protein phosphatase 2, regulatory subunit B', alpha [Source:MGI Symbol;Acc:MGI:2388479]
1719	ENSMUSG00000020477	-0.91897	2.997057	14.21448	0.000163	0.005586	Mrps24	mitochondrial ribosomal protein S24 [Source:MGI Symbol;Acc:MGI:1928142]
2885	ENSMUSG00000024158	-0.91793	4.256131	7.909909	0.004916	0.037705	Hagh	hydroxyacyl glutathione hydrolase [Source:MGI Symbol;Acc:MGI:95745]
79	ENSMUSG00000000805	-0.91768	2.590702	7.773132	0.005303	0.03928	Car4	carbonic anhydrase 4 [Source:MGI Symbol;Acc:MGI:1096574]
1439	ENSMUSG00000019791	-0.91742	2.877942	10.77175	0.001031	0.016989	Hint3	histidine triad nucleotide binding protein 3 [Source:MGI Symbol;Acc:MGI:1914097]
9622	ENSMUSG000000060429	-0.91493	4.437523	16.05713	6.15E-05	0.002722	Sntb1	syntrophin, basic 1 [Source:MGI Symbol;Acc:MGI:101781]
4015	ENSMUSG00000027309	-0.91421	5.820651	16.45827	4.97E-05	0.002326	4930402H24Rik	RIKEN cDNA 4930402H24 gene [Source:MGI Symbol;Acc:MGI:1923029]
6844	ENSMUSG00000037031	-0.91317	3.472238	13.49705	0.000239	0.007017	Tspan15	tetraspanin 15 [Source:MGI Symbol;Acc:MGI:1917673]
1140	ENSMUSG00000016252	-0.9129	4.67577	7.780502	0.005281	0.039253	Atp5e	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit [Source:MGI Symbol;Acc:MGI:1855697]
7375	ENSMUSG00000039166	-0.91241	4.956256	10.03525	0.001536	0.020765	Akap7	A kinase (PRKA) anchor protein 7 [Source:MGI Symbol;Acc:MGI:1859150]
3503	ENSMUSG00000025940	-0.91236	4.326217	9.754118	0.001789	0.02246	Tmem70	transmembrane protein 70 [Source:MGI Symbol;Acc:MGI:1915068]

4650	ENSMUSG00000028975	-0.91223	4.384344	17.80812	2.44E-05	0.001379	Pex14	peroxisomal biogenesis factor 14 [Source:MGI Symbol;Acc:MGI:1927868]
7543	ENSMUSG00000039886	-0.91192	4.987985	12.55499	0.000395	0.009775	Tmem120a	transmembrane protein 120A [Source:MGI Symbol;Acc:MGI:2686991]
7768	ENSMUSG00000040841	-0.90981	2.064055	11.90631	0.000559	0.012143	Six5	sine oculis-related homeobox 5 [Source:MGI Symbol;Acc:MGI:106220]
4490	ENSMUSG00000028603	-0.90834	7.786957	12.78259	0.00035	0.009012	Scp2	sterol carrier protein 2, liver [Source:MGI Symbol;Acc:MGI:98254]
713	ENSMUSG00000006641	-0.90782	4.056627	18.41003	1.78E-05	0.001115	Slc5a6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6 [Source:MGI Symbol;Acc:MGI:2660847]
6306	ENSMUSG000000034308	-0.90528	2.539175	9.977508	0.001585	0.021031	Sdr42e1	short chain dehydrogenase/reductase family 42E, member 1 [Source:MGI Symbol;Acc:MGI:1921282]
43	ENSMUSG00000000440	-0.90498	6.136376	7.594716	0.005854	0.041153	Pparg	peroxisome proliferator activated receptor gamma [Source:MGI Symbol;Acc:MGI:97747]
7211	ENSMUSG000000038539	-0.90419	4.058635	8.726532	0.003136	0.029704	Atf5	activating transcription factor 5 [Source:MGI Symbol;Acc:MGI:2141857]
7407	ENSMUSG000000039253	-0.90348	3.44187	16.93965	3.86E-05	0.001956	Fn3krp	fructosamine 3 kinase related protein [Source:MGI Symbol;Acc:MGI:2679256]
3322	ENSMUSG000000025348	-0.90336	4.63939	10.45461	0.001223	0.018723	Itga7	integrin alpha 7 [Source:MGI Symbol;Acc:MGI:102700]
7577	ENSMUSG000000040006	-0.9032	6.146166	7.317346	0.006829	0.044961	Ginm1	glycoprotein integral membrane 1 [Source:MGI Symbol;Acc:MGI:2384905]
10131	ENSMUSG000000070436	-0.90221	6.66952	16.8148	4.12E-05	0.002014	Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1 [Source:MGI Symbol;Acc:MGI:88283]
6441	ENSMUSG000000034926	-0.90111	3.244254	8.537337	0.003479	0.031355	Dhcr24	24-dehydrocholesterol reductase [Source:MGI Symbol;Acc:MGI:1922004]
9209	ENSMUSG000000054733	-0.89915	3.613622	9.20866	0.002409	0.025807	Msra	methionine sulfoxide reductase A [Source:MGI Symbol;Acc:MGI:106916]
6815	ENSMUSG000000036932	-0.89637	4.521281	10.73361	0.001052	0.017203	Aifm1	apoptosis-inducing factor, mitochondrion-associated 1 [Source:MGI Symbol;Acc:MGI:1349419]
5685	ENSMUSG000000031969	-0.89633	4.000357	8.031925	0.004596	0.036236	Acad8	acyl-Coenzyme A dehydrogenase family, member 8 [Source:MGI Symbol;Acc:MGI:1914198]
3063	ENSMUSG000000024646	-0.89626	6.229064	15.82579	6.94E-05	0.003015	Cyb5a	cytochrome b5 type A (microsomal) [Source:MGI Symbol;Acc:MGI:1926952]
129	ENSMUSG000000001211	-0.8959	6.445282	11.53599	0.000683	0.013672	Agpat3	1-acylglycerol-3-phosphate O-acyltransferase 3 [Source:MGI Symbol;Acc:MGI:1336186]
8235	ENSMUSG000000044026	-0.89572	2.633565	10.6251	0.001116	0.01776	Slc35g1	solute carrier family 35, member G1 [Source:MGI Symbol;Acc:MGI:2444789]
6143	ENSMUSG000000033526	-0.89404	5.092734	15.13494	0.0001	0.00395	Ppip5k1	diphosphoinositol pentakisphosphate kinase 1 [Source:MGI Symbol;Acc:MGI:2443281]
1918	ENSMUSG000000020963	-0.89265	4.464537	14.95908	0.00011	0.004215	Tshr	thyroid stimulating hormone receptor [Source:MGI Symbol;Acc:MGI:98849]
10605	ENSMUSG000000087687	-0.89255	3.637012	16.10915	5.98E-05	0.002676	Pet100	PET100 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:3615306]
8261	ENSMUSG000000044252	-0.89089	5.024219	9.826545	0.00172	0.02196	Osbp1a	oxysterol binding protein-like 1A [Source:MGI Symbol;Acc:MGI:1927551]
4706	ENSMUSG000000029103	-0.88991	5.408885	17.84521	2.40E-05	0.001367	Lrpap1	low density lipoprotein receptor-related protein associated protein 1 [Source:MGI Symbol;Acc:MGI:96829]
4690	ENSMUSG000000029066	-0.88314	3.774126	16.99833	3.74E-05	0.001923	Mrpl20	mitochondrial ribosomal protein L20 [Source:MGI Symbol;Acc:MGI:2137221]
8829	ENSMUSG000000050144	-0.88309	5.072716	22.3633	2.26E-06	0.000276	Slc25a44	solute carrier family 25, member 44 [Source:MGI Symbol;Acc:MGI:2444391]

2339	ENSMUSG000000022106	-0.88189	5.179958	20.4776	6.03E-06	0.00051	Rcbtb2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2 [Source:MGI Symbol;Acc:MGI:1917200]
7142	ENSMUSG000000038301	-0.88145	5.523756	8.781018	0.003044	0.029246	Snx10	sorting nexin 10 [Source:MGI Symbol;Acc:MGI:1919232]
5885	ENSMUSG000000032459	-0.88145	3.234002	13.13434	0.00029	0.007957	Mrps22	mitochondrial ribosomal protein S22 [Source:MGI Symbol;Acc:MGI:1928137]
353	ENSMUSG000000002980	-0.88128	4.983264	9.933582	0.001623	0.021382	Bcam	basal cell adhesion molecule [Source:MGI Symbol;Acc:MGI:1929940]
6763	ENSMUSG000000036667	-0.88065	5.01376	14.39813	0.000148	0.005285	Tcaf1	TRPM8 channel-associated factor 1 [Source:MGI Symbol;Acc:MGI:1914665]
3327	ENSMUSG000000025355	-0.87996	4.257075	11.70774	0.000622	0.012844	Mmp19	matrix metalloproteinase 19 [Source:MGI Symbol;Acc:MGI:1927899]
6219	ENSMUSG000000033917	-0.87982	5.05625	7.297749	0.006904	0.045154	Gde1	glycerophosphodiester phosphodiesterase 1 [Source:MGI Symbol;Acc:MGI:1891827]
3826	ENSMUSG000000026798	-0.87939	1.845749	14.37084	0.00015	0.005335	Coq4	coenzyme Q4 homolog (yeast) [Source:MGI Symbol;Acc:MGI:1098826]
4170	ENSMUSG000000027762	-0.87895	2.185987	9.940109	0.001617	0.021332	Sucnr1	succinate receptor 1 [Source:MGI Symbol;Acc:MGI:1934135]
5003	ENSMUSG000000029998	-0.87747	6.027778	14.77933	0.000121	0.004525	Pcyox1	prenylcysteine oxidase 1 [Source:MGI Symbol;Acc:MGI:1914131]
10212	ENSMUSG000000071657	-0.87661	5.581168	18.11347	2.08E-05	0.001222	Bscl2	Berardinelli-Seip congenital lipodystrophy 2 homolog (seipin) [Source:MGI Symbol;Acc:MGI:1298392]
2279	ENSMUSG000000021951	-0.8766	2.34588	12.70272	0.000365	0.009257	N6amt2	N-6 adenine-specific DNA methyltransferase 2 (putative) [Source:MGI Symbol;Acc:MGI:1915293]
577	ENSMUSG000000005299	-0.87619	5.065989	10.98705	0.000918	0.016148	Letm1	leucine zipper-EF-hand containing transmembrane protein 1 [Source:MGI Symbol;Acc:MGI:1932557]
3442	ENSMUSG000000025780	-0.8756	7.920973	10.2639	0.001357	0.019651	Itih5	inter-alpha (globulin) inhibitor H5 [Source:MGI Symbol;Acc:MGI:1925751]
9639	ENSMUSG000000060675	-0.87527	6.832159	10.89014	0.000967	0.016494	Pla2g16	phospholipase A2, group XVI [Source:MGI Symbol;Acc:MGI:2179715]
1056	ENSMUSG000000015224	-0.87424	2.652774	7.101593	0.007702	0.047915	Cyp2j9	cytochrome P450, family 2, subfamily j, polypeptide 9 [Source:MGI Symbol;Acc:MGI:1921769]
1603	ENSMUSG000000020219	-0.87292	3.272976	12.25295	0.000465	0.010769	Timm13	translocase of inner mitochondrial membrane 13 [Source:MGI Symbol;Acc:MGI:1353432]
4217	ENSMUSG000000027890	-0.87108	2.215046	7.393076	0.006548	0.044038	Gstm4	glutathione S-transferase, mu 4 [Source:MGI Symbol;Acc:MGI:95862]
9104	ENSMUSG000000053460	-0.8704	3.821522	29.53424	5.49E-08	1.66E-05	Ggcx	gamma-glutamyl carboxylase [Source:MGI Symbol;Acc:MGI:1927655]
3778	ENSMUSG000000026676	-0.8699	3.69231	14.24349	0.000161	0.005521	Ccdc3	coiled-coil domain containing 3 [Source:MGI Symbol;Acc:MGI:1921436]
8775	ENSMUSG000000049521	-0.86978	4.094567	16.82832	4.09E-05	0.002008	Cdc42ep1	CDC42 effector protein (Rho GTPase binding) 1 [Source:MGI Symbol;Acc:MGI:1929763]
10225	ENSMUSG000000071847	-0.86978	4.729567	9.123233	0.002524	0.026562	Apccd1	adenomatous polyposis coli down-regulated 1 [Source:MGI Symbol;Acc:MGI:3513977]
179	ENSMUSG000000001576	-0.86933	5.369458	18.28086	1.91E-05	0.001167	Ergic1	endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1 [Source:MGI Symbol;Acc:MGI:1914708]
1374	ENSMUSG000000019066	-0.86788	4.663388	26.10314	3.24E-07	6.65E-05	Rab3d	RAB3D, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:97844]
2805	ENSMUSG000000023960	-0.86686	5.318038	17.14162	3.47E-05	0.001817	Enpp5	ectonucleotide pyrophosphatase/phosphodiesterase 5 [Source:MGI Symbol;Acc:MGI:1933830]
2600	ENSMUSG000000022751	-0.86516	2.891408	8.348636	0.00386	0.033063	Nit2	nitrilase family, member 2 [Source:MGI Symbol;Acc:MGI:1261838]

4101	ENSMUSG000000027555	-0.86437	3.523302	11.70853	0.000622	0.012844	Car13	carbonic anhydrase 13 [Source:MGI Symbol;Acc:MGI:1931322]
4389	ENSMUSG000000028347	-0.86372	3.162808	10.96247	0.00093	0.016244	Tmeff1	transmembrane protein with EGF-like and two follistatin-like domains 1 [Source:MGI Symbol;Acc:MGI:1926810]
10385	ENSMUSG000000075419	-0.86114	4.032744	11.28785	0.00078	0.014835	Dolk	dolichol kinase [Source:MGI Symbol;Acc:MGI:2677836]
1054	ENSMUSG000000015217	-0.85993	2.797273	7.662792	0.005637	0.040409	Hmgb3	high mobility group box 3 [Source:MGI Symbol;Acc:MGI:1098219]
8692	ENSMUSG000000048537	-0.85949	5.725125	16.47646	4.93E-05	0.002313	Phldb1	pleckstrin homology-like domain, family B, member 1 [Source:MGI Symbol;Acc:MGI:2143230]
2161	ENSMUSG000000021662	-0.85829	3.831836	11.3712	0.000746	0.014513	Arhgef28	Rho guanine nucleotide exchange factor (GEF) 28 [Source:MGI Symbol;Acc:MGI:1346016]
8234	ENSMUSG000000044018	-0.85786	4.522369	7.604811	0.005821	0.041054	Mrpl50	mitochondrial ribosomal protein L50 [Source:MGI Symbol;Acc:MGI:107329]
8814	ENSMUSG000000049940	-0.85766	5.196597	18.23281	1.95E-05	0.001183	Pgrmc2	progesterone receptor membrane component 2 [Source:MGI Symbol;Acc:MGI:1918054]
5059	ENSMUSG000000030168	-0.85733	8.330663	10.22991	0.001382	0.019811	Adipor2	adiponectin receptor 2 [Source:MGI Symbol;Acc:MGI:93830]
8572	ENSMUSG000000047409	-0.8557	5.241808	13.86553	0.000196	0.00622	Ctdspl	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like [Source:MGI Symbol;Acc:MGI:1916524]
4963	ENSMUSG000000029776	-0.85523	6.197424	7.514419	0.006121	0.042267	Hibadh	3-hydroxyisobutyrate dehydrogenase [Source:MGI Symbol;Acc:MGI:1889802]
778	ENSMUSG000000007891	-0.85433	9.062504	12.65161	0.000375	0.009464	Ctsd	cathepsin D [Source:MGI Symbol;Acc:MGI:88562]
5787	ENSMUSG000000032251	-0.85368	2.052652	7.450899	0.00634	0.04312	Irak1bp1	interleukin-1 receptor-associated kinase 1 binding protein 1 [Source:MGI Symbol;Acc:MGI:1929475]
4414	ENSMUSG000000028414	-0.8529	3.880388	18.52697	1.68E-05	0.001067	Fktn	fukutin [Source:MGI Symbol;Acc:MGI:2179507]
3065	ENSMUSG000000024659	-0.85275	5.886232	8.391071	0.003771	0.032857	Anxa1	annexin A1 [Source:MGI Symbol;Acc:MGI:96819]
592	ENSMUSG000000005469	-0.85262	5.768411	7.752918	0.005363	0.03956	Prkaca	protein kinase, cAMP dependent, catalytic, alpha [Source:MGI Symbol;Acc:MGI:97592]
10302	ENSMUSG000000073987	-0.85154	3.713279	23.30834	1.38E-06	0.000203	Ggh	gamma-glutamyl hydrolase [Source:MGI Symbol;Acc:MGI:1329035]
1655	ENSMUSG000000020334	-0.84906	2.756871	7.569903	0.005935	0.041454	Slc22a4	solute carrier family 22 (organic cation transporter), member 4 [Source:MGI Symbol;Acc:MGI:1353479]
6184	ENSMUSG000000033735	-0.84872	3.296181	19.02477	1.29E-05	0.000879	Spr	sepiapterin reductase [Source:MGI Symbol;Acc:MGI:103078]
9146	ENSMUSG000000054021	-0.84728	2.070406	11.07046	0.000877	0.015901	Sirt5	sirtuin 5 [Source:MGI Symbol;Acc:MGI:1915596]
5329	ENSMUSG000000030986	-0.84673	4.375321	12.61918	0.000382	0.009564	Dhx32	DEAH (Asp-Glu-Ala-His) box polypeptide 32 [Source:MGI Symbol;Acc:MGI:2141813]
3059	ENSMUSG000000024640	-0.84663	4.045442	13.20318	0.000279	0.007752	Psat1	phosphoserine aminotransferase 1 [Source:MGI Symbol;Acc:MGI:2183441]
4235	ENSMUSG000000027954	-0.84619	2.498331	8.221705	0.004139	0.034271	Efna1	ephrin A1 [Source:MGI Symbol;Acc:MGI:103236]
9686	ENSMUSG000000061306	-0.84413	6.221384	18.16845	2.02E-05	0.0012	Slc38a10	solute carrier family 38, member 10 [Source:MGI Symbol;Acc:MGI:1919305]
7899	ENSMUSG000000041633	-0.84374	4.375846	13.20365	0.000279	0.007752	Kctd12b	potassium channel tetramerisation domain containing 12b [Source:MGI Symbol;Acc:MGI:2444667]
9275	ENSMUSG000000055737	-0.84348	8.029923	11.81001	0.000589	0.012562	Ghr	growth hormone receptor [Source:MGI Symbol;Acc:MGI:95708]

4745	ENSMUSG00000029190	-0.84285	6.254807	10.13241	0.001457	0.020342	D5ErtD579e	DNA segment, Chr 5, ERATO Doi 579, expressed [Source:MGI Symbol;Acc:MGI:1261849]
10604	ENSMUSG000000087679	-0.84231	4.380316	20.13322	7.22E-06	0.00058	C330006A16Rik	RIKEN cDNA C330006A16 gene [Source:MGI Symbol;Acc:MGI:1924939]
904	ENSMUSG00000010911	-0.84205	3.634926	10.2047	0.001401	0.019952	Apip	APAF1 interacting protein [Source:MGI Symbol;Acc:MGI:1926788]
3224	ENSMUSG00000025068	-0.84146	4.644956	13.97648	0.000185	0.005966	Gsto1	glutathione S-transferase omega 1 [Source:MGI Symbol;Acc:MGI:1342273]
6609	ENSMUSG00000035863	-0.84099	3.461258	8.07492	0.004488	0.035895	Palm	paralemmin [Source:MGI Symbol;Acc:MGI:1261814]
10057	ENSMUSG00000068747	-0.84	7.380031	7.507632	0.006144	0.042352	Sort1	sortilin 1 [Source:MGI Symbol;Acc:MGI:1338015]
1550	ENSMUSG00000020087	-0.83976	3.08051	16.41206	5.10E-05	0.002373	Tysnd1	trypsin domain containing 1 [Source:MGI Symbol;Acc:MGI:1919017]
7694	ENSMUSG00000040532	-0.83973	3.5273	13.97021	0.000186	0.005966	Abhd11	abhydrolase domain containing 11 [Source:MGI Symbol;Acc:MGI:1916008]
2094	ENSMUSG00000021474	-0.83899	6.02452	11.82468	0.000585	0.012553	Sfxn1	sideroflexin 1 [Source:MGI Symbol;Acc:MGI:2137677]
411	ENSMUSG00000003438	-0.83817	3.669977	11.07293	0.000876	0.015901	Timm50	translocase of inner mitochondrial membrane 50 [Source:MGI Symbol;Acc:MGI:1913775]
6102	ENSMUSG00000033318	-0.83814	3.027938	9.413513	0.002154	0.024496	Gstt2	glutathione S-transferase, theta 2 [Source:MGI Symbol;Acc:MGI:106188]
2060	ENSMUSG00000021371	-0.83774	5.568191	16.85608	4.03E-05	0.002002	Mcur1	mitochondrial calcium uniporter regulator 1 [Source:MGI Symbol;Acc:MGI:1923387]
3311	ENSMUSG00000025289	-0.83725	2.698878	15.76498	7.17E-05	0.003101	Prdx4	peroxiredoxin 4 [Source:MGI Symbol;Acc:MGI:1859815]
163	ENSMUSG00000001467	-0.83599	4.223691	9.815161	0.001731	0.02198	Cyp51	cytochrome P450, family 51 [Source:MGI Symbol;Acc:MGI:106040]
1486	ENSMUSG00000019917	-0.83447	4.035617	12.30427	0.000452	0.010567	Sep-10	septin 10 [Source:MGI Symbol;Acc:MGI:1918110]
9823	ENSMUSG000000063171	-0.83447	1.969327	12.07337	0.000511	0.011491	Rps4l	ribosomal protein S4-like [Source:MGI Symbol;Acc:MGI:1913434]
6030	ENSMUSG00000032959	-0.83423	5.270586	12.51859	0.000403	0.009844	Pebp1	phosphatidylethanolamine binding protein 1 [Source:MGI Symbol;Acc:MGI:1344408]
10015	ENSMUSG000000067924	-0.83356	2.628067	8.015087	0.004639	0.036412	Cxx1b	CAAX box 1B [Source:MGI Symbol;Acc:MGI:3576504]
10573	ENSMUSG000000085558	-0.83344	2.212941	7.044725	0.00795	0.048641	4930412C18Rik	RIKEN cDNA 4930412C18 gene [Source:MGI Symbol;Acc:MGI:2443490]
2225	ENSMUSG00000021806	-0.82913	4.124933	12.32537	0.000447	0.010493	Nid2	nidogen 2 [Source:MGI Symbol;Acc:MGI:1298229]
10871	ENSMUSG000000107283	-0.82819	3.726767	10.47054	0.001213	0.018667	Mpv17	MpV17 mitochondrial inner membrane protein [Source:MGI Symbol;Acc:MGI:97138]
158	ENSMUSG000000001435	-0.82816	5.496634	8.372463	0.003809	0.032993	Col18a1	collagen, type XVIII, alpha 1 [Source:MGI Symbol;Acc:MGI:88451]
3306	ENSMUSG00000025277	-0.82547	3.207119	9.427057	0.002138	0.02442	Abhd6	abhydrolase domain containing 6 [Source:MGI Symbol;Acc:MGI:1913332]
899	ENSMUSG00000010660	-0.82497	3.049946	17.18261	3.40E-05	0.001796	Plcd1	phospholipase C, delta 1 [Source:MGI Symbol;Acc:MGI:97614]
3231	ENSMUSG00000025092	-0.82367	4.083032	20.23837	6.84E-06	0.00056	Hspa12a	heat shock protein 12A [Source:MGI Symbol;Acc:MGI:1920692]
10398	ENSMUSG000000075704	-0.82327	2.357597	8.651285	0.003268	0.030436	Txnrd2	thioredoxin reductase 2 [Source:MGI Symbol;Acc:MGI:1347023]
8854	ENSMUSG000000050552	-0.82291	3.332571	15.5884	7.87E-05	0.003312	Lamtor4	late endosomal/lysosomal adaptor, MAPK and MTOR activator 4 [Source:MGI Symbol;Acc:MGI:1913346]
8428	ENSMUSG00000045996	-0.82173	2.463104	10.00734	0.001559	0.02082	Polr2k	polymerase (RNA) II (DNA directed) polypeptide K [Source:MGI Symbol;Acc:MGI:102725]
8828	ENSMUSG000000050103	-0.8206	4.68103	7.361026	0.006665	0.044372	Agmo	alkylglycerol monooxygenase [Source:MGI Symbol;Acc:MGI:2442495]

10868	ENSMUSG000000107002	-0.81934	3.427411	7.318983	0.006823	0.044961		NCBP2 antisense RNA 2 (head to head) [Source:HGNC Symbol;Acc:HGNC:25121]
7446	ENSMUSG000000039461	-0.81933	3.159876	14.93522	0.000111	0.004254	Tcta	T cell leukemia translocation altered gene [Source:MGI Symbol;Acc:MGI:1918829]
4701	ENSMUSG000000029094	-0.81866	5.573084	8.89313	0.002862	0.028431	Afap1	actin filament associated protein 1 [Source:MGI Symbol;Acc:MGI:1917542]
1143	ENSMUSG000000016257	-0.81699	4.92257	8.122788	0.004371	0.035485	Slmo2	slowmo homolog 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1913640]
2979	ENSMUSG000000024414	-0.81585	3.27867	7.22551	0.007187	0.045959	Mrpl27	mitochondrial ribosomal protein L27 [Source:MGI Symbol;Acc:MGI:2137224]
546	ENSMUSG000000004931	-0.81471	3.488664	10.99699	0.000913	0.016116	Apba3	amyloid beta (A4) precursor protein-binding, family A, member 3 [Source:MGI Symbol;Acc:MGI:1888527]
8248	ENSMUSG000000044122	-0.81465	3.315134	7.885706	0.004983	0.03794	Proca1	protein interacting with cyclin A1 [Source:MGI Symbol;Acc:MGI:1918274]
4670	ENSMUSG000000029017	-0.81407	5.681829	7.701847	0.005516	0.040018	Pmpcb	peptidase (mitochondrial processing) beta [Source:MGI Symbol;Acc:MGI:1920328]
10027	ENSMUSG000000068184	-0.81268	2.200287	12.53963	0.000398	0.009817	Ndufaf2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2 [Source:MGI Symbol;Acc:MGI:1922847]
8934	ENSMUSG000000051391	-0.81201	6.766683	12.13524	0.000495	0.011208	Ywhag	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide [Source:MGI Symbol;Acc:MGI:108109]
1957	ENSMUSG000000021079	-0.81187	2.481027	11.89849	0.000562	0.01217	Timm9	translocase of inner mitochondrial membrane 9 [Source:MGI Symbol;Acc:MGI:1353436]
5084	ENSMUSG000000030247	-0.81058	2.714662	7.280812	0.00697	0.045391	Kcnj8	potassium inwardly-rectifying channel, subfamily J, member 8 [Source:MGI Symbol;Acc:MGI:1100508]
459	ENSMUSG000000003955	-0.81046	3.52459	7.687252	0.005561	0.040156	Fam162a	family with sequence similarity 162, member A [Source:MGI Symbol;Acc:MGI:1917436]
3381	ENSMUSG000000025510	-0.81033	6.283495	10.52578	0.001177	0.018377	Cd151	CD151 antigen [Source:MGI Symbol;Acc:MGI:1096360]
5693	ENSMUSG000000031985	-0.80978	6.140059	11.44081	0.000718	0.01408	Gnpat	glyceronephosphate O-acyltransferase [Source:MGI Symbol;Acc:MGI:1343460]
1447	ENSMUSG000000019809	-0.80966	4.66353	14.51651	0.000139	0.005098	Pex3	peroxisomal biogenesis factor 3 [Source:MGI Symbol;Acc:MGI:1929646]
4531	ENSMUSG000000028691	-0.80852	6.899534	11.21143	0.000813	0.015325	Prdx1	peroxiredoxin 1 [Source:MGI Symbol;Acc:MGI:99523]
10282	ENSMUSG000000073616	-0.80846	3.922136	8.561018	0.003434	0.031158	Myeov2	myeloma overexpressed 2 [Source:MGI Symbol;Acc:MGI:1914165]
3822	ENSMUSG000000026791	-0.80819	1.929238	9.882416	0.001669	0.021697	Slc2a8	solute carrier family 2, (facilitated glucose transporter), member 8 [Source:MGI Symbol;Acc:MGI:1860103]
8782	ENSMUSG000000049580	-0.80735	2.29499	8.287676	0.003992	0.033688	Tsku	tsukushi, small leucine rich proteoglycan [Source:MGI Symbol;Acc:MGI:2443855]
2308	ENSMUSG000000022018	-0.80726	4.109851	9.664539	0.001879	0.022999	Rgcc	regulator of cell cycle [Source:MGI Symbol;Acc:MGI:1913464]
1085	ENSMUSG000000015575	-0.80691	4.689852	15.18237	9.76E-05	0.003921	Atp6v0e	ATPase, H ⁺ transporting, lysosomal V0 subunit E [Source:MGI Symbol;Acc:MGI:1328318]
6779	ENSMUSG000000036775	-0.80596	3.870696	9.811809	0.001734	0.021994	Decr2	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal [Source:MGI Symbol;Acc:MGI:1347059]

4309	ENSMUSG00000028124	-0.80528	4.264935	8.84097	0.002945	0.028835	Gclm	glutamate-cysteine ligase, modifier subunit [Source:MGI Symbol;Acc:MGI:104995]
10346	ENSMUSG00000074771	-0.80464	5.226435	8.324482	0.003911	0.03327	Ankef1	ankyrin repeat and EF-hand domain containing 1 [Source:MGI Symbol;Acc:MGI:2441685]
2443	ENSMUSG00000022364	-0.80455	3.367707	7.374806	0.006614	0.044268	Tbc1d31	TBC1 domain family, member 31 [Source:MGI Symbol;Acc:MGI:2684931]
209	ENSMUSG00000001829	-0.80412	4.735061	8.844678	0.002939	0.028828	Clpb	ClpB caseinolytic peptidase B [Source:MGI Symbol;Acc:MGI:1100517]
704	ENSMUSG00000006494	-0.80382	6.38545	14.18736	0.000165	0.005617	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1 [Source:MGI Symbol;Acc:MGI:1926119]
1351	ENSMUSG00000018846	-0.80377	8.192444	10.20692	0.001399	0.019952	Pank3	pantothenate kinase 3 [Source:MGI Symbol;Acc:MGI:2387464]
4047	ENSMUSG00000027394	-0.80297	3.264972	18.20508	1.98E-05	0.001193	Ttl	tubulin tyrosine ligase [Source:MGI Symbol;Acc:MGI:1916987]
7794	ENSMUSG00000040997	-0.80198	4.784426	14.86614	0.000115	0.004382	Abhd4	abhydrolase domain containing 4 [Source:MGI Symbol;Acc:MGI:1915938]
580	ENSMUSG00000005354	-0.80195	5.154862	11.95644	0.000545	0.011939	Txn2	thioredoxin 2 [Source:MGI Symbol;Acc:MGI:1929468]
783	ENSMUSG00000008035	-0.80185	3.891156	19.46792	1.02E-05	0.000753	Mid1ip1	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish)) [Source:MGI Symbol;Acc:MGI:1915291]
6155	ENSMUSG00000033585	-0.80089	3.162747	11.81499	0.000588	0.012553	Ndn	necdin [Source:MGI Symbol;Acc:MGI:97290]
6561	ENSMUSG00000035585	-0.79968	2.871702	14.77092	0.000121	0.00453	Tsen34	tRNA splicing endonuclease 34 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1913328]
8971	ENSMUSG00000051950	-0.79854	3.578005	10.5664	0.001152	0.018153	B3glct	beta-3-glucosyltransferase [Source:MGI Symbol;Acc:MGI:2685903]
9120	ENSMUSG00000053644	-0.79731	4.27822	8.543194	0.003468	0.031334	Aldh7a1	aldehyde dehydrogenase family 7, member A1 [Source:MGI Symbol;Acc:MGI:108186]
6140	ENSMUSG00000033488	-0.79691	2.665584	18.58506	1.62E-05	0.001041	BC026585	cDNA sequence BC026585 [Source:MGI Symbol;Acc:MGI:2448516]
6336	ENSMUSG00000034449	-0.7966	2.438886	8.092725	0.004444	0.035791	Dhrs11	dehydrogenase/reductase (SDR family) member 11 [Source:MGI Symbol;Acc:MGI:2652816]
8621	ENSMUSG00000047866	-0.79446	6.190483	24.53805	7.29E-07	0.000128	Lonp2	lon peptidase 2, peroxisomal [Source:MGI Symbol;Acc:MGI:1914137]
5604	ENSMUSG00000031781	-0.79385	2.961386	11.19152	0.000822	0.015437	Ciapi1	cytokine induced apoptosis inhibitor 1 [Source:MGI Symbol;Acc:MGI:1922083]
8640	ENSMUSG00000048040	-0.7936	4.683251	8.318205	0.003925	0.033333	Arxes2	adipocyte-related X-chromosome expressed sequence 2 [Source:MGI Symbol;Acc:MGI:1924226]
3751	ENSMUSG00000026617	-0.79308	4.346907	10.18405	0.001417	0.020072	Bpnt1	bisphosphate 3'-nucleotidase 1 [Source:MGI Symbol;Acc:MGI:1338800]
423	ENSMUSG00000003531	-0.79293	2.265793	7.361586	0.006663	0.044372	Dgcr6	DiGeorge syndrome critical region gene 6 [Source:MGI Symbol;Acc:MGI:1202877]
3069	ENSMUSG00000024665	-0.79235	3.366721	10.52795	0.001176	0.018377	Fads2	fatty acid desaturase 2 [Source:MGI Symbol;Acc:MGI:1930079]
8563	ENSMUSG00000047260	-0.79191	4.372337	8.661829	0.003249	0.030338	Emc6	ER membrane protein complex subunit 6 [Source:MGI Symbol;Acc:MGI:1913298]
3867	ENSMUSG00000026883	-0.79026	5.392895	9.439284	0.002124	0.024379	Dab2ip	disabled 2 interacting protein [Source:MGI Symbol;Acc:MGI:1916851]
4517	ENSMUSG00000028664	-0.7894	2.093357	7.233545	0.007155	0.045855	Ephb2	Eph receptor B2 [Source:MGI Symbol;Acc:MGI:99611]
4566	ENSMUSG00000028792	-0.78846	6.17483	18.26501	1.92E-05	0.00117	Ak2	adenylate kinase 2 [Source:MGI Symbol;Acc:MGI:87978]

4158	ENSMUSG00000027709	-0.7868	4.509901	8.294477	0.003977	0.033624	Mccc1	methylocrotonoyl-Coenzyme A carboxylase 1 (alpha) [Source:MGI Symbol;Acc:MGI:1919289]
7496	ENSMUSG00000039693	-0.78608	2.151506	8.400444	0.003751	0.032824	Msantd3	Myb/SANT-like DNA-binding domain containing 3 [Source:MGI Symbol;Acc:MGI:1913915]
7191	ENSMUSG00000038497	-0.78496	3.68811	9.278911	0.002318	0.025334	Tmco3	transmembrane and coiled-coil domains 3 [Source:MGI Symbol;Acc:MGI:2444946]
957	ENSMUSG00000013622	-0.78453	4.641452	10.34886	0.001296	0.019237	Atraid	all-trans retinoic acid induced differentiation factor [Source:MGI Symbol;Acc:MGI:1918918]
10295	ENSMUSG00000073838	-0.7844	4.570073	7.894532	0.004958	0.037848	Tufm	Tu translation elongation factor, mitochondrial [Source:MGI Symbol;Acc:MGI:1923686]
9414	ENSMUSG00000057531	-0.78431	4.171687	16.88664	3.97E-05	0.001992	Dtnbp1	dystrobrevin binding protein 1 [Source:MGI Symbol;Acc:MGI:2137586]
3359	ENSMUSG00000025464	-0.78382	2.537292	10.67292	0.001087	0.017549	Paox	polyamine oxidase (exo-N4-amino) [Source:MGI Symbol;Acc:MGI:1916983]
5700	ENSMUSG00000031996	-0.78332	8.695188	9.405994	0.002163	0.024496	Aplp2	amyloid beta (A4) precursor-like protein 2 [Source:MGI Symbol;Acc:MGI:88047]
1082	ENSMUSG00000015536	-0.78139	4.778704	11.16262	0.000835	0.015572	Mocs2	molybdenum cofactor synthesis 2 [Source:MGI Symbol;Acc:MGI:1336894]
9188	ENSMUSG00000054519	-0.78083	2.358749	10.15965	0.001435	0.020269	Zfp867	zinc finger protein 867 [Source:MGI Symbol;Acc:MGI:2681848]
10178	ENSMUSG00000071253	-0.78058	4.518233	8.694916	0.003191	0.029999	Slc25a16	solute carrier family 25 (mitochondrial carrier, Graves disease autoantigen), member 16 [Source:MGI Symbol;Acc:MGI:1920382]
1319	ENSMUSG00000018593	-0.78054	8.947941	11.03639	0.000893	0.016032	Sparc	secreted acidic cysteine rich glycoprotein [Source:MGI Symbol;Acc:MGI:98373]
8332	ENSMUSG00000044968	-0.77913	3.324835	8.120416	0.004377	0.035485	Napepld	N-acyl phosphatidylethanolamine phospholipase D [Source:MGI Symbol;Acc:MGI:2140885]
516	ENSMUSG00000004631	-0.77889	4.288814	9.244842	0.002362	0.025553	Sgce	sarcoglycan, epsilon [Source:MGI Symbol;Acc:MGI:1329042]
2474	ENSMUSG00000022437	-0.7777	4.892678	7.219695	0.007211	0.046027	Samm50	sorting and assembly machinery component 50 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1915903]
8087	ENSMUSG00000042569	-0.77685	4.264977	9.000622	0.002699	0.027612	Dhrs7b	dehydrogenase/reductase (SDR family) member 7B [Source:MGI Symbol;Acc:MGI:2384931]
5768	ENSMUSG00000032194	-0.77671	5.035853	8.399278	0.003754	0.032824	Kank2	KN motif and ankyrin repeat domains 2 [Source:MGI Symbol;Acc:MGI:2384568]
2544	ENSMUSG00000022579	-0.77628	3.892144	11.24222	0.0008	0.015099	Gpihbp1	GPI-anchored HDL-binding protein 1 [Source:MGI Symbol;Acc:MGI:1915703]
615	ENSMUSG00000005674	-0.77412	3.712914	12.81716	0.000343	0.00891	Tomm40l	translocase of outer mitochondrial membrane 40 homolog-like (yeast) [Source:MGI Symbol;Acc:MGI:3589112]
10362	ENSMUSG00000074918	-0.774	3.474766	19.42769	1.04E-05	0.000764	Inafm2	InaF motif containing 2 [Source:MGI Symbol;Acc:MGI:1915354]
3517	ENSMUSG00000025980	-0.77376	7.077809	7.911144	0.004913	0.037705	Hspd1	heat shock protein 1 (chaperonin) [Source:MGI Symbol;Acc:MGI:96242]
284	ENSMUSG00000002393	-0.77366	2.630553	12.4447	0.000419	0.010083	Nr2f6	nuclear receptor subfamily 2, group F, member 6 [Source:MGI Symbol;Acc:MGI:1352453]
9410	ENSMUSG00000057497	-0.77281	3.564839	17.1038	3.54E-05	0.001828	Fam136a	family with sequence similarity 136, member A [Source:MGI Symbol;Acc:MGI:1913738]

2385	ENSMUSG00000022217	-0.77256	2.215532	11.74977	0.000609	0.012693	Emc9	ER membrane protein complex subunit 9 [Source:MGI Symbol;Acc:MGI:1934682]
8528	ENSMUSG00000046916	-0.77228	2.784748	7.719565	0.005463	0.039839	Myct1	myc target 1 [Source:MGI Symbol;Acc:MGI:1915882]
3185	ENSMUSG00000024972	-0.77186	5.389185	7.439499	0.006381	0.043264	Lgals12	lectin, galactose binding, soluble 12 [Source:MGI Symbol;Acc:MGI:1929094]
312	ENSMUSG00000002688	-0.77185	3.112804	8.929327	0.002806	0.028208	Prkd1	protein kinase D1 [Source:MGI Symbol;Acc:MGI:99879]
317	ENSMUSG00000002732	-0.77168	3.202723	11.65805	0.000639	0.013087	Fkbp7	FK506 binding protein 7 [Source:MGI Symbol;Acc:MGI:1336879]
5778	ENSMUSG00000032231	-0.7715	6.34227	13.83347	0.0002	0.006289	Anxa2	annexin A2 [Source:MGI Symbol;Acc:MGI:88246]
6278	ENSMUSG00000034203	-0.77058	2.698091	13.20225	0.00028	0.007752	Chchd4	coiled-coil-helix-coiled-coil-helix domain containing 4 [Source:MGI Symbol;Acc:MGI:1919420]
6223	ENSMUSG00000033938	-0.76972	4.963415	7.03707	0.007984	0.048681	Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 [Source:MGI Symbol;Acc:MGI:1914166]
5431	ENSMUSG00000031357	-0.76947	5.45021	25.58746	4.23E-07	8.38E-05	Syap1	synapse associated protein 1 [Source:MGI Symbol;Acc:MGI:1914293]
935	ENSMUSG00000012428	-0.76884	6.434535	16.20226	5.69E-05	0.002574	Steap4	STEAP family member 4 [Source:MGI Symbol;Acc:MGI:1923560]
8706	ENSMUSG00000048755	-0.76791	2.821323	9.457889	0.002102	0.024293	Mcat	malonyl CoA:ACP acyltransferase (mitochondrial) [Source:MGI Symbol;Acc:MGI:2388651]
8594	ENSMUSG00000047617	-0.76771	2.831151	13.64601	0.000221	0.00668	BC029214	cDNA sequence BC029214 [Source:MGI Symbol;Acc:MGI:2442831]
5742	ENSMUSG00000032109	-0.76755	2.753708	8.625055	0.003316	0.030608	Nlr1	NLR family member X1 [Source:MGI Symbol;Acc:MGI:2429611]
8183	ENSMUSG00000043300	-0.76652	2.78653	9.919947	0.001635	0.021386	B3galnt1	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1 [Source:MGI Symbol;Acc:MGI:1349405]
6941	ENSMUSG00000037465	-0.7663	5.2437	7.617582	0.00578	0.040942	Klf10	Kruppel-like factor 10 [Source:MGI Symbol;Acc:MGI:1101353]
4291	ENSMUSG00000028085	-0.76586	3.862148	6.99067	0.008194	0.049434	Gatb	glutamyl-tRNA(Gln) amidotransferase, subunit B [Source:MGI Symbol;Acc:MGI:2442496]
1636	ENSMUSG00000020283	-0.76552	5.534004	15.7257	7.32E-05	0.003141	Pex13	peroxisomal biogenesis factor 13 [Source:MGI Symbol;Acc:MGI:1919379]
1008	ENSMUSG00000014606	-0.76519	5.495625	8.068238	0.004505	0.035895	Slc25a11	solute carrier family 25 (mitochondrial carrier oxoglutarate carrier), member 11 [Source:MGI Symbol;Acc:MGI:1915113]
5730	ENSMUSG00000032067	-0.76292	4.082538	22.24308	2.40E-06	0.000287	Pts	6-pyruvoyl-tetrahydropterin synthase [Source:MGI Symbol;Acc:MGI:1338783]
4428	ENSMUSG00000028443	-0.76149	2.235972	7.281922	0.006965	0.045391	Nudt2	nudix (nucleoside diphosphate linked moiety X)-type motif 2 [Source:MGI Symbol;Acc:MGI:1913651]
3140	ENSMUSG00000024869	-0.75985	2.349548	8.430063	0.003691	0.032528	Nudt8	nudix (nucleoside diphosphate linked moiety X)-type motif 8 [Source:MGI Symbol;Acc:MGI:1913637]
6475	ENSMUSG00000035133	-0.75968	6.969392	11.30953	0.000771	0.014699	Arhgap5	Rho GTPase activating protein 5 [Source:MGI Symbol;Acc:MGI:1332637]
3555	ENSMUSG00000026087	-0.75894	5.040144	7.731919	0.005425	0.039727	Mrpl30	mitochondrial ribosomal protein L30 [Source:MGI Symbol;Acc:MGI:1333820]
6277	ENSMUSG00000034201	-0.75748	3.252516	15.85314	6.85E-05	0.002983	Gas2l1	growth arrest-specific 2 like 1 [Source:MGI Symbol;Acc:MGI:1926176]
5915	ENSMUSG00000032531	-0.75579	5.439484	13.78325	0.000205	0.006387	Amotl2	angiominin-like 2 [Source:MGI Symbol;Acc:MGI:1929286]
6750	ENSMUSG00000036599	-0.7552	3.45114	14.26679	0.000159	0.005487	Chst12	carbohydrate sulfotransferase 12 [Source:MGI Symbol;Acc:MGI:1929064]
4287	ENSMUSG00000028076	-0.75512	5.476621	11.98637	0.000536	0.011868	Cd1d1	CD1d1 antigen [Source:MGI Symbol;Acc:MGI:107674]

7990	ENSMUSG00000042073	-0.75326	3.586132	8.128148	0.004358	0.035485	Abhd14b	abhydrolase domain containing 14b [Source:MGI Symbol;Acc:MGI:1923741]
2566	ENSMUSG00000022665	-0.75217	7.684969	8.994159	0.002708	0.027639	Ccdc80	coiled-coil domain containing 80 [Source:MGI Symbol;Acc:MGI:1915146]
8503	ENSMUSG00000046756	-0.75214	4.223323	10.38844	0.001268	0.018979	Mrps7	mitochondrial ribosomal protein S7 [Source:MGI Symbol;Acc:MGI:1354367]
6938	ENSMUSG00000037455	-0.74986	3.109478	7.249143	0.007093	0.045707	Slc18b1	solute carrier family 18, subfamily B, member 1 [Source:MGI Symbol;Acc:MGI:1923556]
6863	ENSMUSG00000037112	-0.74963	7.313484	8.774266	0.003055	0.029277	Sik2	salt inducible kinase 2 [Source:MGI Symbol;Acc:MGI:2445031]
6010	ENSMUSG00000032867	-0.74826	4.681499	22.90933	1.70E-06	0.000226	Fbxw8	F-box and WD-40 domain protein 8 [Source:MGI Symbol;Acc:MGI:1923041]
3021	ENSMUSG00000024533	-0.74755	4.665388	18.63093	1.59E-05	0.001029	Spire1	spire homolog 1 (Drosophila) [Source:MGI Symbol;Acc:MGI:1915416]
9223	ENSMUSG00000054894	-0.74687	2.171821	9.443042	0.00212	0.024369	Atp5s	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit S [Source:MGI Symbol;Acc:MGI:1915305]
2701	ENSMUSG00000023020	-0.74678	3.30843	7.258373	0.007057	0.045635	Cox14	cytochrome c oxidase assembly protein 14 [Source:MGI Symbol;Acc:MGI:1913629]
3570	ENSMUSG00000026112	-0.74618	6.540196	9.455791	0.002105	0.024295	Coa5	cytochrome C oxidase assembly factor 5 [Source:MGI Symbol;Acc:MGI:1923428]
1516	ENSMUSG00000020003	-0.74601	3.590071	13.38526	0.000254	0.007334	Pex7	peroxisomal biogenesis factor 7 [Source:MGI Symbol;Acc:MGI:1321392]
4653	ENSMUSG00000028980	-0.74585	6.420608	26.94882	2.09E-07	4.87E-05	H6pd	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) [Source:MGI Symbol;Acc:MGI:2140356]
8173	ENSMUSG00000043190	-0.74553	3.062634	13.987	0.000184	0.005966	Rfcsd	Rieske (Fe-S) domain containing [Source:MGI Symbol;Acc:MGI:2145198]
5754	ENSMUSG00000032135	-0.74487	5.433487	7.857289	0.005062	0.038326	Mcam	melanoma cell adhesion molecule [Source:MGI Symbol;Acc:MGI:1933966]
390	ENSMUSG0000003299	-0.74291	4.080744	7.665719	0.005628	0.040397	Mrpl4	mitochondrial ribosomal protein L4 [Source:MGI Symbol;Acc:MGI:2137210]
4528	ENSMUSG00000028684	-0.74027	4.033344	12.18986	0.000481	0.011045	Urod	uroporphyrinogen decarboxylase [Source:MGI Symbol;Acc:MGI:98916]
2895	ENSMUSG00000024181	-0.74	3.33667	8.409322	0.003733	0.03275	Mrpl28	mitochondrial ribosomal protein L28 [Source:MGI Symbol;Acc:MGI:1915861]
900	ENSMUSG00000010663	-0.73981	4.907807	8.827744	0.002967	0.028889	Fads1	fatty acid desaturase 1 [Source:MGI Symbol;Acc:MGI:1923517]
1610	ENSMUSG00000020231	-0.73903	4.094487	16.84266	4.06E-05	0.002002	Dip2a	DIP2 disco-interacting protein 2 homolog A (Drosophila) [Source:MGI Symbol;Acc:MGI:2385920]
4994	ENSMUSG00000029918	-0.73749	3.864661	10.24902	0.001368	0.019737	Mrps33	mitochondrial ribosomal protein S33 [Source:MGI Symbol;Acc:MGI:1338046]
1554	ENSMUSG00000020092	-0.73732	3.782243	10.87602	0.000974	0.016559	Pald1	phosphatase domain containing, paladin 1 [Source:MGI Symbol;Acc:MGI:1351623]
9408	ENSMUSG00000057440	-0.73728	3.898383	10.4867	0.001202	0.018636	Mpp7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7) [Source:MGI Symbol;Acc:MGI:1922989]
1698	ENSMUSG00000020444	-0.73718	3.357855	13.82115	0.000201	0.006295	Guk1	guanylate kinase 1 [Source:MGI Symbol;Acc:MGI:95871]
486	ENSMUSG00000004270	-0.7362	5.12538	12.58498	0.000389	0.009674	Lpcat3	lysophosphatidylcholine acyltransferase 3 [Source:MGI Symbol;Acc:MGI:1315211]
2767	ENSMUSG00000023707	-0.73524	2.132825	9.121995	0.002526	0.026562	Ogfod2	2-oxoglutarate and iron-dependent oxygenase domain containing 2 [Source:MGI Symbol;Acc:MGI:1913877]
4474	ENSMUSG00000028563	-0.73449	2.77748	7.12712	0.007593	0.047491	Tm2d1	TM2 domain containing 1 [Source:MGI Symbol;Acc:MGI:2137022]

8370	ENSMUSG00000045410	-0.73318	3.888768	8.946618	0.00278	0.02812	Akr1e1	aldo-keto reductase family 1, member E1 [Source:MGI Symbol;Acc:MGI:1914758]
10351	ENSMUSG00000074797	-0.73293	3.199477	10.23039	0.001381	0.019811	Itpa	inosine triphosphatase (nucleoside triphosphate pyrophosphatase) [Source:MGI Symbol;Acc:MGI:96622]
2078	ENSMUSG00000021420	-0.73256	3.703562	8.291839	0.003982	0.033637	Fars2	phenylalanine-tRNA synthetase 2 (mitochondrial) [Source:MGI Symbol;Acc:MGI:1917205]
2214	ENSMUSG00000021772	-0.73175	3.191398	10.71641	0.001062	0.017269	Nkiras1	NFKB inhibitor interacting Ras-like protein 1 [Source:MGI Symbol;Acc:MGI:1916971]
2687	ENSMUSG00000022982	-0.73171	5.957498	9.354424	0.002224	0.024783	Sod1	superoxide dismutase 1, soluble [Source:MGI Symbol;Acc:MGI:98351]
8949	ENSMUSG00000051579	-0.73165	3.877558	12.45462	0.000417	0.010051	Tceal8	transcription elongation factor A (SII)-like 8 [Source:MGI Symbol;Acc:MGI:1913934]
3303	ENSMUSG00000025268	-0.73151	3.363859	13.07131	0.0003	0.008107	Maged2	melanoma antigen, family D, 2 [Source:MGI Symbol;Acc:MGI:1933391]
9642	ENSMUSG00000060681	-0.73003	4.803854	9.487262	0.002069	0.024086	Slc9a6	solute carrier family 9 (sodium/hydrogen exchanger), member 6 [Source:MGI Symbol;Acc:MGI:2443511]
2764	ENSMUSG00000023495	-0.73002	3.203984	10.32775	0.00131	0.019348	Pcbp4	poly(rC) binding protein 4 [Source:MGI Symbol;Acc:MGI:1890471]
3464	ENSMUSG00000025854	-0.72995	2.566867	13.86799	0.000196	0.00622	Fam20c	family with sequence similarity 20, member C [Source:MGI Symbol;Acc:MGI:2136853]
892	ENSMUSG00000010476	-0.72952	4.035884	7.867537	0.005033	0.038163	Ebf3	early B cell factor 3 [Source:MGI Symbol;Acc:MGI:894289]
5902	ENSMUSG00000032501	-0.72927	3.717154	9.324273	0.002261	0.02501	Trib1	tribbles homolog 1 (Drosophila) [Source:MGI Symbol;Acc:MGI:2443397]
8736	ENSMUSG00000049091	-0.72766	4.897927	8.563641	0.003429	0.031139	Sephs2	selenophosphate synthetase 2 [Source:MGI Symbol;Acc:MGI:108388]
10165	ENSMUSG00000070972	-0.72761	2.773345	11.1125	0.000857	0.015837	Dnajc25	DnaJ (Hsp40) homolog, subfamily C, member 25 [Source:MGI Symbol;Acc:MGI:1919679]
10388	ENSMUSG00000075486	-0.72751	3.037245	10.11166	0.001473	0.020395	Comm6	COMM domain containing 6 [Source:MGI Symbol;Acc:MGI:1913450]
2725	ENSMUSG00000023079	-0.72735	2.808967	8.701489	0.00318	0.029956	Gtf2ird1	general transcription factor II I repeat domain-containing 1 [Source:MGI Symbol;Acc:MGI:1861942]
2585	ENSMUSG00000022706	-0.72735	3.614663	9.563535	0.001985	0.023456	Mrpl40	mitochondrial ribosomal protein L40 [Source:MGI Symbol;Acc:MGI:1332635]
7085	ENSMUSG00000038122	-0.72731	3.801739	9.522887	0.002029	0.023775	Tbc1d32	TBC1 domain family, member 32 [Source:MGI Symbol;Acc:MGI:2442827]
2220	ENSMUSG00000021786	-0.72724	3.586331	8.814406	0.002989	0.028983	Oxsm	3-oxoacyl-ACP synthase, mitochondrial [Source:MGI Symbol;Acc:MGI:1918397]
6213	ENSMUSG00000033900	-0.72701	2.074438	7.842169	0.005104	0.038541	Map9	microtubule-associated protein 9 [Source:MGI Symbol;Acc:MGI:2442208]
10177	ENSMUSG00000071252	-0.72641	2.387451	11.68785	0.000629	0.012933	2210408I21Rik	RIKEN cDNA 2210408I21 gene [Source:MGI Symbol;Acc:MGI:1919621]
7965	ENSMUSG00000041939	-0.72351	2.286178	12.15597	0.000489	0.011131	Mvk	mevalonate kinase [Source:MGI Symbol;Acc:MGI:107624]
4728	ENSMUSG00000029162	-0.72331	2.427818	8.242836	0.004091	0.034056	Khk	ketoheokinase [Source:MGI Symbol;Acc:MGI:1096353]
3141	ENSMUSG00000024870	-0.72284	5.633271	10.13928	0.001451	0.020342	Rab1b	RAB1B, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:1923558]
10033	ENSMUSG00000068264	-0.7228	2.468647	7.623655	0.005761	0.040898	Ap5s1	adaptor-related protein 5 complex, sigma 1 subunit [Source:MGI Symbol;Acc:MGI:1916846]
7658	ENSMUSG00000040374	-0.72212	3.958252	13.21589	0.000278	0.007752	Pex2	peroxisomal biogenesis factor 2 [Source:MGI Symbol;Acc:MGI:107486]

466	ENSMUSG00000004044	-0.72189	6.668384	7.574481	0.00592	0.04141	Ptrf	polymerase I and transcript release factor [Source:MGI Symbol;Acc:MGI:1277968]
8104	ENSMUSG000000042642	-0.72105	3.782197	12.59299	0.000387	0.009654	Flad1	RFad1, flavin adenine dinucleotide synthetase, homolog (yeast) [Source:MGI Symbol;Acc:MGI:2443030]
6408	ENSMUSG000000034781	-0.72073	5.022923	11.07366	0.000876	0.015901	Gna11	guanine nucleotide binding protein, alpha 11 [Source:MGI Symbol;Acc:MGI:95766]
10693	ENSMUSG000000093661	-0.7204	4.730247	8.510497	0.003531	0.031584	Eif4e3	eukaryotic translation initiation factor 4E member 3 [Source:MGI Symbol;Acc:MGI:1914142]
5401	ENSMUSG000000031246	-0.71967	6.547679	20.59092	5.69E-06	0.000487	Sh3bgrl	SH3-binding domain glutamic acid-rich protein like [Source:MGI Symbol;Acc:MGI:1930849]
712	ENSMUSG000000006611	-0.71926	4.222581	8.725577	0.003138	0.029704	Hfe	hemochromatosis [Source:MGI Symbol;Acc:MGI:109191]
2619	ENSMUSG000000022797	-0.71893	4.900132	7.631436	0.005736	0.040814	Tfrc	transferrin receptor [Source:MGI Symbol;Acc:MGI:98822]
236	ENSMUSG000000002015	-0.71856	5.839926	27.26293	1.78E-07	4.40E-05	Bcap31	B cell receptor associated protein 31 [Source:MGI Symbol;Acc:MGI:1350933]
9067	ENSMUSG000000052962	-0.71823	3.855891	7.333533	0.006768	0.044775	Mrpl35	mitochondrial ribosomal protein L35 [Source:MGI Symbol;Acc:MGI:1913473]
8816	ENSMUSG000000049960	-0.71805	3.420942	7.250181	0.007089	0.045707	Mrps16	mitochondrial ribosomal protein S16 [Source:MGI Symbol;Acc:MGI:1913492]
6861	ENSMUSG000000037110	-0.71753	6.629592	8.718513	0.00315	0.029786	Ralgapa2	Ral GTPase activating protein, alpha subunit 2 (catalytic) [Source:MGI Symbol;Acc:MGI:3036245]
7821	ENSMUSG000000041168	-0.7175	5.490952	9.265408	0.002335	0.025361	Lonp1	lon peptidase 1, mitochondrial [Source:MGI Symbol;Acc:MGI:1921392]
3607	ENSMUSG000000026209	-0.71681	4.398749	21.3862	3.75E-06	0.000359	Dnpep	aspartyl aminopeptidase [Source:MGI Symbol;Acc:MGI:1278328]
6051	ENSMUSG000000033059	-0.71584	5.985919	11.30824	0.000772	0.014699	Pygb	brain glycogen phosphorylase [Source:MGI Symbol;Acc:MGI:97828]
9489	ENSMUSG000000058586	-0.71581	3.35646	19.09905	1.24E-05	0.00087	Serhl	serine hydrolase-like [Source:MGI Symbol;Acc:MGI:1890404]
243	ENSMUSG000000002058	-0.71554	2.947368	8.14769	0.004312	0.035283	Unc119	unc-119 homolog (C. elegans) [Source:MGI Symbol;Acc:MGI:1328357]
5752	ENSMUSG000000032126	-0.7136	3.284726	8.321654	0.003918	0.033296	Hmbs	hydroxymethylbilane synthase [Source:MGI Symbol;Acc:MGI:96112]
6299	ENSMUSG000000034285	-0.71333	2.295156	9.237529	0.002371	0.025584	Nipsnap1	4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (C. elegans) [Source:MGI Symbol;Acc:MGI:1278344]
5119	ENSMUSG000000030339	-0.71273	4.335116	7.745503	0.005385	0.039679	Ltbr	lymphotoxin B receptor [Source:MGI Symbol;Acc:MGI:104875]
10666	ENSMUSG000000090952	-0.71207	1.753055	8.267343	0.004036	0.033806	Gm17251	predicted gene, 17251 [Source:MGI Symbol;Acc:MGI:4936885]
1962	ENSMUSG000000021102	-0.71196	3.612325	8.17199	0.004254	0.034938	Glrx5	glutaredoxin 5 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1920296]
159	ENSMUSG000000001436	-0.71162	2.503075	10.29128	0.001337	0.019575	Slc19a1	solute carrier family 19 (folate transporter), member 1 [Source:MGI Symbol;Acc:MGI:103182]
569	ENSMUSG000000005161	-0.71118	5.53802	8.944092	0.002784	0.02812	Prdx2	peroxiredoxin 2 [Source:MGI Symbol;Acc:MGI:109486]
4568	ENSMUSG000000028795	-0.71115	2.867173	8.660348	0.003252	0.030338	Ccdc28b	coiled coil domain containing 28B [Source:MGI Symbol;Acc:MGI:1913514]
9770	ENSMUSG000000062376	-0.71063	2.665763	10.0968	0.001485	0.020485	Borcs7	BLOC-1 related complex subunit 7 [Source:MGI Symbol;Acc:MGI:1913689]
10136	ENSMUSG000000070476	-0.71052	2.407511	9.468605	0.00209	0.024276	Fam217b	family with sequence similarity 217, member B [Source:MGI Symbol;Acc:MGI:1918782]

8296	ENSMUSG00000044627	-0.70998	5.838341	7.247024	0.007102	0.045734	Swi5	SWI5 recombination repair homolog (yeast) [Source:MGI Symbol;Acc:MGI:1920181]
3424	ENSMUSG00000025728	-0.70928	4.972012	20.90421	4.83E-06	0.000438	Pigq	phosphatidylinositol glycan anchor biosynthesis, class Q [Source:MGI Symbol;Acc:MGI:1333114]
2797	ENSMUSG00000023939	-0.70817	2.277588	6.984925	0.00822	0.049538	Mrpl14	mitochondrial ribosomal protein L14 [Source:MGI Symbol;Acc:MGI:1333864]
6954	ENSMUSG00000037531	-0.70652	3.334777	11.18535	0.000824	0.015442	Mrpl47	mitochondrial ribosomal protein L47 [Source:MGI Symbol;Acc:MGI:1921850]
4129	ENSMUSG00000027637	-0.70496	4.495645	8.055773	0.004536	0.03599	1110008F13Rik	RIKEN cDNA 1110008F13 gene [Source:MGI Symbol;Acc:MGI:1914638]
4281	ENSMUSG00000028063	-0.70346	5.685393	7.432581	0.006405	0.043376	Lmna	lamin A [Source:MGI Symbol;Acc:MGI:96794]
9464	ENSMUSG00000058267	-0.70195	3.203094	8.2442	0.004088	0.034056	Mrps14	mitochondrial ribosomal protein S14 [Source:MGI Symbol;Acc:MGI:1928141]
2984	ENSMUSG00000024424	-0.70172	2.301122	10.60745	0.001126	0.017852	Ttc39c	tetratricopeptide repeat domain 39C [Source:MGI Symbol;Acc:MGI:1919997]
10740	ENSMUSG00000097006	-0.70077	2.0908	7.344484	0.006727	0.044584	9530082P21Rik	RIKEN cDNA 9530082P21 gene [Source:MGI Symbol;Acc:MGI:3603340]
4265	ENSMUSG00000028032	-0.70064	4.555625	12.82726	0.000342	0.008884	Papss1	3'-phosphoadenosine 5'-phosphosulfate synthase 1 [Source:MGI Symbol;Acc:MGI:1330587]
6625	ENSMUSG00000035944	-0.70042	3.629276	10.82576	0.001001	0.016748	Ttc38	tetratricopeptide repeat domain 38 [Source:MGI Symbol;Acc:MGI:2146198]
7448	ENSMUSG00000039470	-0.6998	2.392215	8.639494	0.00329	0.030478	Zdhhc2	zinc finger, DHHC domain containing 2 [Source:MGI Symbol;Acc:MGI:1923452]
775	ENSMUSG00000007872	-0.69938	3.484179	9.341341	0.00224	0.024872	Id3	inhibitor of DNA binding 3 [Source:MGI Symbol;Acc:MGI:96398]
4751	ENSMUSG00000029199	-0.69799	4.469186	8.840986	0.002945	0.028835	Lias	lipoic acid synthetase [Source:MGI Symbol;Acc:MGI:1934604]
3886	ENSMUSG00000026932	-0.69667	4.887374	12.88887	0.000331	0.008721	Nacc2	nucleus accumbens associated 2, BEN and BTB (POZ) domain containing [Source:MGI Symbol;Acc:MGI:1915241]
1505	ENSMUSG00000019978	-0.69656	6.805472	34.19286	4.99E-09	2.47E-06	Epb41l2	erythrocyte membrane protein band 4.1 like 2 [Source:MGI Symbol;Acc:MGI:103009]
3695	ENSMUSG00000026471	-0.69613	4.614719	9.271979	0.002327	0.025334	Mr1	major histocompatibility complex, class I-related [Source:MGI Symbol;Acc:MGI:1195463]
6489	ENSMUSG00000035198	-0.69348	3.55527	7.515223	0.006118	0.042267	Tubg1	tubulin, gamma 1 [Source:MGI Symbol;Acc:MGI:101834]
8197	ENSMUSG00000043542	-0.69137	3.635503	8.549847	0.003456	0.031246	Zc2hc1a	zinc finger, C2HC-type containing 1A [Source:MGI Symbol;Acc:MGI:1914556]
4065	ENSMUSG00000027439	-0.69033	4.801835	8.578018	0.003402	0.031017	Gzf1	GDNF-inducible zinc finger protein 1 [Source:MGI Symbol;Acc:MGI:1921783]
2836	ENSMUSG00000024048	-0.68981	6.157345	14.24491	0.00016	0.005521	Myl12a	myosin, light chain 12A, regulatory, non-sarcomeric [Source:MGI Symbol;Acc:MGI:1914518]
3935	ENSMUSG00000027086	-0.68975	3.093525	14.32347	0.000154	0.005376	Fastkd1	FAST kinase domains 1 [Source:MGI Symbol;Acc:MGI:2444596]
5475	ENSMUSG00000031482	-0.68718	2.866867	10.99862	0.000912	0.016116	Slc25a15	solute carrier family 25 (mitochondrial carrier ornithine transporter), member 15 [Source:MGI Symbol;Acc:MGI:1342274]
4661	ENSMUSG00000028998	-0.68682	4.318414	11.8161	0.000587	0.012553	Tomm7	translocase of outer mitochondrial membrane 7 homolog (yeast) [Source:MGI Symbol;Acc:MGI:1913419]
321	ENSMUSG00000002763	-0.6865	4.058562	9.322796	0.002263	0.02501	Pex6	peroxisomal biogenesis factor 6 [Source:MGI Symbol;Acc:MGI:2385054]
6846	ENSMUSG00000037049	-0.68542	4.723103	9.669398	0.001874	0.022999	Smpd1	sphingomyelin phosphodiesterase 1, acid lysosomal [Source:MGI Symbol;Acc:MGI:98325]

4658	ENSMUSG00000028991	-0.68473	5.792634	10.46004	0.00122	0.018703	Mtor	mechanistic target of rapamycin (serine/threonine kinase) [Source:MGI Symbol;Acc:MGI:1928394]
10271	ENSMUSG00000073481	-0.68458	5.096893	26.28691	2.94E-07	6.29E-05	Mar-02	mitochondrial amidoxime reducing component 2 [Source:MGI Symbol;Acc:MGI:1914497]
7521	ENSMUSG00000039787	-0.68242	2.493941	7.548688	0.006005	0.041785	Cercam	cerebral endothelial cell adhesion molecule [Source:MGI Symbol;Acc:MGI:2139134]
2883	ENSMUSG00000024150	-0.68123	4.843993	18.69343	1.54E-05	0.001002	Mcfd2	multiple coagulation factor deficiency 2 [Source:MGI Symbol;Acc:MGI:2183439]
6002	ENSMUSG00000032840	-0.68091	3.415829	10.86376	0.000981	0.016592	2410131K14Rik	RIKEN cDNA 2410131K14 gene [Source:MGI Symbol;Acc:MGI:1924042]
4984	ENSMUSG00000029851	-0.68023	2.65943	7.912493	0.004909	0.037705	Tcaf2	TRPM8 channel-associated factor 2 [Source:MGI Symbol;Acc:MGI:2385258]
634	ENSMUSG00000005873	-0.67975	6.230768	12.80771	0.000345	0.008934	Reep5	receptor accessory protein 5 [Source:MGI Symbol;Acc:MGI:1270152]
6662	ENSMUSG00000036138	-0.67912	4.304428	20.57938	5.72E-06	0.000487	Acaa1a	acetyl-Coenzyme A acyltransferase 1A [Source:MGI Symbol;Acc:MGI:2148491]
984	ENSMUSG00000014301	-0.6787	2.642302	7.277227	0.006983	0.045428	Pam16	presequence translocase-associated motor 16 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1913699]
10103	ENSMUSG00000069844	-0.67522	3.17416	10.83255	0.000997	0.016744	Sco1	SCO cytochrome oxidase deficient homolog 1 (yeast) [Source:MGI Symbol;Acc:MGI:106362]
9352	ENSMUSG00000056698	-0.67522	3.772863	8.803115	0.003007	0.029074	Elmod3	ELMO/CED-12 domain containing 3 [Source:MGI Symbol;Acc:MGI:2445168]
9715	ENSMUSG000000061650	-0.67516	2.620519	7.20826	0.007257	0.046186	Med9	mediator complex subunit 9 [Source:MGI Symbol;Acc:MGI:2183151]
10331	ENSMUSG00000074457	-0.67482	3.955204	8.576876	0.003405	0.031017	S100a16	S100 calcium binding protein A16 [Source:MGI Symbol;Acc:MGI:1915110]
1256	ENSMUSG00000018042	-0.67281	5.931606	9.076579	0.002589	0.02697	Cyb5r3	cytochrome b5 reductase 3 [Source:MGI Symbol;Acc:MGI:94893]
3018	ENSMUSG00000024527	-0.6728	4.878865	8.031907	0.004596	0.036236	Afg3l2	AFG3-like AAA ATPase 2 [Source:MGI Symbol;Acc:MGI:1916847]
3358	ENSMUSG00000025453	-0.67274	4.146881	13.22967	0.000276	0.007752	Nnt	nicotinamide nucleotide transhydrogenase [Source:MGI Symbol;Acc:MGI:109279]
3004	ENSMUSG00000024480	-0.67274	5.212875	13.55483	0.000232	0.006898	Ap3s1	adaptor-related protein complex 3, sigma 1 subunit [Source:MGI Symbol;Acc:MGI:1337062]
5830	ENSMUSG00000032342	-0.67245	3.652523	8.762327	0.003075	0.029381	Mto1	mitochondrial translation optimization 1 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1915541]
5162	ENSMUSG00000030530	-0.67227	5.179456	11.8226	0.000585	0.012553	Furin	furin (paired basic amino acid cleaving enzyme) [Source:MGI Symbol;Acc:MGI:97513]
3373	ENSMUSG00000025495	-0.66976	2.681736	7.104327	0.00769	0.047915	Ptdss2	phosphatidylserine synthase 2 [Source:MGI Symbol;Acc:MGI:1351664]
4688	ENSMUSG00000029063	-0.66914	6.403849	19.7632	8.77E-06	0.000677	Nadk	NAD kinase [Source:MGI Symbol;Acc:MGI:2183149]
7324	ENSMUSG00000038982	-0.66792	2.977178	10.21934	0.00139	0.019861	Bloc1s5	biogenesis of lysosomal organelles complex-1, subunit 5, muted [Source:MGI Symbol;Acc:MGI:2178598]
801	ENSMUSG00000008435	-0.66767	2.989863	8.447382	0.003656	0.032273	Rdh13	retinol dehydrogenase 13 (all-trans and 9-cis) [Source:MGI Symbol;Acc:MGI:1918732]
162	ENSMUSG00000001445	-0.66642	4.43832	12.3994	0.000429	0.010262	Mrpl10	mitochondrial ribosomal protein L10 [Source:MGI Symbol;Acc:MGI:1333801]

1387	ENSMUSG00000019210	-0.66529	5.375863	9.596064	0.00195	0.023296	Atp6v1e1	ATPase, H ⁺ transporting, lysosomal V1 subunit E1 [Source:MGI Symbol;Acc:MGI:894326]
2552	ENSMUSG00000022617	-0.66504	4.029152	10.45919	0.00122	0.018703	Chkb	choline kinase beta [Source:MGI Symbol;Acc:MGI:1328313]
3425	ENSMUSG00000025731	-0.66434	2.641682	7.179525	0.007374	0.046659	0610011F06Rik	RIKEN cDNA 0610011F06 gene [Source:MGI Symbol;Acc:MGI:1915597]
6412	ENSMUSG00000034795	-0.66422	2.107251	6.967447	0.008301	0.049804	Ccdc122	coiled-coil domain containing 122 [Source:MGI Symbol;Acc:MGI:1918358]
5485	ENSMUSG00000031503	-0.66246	7.509011	11.06538	0.00088	0.015901	Col4a2	collagen, type IV, alpha 2 [Source:MGI Symbol;Acc:MGI:88455]
2592	ENSMUSG00000022722	-0.66209	1.983333	8.210585	0.004165	0.034417	Arl6	ADP-ribosylation factor-like 6 [Source:MGI Symbol;Acc:MGI:1927136]
1606	ENSMUSG00000020225	-0.66167	3.846078	16.26731	5.50E-05	0.002529	Tmbim4	transmembrane BAX inhibitor motif containing 4 [Source:MGI Symbol;Acc:MGI:1915462]
1148	ENSMUSG00000016382	-0.66079	4.932435	9.465994	0.002093	0.024276	Pls3	plastin 3 (T-isoform) [Source:MGI Symbol;Acc:MGI:104807]
6902	ENSMUSG00000037316	-0.66059	4.98574	13.54697	0.000233	0.006908	Bag4	BCL2-associated athanogene 4 [Source:MGI Symbol;Acc:MGI:1914634]
10296	ENSMUSG00000073889	-0.65965	3.837282	16.94517	3.85E-05	0.001956	Il11ra1	interleukin 11 receptor, alpha chain 1 [Source:MGI Symbol;Acc:MGI:107426]
3789	ENSMUSG00000026709	-0.65958	3.232029	13.12091	0.000292	0.007995	Dars2	aspartyl-tRNA synthetase 2 (mitochondrial) [Source:MGI Symbol;Acc:MGI:2442510]
1943	ENSMUSG00000021040	-0.65926	3.716154	10.26446	0.001356	0.019651	Slirp	SRA stem-loop interacting RNA binding protein [Source:MGI Symbol;Acc:MGI:1916394]
742	ENSMUSG00000007039	-0.65871	2.678354	10.28568	0.001341	0.019582	Ddah2	dimethylarginine dimethylaminohydrolase 2 [Source:MGI Symbol;Acc:MGI:1859016]
4795	ENSMUSG00000029335	-0.65855	3.597291	12.43426	0.000422	0.010117	Bmp3	bone morphogenetic protein 3 [Source:MGI Symbol;Acc:MGI:88179]
2089	ENSMUSG00000021460	-0.65719	4.693509	9.683409	0.001859	0.02296	Auh	AU RNA binding protein/enoyl-coenzyme A hydratase [Source:MGI Symbol;Acc:MGI:1338011]
10328	ENSMUSG00000074364	-0.65703	6.46418	9.37254	0.002203	0.024666	Ehd2	EH-domain containing 2 [Source:MGI Symbol;Acc:MGI:2154274]
2823	ENSMUSG00000024012	-0.65686	5.172069	9.430454	0.002134	0.0244	Mtch1	mitochondrial carrier homolog 1 (C. elegans) [Source:MGI Symbol;Acc:MGI:1929261]
7353	ENSMUSG00000039096	-0.65674	2.164232	9.873398	0.001677	0.021725	Rsad1	radical S-adenosyl methionine domain containing 1 [Source:MGI Symbol;Acc:MGI:3039628]
4562	ENSMUSG00000028779	-0.65643	3.836516	10.98209	0.00092	0.016148	Pef1	penta-EF hand domain containing 1 [Source:MGI Symbol;Acc:MGI:1915148]
4534	ENSMUSG00000028696	-0.6545	2.404455	10.27759	0.001347	0.019612	Ipp	IAP promoted placental gene [Source:MGI Symbol;Acc:MGI:96581]
9698	ENSMUSG00000061461	-0.65445	3.532374	9.692162	0.001851	0.022913	Smim20	small integral membrane protein 20 [Source:MGI Symbol;Acc:MGI:1913528]
864	ENSMUSG00000009905	-0.65344	4.802115	11.74494	0.00061	0.012693	Kdsr	3-ketodihydrosphingosine reductase [Source:MGI Symbol;Acc:MGI:1918000]
5582	ENSMUSG00000031723	-0.65312	2.875395	8.032606	0.004594	0.036236	Txn14b	thioredoxin-like 4B [Source:MGI Symbol;Acc:MGI:2443724]
2858	ENSMUSG00000024082	-0.65251	3.138988	10.96918	0.000926	0.016228	Ndufaf7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex assembly factor 7 [Source:MGI Symbol;Acc:MGI:1920944]
3382	ENSMUSG00000025511	-0.65095	3.426033	8.726298	0.003137	0.029704	Tspan4	tetraspanin 4 [Source:MGI Symbol;Acc:MGI:1928097]
9881	ENSMUSG00000063931	-0.64995	4.745434	7.008516	0.008112	0.049188	Pepd	peptidase D [Source:MGI Symbol;Acc:MGI:97542]
8540	ENSMUSG00000047067	-0.6487	1.947813	7.10028	0.007707	0.047915	Dusp28	dual specificity phosphatase 28 [Source:MGI Symbol;Acc:MGI:1914696]

5336	ENSMUSG00000031012	-0.64856	4.551228	7.432685	0.006405	0.043376	Cask	calcium/calmodulin-dependent serine protein kinase (MAGUK family) [Source:MGI Symbol;Acc:MGI:1309489]
1892	ENSMUSG00000020900	-0.64738	4.797545	8.233922	0.004111	0.034145	Myh10	myosin, heavy polypeptide 10, non-muscle [Source:MGI Symbol;Acc:MGI:1930780]
8747	ENSMUSG00000049287	-0.64719	2.101472	8.558379	0.003439	0.031177	Iba57	IBA57, iron-sulfur cluster assembly homolog (<i>S. cerevisiae</i>) [Source:MGI Symbol;Acc:MGI:3041174]
6952	ENSMUSG00000037523	-0.64567	4.968288	11.06445	0.00088	0.015901	Mavs	mitochondrial antiviral signaling protein [Source:MGI Symbol;Acc:MGI:2444773]
8900	ENSMUSG00000051098	-0.64531	3.066568	8.898138	0.002855	0.028431	Mblac2	metallo-beta-lactamase domain containing 2 [Source:MGI Symbol;Acc:MGI:1920102]
1039	ENSMUSG00000015092	-0.64465	4.82921	13.45021	0.000245	0.00714	Edf1	endothelial differentiation-related factor 1 [Source:MGI Symbol;Acc:MGI:1891227]
3062	ENSMUSG00000024645	-0.64439	3.797823	8.355152	0.003846	0.033062	Timm21	translocase of inner mitochondrial membrane 21 [Source:MGI Symbol;Acc:MGI:1920595]
3816	ENSMUSG00000026781	-0.64363	5.746011	9.902369	0.001651	0.02154	Acbd5	acyl-Coenzyme A binding domain containing 5 [Source:MGI Symbol;Acc:MGI:1921409]
3348	ENSMUSG00000025410	-0.64341	5.552539	15.08252	0.000103	0.004019	Dctn2	dynactin 2 [Source:MGI Symbol;Acc:MGI:107733]
1358	ENSMUSG00000018900	-0.64339	3.743031	8.939351	0.002791	0.02812	Slc22a5	solute carrier family 22 (organic cation transporter), member 5 [Source:MGI Symbol;Acc:MGI:1329012]
401	ENSMUSG00000003380	-0.6427	4.048872	15.42101	8.60E-05	0.003564	Rabac1	Rab acceptor 1 (prenylated) [Source:MGI Symbol;Acc:MGI:1201692]
3460	ENSMUSG00000025817	-0.64219	4.259746	7.06558	0.007858	0.048373	Nudt5	nudix (nucleoside diphosphate linked moiety X)-type motif 5 [Source:MGI Symbol;Acc:MGI:1858232]
8749	ENSMUSG00000049295	-0.64216	3.214407	12.8974	0.000329	0.008714	Zfp219	zinc finger protein 219 [Source:MGI Symbol;Acc:MGI:1917140]
1431	ENSMUSG00000019763	-0.64168	4.652848	7.93719	0.004843	0.037453	Rmnd1	required for meiotic nuclear division 1 homolog (<i>S. cerevisiae</i>) [Source:MGI Symbol;Acc:MGI:1913334]
4495	ENSMUSG00000028618	-0.64123	5.806453	20.05979	7.51E-06	0.000597	Tmem59	transmembrane protein 59 [Source:MGI Symbol;Acc:MGI:1929278]
2414	ENSMUSG00000022299	-0.64081	3.931478	10.05234	0.001522	0.020698	Slc25a32	solute carrier family 25, member 32 [Source:MGI Symbol;Acc:MGI:1917156]
7038	ENSMUSG00000037949	-0.63918	4.725471	10.78819	0.001021	0.016915	Ano10	anoctamin 10 [Source:MGI Symbol;Acc:MGI:2143103]
1548	ENSMUSG00000020079	-0.63909	3.670511	11.32267	0.000766	0.014673	Supv3l1	suppressor of var1, 3-like 1 (<i>S. cerevisiae</i>) [Source:MGI Symbol;Acc:MGI:2441711]
10508	ENSMUSG00000079316	-0.63767	4.333402	9.528601	0.002023	0.023752	Rab9	RAB9, member RAS oncogene family [Source:MGI Symbol;Acc:MGI:1890695]
2755	ENSMUSG00000023307	-0.63751	5.642859	9.740687	0.001802	0.022573	Mar-05	membrane-associated ring finger (C3HC4) 5 [Source:MGI Symbol;Acc:MGI:1915207]
9459	ENSMUSG00000058230	-0.63697	6.015401	8.336402	0.003886	0.033182	Arhgap35	Rho GTPase activating protein 35 [Source:MGI Symbol;Acc:MGI:1929494]
5040	ENSMUSG00000030095	-0.63687	6.179329	8.939934	0.00279	0.02812	Tmem43	transmembrane protein 43 [Source:MGI Symbol;Acc:MGI:1921372]
4678	ENSMUSG00000029036	-0.63675	4.21871	14.51561	0.000139	0.005098	Atad3a	ATPase family, AAA domain containing 3A [Source:MGI Symbol;Acc:MGI:1919214]
9892	ENSMUSG00000064120	-0.63672	3.841725	7.760613	0.00534	0.039499	Mocs1	molybdenum cofactor synthesis 1 [Source:MGI Symbol;Acc:MGI:1928904]

4955	ENSMUSG00000029761	-0.63663	6.467831	10.19198	0.001411	0.020034	Cald1	caldesmon 1 [Source:MGI Symbol;Acc:MGI:88250]
7527	ENSMUSG00000039826	-0.63452	3.525711	14.07401	0.000176	0.005849	Trub2	TruB pseudouridine (psi) synthase homolog 2 (E. coli) [Source:MGI Symbol;Acc:MGI:2442186]
8791	ENSMUSG00000049680	-0.63448	4.335357	10.3683	0.001282	0.019135	Urgcp	upregulator of cell proliferation [Source:MGI Symbol;Acc:MGI:1919296]
2191	ENSMUSG00000021716	-0.6325	3.438033	9.297345	0.002295	0.025249	Srek1ip1	splicing regulatory glutamine/lysine-rich protein 1interacting protein 1 [Source:MGI Symbol;Acc:MGI:1914538]
7007	ENSMUSG00000037815	-0.63125	6.914612	10.52247	0.001179	0.018383	Ctnna1	catenin (cadherin associated protein), alpha 1 [Source:MGI Symbol;Acc:MGI:88274]
5759	ENSMUSG00000032180	-0.63123	2.58356	9.397269	0.002173	0.024562	Tmed1	transmembrane emp24 domain containing 1 [Source:MGI Symbol;Acc:MGI:106201]
6254	ENSMUSG00000034108	-0.63029	2.944175	8.657052	0.003258	0.030367	Ccs	copper chaperone for superoxide dismutase [Source:MGI Symbol;Acc:MGI:1333783]
7580	ENSMUSG00000040018	-0.62725	4.044945	8.538642	0.003477	0.031355	Cox15	cytochrome c oxidase assembly protein 15 [Source:MGI Symbol;Acc:MGI:1920112]
1751	ENSMUSG00000020553	-0.62639	2.418182	9.647019	0.001897	0.023116	Pctp	phosphatidylcholine transfer protein [Source:MGI Symbol;Acc:MGI:107375]
5500	ENSMUSG00000031534	-0.62285	3.604096	7.360969	0.006665	0.044372	Smim19	small integral membrane protein 19 [Source:MGI Symbol;Acc:MGI:2142501]
3375	ENSMUSG00000025499	-0.62224	3.719718	11.51177	0.000692	0.013701	Hras	Harvey rat sarcoma virus oncogene [Source:MGI Symbol;Acc:MGI:96224]
5284	ENSMUSG00000030842	-0.62212	4.59295	8.067355	0.004507	0.035895	Lamtor1	late endosomal/lysosomal adaptor, MAPK and MTOR activator 1 [Source:MGI Symbol;Acc:MGI:1913758]
7475	ENSMUSG00000039616	-0.62069	2.601535	7.630606	0.005739	0.040814	Mocos	molybdenum cofactor sulfurase [Source:MGI Symbol;Acc:MGI:1915841]
8590	ENSMUSG00000047554	-0.62023	4.137637	13.3842	0.000254	0.007334	Tmem41b	transmembrane protein 41B [Source:MGI Symbol;Acc:MGI:1289225]
7178	ENSMUSG00000038429	-0.61991	5.246245	14.48024	0.000142	0.005159	Usp5	ubiquitin specific peptidase 5 (isopeptidase T) [Source:MGI Symbol;Acc:MGI:1347343]
3047	ENSMUSG00000024601	-0.61988	5.451849	10.91592	0.000953	0.016433	Isoc1	isochorismatase domain containing 1 [Source:MGI Symbol;Acc:MGI:1913557]
4432	ENSMUSG00000028455	-0.61958	4.276922	8.368863	0.003817	0.032993	Stoml2	stomatin (Epb7.2)-like 2 [Source:MGI Symbol;Acc:MGI:1913842]
1355	ENSMUSG00000018882	-0.61926	4.072836	9.378651	0.002195	0.02462	Mrpl45	mitochondrial ribosomal protein L45 [Source:MGI Symbol;Acc:MGI:1914286]
5687	ENSMUSG00000031974	-0.61905	3.410012	7.741857	0.005396	0.039679	Abcb10	ATP-binding cassette, sub-family B (MDR/TAP), member 10 [Source:MGI Symbol;Acc:MGI:1860508]
3858	ENSMUSG00000026860	-0.61699	5.074655	15.13592	0.0001	0.00395	Sh3glb2	SH3-domain GRB2-like endophilin B2 [Source:MGI Symbol;Acc:MGI:2385131]
5743	ENSMUSG00000032112	-0.61446	3.048272	6.999965	0.008151	0.049302	Trappc4	trafficking protein particle complex 4 [Source:MGI Symbol;Acc:MGI:1926211]
7611	ENSMUSG00000040174	-0.61397	3.214193	8.160356	0.004282	0.03513	Alkbh3	alkB, alkylation repair homolog 3 (E. coli) [Source:MGI Symbol;Acc:MGI:1916363]
4160	ENSMUSG00000027712	-0.61291	7.003685	11.92023	0.000555	0.012076	Anxa5	annexin A5 [Source:MGI Symbol;Acc:MGI:106008]
1337	ENSMUSG00000018761	-0.61209	4.009142	13.88757	0.000194	0.006183	Mpdu1	mannose-P-dolichol utilization defect 1 [Source:MGI Symbol;Acc:MGI:1346040]
7523	ENSMUSG00000039795	-0.61165	3.335651	11.02907	0.000897	0.016032	Zfand1	zinc finger, AN1-type domain 1 [Source:MGI Symbol;Acc:MGI:1913611]
4477	ENSMUSG00000028568	-0.61071	5.074779	15.02697	0.000106	0.004097	Btf3l4	basic transcription factor 3-like 4 [Source:MGI Symbol;Acc:MGI:1915312]

6133	ENSMUSG000000033446	-0.60946	4.658199	7.26463	0.007033	0.045598	Lpar6	lysophosphatidic acid receptor 6 [Source:MGI Symbol;Acc:MGI:1914418]
696	ENSMUSG000000006423	-0.60883	5.504481	13.02014	0.000308	0.00825	C330007P06Rik	RIKEN cDNA C330007P06 gene [Source:MGI Symbol;Acc:MGI:1924894]
5854	ENSMUSG000000032388	-0.60705	4.986938	14.36212	0.000151	0.005335	Spg21	spastic paraplegia 21 homolog (human) [Source:MGI Symbol;Acc:MGI:106403]
1559	ENSMUSG000000020102	-0.6067	3.670622	11.94217	0.000549	0.011971	Slc16a7	solute carrier family 16 (monocarboxylic acid transporters), member 7 [Source:MGI Symbol;Acc:MGI:1330284]
9806	ENSMUSG000000062963	-0.60552	4.378985	9.175781	0.002452	0.026114	Ufc1	ubiquitin-fold modifier conjugating enzyme 1 [Source:MGI Symbol;Acc:MGI:1913405]
1637	ENSMUSG000000020284	-0.60474	2.451326	9.1434	0.002496	0.026415	1810043G02Rik	RIKEN cDNA 1810043G02 gene [Source:MGI Symbol;Acc:MGI:1915134]
5484	ENSMUSG000000031502	-0.6045	8.196556	8.585099	0.003389	0.031014	Col4a1	collagen, type IV, alpha 1 [Source:MGI Symbol;Acc:MGI:88454]
8620	ENSMUSG000000047843	-0.60403	2.869703	9.37784	0.002196	0.02462	Bri3	brain protein I3 [Source:MGI Symbol;Acc:MGI:1933174]
10095	ENSMUSG000000069744	-0.60299	4.668604	7.832735	0.005131	0.038556	Psbmb3	proteasome (prosome, macropain) subunit, beta type 3 [Source:MGI Symbol;Acc:MGI:1347014]
5617	ENSMUSG000000031807	-0.60244	3.706013	10.7916	0.00102	0.016915	Pgls	6-phosphogluconolactonase [Source:MGI Symbol;Acc:MGI:1913421]
10004	ENSMUSG000000067722	-0.60214	3.306426	12.4798	0.000411	0.009961	BC003965	cDNA sequence BC003965 [Source:MGI Symbol;Acc:MGI:2670966]
10549	ENSMUSG000000081534	-0.60199	5.115854	7.093256	0.007737	0.048023	Slc48a1	solute carrier family 48 (heme transporter), member 1 [Source:MGI Symbol;Acc:MGI:1914989]
1093	ENSMUSG000000015668	-0.59977	3.413157	10.12697	0.001461	0.020342	Pdzd11	PDZ domain containing 11 [Source:MGI Symbol;Acc:MGI:1919871]
9996	ENSMUSG000000067369	-0.59922	4.13669	13.37283	0.000255	0.007359	Trmt2b	TRM2 tRNA methyltransferase 2B [Source:MGI Symbol;Acc:MGI:2442530]
2143	ENSMUSG000000021607	-0.59875	3.022881	8.255696	0.004062	0.033971	Mrpl36	mitochondrial ribosomal protein L36 [Source:MGI Symbol;Acc:MGI:2137228]
4545	ENSMUSG000000028719	-0.59804	5.310868	12.89475	0.00033	0.008714	Cmpk1	cytidine monophosphate (UMP-CMP) kinase 1 [Source:MGI Symbol;Acc:MGI:1913838]
707	ENSMUSG000000006526	-0.59669	3.282198	11.6563	0.00064	0.013087	Tmem110	transmembrane protein 110 [Source:MGI Symbol;Acc:MGI:1921500]
5428	ENSMUSG000000031352	-0.59638	3.806088	8.49729	0.003557	0.031766	Hccs	holocytochrome c synthetase [Source:MGI Symbol;Acc:MGI:106911]
1201	ENSMUSG000000017386	-0.59449	3.584767	7.651807	0.005672	0.040567	Traf4	TNF receptor associated factor 4 [Source:MGI Symbol;Acc:MGI:1202880]
1246	ENSMUSG000000017837	-0.59238	3.874208	7.371824	0.006625	0.044268	Nkiras2	NFKB inhibitor interacting Ras-like protein 2 [Source:MGI Symbol;Acc:MGI:1919216]
9767	ENSMUSG000000062352	-0.59161	3.85919	13.43417	0.000247	0.007179	Itgb1bp1	integrin beta 1 binding protein 1 [Source:MGI Symbol;Acc:MGI:1306802]
6641	ENSMUSG000000036026	-0.5912	4.350983	8.079055	0.004478	0.035895	Tmem63b	transmembrane protein 63b [Source:MGI Symbol;Acc:MGI:2387609]
2736	ENSMUSG000000023147	-0.59038	2.585812	8.925638	0.002812	0.028239	Wrb	tryptophan rich basic protein [Source:MGI Symbol;Acc:MGI:2136882]
9710	ENSMUSG000000061559	-0.58855	3.758637	15.02601	0.000106	0.004097	Wdr61	WD repeat domain 61 [Source:MGI Symbol;Acc:MGI:1917493]
3428	ENSMUSG000000025736	-0.58661	3.861137	10.80785	0.001011	0.016839	Jmjd8	jumonji domain containing 8 [Source:MGI Symbol;Acc:MGI:1919356]
1463	ENSMUSG000000019846	-0.5858	6.645368	7.514262	0.006121	0.042267	Lama4	laminin, alpha 4 [Source:MGI Symbol;Acc:MGI:109321]
8023	ENSMUSG000000042271	-0.58561	3.432578	8.822394	0.002976	0.028896	Nxt2	nuclear transport factor 2-like export factor 2 [Source:MGI Symbol;Acc:MGI:2147914]
8065	ENSMUSG000000042462	-0.5856	2.268438	7.513061	0.006125	0.042268	Dctpp1	dCTP pyrophosphatase 1 [Source:MGI Symbol;Acc:MGI:1913672]

1488	ENSMUSG00000019923	-0.5853	5.190421	8.1143	0.004392	0.035578	Zwint	ZW10 interactor [Source:MGI Symbol;Acc:MGI:1289227]
3130	ENSMUSG00000024845	-0.58505	5.095151	16.10479	5.99E-05	0.002676	Tmem134	transmembrane protein 134 [Source:MGI Symbol;Acc:MGI:1914240]
8671	ENSMUSG00000048307	-0.5842	4.024938	9.114951	0.002535	0.026608	Ankrd46	ankyrin repeat domain 46 [Source:MGI Symbol;Acc:MGI:1916089]
3752	ENSMUSG00000026618	-0.58201	5.959342	8.569539	0.003418	0.031102	lars2	isoleucine-tRNA synthetase 2, mitochondrial [Source:MGI Symbol;Acc:MGI:1919586]
8211	ENSMUSG00000043733	-0.58192	5.987127	17.432	2.98E-05	0.001622	Ptpn11	protein tyrosine phosphatase, non-receptor type 11 [Source:MGI Symbol;Acc:MGI:99511]
8112	ENSMUSG00000042682	-0.58102	4.252636	7.979663	0.004731	0.03687	Selk	selenoprotein K [Source:MGI Symbol;Acc:MGI:1931466]
1059	ENSMUSG00000015289	-0.5801	2.639091	7.64982	0.005678	0.040567	Lage3	L antigen family, member 3 [Source:MGI Symbol;Acc:MGI:1913442]
2186	ENSMUSG00000021710	-0.57945	4.159035	15.71713	7.36E-05	0.003143	Nln	neurolysin (metallopeptidase M3 family) [Source:MGI Symbol;Acc:MGI:1923055]
10114	ENSMUSG00000070044	-0.57924	3.257512	11.09815	0.000864	0.015878	Fam149a	family with sequence similarity 149, member A [Source:MGI Symbol;Acc:MGI:2387177]
1112	ENSMUSG00000015806	-0.57871	3.186148	7.664273	0.005632	0.040403	Qdpr	quinoid dihydropteridine reductase [Source:MGI Symbol;Acc:MGI:97836]
9199	ENSMUSG00000054676	-0.57798	5.424604	13.01029	0.00031	0.008273	1600014C10Rik	RIKEN cDNA 1600014C10 gene [Source:MGI Symbol;Acc:MGI:1919494]
10117	ENSMUSG00000070283	-0.57772	2.599941	7.30385	0.006881	0.045137	Ndufaf3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 3 [Source:MGI Symbol;Acc:MGI:1913956]
9744	ENSMUSG00000062054	-0.57688	3.518816	9.543623	0.002006	0.023642	lah1	isoamyl acetate-hydrolyzing esterase 1 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1914982]
10068	ENSMUSG00000068921	-0.57561	4.201222	12.17747	0.000484	0.011096	Dap3	death associated protein 3 [Source:MGI Symbol;Acc:MGI:1929538]
3366	ENSMUSG00000025484	-0.57475	3.027816	7.032736	0.008003	0.048745	Bet1l	blocked early in transport 1 homolog (S. cerevisiae)-like [Source:MGI Symbol;Acc:MGI:1913128]
4494	ENSMUSG00000028614	-0.57464	4.137991	10.12716	0.001461	0.020342	Ndc1	NDC1 transmembrane nucleoporin [Source:MGI Symbol;Acc:MGI:1920037]
3713	ENSMUSG00000026509	-0.57461	6.367099	10.17127	0.001426	0.020185	Capn2	calpain 2 [Source:MGI Symbol;Acc:MGI:88264]
8268	ENSMUSG00000044345	-0.57373	3.856229	9.278565	0.002319	0.025334	Marveld1	MARVEL (membrane-associating) domain containing 1 [Source:MGI Symbol;Acc:MGI:2147570]
6756	ENSMUSG00000036620	-0.57371	4.043275	10.92137	0.000951	0.016415	Mgat4b	mannoside acetylglucosaminyltransferase 4, isoenzyme B [Source:MGI Symbol;Acc:MGI:2143974]
863	ENSMUSG00000009894	-0.57367	3.363655	7.977281	0.004737	0.036892	Snap47	synaptosomal-associated protein, 47 [Source:MGI Symbol;Acc:MGI:1915076]
9537	ENSMUSG00000059316	-0.57241	3.641458	7.666101	0.005627	0.040397	Slc27a4	solute carrier family 27 (fatty acid transporter), member 4 [Source:MGI Symbol;Acc:MGI:1347347]
9293	ENSMUSG00000055943	-0.57221	4.821407	7.337655	0.006752	0.044699	Emc7	ER membrane protein complex subunit 7 [Source:MGI Symbol;Acc:MGI:1920274]
4599	ENSMUSG00000028864	-0.57172	3.057346	7.274682	0.006993	0.045465	Hgf	hepatocyte growth factor [Source:MGI Symbol;Acc:MGI:96079]
2796	ENSMUSG00000023938	-0.56853	2.86494	7.458672	0.006313	0.043019	Aars2	alanyl-tRNA synthetase 2, mitochondrial (putative) [Source:MGI Symbol;Acc:MGI:2681839]
8642	ENSMUSG00000048058	-0.56804	3.47846	8.029989	0.004601	0.036248	Ldlrad3	low density lipoprotein receptor class A domain containing 3 [Source:MGI Symbol;Acc:MGI:2138856]

3437	ENSMUSG000000025759	-0.56781	3.968468	11.78698	0.000596	0.012617	Mfsd8	major facilitator superfamily domain containing 8 [Source:MGI Symbol;Acc:MGI:1919425]
7762	ENSMUSG000000040813	-0.565	4.000196	8.212475	0.00416	0.034417	Tex264	testis expressed gene 264 [Source:MGI Symbol;Acc:MGI:1096570]
1296	ENSMUSG000000018427	-0.56456	3.660672	9.774593	0.001769	0.02234	Ypel2	yippee-like 2 (Drosophila) [Source:MGI Symbol;Acc:MGI:1925114]
5013	ENSMUSG000000030035	-0.56448	4.025116	10.47935	0.001207	0.018651	Wbp1	WW domain binding protein 1 [Source:MGI Symbol;Acc:MGI:104710]
6041	ENSMUSG000000033020	-0.56348	2.93603	8.070057	0.0045	0.035895	Polr2f	polymerase (RNA) II (DNA directed) polypeptide F [Source:MGI Symbol;Acc:MGI:1349393]
2458	ENSMUSG000000022401	-0.56235	3.854166	10.07818	0.0015	0.020549	Xpnpep3	X-prolyl aminopeptidase (aminopeptidase P) 3, putative [Source:MGI Symbol;Acc:MGI:2445217]
55	ENSMUSG000000000581	-0.56041	4.030509	9.214512	0.002401	0.02575	C1d	C1D nuclear receptor co-repressor [Source:MGI Symbol;Acc:MGI:1927354]
7836	ENSMUSG000000041241	-0.56041	3.653435	9.590687	0.001956	0.023296	Mul1	mitochondrial ubiquitin ligase activator of NFkB 1 [Source:MGI Symbol;Acc:MGI:1915600]
2099	ENSMUSG000000021484	-0.55987	6.52618	19.53988	9.85E-06	0.000735	Lman2	lectin, mannose-binding 2 [Source:MGI Symbol;Acc:MGI:1914140]
5623	ENSMUSG000000031819	-0.55776	3.782888	14.00964	0.000182	0.005933	Emc8	ER membrane protein complex subunit 8 [Source:MGI Symbol;Acc:MGI:1343095]
5001	ENSMUSG000000029993	-0.5573	4.127245	8.522873	0.003507	0.0314	Nfu1	NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1913290]
2012	ENSMUSG000000021236	-0.55617	5.373005	10.11567	0.00147	0.02038	Entpd5	ectonucleoside triphosphate diphosphohydrolase 5 [Source:MGI Symbol;Acc:MGI:1321385]
4656	ENSMUSG000000028988	-0.55541	2.900004	10.27063	0.001352	0.019637	Ctnnbip1	catenin beta interacting protein 1 [Source:MGI Symbol;Acc:MGI:1915756]
3044	ENSMUSG000000024592	-0.55512	3.540208	11.5819	0.000666	0.013488	C330018D20Rik	RIKEN cDNA C330018D20 gene [Source:MGI Symbol;Acc:MGI:1924672]
2401	ENSMUSG000000022257	-0.55441	4.528956	11.362	0.00075	0.014541	Laptm4b	lysosomal-associated protein transmembrane 4B [Source:MGI Symbol;Acc:MGI:1890494]
7284	ENSMUSG000000038816	-0.55345	3.239558	8.183179	0.004228	0.034769	Ctnnal1	catenin (cadherin associated protein), alpha-like 1 [Source:MGI Symbol;Acc:MGI:1859649]
7600	ENSMUSG000000040112	-0.55253	4.120973	8.752928	0.003091	0.029441	Mrps35	mitochondrial ribosomal protein S35 [Source:MGI Symbol;Acc:MGI:2385255]
6725	ENSMUSG000000036473	-0.55117	3.447751	7.732521	0.005423	0.039727	Tbc1d24	TBC1 domain family, member 24 [Source:MGI Symbol;Acc:MGI:2443456]
3916	ENSMUSG000000027007	-0.55043	5.236445	11.94021	0.000549	0.011971	Ssfa2	sperm specific antigen 2 [Source:MGI Symbol;Acc:MGI:1917849]
5253	ENSMUSG000000030763	-0.5498	3.217244	7.000998	0.008146	0.049302	Lcmt1	leucine carboxyl methyltransferase 1 [Source:MGI Symbol;Acc:MGI:1353593]
9013	ENSMUSG000000052459	-0.54953	5.72195	13.56696	0.00023	0.006885	Atp6v1a	ATPase, H ⁺ transporting, lysosomal V1 subunit A [Source:MGI Symbol;Acc:MGI:1201780]
432	ENSMUSG000000003617	-0.54905	7.030722	11.00837	0.000907	0.01607	Cp	ceruloplasmin [Source:MGI Symbol;Acc:MGI:88476]
8824	ENSMUSG000000050052	-0.54695	4.570951	8.04881	0.004553	0.036082	Tdrp	testis development related protein [Source:MGI Symbol;Acc:MGI:1919398]
6889	ENSMUSG000000037260	-0.54693	4.367713	9.628394	0.001916	0.02312	Hgsnat	heparan-alpha-glucosaminide N-acetyltransferase [Source:MGI Symbol;Acc:MGI:1196297]
6852	ENSMUSG000000037072	-0.54587	6.669144	9.053314	0.002622	0.027152	Sep-15	selenoprotein [Source:MGI Symbol;Acc:MGI:1927947]
7256	ENSMUSG000000038712	-0.5452	4.15085	8.777367	0.00305	0.029277	Fam63a	family with sequence similarity 63, member A [Source:MGI Symbol;Acc:MGI:1922257]

4522	ENSMUSG00000028672	-0.54314	3.366269	8.624012	0.003318	0.030608	Hmgcl	3-hydroxy-3-methylglutaryl-Coenzyme A lyase [Source:MGI Symbol;Acc:MGI:96158]
10007	ENSMUSG00000067787	-0.54162	4.529396	8.071885	0.004496	0.035895	Blcap	bladder cancer associated protein homolog (human) [Source:MGI Symbol;Acc:MGI:1858907]
7516	ENSMUSG00000039768	-0.54091	4.680779	9.007705	0.002688	0.027583	Dnajc11	DnaJ (Hsp40) homolog, subfamily C, member 11 [Source:MGI Symbol;Acc:MGI:2443386]
703	ENSMUSG00000006476	-0.54041	3.58599	7.890626	0.004969	0.037863	Nsmf	NMDA receptor synaptonuclear signaling and neuronal migration factor [Source:MGI Symbol;Acc:MGI:1861755]
5726	ENSMUSG00000032058	-0.5403	5.209283	11.32128	0.000766	0.014673	Ppp2r1b	protein phosphatase 2, regulatory subunit A, beta [Source:MGI Symbol;Acc:MGI:1920949]
4961	ENSMUSG00000029772	-0.53961	5.88824	13.56343	0.000231	0.006885	Ahcyl2	S-adenosylhomocysteine hydrolase-like 2 [Source:MGI Symbol;Acc:MGI:1921590]
9968	ENSMUSG00000066735	-0.53915	5.03015	18.04156	2.16E-05	0.001253	Vkorc11l	vitamin K epoxide reductase complex, subunit 1-like 1 [Source:MGI Symbol;Acc:MGI:1916818]
6983	ENSMUSG00000037706	-0.53838	6.579629	8.854239	0.002924	0.028781	Cd81	CD81 antigen [Source:MGI Symbol;Acc:MGI:1096398]
10386	ENSMUSG00000075467	-0.53724	3.542953	8.150148	0.004306	0.035283	Dnlz	DNL-type zinc finger [Source:MGI Symbol;Acc:MGI:106559]
433	ENSMUSG00000003623	-0.537	5.161936	12.2699	0.00046	0.010717	Crot	carnitine O-octanoyltransferase [Source:MGI Symbol;Acc:MGI:1921364]
2267	ENSMUSG00000021917	-0.5363	4.446583	7.446402	0.006356	0.043179	Spcs1	signal peptidase complex subunit 1 homolog (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1916269]
3780	ENSMUSG00000026687	-0.53615	4.595861	7.947027	0.004817	0.037394	Aldh9a1	aldehyde dehydrogenase 9, subfamily A1 [Source:MGI Symbol;Acc:MGI:1861622]
225	ENSMUSG00000001946	-0.53386	3.817281	7.0042	0.008132	0.04928	Esam	endothelial cell-specific adhesion molecule [Source:MGI Symbol;Acc:MGI:1916774]
356	ENSMUSG00000002984	-0.53146	3.708149	11.65359	0.000641	0.013087	Tomm40	translocase of outer mitochondrial membrane 40 homolog (yeast) [Source:MGI Symbol;Acc:MGI:1858259]
4505	ENSMUSG00000028641	-0.53088	2.704489	7.040538	0.007969	0.048641	P3h1	prolyl 3-hydroxylase 1 [Source:MGI Symbol;Acc:MGI:1888921]
5227	ENSMUSG00000030706	-0.53081	4.299533	9.607841	0.001937	0.023224	Mrpl48	mitochondrial ribosomal protein L48 [Source:MGI Symbol;Acc:MGI:1289321]
6649	ENSMUSG00000036078	-0.52975	4.109397	7.995698	0.004689	0.03665	Sigmar1	sigma non-opioid intracellular receptor 1 [Source:MGI Symbol;Acc:MGI:1195268]
9331	ENSMUSG00000056429	-0.52905	7.597219	8.396214	0.00376	0.032828	Tgoln1	trans-golgi network protein [Source:MGI Symbol;Acc:MGI:105080]
7107	ENSMUSG00000038188	-0.52828	2.263776	7.387542	0.006568	0.044092	Scarf1	scavenger receptor class F, member 1 [Source:MGI Symbol;Acc:MGI:2449455]
316	ENSMUSG00000002731	-0.52676	3.033972	7.779009	0.005286	0.039259	Prkra	protein kinase, interferon inducible double stranded RNA dependent activator [Source:MGI Symbol;Acc:MGI:1344375]
3891	ENSMUSG00000026944	-0.52644	5.634804	7.394575	0.006542	0.044038	Abca2	ATP-binding cassette, sub-family A (ABC1), member 2 [Source:MGI Symbol;Acc:MGI:99606]
1973	ENSMUSG00000021124	-0.52214	4.786528	7.581639	0.005897	0.041344	Vti1b	vesicle transport through interaction with t-SNAREs 1B [Source:MGI Symbol;Acc:MGI:1855688]
6692	ENSMUSG00000036299	-0.52141	4.457832	9.673617	0.001869	0.022999	BC031181	cDNA sequence BC031181 [Source:MGI Symbol;Acc:MGI:3039614]

2105	ENSMUSG000000021497	-0.51978	4.178172	6.972976	0.008275	0.049759	Txndc15	thioredoxin domain containing 15 [Source:MGI Symbol;Acc:MGI:1916922]
148	ENSMUSG000000001366	-0.51972	4.255393	7.940866	0.004833	0.037453	Fbxo9	f-box protein 9 [Source:MGI Symbol;Acc:MGI:1918788]
825	ENSMUSG000000009073	-0.51941	5.376228	9.713277	0.001829	0.022728	Nf2	neurofibromatosis 2 [Source:MGI Symbol;Acc:MGI:97307]
2298	ENSMUSG000000021996	-0.51909	4.76736	12.36559	0.000437	0.01036	Esd	esterase D/formylglutathione hydrolase [Source:MGI Symbol;Acc:MGI:95421]
10461	ENSMUSG000000078861	-0.51619	3.146984	7.299735	0.006896	0.045154	Zfp931	zinc finger protein 931 [Source:MGI Symbol;Acc:MGI:2441662]
8294	ENSMUSG000000044600	-0.51519	4.135093	8.061167	0.004522	0.035916	Smim7	small integral membrane protein 7 [Source:MGI Symbol;Acc:MGI:1914068]
3092	ENSMUSG000000024759	-0.51499	6.796552	12.10709	0.000502	0.011308	Atl3	atlastin GTPase 3 [Source:MGI Symbol;Acc:MGI:1924270]
2016	ENSMUSG000000021242	-0.51477	6.618806	8.783543	0.00304	0.029246	Npc2	Niemann-Pick type C2 [Source:MGI Symbol;Acc:MGI:1915213]
2230	ENSMUSG000000021814	-0.51419	5.753593	10.34587	0.001298	0.019237	Anxa7	annexin A7 [Source:MGI Symbol;Acc:MGI:88031]
562	ENSMUSG000000005078	-0.51249	3.641696	11.12372	0.000852	0.015794	Jkamp	JNK1/MAPK8-associated membrane protein [Source:MGI Symbol;Acc:MGI:1915057]
1365	ENSMUSG000000018931	-0.5113	4.008678	7.402271	0.006514	0.043977	Natd1	N-acetyltransferase domain containing 1 [Source:MGI Symbol;Acc:MGI:1344388]
8722	ENSMUSG000000048911	-0.50937	3.339749	7.542248	0.006027	0.041907	Rnf24	ring finger protein 24 [Source:MGI Symbol;Acc:MGI:1261771]
83	ENSMUSG000000000827	-0.50877	4.85259	13.11222	0.000293	0.008012	Tpd52l2	tumor protein D52-like 2 [Source:MGI Symbol;Acc:MGI:1913564]
654	ENSMUSG000000006024	-0.50779	5.606242	7.580011	0.005902	0.041355	Napa	N-ethylmaleimide sensitive fusion protein attachment protein alpha [Source:MGI Symbol;Acc:MGI:104563]
6201	ENSMUSG000000033819	-0.50693	3.152557	8.367394	0.00382	0.032993	Ppp1r16a	protein phosphatase 1, regulatory (inhibitor) subunit 16A [Source:MGI Symbol;Acc:MGI:1920312]
6317	ENSMUSG000000034345	-0.50412	4.163757	7.390346	0.006557	0.044051	Gtf2h5	general transcription factor IIH, polypeptide 5 [Source:MGI Symbol;Acc:MGI:107227]
724	ENSMUSG000000006736	-0.50351	4.650433	8.64154	0.003286	0.03047	Tspan31	tetraspanin 31 [Source:MGI Symbol;Acc:MGI:1914375]
4373	ENSMUSG000000028293	-0.50253	2.932182	7.140315	0.007537	0.047306	Slc35a1	solute carrier family 35 (CMP-sialic acid transporter), member 1 [Source:MGI Symbol;Acc:MGI:1345622]
4429	ENSMUSG000000028447	-0.50195	3.968566	7.251462	0.007084	0.045702	Dctn3	dynactin 3 [Source:MGI Symbol;Acc:MGI:1859251]
1411	ENSMUSG000000019518	-0.50083	3.881784	9.682197	0.001861	0.02296	Ap4m1	adaptor-related protein complex AP-4, mu 1 [Source:MGI Symbol;Acc:MGI:1337063]
9735	ENSMUSG000000061904	-0.49971	6.830027	8.682299	0.003213	0.030155	Slc25a3	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 3 [Source:MGI Symbol;Acc:MGI:1353498]
5677	ENSMUSG000000031950	-0.49826	4.535818	7.271072	0.007007	0.045524	Gabarapl2	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 2 [Source:MGI Symbol;Acc:MGI:1890602]
3566	ENSMUSG000000026107	-0.49554	5.941031	10.43178	0.001239	0.018821	Nabp1	nucleic acid binding protein 1 [Source:MGI Symbol;Acc:MGI:1923258]
4069	ENSMUSG000000027465	-0.49495	5.857822	7.597713	0.005844	0.041153	Tbc1d20	TBC1 domain family, member 20 [Source:MGI Symbol;Acc:MGI:1914481]
3593	ENSMUSG000000026179	-0.49394	4.026324	7.214543	0.007232	0.046106	Pnkd	paroxysmal nonkinesigenic dyskinesia [Source:MGI Symbol;Acc:MGI:1930773]
2034	ENSMUSG000000021276	-0.49391	2.761427	7.079414	0.007797	0.048164	Cinp	cyclin-dependent kinase 2 interacting protein [Source:MGI Symbol;Acc:MGI:1914486]

2122	ENSMUSG000000021550	-0.49344	3.862032	8.646683	0.003277	0.030436	2210016F16Rik	RIKEN cDNA 2210016F16 gene [Source:MGI Symbol;Acc:MGI:1917403]
9491	ENSMUSG000000058594	-0.49167	5.334942	12.83947	0.000339	0.008868	Fbxo18	F-box protein 18 [Source:MGI Symbol;Acc:MGI:1354699]
1924	ENSMUSG000000020993	-0.49122	4.520174	8.390243	0.003772	0.032857	Trappc6b	trafficking protein particle complex 6B [Source:MGI Symbol;Acc:MGI:1925482]
8902	ENSMUSG000000051154	-0.49067	5.048116	7.484603	0.006223	0.042698	Commdd3	COMM domain containing 3 [Source:MGI Symbol;Acc:MGI:88218]
4906	ENSMUSG000000029621	-0.49047	4.35467	8.370133	0.003814	0.032993	Arpc1a	actin related protein 2/3 complex, subunit 1A [Source:MGI Symbol;Acc:MGI:1928896]
6635	ENSMUSG000000036002	-0.49016	3.428731	7.558116	0.005974	0.041627	Fam214b	family with sequence similarity 214, member B [Source:MGI Symbol;Acc:MGI:2441854]
537	ENSMUSG000000004846	-0.48594	4.358803	7.916753	0.004898	0.037705	Plod3	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 [Source:MGI Symbol;Acc:MGI:1347008]
1619	ENSMUSG000000020253	-0.48398	4.441944	9.578265	0.001969	0.023335	Ppm1m	protein phosphatase 1M [Source:MGI Symbol;Acc:MGI:1915155]
3527	ENSMUSG000000026005	-0.48023	5.174767	9.37844	0.002196	0.02462	Rpe	ribulose-5-phosphate-3-epimerase [Source:MGI Symbol;Acc:MGI:1913896]
10660	ENSMUSG000000090841	-0.4796	7.157677	12.7768	0.000351	0.009019	Myl6	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle [Source:MGI Symbol;Acc:MGI:109318]
1614	ENSMUSG000000020241	-0.47858	6.530771	8.535946	0.003482	0.031355	Col6a2	collagen, type VI, alpha 2 [Source:MGI Symbol;Acc:MGI:88460]
8720	ENSMUSG000000048878	-0.47735	4.688912	11.475	0.000705	0.013899	Hexim1	hexamethylene bis-acetamide inducible 1 [Source:MGI Symbol;Acc:MGI:2385923]
4081	ENSMUSG000000027502	-0.47713	5.077264	8.849303	0.002932	0.028802	Rtfcd1	replication termination factor 2 domain containing 1 [Source:MGI Symbol;Acc:MGI:1913654]
4899	ENSMUSG000000029598	-0.47673	4.286947	11.01809	0.000902	0.016039	Plbd2	phospholipase B domain containing 2 [Source:MGI Symbol;Acc:MGI:1919022]
1535	ENSMUSG000000020057	-0.47658	5.449201	6.974223	0.008269	0.049752	Dram1	DNA-damage regulated autophagy modulator 1 [Source:MGI Symbol;Acc:MGI:1918962]
833	ENSMUSG000000009291	-0.47545	5.930522	10.06679	0.00151	0.020587	Pttg1ip	pituitary tumor-transforming 1 interacting protein [Source:MGI Symbol;Acc:MGI:2652132]
1163	ENSMUSG000000016541	-0.47519	5.170713	8.760262	0.003079	0.029381	Atxn10	ataxin 10 [Source:MGI Symbol;Acc:MGI:1859293]
2679	ENSMUSG000000022964	-0.47403	4.434314	7.944628	0.004823	0.037403	Tmem50b	transmembrane protein 50B [Source:MGI Symbol;Acc:MGI:1925225]
1191	ENSMUSG000000017286	-0.47204	4.339716	9.620126	0.001925	0.02312	Glod4	glyoxalase domain containing 4 [Source:MGI Symbol;Acc:MGI:1914451]
5477	ENSMUSG000000031485	-0.46871	4.544623	7.722912	0.005452	0.039792	Prosc	proline synthetase co-transcribed [Source:MGI Symbol;Acc:MGI:1891207]
5929	ENSMUSG000000032563	-0.46826	4.323573	10.11958	0.001467	0.020363	Mrpl3	mitochondrial ribosomal protein L3 [Source:MGI Symbol;Acc:MGI:2137204]
4316	ENSMUSG000000028138	-0.46752	4.995936	9.871159	0.001679	0.021726	Adh5	alcohol dehydrogenase 5 (class III), chi polypeptide [Source:MGI Symbol;Acc:MGI:87929]
5848	ENSMUSG000000032381	-0.46611	4.417935	7.255418	0.007069	0.045663	Fam96a	family with sequence similarity 96, member A [Source:MGI Symbol;Acc:MGI:1915500]
4714	ENSMUSG000000029125	-0.46421	3.767692	7.331136	0.006777	0.04478	Stx18	syntaxin 18 [Source:MGI Symbol;Acc:MGI:1918366]
5110	ENSMUSG000000030314	-0.46216	3.790224	7.804358	0.005212	0.038871	Atg7	autophagy related 7 [Source:MGI Symbol;Acc:MGI:1921494]
3435	ENSMUSG000000025757	-0.46189	4.76917	9.573912	0.001974	0.023349	Hspa4l	heat shock protein 4 like [Source:MGI Symbol;Acc:MGI:107422]

824	ENSMUSG00000009035	-0.461	4.794517	7.099853	0.007709	0.047915	Tmem184b	transmembrane protein 184b [Source:MGI Symbol;Acc:MGI:2445179]
1598	ENSMUSG000000020190	-0.46025	7.239374	9.435907	0.002128	0.024379	Mknk2	MAP kinase-interacting serine/threonine kinase 2 [Source:MGI Symbol;Acc:MGI:894279]
10262	ENSMUSG000000073155	-0.45988	4.196982	7.451528	0.006338	0.04312	1810058I24Rik	RIKEN cDNA 1810058I24 gene [Source:MGI Symbol;Acc:MGI:1914955]
4893	ENSMUSG000000029578	-0.4535	5.340953	8.982256	0.002726	0.027735	Wipi2	WD repeat domain, phosphoinositide interacting 2 [Source:MGI Symbol;Acc:MGI:1923831]
6151	ENSMUSG000000033557	-0.45151	5.517978	14.61212	0.000132	0.004878	Fam20b	family with sequence similarity 20, member B [Source:MGI Symbol;Acc:MGI:2443990]
6976	ENSMUSG000000037669	-0.45052	4.655564	7.196867	0.007303	0.046372	Ldah	lipid droplet associated hydrolase [Source:MGI Symbol;Acc:MGI:1916082]
3293	ENSMUSG000000025240	-0.44696	5.8459	7.185524	0.007349	0.046558	Sacm1l	SAC1 (suppressor of actin mutations 1, homolog)-like (S. cerevisiae) [Source:MGI Symbol;Acc:MGI:1933169]
1098	ENSMUSG000000015714	-0.44544	5.681424	8.229397	0.004122	0.034187	Cers2	ceramide synthase 2 [Source:MGI Symbol;Acc:MGI:1924143]
3649	ENSMUSG000000026335	-0.4445	6.102237	7.341226	0.006739	0.044638	Pam	peptidylglycine alpha-amidating monooxygenase [Source:MGI Symbol;Acc:MGI:97475]
6081	ENSMUSG000000033191	-0.44236	4.330654	7.834305	0.005126	0.038556	Tie1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1 [Source:MGI Symbol;Acc:MGI:99906]
7598	ENSMUSG000000040105	-0.44218	3.720571	8.73938	0.003114	0.029602	Ppapdc2	phosphatidic acid phosphatase type 2 domain containing 2 [Source:MGI Symbol;Acc:MGI:1921661]
5024	ENSMUSG000000030061	-0.43342	5.182753	9.322938	0.002263	0.02501	Uba3	ubiquitin-like modifier activating enzyme 3 [Source:MGI Symbol;Acc:MGI:1341217]
4959	ENSMUSG000000029767	-0.43329	6.946928	7.574157	0.005921	0.04141	Calu	calumenin [Source:MGI Symbol;Acc:MGI:1097158]
5453	ENSMUSG000000031402	-0.43125	5.272914	7.245109	0.007109	0.04575	Mpp1	membrane protein, palmitoylated [Source:MGI Symbol;Acc:MGI:105941]
7562	ENSMUSG000000039959	-0.42982	5.218161	10.3483	0.001296	0.019237	Hip1	huntingtin interacting protein 1 [Source:MGI Symbol;Acc:MGI:1099804]
5592	ENSMUSG000000031751	-0.42921	6.199936	7.489696	0.006205	0.042604	Amfr	autocrine motility factor receptor [Source:MGI Symbol;Acc:MGI:1345634]
5476	ENSMUSG000000031483	-0.4251	4.898402	9.63513	0.001909	0.02312	Erlin2	ER lipid raft associated 2 [Source:MGI Symbol;Acc:MGI:2387215]
3103	ENSMUSG000000024781	-0.42451	5.564183	10.13118	0.001458	0.020342	Lipa	lysosomal acid lipase A [Source:MGI Symbol;Acc:MGI:96789]
4476	ENSMUSG000000028567	-0.42267	3.949973	7.394315	0.006543	0.044038	Txndc12	thioredoxin domain containing 12 (endoplasmic reticulum) [Source:MGI Symbol;Acc:MGI:1913323]
10024	ENSMUSG000000068040	-0.41676	4.956622	8.054781	0.004538	0.03599	Tm9sf4	transmembrane 9 superfamily protein member 4 [Source:MGI Symbol;Acc:MGI:2139220]
5376	ENSMUSG000000031166	-0.41625	4.606122	7.478802	0.006243	0.042782	Wdr13	WD repeat domain 13 [Source:MGI Symbol;Acc:MGI:1914661]
146	ENSMUSG000000001348	-0.41523	5.784105	8.615754	0.003333	0.030696	Acp5	acid phosphatase 5, tartrate resistant [Source:MGI Symbol;Acc:MGI:87883]
6230	ENSMUSG000000033964	-0.40733	5.174064	7.621748	0.005767	0.040909	Zbtb41	zinc finger and BTB domain containing 41 homolog [Source:MGI Symbol;Acc:MGI:2444487]
4532	ENSMUSG000000028692	-0.40647	6.427812	9.738285	0.001805	0.022576	Akr1a1	aldo-keto reductase family 1, member A1 (aldehyde reductase) [Source:MGI Symbol;Acc:MGI:1929955]
675	ENSMUSG000000006299	-0.40427	5.436572	8.834122	0.002956	0.028861	Aamp	angio-associated migratory protein [Source:MGI Symbol;Acc:MGI:107809]
9624	ENSMUSG000000060450	-0.40346	5.152454	7.346742	0.006718	0.044555	Rnf14	ring finger protein 14 [Source:MGI Symbol;Acc:MGI:1929668]

7981	ENSMUSG00000042032	-0.40057	4.91626	7.232692	0.007159	0.045855	Mat2b	methionine adenosyltransferase II, beta [Source:MGI Symbol;Acc:MGI:1913667]
7517	ENSMUSG00000039770	-0.39994	4.988209	9.339171	0.002243	0.024872	Ypel5	yippee-like 5 (Drosophila) [Source:MGI Symbol;Acc:MGI:1916937]
5993	ENSMUSG00000032788	-0.387	4.620327	8.809856	0.002996	0.028992	Pdxk	pyridoxal (pyridoxine, vitamin B6) kinase [Source:MGI Symbol;Acc:MGI:1351869]
9811	ENSMUSG00000063015	-0.38381	6.974863	8.487022	0.003577	0.031861	Ccni	cyclin I [Source:MGI Symbol;Acc:MGI:1341077]
735	ENSMUSG00000006932	-0.36121	7.529425	8.267457	0.004036	0.033806	Ctnnb1	catenin (cadherin associated protein), beta 1 [Source:MGI Symbol;Acc:MGI:88276]
3966	ENSMUSG00000027189	-0.31902	6.255658	6.988825	0.008202	0.049457	Trim44	tripartite motif-containing 44 [Source:MGI Symbol;Acc:MGI:1931835]
4695	ENSMUSG00000029076	-0.30701	6.483861	8.540784	0.003473	0.03135	Sdf4	stromal cell derived factor 4 [Source:MGI Symbol;Acc:MGI:108079]

Appendix 3 Differentially Expressed Genes Upregulated in Mice Fed High Folic Acid during Adulthood

ID	Gene	LogFC	LogCPM	LR	PValue	FDR	Mgi_symbol	Description
6075	ENSMUSG000000031906	3.752212	3.48748	17.47893	2.91E-05	0.005139	Smpd3	sphingomyelin phosphodiesterase 3, neutral [Source:MGI Symbol;Acc:MGI:1927578]
3035	ENSMUSG000000024011	3.698289	6.129371	15.62598	7.72E-05	0.009287	Pi16	peptidase inhibitor 16 [Source:MGI Symbol;Acc:MGI:1921366]
10169	ENSMUSG000000056481	3.33967	5.79263	29.02017	7.16E-08	7.99E-05	Cd248	CD248 antigen, endosialin [Source:MGI Symbol;Acc:MGI:1917695]
5825	ENSMUSG000000031343	3.202282	2.975694	34.60942	4.03E-09	1.24E-05	Gabra3	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3 [Source:MGI Symbol;Acc:MGI:95615]
6204	ENSMUSG000000032243	3.179464	3.102242	29.20908	6.50E-08	7.97E-05	Itga11	integrin alpha 11 [Source:MGI Symbol;Acc:MGI:2442114]
8891	ENSMUSG000000044017	3.095523	5.18673	26.88592	2.16E-07	0.000166	Adgrd1	adhesion G protein-coupled receptor D1 [Source:MGI Symbol;Acc:MGI:3041203]
889	ENSMUSG000000008845	3.070336	5.54632	26.13873	3.18E-07	0.0002	Cd163	CD163 antigen [Source:MGI Symbol;Acc:MGI:2135946]
9763	ENSMUSG000000051906	3.04923	4.77736	32.36688	1.28E-08	2.24E-05	Cd209f	CD209f antigen [Source:MGI Symbol;Acc:MGI:1916392]
7921	ENSMUSG000000039109	3.039735	6.483796	22.41154	2.20E-06	0.0009	F13a1	coagulation factor XIII, A1 subunit [Source:MGI Symbol;Acc:MGI:1921395]
1471	ENSMUSG000000018927	2.989764	6.262301	23.39205	1.32E-06	0.000649	Ccl6	chemokine (C-C motif) ligand 6 [Source:MGI Symbol;Acc:MGI:98263]
11220	ENSMUSG000000071984	2.989204	6.323003	12.9753	0.000316	0.023618	Fndc1	fibronectin type III domain containing 1 [Source:MGI Symbol;Acc:MGI:1915905]
3869	ENSMUSG000000026193	2.981304	9.033849	20.23284	6.86E-06	0.001912	Fn1	fibronectin 1 [Source:MGI Symbol;Acc:MGI:95566]
7220	ENSMUSG000000036412	2.940951	2.881137	16.92585	3.89E-05	0.005942	Arsi	arylsulfatase i [Source:MGI Symbol;Acc:MGI:2670959]
11552	ENSMUSG000000079168	2.787528	3.45379	36.41113	1.60E-09	9.80E-06	Cd209g	CD209g antigen [Source:MGI Symbol;Acc:MGI:1917442]
4261	ENSMUSG000000027204	2.782546	7.456399	14.40969	0.000147	0.014469	Fbn1	fibrillin 1 [Source:MGI Symbol;Acc:MGI:95489]
10559	ENSMUSG000000061100	2.769118	7.608786	18.63869	1.58E-05	0.003343	Retnla	resistin like alpha [Source:MGI Symbol;Acc:MGI:1888504]
1487	ENSMUSG000000019122	2.761874	5.51696	27.35506	1.69E-07	0.000166	Ccl9	chemokine (C-C motif) ligand 9 [Source:MGI Symbol;Acc:MGI:104533]
1602	ENSMUSG000000019899	2.75248	6.405612	20.10779	7.32E-06	0.001996	Lama2	laminin, alpha 2 [Source:MGI Symbol;Acc:MGI:99912]
8978	ENSMUSG000000044749	2.746114	3.688474	18.92436	1.36E-05	0.003125	Abca6	ATP-binding cassette, sub-family A (ABC1), member 6 [Source:MGI Symbol;Acc:MGI:1923434]
3053	ENSMUSG000000024053	2.732957	5.345138	25.12778	5.37E-07	0.000299	Emilin2	elastin microfibril interfacer 2 [Source:MGI Symbol;Acc:MGI:2389136]
181	ENSMUSG000000001506	2.726791	9.084521	20.25797	6.77E-06	0.001912	Col1a1	collagen, type I, alpha 1 [Source:MGI Symbol;Acc:MGI:88467]

2914	ENSMUSG00000023046	2.704725	6.225446	15.05785	0.000104	0.011127	Igfbp6	insulin-like growth factor binding protein 6 [Source:MGI Symbol;Acc:MGI:96441]
7453	ENSMUSG00000037379	2.69445	4.877884	14.29447	0.000156	0.014871	Spon2	spondin 2, extracellular matrix protein [Source:MGI Symbol;Acc:MGI:1923724]
368	ENSMUSG00000002847	2.687844	5.34668	14.97259	0.000109	0.011374	Pla1a	phospholipase A1 member A [Source:MGI Symbol;Acc:MGI:1934677]
9481	ENSMUSG000000049130	2.681699	3.540737	31.60094	1.89E-08	2.90E-05	C5ar1	complement component 5a receptor 1 [Source:MGI Symbol;Acc:MGI:88232]
3809	ENSMUSG000000026043	2.651278	10.96347	13.91203	0.000192	0.01716	Col3a1	collagen, type III, alpha 1 [Source:MGI Symbol;Acc:MGI:88453]
8857	ENSMUSG000000043613	2.61734	5.539506	13.17723	0.000283	0.022292	Mmp3	matrix metalloproteinase 3 [Source:MGI Symbol;Acc:MGI:97010]
6548	ENSMUSG000000033327	2.614372	7.216023	21.46202	3.61E-06	0.001265	Tnxb	tenascin XB [Source:MGI Symbol;Acc:MGI:1932137]
8394	ENSMUSG000000040950	2.605377	4.796281	20.89588	4.85E-06	0.001526	Mgl2	macrophage galactose N-acetyl-galactosamine specific lectin 2 [Source:MGI Symbol;Acc:MGI:2385729]
6181	ENSMUSG000000032181	2.577384	4.810375	11.83029	0.000583	0.037839	Scg3	secretogranin III [Source:MGI Symbol;Acc:MGI:103032]
4171	ENSMUSG000000026938	2.535281	3.265522	13.77158	0.000206	0.018096	Fcna	ficolin A [Source:MGI Symbol;Acc:MGI:1340905]
2234	ENSMUSG000000021390	2.524996	6.698699	16.24292	5.57E-05	0.007683	Ogn	osteoglycin [Source:MGI Symbol;Acc:MGI:109278]
8119	ENSMUSG000000039883	2.508339	4.035091	16.90883	3.92E-05	0.005942	Lrrc17	leucine rich repeat containing 17 [Source:MGI Symbol;Acc:MGI:1921761]
11204	ENSMUSG000000071656	2.499824	4.327414	18.89552	1.38E-05	0.003125	Lrrn4cl	LRRN4 C-terminal like [Source:MGI Symbol;Acc:MGI:1916102]
10860	ENSMUSG000000066113	2.492664	3.572922	14.75206	0.000123	0.012233	Adamts1	ADAMTS-like 1 [Source:MGI Symbol;Acc:MGI:1924989]
741	ENSMUSG000000006369	2.476519	4.079106	11.26274	0.000791	0.046506	Fbln1	fibulin 1 [Source:MGI Symbol;Acc:MGI:95487]
5286	ENSMUSG000000029661	2.460243	9.603296	16.35348	5.26E-05	0.0075	Col1a2	collagen, type I, alpha 2 [Source:MGI Symbol;Acc:MGI:88468]
6001	ENSMUSG000000031740	2.447916	7.328671	13.40166	0.000251	0.020706	Mmp2	matrix metalloproteinase 2 [Source:MGI Symbol;Acc:MGI:97009]
5366	ENSMUSG000000029925	2.437321	2.808387	26.89115	2.15E-07	0.000166	Tbxas1	thromboxane A synthase 1, platelet [Source:MGI Symbol;Acc:MGI:98497]
2624	ENSMUSG000000022371	2.425037	6.578245	17.45681	2.94E-05	0.005139	Col14a1	collagen, type XIV, alpha 1 [Source:MGI Symbol;Acc:MGI:1341272]
7739	ENSMUSG000000038463	2.41534	4.599795	16.51033	4.84E-05	0.007069	Olfml2b	olfactomedin-like 2B [Source:MGI Symbol;Acc:MGI:2443310]
4127	ENSMUSG000000026837	2.366063	7.152286	19.13053	1.22E-05	0.002881	Col5a1	collagen, type V, alpha 1 [Source:MGI Symbol;Acc:MGI:88457]
1331	ENSMUSG000000017737	2.355823	3.806054	19.15407	1.21E-05	0.002881	Mmp9	matrix metalloproteinase 9 [Source:MGI Symbol;Acc:MGI:97011]
3815	ENSMUSG000000026069	2.317838	3.14626	19.61955	9.45E-06	0.002416	Il1rl1	interleukin 1 receptor-like 1 [Source:MGI Symbol;Acc:MGI:98427]
8066	ENSMUSG000000039683	2.316999	3.500933	15.71009	7.38E-05	0.009184	Sdk1	sidekick homolog 1 (chicken) [Source:MGI Symbol;Acc:MGI:2444413]
1352	ENSMUSG000000017969	2.31129	2.529852	16.32989	5.32E-05	0.007507	Ptgis	prostaglandin I2 (prostacyclin) synthase [Source:MGI Symbol;Acc:MGI:1097156]
8226	ENSMUSG000000040310	2.305193	2.859775	15.70344	7.41E-05	0.009184	Alx4	aristaless-like homeobox 4 [Source:MGI Symbol;Acc:MGI:108359]
3894	ENSMUSG000000026255	2.301714	3.098082	17.27453	3.23E-05	0.005293	Efhd1	EF hand domain containing 1 [Source:MGI Symbol;Acc:MGI:1921607]
4585	ENSMUSG000000028047	2.282829	4.075008	13.95302	0.000187	0.016913	Thbs3	thrombospondin 3 [Source:MGI Symbol;Acc:MGI:98739]
5155	ENSMUSG000000029373	2.273035	3.933722	26.29049	2.94E-07	0.0002	Pf4	platelet factor 4 [Source:MGI Symbol;Acc:MGI:1888711]
1782	ENSMUSG000000020312	2.236769	3.667586	20.91792	4.79E-06	0.001526	Shc2	SHC (Src homology 2 domain containing) transforming protein 2 [Source:MGI Symbol;Acc:MGI:106180]
9370	ENSMUSG000000048126	2.228025	7.933948	30.04454	4.22E-08	5.76E-05	Col6a3	collagen, type VI, alpha 3 [Source:MGI Symbol;Acc:MGI:88461]
4010	ENSMUSG000000026574	2.214016	8.258823	17.95963	2.26E-05	0.004558	Dpt	dermatopontin [Source:MGI Symbol;Acc:MGI:1928392]

9283	ENSMUSG00000047414	2.203809	4.135217	13.05779	0.000302	0.023059	Flrt2	fibronectin leucine rich transmembrane protein 2 [Source:MGI Symbol;Acc:MGI:3603594]
3835	ENSMUSG00000026109	2.194588	4.050617	13.02003	0.000308	0.023202	Tmeff2	transmembrane protein with EGF-like and two follistatin-like domains 2 [Source:MGI Symbol;Acc:MGI:1861735]
9975	ENSMUSG00000054203	2.172726	4.993258	15.23783	9.48E-05	0.010253	Ifi205	interferon activated gene 205 [Source:MGI Symbol;Acc:MGI:101847]
8292	ENSMUSG00000040552	2.169676	3.515383	13.02901	0.000307	0.023202	C3ar1	complement component 3a receptor 1 [Source:MGI Symbol;Acc:MGI:1097680]
1305	ENSMUSG00000017446	2.165937	5.246487	17.92108	2.30E-05	0.004558	C1qtnf1	C1q and tumor necrosis factor related protein 1 [Source:MGI Symbol;Acc:MGI:1919254]
3703	ENSMUSG00000025784	2.159297	6.631845	14.96766	0.000109	0.011374	Clec3b	C-type lectin domain family 3, member b [Source:MGI Symbol;Acc:MGI:104540]
2349	ENSMUSG00000021702	2.154367	4.427459	14.87763	0.000115	0.01183	Thbs4	thrombospondin 4 [Source:MGI Symbol;Acc:MGI:1101779]
2189	ENSMUSG00000021268	2.145635	4.239677	11.65809	0.000639	0.039699	Meg3	maternally expressed 3 [Source:MGI Symbol;Acc:MGI:1202886]
573	ENSMUSG00000004814	2.13443	2.619005	13.31715	0.000263	0.021094	Ccl24	chemokine (C-C motif) ligand 24 [Source:MGI Symbol;Acc:MGI:1928953]
9294	ENSMUSG00000047497	2.128152	5.511698	12.57861	0.00039	0.028502	Adamts12	a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 12 [Source:MGI Symbol;Acc:MGI:2146046]
2418	ENSMUSG00000021876	2.113216	6.940739	16.99484	3.75E-05	0.005822	Rnase4	ribonuclease, RNase A family 4 [Source:MGI Symbol;Acc:MGI:1926217]
1207	ENSMUSG00000015852	2.110559	4.669107	14.2682	0.000159	0.014964	Fcrls	Fc receptor-like 5, scavenger receptor [Source:MGI Symbol;Acc:MGI:1933397]
1953	ENSMUSG00000020695	2.106666	4.900997	26.49322	2.64E-07	0.000191	Mrc2	mannose receptor, C type 2 [Source:MGI Symbol;Acc:MGI:107818]
4616	ENSMUSG00000028108	2.105854	5.941667	23.14	1.51E-06	0.000711	Ecm1	extracellular matrix protein 1 [Source:MGI Symbol;Acc:MGI:103060]
1946	ENSMUSG00000020681	2.10306	5.866796	21.56994	3.41E-06	0.001231	Ace	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 [Source:MGI Symbol;Acc:MGI:87874]
79	ENSMUSG00000000753	2.098949	7.119476	19.64162	9.34E-06	0.002416	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1 [Source:MGI Symbol;Acc:MGI:108080]
7228	ENSMUSG00000036446	2.09554	7.833338	11.38824	0.000739	0.044462	Lum	lumican [Source:MGI Symbol;Acc:MGI:109347]
4056	ENSMUSG00000026674	2.093032	6.709931	21.32484	3.88E-06	0.001286	Ddr2	discoidin domain receptor family, member 2 [Source:MGI Symbol;Acc:MGI:1345277]
5911	ENSMUSG00000031538	2.083476	5.046761	11.9727	0.00054	0.036603	Plat	plasminogen activator, tissue [Source:MGI Symbol;Acc:MGI:97610]
2993	ENSMUSG00000023885	2.080238	6.428798	15.82645	6.94E-05	0.008803	Thbs2	thrombospondin 2 [Source:MGI Symbol;Acc:MGI:98738]
10165	ENSMUSG00000056427	2.075112	6.156537	13.5804	0.000229	0.019244	Slit3	slit homolog 3 (Drosophila) [Source:MGI Symbol;Acc:MGI:1315202]
511	ENSMUSG00000004098	2.068609	7.546755	15.02607	0.000106	0.011218	Col5a3	collagen, type V, alpha 3 [Source:MGI Symbol;Acc:MGI:1858212]
7401	ENSMUSG00000037206	2.068572	5.56149	22.11005	2.57E-06	0.001019	Islr	immunoglobulin superfamily containing leucine-rich repeat [Source:MGI Symbol;Acc:MGI:1349645]
6997	ENSMUSG00000035279	2.054824	4.475645	16.02481	6.25E-05	0.00825	Ssc5d	scavenger receptor cysteine rich family, 5 domains [Source:MGI Symbol;Acc:MGI:3606211]
2820	ENSMUSG00000022816	2.054723	8.04454	13.58778	0.000228	0.019244	Fstl1	follistatin-like 1 [Source:MGI Symbol;Acc:MGI:102793]
11910	ENSMUSG00000090084	2.045239	5.126926	11.86195	0.000573	0.037599	Srpx	sushi-repeat-containing protein [Source:MGI Symbol;Acc:MGI:1858306]
8559	ENSMUSG00000041797	2.04293	5.230155	15.42904	8.57E-05	0.010012	Abca9	ATP-binding cassette, sub-family A (ABC1), member 9 [Source:MGI Symbol;Acc:MGI:2386796]

7244	ENSMUSG00000036545	2.034725	6.242903	13.88106	0.000195	0.017319	Adamts2	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2 [Source:MGI Symbol;Acc:MGI:1347356]
6568	ENSMUSG00000033420	2.001399	6.313593	14.3173	0.000154	0.014807	Antxr1	anthrax toxin receptor 1 [Source:MGI Symbol;Acc:MGI:1916788]
5285	ENSMUSG00000029659	1.992774	3.924524	20.25019	6.79E-06	0.001912	Medag	mesenteric estrogen dependent adipogenesis [Source:MGI Symbol;Acc:MGI:1917967]
4339	ENSMUSG00000027386	1.990433	3.705387	11.66564	0.000637	0.039699	Fbln7	fibulin 7 [Source:MGI Symbol;Acc:MGI:1917620]
1746	ENSMUSG00000020241	1.980787	7.699172	32.82925	1.01E-08	2.06E-05	Col6a2	collagen, type VI, alpha 2 [Source:MGI Symbol;Acc:MGI:88460]
6248	ENSMUSG00000032334	1.978786	5.938506	17.52981	2.83E-05	0.005139	Loxl1	lysyl oxidase-like 1 [Source:MGI Symbol;Acc:MGI:106096]
1609	ENSMUSG00000019929	1.976795	9.441874	15.26183	9.36E-05	0.010253	Dcn	decorin [Source:MGI Symbol;Acc:MGI:94872]
8420	ENSMUSG00000041120	1.976255	5.046601	15.36747	8.85E-05	0.01015	Nbl1	neuroblastoma, suppression of tumorigenicity 1 [Source:MGI Symbol;Acc:MGI:104591]
10682	ENSMUSG00000062591	1.970152	2.345598	34.66489	3.92E-09	1.24E-05	Tubb4a	tubulin, beta 4A class IVA [Source:MGI Symbol;Acc:MGI:107848]
4121	ENSMUSG00000026825	1.962515	4.748955	37.7821	7.91E-10	9.71E-06	Dnm1	dynamain 1 [Source:MGI Symbol;Acc:MGI:107384]
6735	ENSMUSG00000034205	1.961196	5.07309	17.40156	3.03E-05	0.005139	Loxl2	lysyl oxidase-like 2 [Source:MGI Symbol;Acc:MGI:2137913]
3960	ENSMUSG00000026443	1.945743	2.869033	11.69682	0.000626	0.039604	Lrrn2	leucine rich repeat protein 2, neuronal [Source:MGI Symbol;Acc:MGI:106037]
125	ENSMUSG00000001119	1.929115	7.841947	25.89896	3.60E-07	0.00021	Col6a1	collagen, type VI, alpha 1 [Source:MGI Symbol;Acc:MGI:88459]
239	ENSMUSG00000001930	1.928711	6.833128	11.88739	0.000565	0.037288	Vwf	Von Willebrand factor homolog [Source:MGI Symbol;Acc:MGI:98941]
1250	ENSMUSG00000016494	1.926517	6.968006	21.96464	2.78E-06	0.001065	Cd34	CD34 antigen [Source:MGI Symbol;Acc:MGI:88329]
6805	ENSMUSG00000034463	1.918364	5.197395	15.64281	7.65E-05	0.009287	Scara3	scavenger receptor class A, member 3 [Source:MGI Symbol;Acc:MGI:2444418]
1943	ENSMUSG00000020676	1.908838	5.214579	21.57822	3.40E-06	0.001231	Ccl11	chemokine (C-C motif) ligand 11 [Source:MGI Symbol;Acc:MGI:103576]
8930	ENSMUSG00000044337	1.898499	5.431685	33.14185	8.57E-09	2.06E-05	Ackr3	atypical chemokine receptor 3 [Source:MGI Symbol;Acc:MGI:109562]
3073	ENSMUSG00000024084	1.892018	3.53439	16.69532	4.39E-05	0.006568	Qpct	glutaminy-peptide cyclotransferase (glutaminy cyclase) [Source:MGI Symbol;Acc:MGI:1917786]
4617	ENSMUSG00000028111	1.890661	4.960919	13.67665	0.000217	0.018899	Ctsk	cathepsin K [Source:MGI Symbol;Acc:MGI:107823]
1694	ENSMUSG00000020122	1.884449	6.071852	17.40253	3.02E-05	0.005139	Egfr	epidermal growth factor receptor [Source:MGI Symbol;Acc:MGI:95294]
12093	ENSMUSG00000097451	1.880883	4.152567	17.0461	3.65E-05	0.00574	Rian	RNA imprinted and accumulated in nucleus [Source:MGI Symbol;Acc:MGI:1922995]
4117	ENSMUSG00000026819	1.879951	5.744396	12.42104	0.000425	0.030646	Slc25a25	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25 [Source:MGI Symbol;Acc:MGI:1915913]
9753	ENSMUSG000000051748	1.868341	2.919475	14.10478	0.000173	0.015954	Wfdc21	WAP four-disulfide core domain 21 [Source:MGI Symbol;Acc:MGI:1913357]
8335	ENSMUSG00000040711	1.822415	6.225416	11.7515	0.000608	0.038783	Sh3pxd2b	SH3 and PX domains 2B [Source:MGI Symbol;Acc:MGI:2442062]
6489	ENSMUSG00000033066	1.813053	5.791855	13.08969	0.000297	0.022917	Gas7	growth arrest specific 7 [Source:MGI Symbol;Acc:MGI:1202388]
9331	ENSMUSG00000047793	1.79296	5.46476	26.94582	2.09E-07	0.000166	Sned1	sushi, nidogen and EGF-like domains 1 [Source:MGI Symbol;Acc:MGI:3045960]
6102	ENSMUSG00000031963	1.774257	3.993765	15.24516	9.44E-05	0.010253	Bmper	BMP-binding endothelial regulator [Source:MGI Symbol;Acc:MGI:1920480]
4251	ENSMUSG00000027188	1.741792	4.370318	13.05475	0.000303	0.023059	Pamr1	peptidase domain containing associated with muscle regeneration 1 [Source:MGI Symbol;Acc:MGI:2445082]
11141	ENSMUSG00000070942	1.741307	4.748557	16.13236	5.91E-05	0.007965	Il1rl2	interleukin 1 receptor-like 2 [Source:MGI Symbol;Acc:MGI:1913107]

5112	ENSMUSG00000029231	1.738631	6.563381	20.49356	5.98E-06	0.001791	Pdgfra	platelet derived growth factor receptor, alpha polypeptide [Source:MGI Symbol;Acc:MGI:97530]
4776	ENSMUSG00000028517	1.737354	7.381509	19.43184	1.04E-05	0.002611	Ppap2b	phosphatidic acid phosphatase type 2B [Source:MGI Symbol;Acc:MGI:1915166]
4700	ENSMUSG00000028339	1.736581	7.598993	16.182	5.75E-05	0.007846	Col15a1	collagen, type XV, alpha 1 [Source:MGI Symbol;Acc:MGI:88449]
1501	ENSMUSG00000019278	1.732795	6.348602	12.63441	0.000379	0.02783	Dpep1	dipeptidase 1 (renal) [Source:MGI Symbol;Acc:MGI:94917]
9840	ENSMUSG00000052727	1.693269	5.578937	11.74543	0.00061	0.038783	Map1b	microtubule-associated protein 1B [Source:MGI Symbol;Acc:MGI:1306778]
1306	ENSMUSG00000017466	1.689254	8.292043	17.14842	3.46E-05	0.005511	Timp2	tissue inhibitor of metalloproteinase 2 [Source:MGI Symbol;Acc:MGI:98753]
632	ENSMUSG00000005397	1.681812	8.892806	16.28141	5.46E-05	0.007614	Nid1	nidogen 1 [Source:MGI Symbol;Acc:MGI:97342]
8859	ENSMUSG00000043629	1.666067	2.74759	11.09307	0.000867	0.04946	1700019D03Rik	RIKEN cDNA 1700019D03 gene [Source:MGI Symbol;Acc:MGI:1914330]
623	ENSMUSG00000005338	1.659054	4.23361	26.09148	3.26E-07	0.0002	Cadm3	cell adhesion molecule 3 [Source:MGI Symbol;Acc:MGI:2137858]
4015	ENSMUSG00000026580	1.647426	2.852368	11.58162	0.000666	0.04087	Selp	selectin, platelet [Source:MGI Symbol;Acc:MGI:98280]
6521	ENSMUSG00000033207	1.62894	2.322513	12.42759	0.000423	0.030646	Mamdc2	MAM domain containing 2 [Source:MGI Symbol;Acc:MGI:1918988]
6952	ENSMUSG00000035107	1.604302	4.343177	19.70043	9.06E-06	0.002416	Dcbld2	discoidin, CUB and LCCL domain containing 2 [Source:MGI Symbol;Acc:MGI:1920629]
2507	ENSMUSG00000022098	1.593013	5.878186	12.10955	0.000502	0.03478	Bmp1	bone morphogenetic protein 1 [Source:MGI Symbol;Acc:MGI:88176]
4128	ENSMUSG00000026840	1.592717	4.320906	15.48059	8.34E-05	0.009836	Lamc3	laminin gamma 3 [Source:MGI Symbol;Acc:MGI:1344394]
4281	ENSMUSG00000027254	1.579555	3.014428	17.932	2.29E-05	0.004558	Map1a	microtubule-associated protein 1 A [Source:MGI Symbol;Acc:MGI:1306776]
2521	ENSMUSG00000022122	1.577547	4.918146	17.41461	3.01E-05	0.005139	Ednrb	endothelin receptor type B [Source:MGI Symbol;Acc:MGI:102720]
9115	ENSMUSG00000045934	1.52195	2.510254	15.29564	9.19E-05	0.010253	Mtmr11	myotubularin related protein 11 [Source:MGI Symbol;Acc:MGI:2652817]
6185	ENSMUSG00000032186	1.512622	4.448507	23.67273	1.14E-06	0.000584	Tmod2	tropomodulin 2 [Source:MGI Symbol;Acc:MGI:1355335]
5888	ENSMUSG00000031494	1.510783	3.064601	17.49496	2.88E-05	0.005139	Cd209a	CD209a antigen [Source:MGI Symbol;Acc:MGI:2157942]
2152	ENSMUSG00000021186	1.509179	5.380223	15.26738	9.33E-05	0.010253	Fbln5	fibulin 5 [Source:MGI Symbol;Acc:MGI:1346091]
9702	ENSMUSG000000051323	1.507856	4.093811	12.04368	0.00052	0.035829	Pcdh19	protocadherin 19 [Source:MGI Symbol;Acc:MGI:2685563]
7369	ENSMUSG00000037060	1.497985	5.706988	13.63056	0.000223	0.019232	Prkcdbp	protein kinase C, delta binding protein [Source:MGI Symbol;Acc:MGI:1923422]
9850	ENSMUSG000000052821	1.462333	3.014606	16.47957	4.92E-05	0.0071	Cysltr1	cysteinyl leukotriene receptor 1 [Source:MGI Symbol;Acc:MGI:1926218]
1653	ENSMUSG00000020044	1.456522	7.475163	15.64157	7.66E-05	0.009287	Timp3	tissue inhibitor of metalloproteinase 3 [Source:MGI Symbol;Acc:MGI:98754]
8884	ENSMUSG00000043943	1.449324	3.227319	17.55948	2.78E-05	0.005139	Naalad2	N-acetylated alpha-linked acidic dipeptidase 2 [Source:MGI Symbol;Acc:MGI:1919810]
4809	ENSMUSG00000028583	1.447742	4.308614	11.64665	0.000643	0.039699	Pdpn	podoplanin [Source:MGI Symbol;Acc:MGI:103098]
2447	ENSMUSG00000021943	1.432369	3.973241	14.37171	0.00015	0.014503	Gdf10	growth differentiation factor 10 [Source:MGI Symbol;Acc:MGI:95684]
9017	ENSMUSG00000045094	1.402908	2.159553	11.67188	0.000635	0.039699	Arhgef37	Rho guanine nucleotide exchange factor (GEF) 37 [Source:MGI Symbol;Acc:MGI:3045339]
10732	ENSMUSG000000063160	1.399783	3.818775	21.35407	3.82E-06	0.001286	Numb1	numb-like [Source:MGI Symbol;Acc:MGI:894702]
8030	ENSMUSG00000039529	1.396946	4.196309	15.49921	8.25E-05	0.009834	Atp8b1	ATPase, class I, type 8B, member 1 [Source:MGI Symbol;Acc:MGI:1859665]
2947	ENSMUSG00000023224	1.396351	7.315028	11.79508	0.000594	0.038157	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1 [Source:MGI Symbol;Acc:MGI:894696]

6971	ENSMUSG00000035184	1.394884	3.035645	22.87992	1.72E-06	0.000784	Fam124a	family with sequence similarity 124, member A [Source:MGI Symbol;Acc:MGI:3645930]
4846	ENSMUSG00000028664	1.392228	3.52293	11.89252	0.000564	0.037288	Ephb2	Eph receptor B2 [Source:MGI Symbol;Acc:MGI:99611]
5001	ENSMUSG00000028996	1.389736	4.450239	17.14791	3.46E-05	0.005511	Rbp7	retinol binding protein 7, cellular [Source:MGI Symbol;Acc:MGI:1890409]
5308	ENSMUSG00000029718	1.380253	5.855088	20.67405	5.44E-06	0.00167	Pcolce	procollagen C-endopeptidase enhancer protein [Source:MGI Symbol;Acc:MGI:105099]
1333	ENSMUSG00000017754	1.379215	6.081547	14.19987	0.000164	0.015399	Pltp	phospholipid transfer protein [Source:MGI Symbol;Acc:MGI:103151]
8811	ENSMUSG00000043099	1.376376	3.614396	16.07515	6.09E-05	0.008121	Hic1	hypermethylated in cancer 1 [Source:MGI Symbol;Acc:MGI:1338010]
10008	ENSMUSG00000054580	1.352433	3.559025	15.83018	6.93E-05	0.008803	Pla2r1	phospholipase A2 receptor 1 [Source:MGI Symbol;Acc:MGI:102468]
10221	ENSMUSG00000057123	1.349867	2.77672	12.9211	0.000325	0.024165	Gja5	gap junction protein, alpha 5 [Source:MGI Symbol;Acc:MGI:95716]
4335	ENSMUSG00000027377	1.345486	3.168061	16.53701	4.77E-05	0.007054	Mall	mal, T cell differentiation protein-like [Source:MGI Symbol;Acc:MGI:2385152]
2945	ENSMUSG00000023191	1.335201	4.361844	13.09534	0.000296	0.022917	P3h3	prolyl 3-hydroxylase 3 [Source:MGI Symbol;Acc:MGI:1315208]
2851	ENSMUSG00000022893	1.328471	5.554274	11.95454	0.000545	0.036758	Adamts1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1 [Source:MGI Symbol;Acc:MGI:109249]
4888	ENSMUSG00000028763	1.321204	8.621468	12.39227	0.000431	0.030939	Hspg2	perlecan (heparan sulfate proteoglycan 2) [Source:MGI Symbol;Acc:MGI:96257]
7778	ENSMUSG00000038587	1.31517	5.791619	18.75719	1.48E-05	0.003226	Akap12	A kinase (PRKA) anchor protein (gravin) 12 [Source:MGI Symbol;Acc:MGI:1932576]
2506	ENSMUSG00000022096	1.309536	5.870832	11.39629	0.000736	0.044462	Hr	hairless [Source:MGI Symbol;Acc:MGI:96223]
2748	ENSMUSG00000022656	1.304421	3.503316	13.52947	0.000235	0.019606	Pvrl3	poliovirus receptor-related 3 [Source:MGI Symbol;Acc:MGI:1930171]
5416	ENSMUSG00000030110	1.294324	2.570919	11.25982	0.000792	0.046506	Ret	ret proto-oncogene [Source:MGI Symbol;Acc:MGI:97902]
6858	ENSMUSG00000034675	1.288639	4.590418	11.12199	0.000853	0.049151	Dbn1	drebrin 1 [Source:MGI Symbol;Acc:MGI:1931838]
1070	ENSMUSG00000014329	1.287122	4.451375	11.45746	0.000712	0.04326	Bicc1	bicaudal C homolog 1 (Drosophila) [Source:MGI Symbol;Acc:MGI:1933388]
2541	ENSMUSG00000022180	1.255186	3.490614	17.3933	3.04E-05	0.005139	Slc7a8	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8 [Source:MGI Symbol;Acc:MGI:1355323]
177	ENSMUSG00000001473	1.241074	4.894148	11.28874	0.00078	0.04623	Tubb6	tubulin, beta 6 class V [Source:MGI Symbol;Acc:MGI:1915201]
3918	ENSMUSG00000026315	1.23721	3.037188	17.29196	3.21E-05	0.005293	Serpnb8	serine (or cysteine) peptidase inhibitor, clade B, member 8 [Source:MGI Symbol;Acc:MGI:894657]
5883	ENSMUSG00000031486	1.23638	4.345151	11.49601	0.000697	0.042583	Adgra2	adhesion G protein-coupled receptor A2 [Source:MGI Symbol;Acc:MGI:1925810]
6123	ENSMUSG00000032006	1.232254	3.643233	12.18794	0.000481	0.033924	Pdgfd	platelet-derived growth factor, D polypeptide [Source:MGI Symbol;Acc:MGI:1919035]
57	ENSMUSG00000000555	1.230958	4.961901	24.99609	5.74E-07	0.000307	Itga5	integrin alpha 5 (fibronectin receptor alpha) [Source:MGI Symbol;Acc:MGI:96604]
7736	ENSMUSG00000038456	1.221939	4.237789	12.12478	0.000498	0.034694	Dennd2a	DENN/MADD domain containing 2A [Source:MGI Symbol;Acc:MGI:2444961]
5059	ENSMUSG00000029122	1.221093	3.957942	15.22875	9.52E-05	0.010253	Evc	Ellis van Creveld gene syndrome [Source:MGI Symbol;Acc:MGI:1890596]
10347	ENSMUSG00000058624	1.219928	6.460683	14.80903	0.000119	0.012004	Gda	guanine deaminase [Source:MGI Symbol;Acc:MGI:95678]
11793	ENSMUSG00000084960	1.186977	2.709351	13.42419	0.000248	0.020597	B430010I23Rik	RIKEN cDNA B430010I23 gene [Source:MGI Symbol;Acc:MGI:1926099]
5680	ENSMUSG00000030865	1.16902	4.071625	14.13332	0.00017	0.015833	Chp2	calcineurin-like EF hand protein 2 [Source:MGI Symbol;Acc:MGI:1917511]
7869	ENSMUSG00000038894	1.163727	5.165967	12.01961	0.000526	0.035989	Irs2	insulin receptor substrate 2 [Source:MGI Symbol;Acc:MGI:109334]

11982	ENSMUSG00000092341	1.153621	8.718688	13.36723	0.000256	0.020949	Malat1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA) [Source:MGI Symbol;Acc:MGI:1919539]
4786	ENSMUSG00000028541	1.14673	2.977523	11.22	0.000809	0.04727	B4galt2	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2 [Source:MGI procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide [Source:MGI Symbol;Acc:MGI:894286]
1466	ENSMUSG00000018906	1.102495	4.485631	15.99043	6.37E-05	0.008311	P4ha2	STEAP family member 3 [Source:MGI Symbol;Acc:MGI:1915678]
3940	ENSMUSG00000026389	1.095449	5.33976	13.34084	0.00026	0.020966	Steap3	biglycan [Source:MGI Symbol;Acc:MGI:88158]
5840	ENSMUSG00000031375	1.075354	7.617972	11.20743	0.000815	0.04727	Bgn	START domain containing 9 [Source:MGI Symbol;Acc:MGI:3045258]
6619	ENSMUSG00000033705	1.062688	5.048199	11.02464	0.000899	0.04987	Stard9	dual specificity phosphatase 1 [Source:MGI Symbol;Acc:MGI:105120]
3114	ENSMUSG00000024190	1.05063	5.209332	11.08258	0.000871	0.04951	Dusp1	Kruppel-like factor 4 (gut) [Source:MGI Symbol;Acc:MGI:1342287]
391	ENSMUSG00000003032	1.04926	5.518009	13.83582	0.000199	0.017613	Klf4	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 4 [Source:MGI Symbol;Acc:MGI:1920431]
2967	ENSMUSG00000023411	1.041771	3.830791	15.82229	6.96E-05	0.008803	Nfatc4	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9 [Source:MGI Symbol;Acc:MGI:1916320]
5378	ENSMUSG00000030022	1.001226	5.203017	11.35584	0.000752	0.045024	Adamts9	neuron navigator 1 [Source:MGI Symbol;Acc:MGI:2183683]
913	ENSMUSG00000009418	0.992554	6.064068	17.88578	2.35E-05	0.004569	Nav1	t-complex 11 like 1 [Source:MGI Symbol;Acc:MGI:2444263]
4243	ENSMUSG00000027175	0.960828	2.766366	11.05624	0.000884	0.049817	Tcp11l1	CCCTC-binding factor (zinc finger protein)-like, opposite strand [Source:MGI Symbol;Acc:MGI:1921411]
11861	ENSMUSG00000087382	0.956693	3.768495	12.14257	0.000493	0.034561	Ctcflos	interferon induced transmembrane protein 3 [Source:MGI Symbol;Acc:MGI:1913391]
3624	ENSMUSG00000025492	0.932351	6.805236	15.24905	9.42E-05	0.010253	Ifitm3	RIKEN cDNA 6430548M08 gene [Source:MGI Symbol;Acc:MGI:2443793]
6040	ENSMUSG00000031824	0.904635	3.895292	11.20313	0.000817	0.04727	6430548M08Rik	prolyl 3-hydroxylase 1 [Source:MGI Symbol;Acc:MGI:1888921]
4834	ENSMUSG00000028641	0.899637	4.476635	11.94361	0.000548	0.036773	P3h1	cytoglobin [Source:MGI Symbol;Acc:MGI:2149481]
2005	ENSMUSG00000020810	0.8674	5.568773	13.57718	0.000229	0.019244	Cygb	deleted in liver cancer 1 [Source:MGI Symbol;Acc:MGI:1354949]
5902	ENSMUSG00000031523	0.798592	7.835711	11.80493	0.000591	0.038156	Dlc1	plexin A2 [Source:MGI Symbol;Acc:MGI:107684]
4042	ENSMUSG00000026640	0.725771	5.114586	11.01835	0.000902	0.04987	Plxna2	solute carrier family 12, member 4 [Source:MGI Symbol;Acc:MGI:1309465]
1337	ENSMUSG00000017765	0.725287	5.024759	11.83849	0.00058	0.037839	Slc12a4	

Appendix 4 Differentially Expressed Genes Downregulated in Mice Fed High Folic Acid during Adulthood

ID	Gene	LogFC	LogCPM	LR	PValue	FDR	Mgi_symbol	Description
11395	ENSMUSG00000074873	-1.97643	2.687092	13.10335	0.000295	0.022917	Al606181	expressed sequence Al606181 [Source:MGI Symbol;Acc:MGI:2147586]
265	ENSMUSG00000002083	-1.76456	2.5646	27.15255	1.88E-07	0.000166	Bbc3	BCL2 binding component 3 [Source:MGI Symbol;Acc:MGI:2181667]
7960	ENSMUSG00000039221	-1.74953	2.926932	22.50993	2.09E-06	0.000885	Rpl22l1	ribosomal protein L22 like 1 [Source:MGI Symbol;Acc:MGI:1915278]
12220	ENSMUSG00000105814	-1.69341	3.11809	18.86833	1.40E-05	0.003125	mmu-mir-703	mmu-mir-703 [Source:miRBase;Acc:MI0004687]
9787	ENSMUSG00000052192	-1.51961	5.098816	13.25103	0.000272	0.021709	Gm5963	predicted pseudogene 5963 [Source:MGI Symbol;Acc:MGI:3648418]
11826	ENSMUSG00000086290	-1.39266	4.026394	22.74537	1.85E-06	0.000811	Snhg12	small nucleolar RNA host gene 12 [Source:MGI Symbol;Acc:MGI:1916721]
1252	ENSMUSG00000016496	-1.26747	4.583259	13.57863	0.000229	0.019244	Cd274	CD274 antigen [Source:MGI Symbol;Acc:MGI:1926446]
2868	ENSMUSG00000022948	-1.23327	2.525636	13.35105	0.000258	0.020966	Setd4	SET domain containing 4 [Source:MGI Symbol;Acc:MGI:2136890] tumor protein, translationally-controlled 1 [Source:MGI Symbol;Acc:MGI:104890]
10478	ENSMUSG00000060126	-1.20074	4.43401	15.40801	8.66E-05	0.010028	Tpt1	M phase phosphoprotein 6 [Source:MGI Symbol;Acc:MGI:1915783]
6053	ENSMUSG00000031843	-1.19544	2.627949	18.73989	1.50E-05	0.003226	Mphosph6	deoxyuridine triphosphatase [Source:MGI Symbol;Acc:MGI:1346051]
4260	ENSMUSG00000027203	-1.12815	3.66665	11.02542	0.000899	0.04987	Dut	stem-loop binding protein [Source:MGI Symbol;Acc:MGI:108402]
557	ENSMUSG00000004642	-1.12055	4.498299	11.88817	0.000565	0.037288	Slbp	predicted gene 6158 [Source:MGI Symbol;Acc:MGI:3779562]
11923	ENSMUSG00000090381	-1.07958	4.214796	12.20773	0.000476	0.03376	Gm6158	prothymosin alpha [Source:MGI Symbol;Acc:MGI:97803]
3887	ENSMUSG00000026238	-1.05222	7.358877	11.02984	0.000897	0.04987	Ptma	centrosomal protein 57 [Source:MGI Symbol;Acc:MGI:1915551]
6081	ENSMUSG00000031922	-1.04211	4.593611	12.25561	0.000464	0.033096	Cep57	inosine 5'-phosphate dehydrogenase 1 [Source:MGI Symbol;Acc:MGI:96567]
452	ENSMUSG00000003500	-0.98072	5.486475	17.38206	3.06E-05	0.005139	Impdh1	tubulin cofactor A [Source:MGI Symbol;Acc:MGI:107549]
8607	ENSMUSG00000042043	-0.97069	4.514417	19.34789	1.09E-05	0.002674	Tbca	hairy and enhancer of split 6 [Source:MGI Symbol;Acc:MGI:1859852]
10921	ENSMUSG00000067071	-0.95864	3.584065	11.32856	0.000763	0.045468	Hes6	

9689	ENSMUSG00000051224	-0.95393	2.949081	14.01085	0.000182	0.016642	Tceanc	transcription elongation factor A (SII) N-terminal and central domain containing [Source:MGI Symbol;Acc:MGI:2685236]
3168	ENSMUSG00000024317	-0.90775	4.838313	12.01452	0.000528	0.035989	Rnf138	ring finger protein 138 [Source:MGI Symbol;Acc:MGI:1929211]
5479	ENSMUSG00000030287	-0.90467	5.879623	17.97486	2.24E-05	0.004558	Itpr2	inositol 1,4,5-triphosphate receptor 2 [Source:MGI Symbol;Acc:MGI:99418]
8019	ENSMUSG00000039480	-0.85606	4.580229	14.3711	0.00015	0.014503	Nt5dc1	5'-nucleotidase domain containing 1 [Source:MGI Symbol;Acc:MGI:2442446]
7132	ENSMUSG00000035958	-0.8294	4.486807	14.82658	0.000118	0.012004	Tdp2	tyrosyl-DNA phosphodiesterase 2 [Source:MGI Symbol;Acc:MGI:1860486]
1615	ENSMUSG00000019948	-0.80862	3.868371	14.80317	0.000119	0.012004	Actr6	ARP6 actin-related protein 6 [Source:MGI Symbol;Acc:MGI:1914269]
9574	ENSMUSG00000050002	-0.80725	4.135431	11.64502	0.000644	0.039699	Idnk	idnK gluconokinase homolog (E. coli) [Source:MGI Symbol;Acc:MGI:1922981]
6455	ENSMUSG00000032902	-0.80707	5.78607	13.99736	0.000183	0.016642	Slc16a1	solute carrier family 16 (monocarboxylic acid transporters), member 1 [Source:MGI Symbol;Acc:MGI:106013]
3814	ENSMUSG00000026064	-0.805	5.838342	14.40537	0.000147	0.014469	Ptp4a1	protein tyrosine phosphatase 4a1 [Source:MGI Symbol;Acc:MGI:1277096]
11131	ENSMUSG00000070738	-0.76921	7.572338	11.05403	0.000885	0.049817	Dgkd	diacylglycerol kinase, delta [Source:MGI Symbol;Acc:MGI:2138334]
3680	ENSMUSG00000025722	-0.76345	4.294352	11.09554	0.000865	0.04946	Wdr73	WD repeat domain 73 [Source:MGI Symbol;Acc:MGI:1919218]
7982	ENSMUSG00000039304	-0.69077	5.068961	12.86937	0.000334	0.024692	Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10 [Source:MGI Symbol;Acc:MGI:107414]
3955	ENSMUSG00000026434	-0.62683	6.797209	13.22669	0.000276	0.021851	Nucks1	nuclear casein kinase and cyclin-dependent kinase substrate 1 [Source:MGI Symbol;Acc:MGI:1934811]

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