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**UNIVERSITY OF SOUTHAMPTON**

FACULTY OF MEDICINE

Cancer Sciences

**Regulation of the endocytic adaptor protein Eps15 by the CtBP family of  
metabolic sensors**

by

**Abbie Mead**

Thesis for the degree of Doctor of Philosophy

September 2016



UNIVERSITY OF SOUTHAMPTON

## **ABSTRACT**

FACULTY OF MEDICINE

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### **REGULATION OF THE ENDOCYTIC ADAPTOR PROTEIN EPS15 BY THE CTBP FAMILY OF METABOLIC SENSORS**

by Abbie Mead

Tumour cells metabolise glucose via ATP-inefficient aerobic glycolysis providing biosynthetic advantage to proliferating cells and increasing the ratio of the reduced form of the glycolytic coenzyme nicotinamide adenine dinucleotide (NADH) to its oxidised form (NAD<sup>+</sup>), resulting in activation of glycolytic sensors; C-terminal binding proteins (CtBPs). Our group previously showed that CtBPs regulate integrin-dependent cancer cell motility, an important observation since >90% cancer-related deaths are due to metastasis. Cell motility depends on integrin expression and localisation, regulated by endocytosis driving re-localisation of integrins from old to new adhesion complexes. A gene microarray showed that the endocytic adaptor protein, Eps15, is down-regulated upon CtBP1 knockdown, therefore our aim was to investigate whether CtBP1 could regulate cell movement through Eps15.

We confirmed this relationship at RNA and protein level in multiple cancer cell lines and tumour tissues and Chromatin Immunoprecipitation showed a functional link between the two proteins. This suggests that CtBP1 is not only a transcriptional co-repressor but can act as a transcriptional activator. *In vitro* migration and invasion assays revealed that, like CtBP1, Eps15 knockdown significantly inhibits tumour cell motility. Furthermore, *in vivo* stable Eps15 knockdown cells produce significantly smaller tumours compared to control cells. The best-described function of Eps15 is regulation of clathrin-dependent receptor endocytosis. Endocytosis assays following Eps15 knockdown, however showed no consistent effect on internalised or cell-surface levels of integrin  $\alpha v \beta 6$  or  $\alpha 5 \beta 1$ . Notably, Eps15 down-regulation significantly inhibited cell adhesion and spreading, and this effect appeared to result from a poorly-organised actin-cytoskeleton. Consistent with this, RNA sequencing using an oral squamous carcinoma cell line has revealed a novel role of Eps15 in the regulation of several cell adhesion regulators.

In summary, the metabolic sensor CtBP1 regulates tumour cell motility and positively regulates Eps15, which exerts a novel endocytosis-independent effect on cell adhesion to modulate cell movement.



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## DECLARATION OF AUTHORSHIP

I, Abbie Mead declare that this thesis entitled, Regulation of the endocytic adaptor protein Eps15 by the CtBP family of metabolic sensors, and the work presented in it are my own and has been generated by me as the result of my own original research.

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;
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. None of this work has been published before submission;

Signed: .....

Date: .....



## Acknowledgements

Throughout this PhD I have learnt a great deal, and have learnt the true importance of patience, perseverance and the multi-channel pipette. Without the continued advice and support of friends, family and colleagues the completion of this work would not have been possible and while one page does not seem enough to acknowledge everyone that I would like to, I will name those key people who have helped me immeasurably over the past 3 years.

First and foremost, I would like to thank my supervisors Gareth Thomas and Jeremy Blaydes for their continued support throughout my PhD. They have helped guide this project through every trial and been ready to celebrate every success. Thanks also to Dr. Emre Sayan and his group, who made the *in vivo* study possible.

I am most grateful to Veronika Jenei, who taught me the finesse and skill required to produce good data. Her patience never faltered and while she began a colleague and a mentor I would like to think that she has ended this PhD a friend. Without her support and genuine enthusiasm for science I would not have been able to complete this PhD.

I would also like to thank members of both the Blaydes group and the Experimental Pathology group; Caroline Barker, Charlie Birts, Arindam Banerjee, Kate Packwood, Chris Hanley, Steve Frampton, Karwan Moutasim, Jo Todd, Toby Mellows, Monette Lopez, Hollie Robinson and Jason Fleming. Their support, friendship and fancy dress outfits have made the last 3 years fly-by!

Finally, I would like to thank my dearest family and my oldest friends for their continued support and belief in me, and for keeping me on track.



## Definitions and Abbreviations

<b>Abbreviation</b>	<b>Definition</b>
ACB	Actin Cytoskeleton Binding
AMPK	AMP-activated Protein Kinase
AMPS	2-Acrylamido-2-Methylpropane Sulfonic acid
ATP	Adenosine 5'-Triphosphate
AU	Arbitrary Units
BAR	Bin-Amphiphysin-Rvs
BDP	BAR-Domain Protein
BSA	Bovine Serum Albumin
CAV	Caveolin
CC	Coiled-Coil
CCS	Clathrin Coated Structure
CDR	Circular Dorsal Ruffle
CFP	Cyan Fluorescent Protein
CI	Cell Index
CIE	Clathrin-Independent Endocytosis
CLASP	Clathrin Associated Sorting Protein
CLT	Clathrin
CME	Clathrin-Mediated Endocytosis
COLI	Collagen I
CtBP	C-terminal Binding Protein
DAPI	4',6-Diamidino-2-Phenylindole
dH <sub>2</sub> O	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylene Diamino Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EHD	Eps15-Homology Domain
EMT	Epithelial to Mesenchymal Transition
EPEC	Escherichia coli
EPS15	EGFR Pathway Substrate 15

ESCRT	Endosomal Sorting Complex Required for Transport
FA	Focal Adhesion
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FN	Fibronectin
FRET	Fluorescence Resonance Energy Transfer
GAP	GTPase Activating Protein
GEF	Guanine nucleotide Exchange Factor
GJ	Gap Junction
GTP	Guanosine Triphosphate
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylases
HMT	Histone Methyltransferase
HSC70	Heat Shock Chaperone protein 70
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
KGM	Keratinocyte Growth Medium
LAP	TGF- $\beta$ Latency Associated Peptide
LTBP	Latent TGF- $\beta$ Binding Protein
MAP	Mitogen-Activated Protein
MEF	Mouse Embryonic Fibroblast
MLEC	Mink Lung Epithelial Cells
NADH	Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide
NPF	Nucleation Promoting Factor
OSCC	Oral Squamous Cell Carcinoma
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PBS-Tween	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PTPN3	Protein Tyrosine Phosphatase 3
RFU	Relative Fluorescence Unit
RLU	Relative Light Unit
RNA	Ribonucleic Acid

RNAi	RNA interference
RTCA	Real Time Cell Analyser
RTK	Receptor Tyrosine Kinase
SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	small interfering RNA
TEMED	Tetramethylethylenediamine
TGF- $\beta$	Transforming Growth Factor-Beta
UIM	Ubiquitin Interacting Motif
UTR	Untranslated Region
WASP	Whiskott-Aldrich Syndrome Protein
WAVE	WASP-family Verprolin-homologous protein
WIP	WASP Interacting Protein
YFP	Yellow Fluorescent Protein





# Chapter 1: Introduction

## 1.1 The Hallmarks of Cancer

Cancer cells are able to develop and thrive due to a number of changes that occur to allow survival where normal cells would perish. A review by Hanahan and Weinberg in 2000 concluded six key hallmarks of cancer; sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis all of which begin with underlying genomic instability (Hanahan et al. 2000). A more recent review has added two emerging hallmarks; reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg 2011).

The dysregulation of cell motility and cell metabolism are the foundations of this project. In a previous project increased aerobic glycolysis, which is known to activate the metabolic sensors C-terminal binding proteins (CtBPs), was found to induce integrin-dependent cell motility (Chrzan 2014). Furthermore, it was also found that the down-regulation of CtBPs, inhibits integrin-dependent cell motility. Gene microarray analysis suggested a link between metabolism and clathrin-dependent integrin endocytosis as the protein Eps15, vital for clathrin-mediated endocytosis, was found to be down-regulated following knockdown of CtBP1.

## 1.2 Metabolism; the Warburg effect

In 1924 Otto Warburg discovered that the metabolism of cancer cells differs to that of normal cells (Upadhyay et al. 2013). He discovered that, regardless of surrounding oxygen levels, cancer cells metabolise glucose via glycolysis rather than via oxidative phosphorylation. Oxidative phosphorylation is utilised by normal cells for maximum adenosine 5'-triphosphate (ATP) production but glycolysis results in a greater production of lactate rather than ATP and this phenomenon was termed 'The Warburg Effect' (Figure 1.1).

The reason for this metabolic shift remains unclear. One theory suggests that this is important to maintain cell proliferation, as glycolysis supports the uptake and incorporation of nutrients into the biomass, rather than utilising them for ATP production. Proliferating cells require a large amount of nucleotides, amino acids and lipids in their biomass ready for mitosis; production of which requires the consumption of more carbon equivalents and nicotinamide adenine dinucleotide (NADH) than of ATP; both of which are produced more rapidly by aerobic glycolysis. Consistent with this, normal cells have been shown to utilise aerobic glycolysis when undergoing

rapid proliferation (Vander Heiden et al. 2009). Glycolysis therefore results in increased NADH, carbon equivalents as well as increased lactate production. Additionally, increased lactate production could benefit tumour cells by reducing the pH of the surrounding tumour environment to the detriment of tumour-attacking immune cells, as well as disrupting normal tissue architecture to enhance tumour cell invasion (Locasale & Cantley 2010).

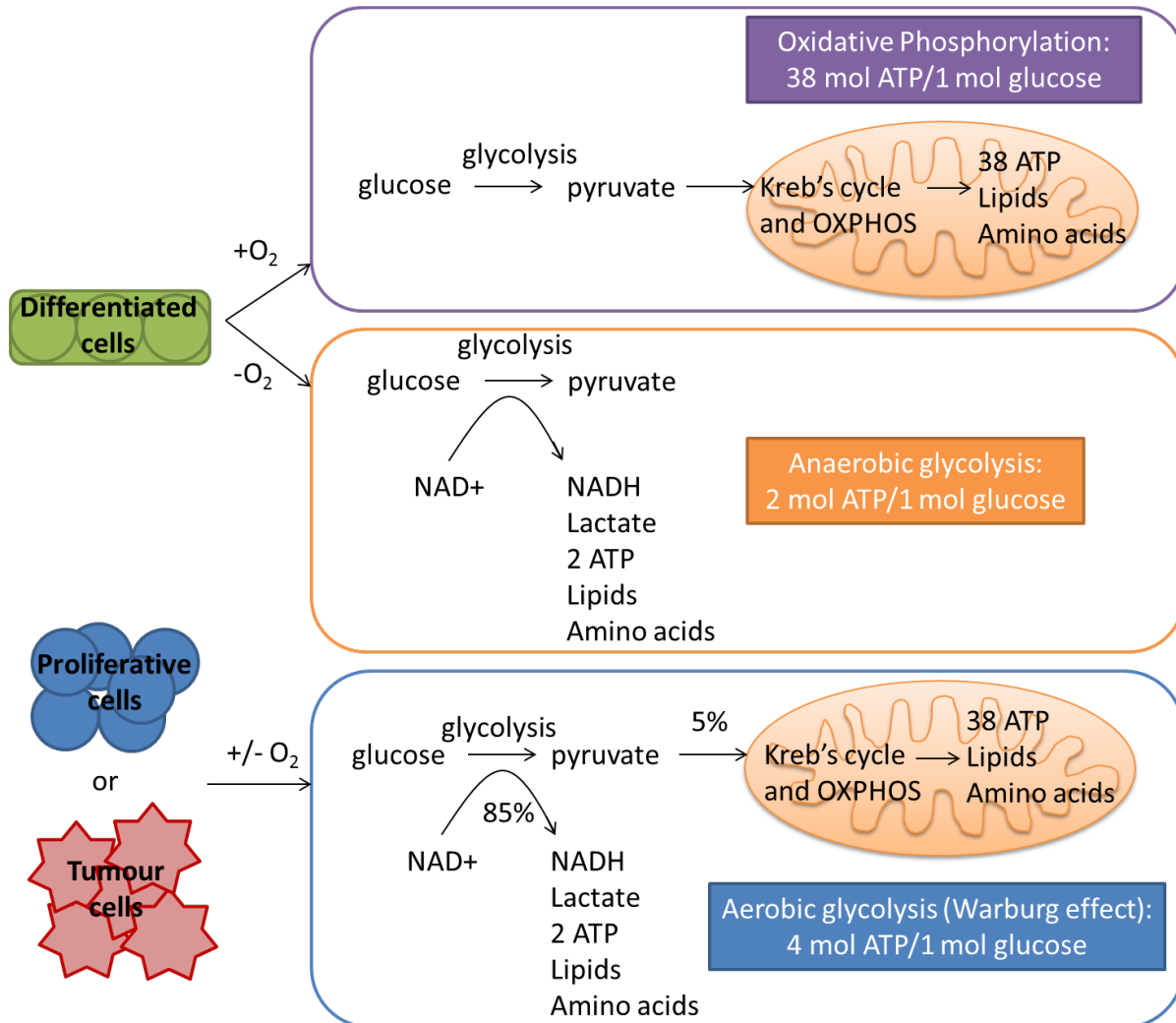


Figure 1.1 Difference in metabolism between normal and cancer cells  
Schematic representation of oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis and the cells that utilise them. There are many similarities between the methods of metabolism. Image adapted from Vander Heiden *et al* (Vander Heiden et al. 2009).

## 1.3 C-terminal binding proteins

### 1.3.1 CtBP1 and CtBP2 structure and function

C-terminal binding proteins (CtBPs) are oncogenic binding proteins discovered through their interaction with the C-terminus of the adenovirus E1A protein (Boyd et al. 1993). Vertebrates contain two loci; CtBP1 and CtBP2 (CtBP1 is mapped to the human chromosome 4p16 while CtBP2

is mapped at 21q21.3 (Katsanis & Fisher 1998) which have a variety of functions during development, cell cycle regulation and transformation (Chinnadurai 2002). The two variants of CtBPs share 78% amino acid identity (Katsanis & Fisher 1998) but have distinct roles *in vivo*; while CtBP1-deficient mice are able to survive but are very small and die young, CtBP2-deficient mice die *in utero* (Hildebrand & Soriano 2002). Hildebrand and Soriano suggest that the phenotypes they observed in their CtBP deficient mice highlight the large number of transcription factors affected by the absence of CtBP. The authors found that loss of CtBP caused embryo-wide deregulation of gene expression and suggest that this is due to the loss of transcription factor function; both repressor and activator function (Hildebrand & Soriano 2002).

Activation of CtBPs is not fully understood however, CtBPs have been shown to bind NADH so it's thought that CtBP activation is modulated by cellular energy homeostasis (Chinnadurai 2002). Therefore, a change in tissue homeostasis, such as an increase in NADH due to the metabolic switch that occurs during aerobic glycolysis, could lead to CtBP activation. In this way CtBPs have been described as redox sensors for transcription and an increasing amount of evidence supports this. CtBPs contain a sequence matching that of NAD<sup>+</sup>/NADH in their central domain (Figure 1.2) (Zhang et al. 2002) and have >100-fold greater affinity for NADH than NAD (Fjeld et al. 2003). Binding to NADH induces dimerisation of CtBP monomers at their dimerisation domain (Figure 1.2); both homo- and hetero-dimers which provides a scaffold for further transcriptional repressor proteins to bind to such as histone deacetylases (HDAC), histone methyltransferases (HMT) and histone acetyltransferases (HAT) (Nardini et al. 2009; Kumar et al. 2002; Kim et al. 2005).

CtBP protein structure can be separated into four key domains; the substrate binding domain; which contains a PXDLS motif interacting pocket which mediates interaction with a variety of transcription factors (Chinnadurai 2002), the central domain; containing the NAD<sup>+</sup>/NADH binding site (Zhang et al. 2002), the C-terminal domain which is intrinsically unstructured and is targeted by a variety of post-translational modifications (Nardini et al. 2006) and the N-terminal domain which contains the greatest variability. Most important here is the availability of the nuclear localisation sequence (NLS) available in CtBP2-L which is absent from all other CtBP forms (Verger et al. 2006). Additionally, in retinal cells, use of an alternative, tissue-specific promoter in the first intron of the CtBP2 gene leads to the production of mRNA encoding the synaptic ribbon protein RIBEYE, which consists of a large, unique N-terminal domain fused to all but the N-terminal 20 amino acids of CtBP2 (Figure 1.2) (Bergman & Blaydes 2006). CtBP1-S is the predominantly expressed isoform of the two CtBP1 isoforms and is usually the only isoform able to be distinguished via Western blotting techniques as CtBP1-L is so minimally expressed and the two differ by just 11 amino acids in size. On the other hand, CtBP2-S and CtBP2-L often appear as two

distinct bands as they are expressed at relatively similar levels and they differ by a larger 25 amino acids (Birts et al. 2010)

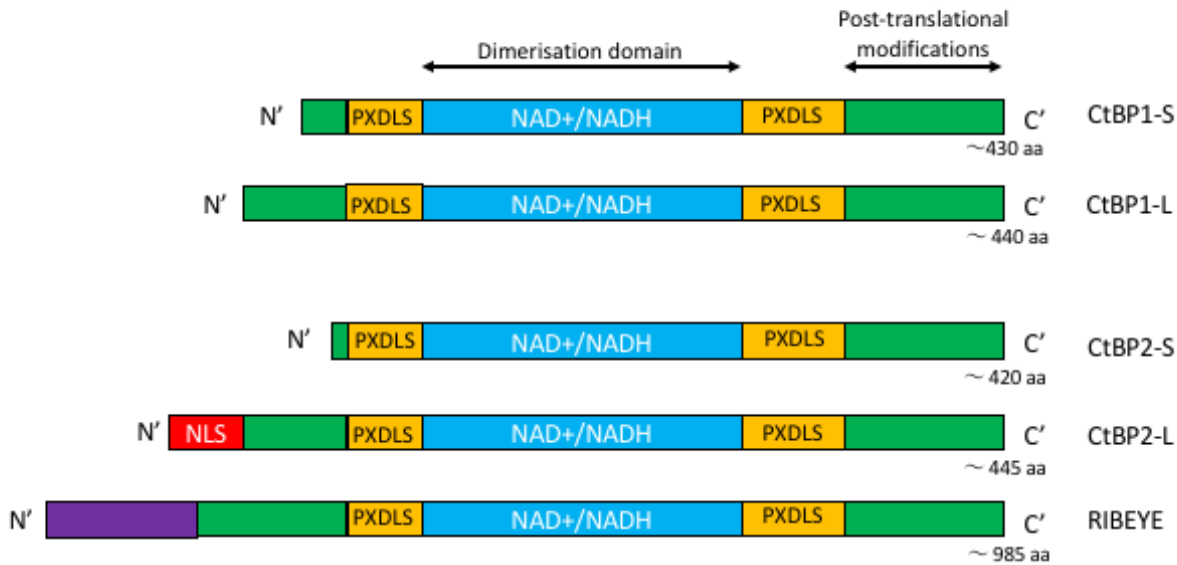


Figure 1.2 Schematic representation of different CtBP1 and CtBP2 isoforms

CtBP1 gene encodes two isoforms; short (CtBP1-S) and long (CtBP1-L) while alternative mRNA splicing produces two CtBP2 isoforms; short (CtBP2-S) and long (CtBP2-L). All CtBP forms have highly conserved domains including PXDLS motifs, an NAD<sup>+</sup>/NADH binding central domain, a C-terminal domain with sites targeted by post-translational modifications and an N-terminal domain. The N-terminal domain differs most between the CtBP isoforms, most important here is the availability of an NLS sequence in the N-terminal of CtBP2-L. Use of an alternative promoter, specific in retinal tissue, produces the RIBEYE isoform of CtBP2 which contains a much larger N-terminal. Image composed from information from a number of papers (Birts et al. 2010; Bergman & Blaydes 2006; Verger et al. 2006; Nardini et al. 2006).

CtBP function is not only regulated by NADH fluctuations directly but can also be attenuated by phosphorylation in response to activation of other kinases including AMPK and Pak1. CtBP1 is phosphorylated by AMP-activated protein kinase (AMPK) under metabolic stress, inducing CtBP ubiquitination (Kim et al. 2013). AMPK-mediated phosphorylation of CtBP1 at serine 158 site (Ser158) attenuates the repressive function of CtBP1 on a number of apoptotic genes including *Noxa* and *Bax*. These results indicate a role for CtBP1 regulation in mediating apoptosis to cause cell death upon glucose deprivation in AMPK-activating conditions (Kim et al. 2013). Additionally, p21-activated kinase 1 (Pak1) also phosphorylates CtBP selectively on Ser158 triggering CtBP cellular redistribution and blocking CtBP co-repressor functions (Barnes et al. 2003). Barnes *et al* found that a Ser158A substitution in CtBP or Pak1 knockdown by short interference RNA blocked CtBP phosphorylation, redistribution and attenuation of CtBP co-repressor functions in reporter and chromatin assays. Pak1 is activated by a number of factors; growth-factor receptor tyrosine kinases via Rac1 or Cdc42, by non-receptor tyrosine kinases and by lipids. Additionally, the authors found that in the presence of NADH, Pak1 super-phosphorylates CtBP and inhibits CtBP

dehydrogenase activity, suggesting that preferential phosphorylation of active CtBP may alter secondary structures and influence both enzymatic and co-repressor functions (Barnes et al. 2003).

The two best-characterised functions of CtBPs are as transcriptional co-repressors and metabolic sensors. The mechanism by which CtBPs act as transcriptional co-repressors is still not fully understood but it is thought to occur in a HDAC-dependent manner as CtBPs have been shown to bind to histone deacetylase (HDAC1) in co-localisation experiments, thus transcriptional repression via de-acetylation is plausible (Sundqvist et al. 1998; Kim et al. 2005). CtBP can serve as a transcriptional co-repressor by recruiting histone deacetylases (Kim et al. 2005). Histone acetyltransferase co-activators bind to acetylated histones through their bromodomains and catalyse the acetylation of histone tails (H3 and H4) for transcriptional activation. Kim *et al* found that CtBP inhibits the histone acetyltransferase p300 in an NADH-sensitive manner consequently blocking transcriptional activation. The dissociation of NADH from CtBP1 allows CtBP1 to inhibit p300 and leads to the repression of p300-driven transcriptional activity. Kim *et al* thus suggested CtBP1 serves as an energy-sensing repressor of histone acetyltransferase(s) to affect general transcription (Kim et al. 2005).

Additionally, Sundqvist *et al* studied a Gal4-E1A fusion protein, a potent transcriptional activator when expressed, and discovered that upon CtBP binding to the E1A subunit, E1A dependent transcription activation was abolished. Sundqvist *et al* expanded their research and discovered that CtBP and E1A were able to compete to regulate transcription of the proliferating cell nuclear antigen (PCNA). PCNA transcription was repressed due to a CtBP-HDAC1 complex bound to its promoter. An exon of E1A containing just a CtBP binding site was sufficient to alleviate repression by disrupting the promoter bound CtBP-HDAC1 complex from the promoter (Sundqvist et al. 1998).

Shi *et al* performed glycerol-gradient sedimentation and gel-filtration experiments to identify proteins found bound to CtBPs (Shi et al. 2003). Shi *et al* identified both histone deacetylases (HDAC) and methyltransferases (HMT) and suggested that the CtBP complex coordinates histone modifications to result in a repressive chromatin environment. Furthermore, following histone and CtBP complex incubation, Shi *et al* showed that the CtBP complex could efficiently and specifically methylate histone H3 and that the CtBP complex has HMT activity that can methylate both free and mono-nucleosomal histones *in vitro* (Shi et al. 2003).

The role of CtBPs as transcriptional activators has also been discussed in the literature (Phippen et al. 2000; Paliwal et al. 2012). Phippen *et al* found that *Drosophila* CtBP (dCtBP) can both activate and repress transcription by utilising distinct dCtBP domains for each function (Phippen et al.

2000). The authors found the role of CtBP as either activator or repressor to be cell-type specific and found that the regions of dCtBP required for each transcriptional activity did not overlap. dCtBP activates transcription in B78 mouse melanoma cells and represses transcription in CV-1 green monkey kidney cells. Efficient activation in 293 human embryonic kidney cells maps to amino acids 255-325 on dCtBP while the region required for efficient repression in NIH-3T3 mouse embryonic fibroblast cells maps to amino acids 190-273, suggesting these cell type-dependent differences to be due to differences in protein interactions (Phippen et al. 2000). Furthermore, interaction with other proteins was able to alter the function of dCtBP significantly. Co-expression of the oncogene E1A turned CtBP from a strong repressor into a weak-activator in NIH-3T3 cells and enhanced dCtBP's activator function in 293 cells. Based on their results Phippen *et al* suggest that dCtBP could be part of different multi-protein complexes that carry out distinct functions (Phippen et al. 2000). Additionally, Paliwal *et al* found that CtBP2 is able to promote human cancer cell migration via transcriptional activation of T-cell lymphoma and metastasis 1 (Tiam1) protein (Paliwal et al. 2012). CtBP2 knockdown via RNAi led to down-regulation of Tiam1 in response, and vice versa, over-expression of CtBP2 led to increased expression of Tiam1. Paliwal *et al* found that activation occurred in conjunction with the CtBP-interacting Kruppel-like factor 8 (KLF8) which binds to the promoter of Tiam1, providing a scaffold for CtBP2 interaction and consequent activation of Tiam1 (Paliwal et al. 2012).

Bhambhani *et al* found that the role of CtBPs as a transcriptional co-activator or co-repressor alters depending on its oligomeric state. CtBP can both repress and activate Wingless (Wg) nuclear targets in *Drosophila*. Bhambhani *et al* suggest that CtBP is a gene-specific regulator of Wg signalling where some targets require CtBP dimers for inhibition and other targets require CtBP monomers to activate their expression (Bhambhani et al. 2011). Bhambhani *et al* found that mutant forms of CtBP which were unable to dimerise were still able to activate Wg targets but were no longer capable of repression while co-expression of different monomeric forms of CtBP capable of hetero-dimerisation were able to restore repression activity (Bhambhani et al. 2011).

### 1.3.2 CtBPs, cell motility and tumour progression

CtBPs are linked to tumour progression due to their roles in epithelial to mesenchymal transition (EMT), as apoptotic antagonists, through their repression of several tumour suppressor genes, and also their interaction with adhesion molecules, including E-cadherin, and other downstream targets important in actin organisation (Chinnadurai 2009). It has been found that in hypoxic conditions, or an increase in NADH (seen with glycolysis), CtBPs are activated and bind to the E-cadherin promoter, causing a decrease in E-cadherin that results in loss of cell-cell contact and increased tumour cell migration (Zhang et al. 2006). Additionally, Pak1 phosphorylation of down-

stream substrates, which includes CtBP1, has been linked to a wide range of biological activities including; cell cytoskeletal reorganisation, leading to increased motility and enhanced cell survival (Barnes et al. 2003). CtBP1 may even bind actin directly to affect actin re-organisation during cell migration, as the brain specific actin-related protein; ArpN $\alpha$  has been shown to interact with CtBP1 (Oma et al. 2003). Furthermore, as discussed previously, CtBP2 has been shown to promote human cancer cell migration through transcriptional activation of Tiam1 (Paliwal et al. 2012). Tiam1 is a guanine nucleotide exchange factor (GEF) for Rac GTPase that plays a critical role in regulating cell adhesion, invasion, and migration and has been directly implicated in the promotion of cancer progression and metastasis (Paliwal et al. 2012). Furthermore, both CtBPs have been linked to the Alternative Reading Frame Tumour Suppressor (p19<sup>Arf</sup>) in the modulation of CtBP-dependant tumour cell motility (Chen et al. 2008; Paliwal et al. 2007). Some research has shown the potential for CtBPs as therapeutic targets in human cancer. Straza *et al* found that treatment with a CtBP inhibitor, CtBP dehydrogenase substrate 4-methylthio-2-oxobutyric acid (MTOB), was able to decrease tumour burden and induce tumour cell apoptosis in human colon cancer cell peritoneal xenographs (Straza et al. 2010). CtBPs have also been shown to regulate resistance to chemotherapy through regulation of p53 in breast cancer cells (Birts et al. 2010).

## 1.4 Integrins and tumour cell motility

Integrins are the main extracellular matrix (ECM) receptors expressed by cells, and most forms of cell movement through ECM is integrin-dependent. Integrins are heterodimers composed of an  $\alpha$  and  $\beta$  chain of which 24 different combinations have been identified (Barczyk et al. 2010) allowing interaction with different ECM proteins throughout the body and providing tissue-specificity through their selectivity (Figure 1.3). For example,  $\alpha\beta6$  integrin is able to bind to the TGF- $\beta$  latency associated protein (LAP) and fibronectin (FN) but not to collagen I (COLI) (Margadant et al. 2011).

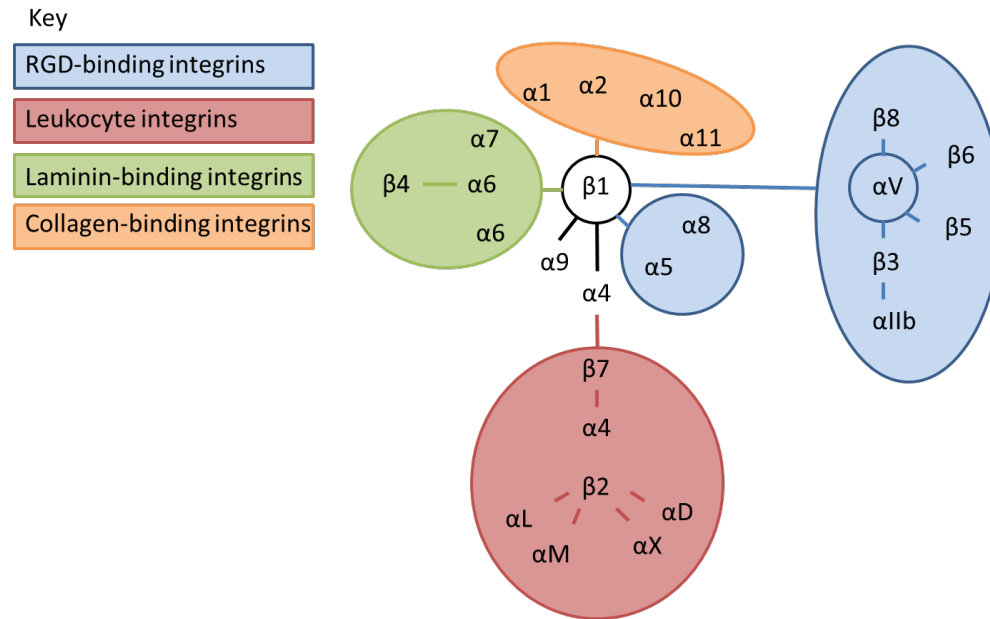


Figure 1.3 Integrin binding and their ligands

Different combinations of integrins bind to alternate ECM ligands, including laminin, collagen, leukocyte specific and RGD-motif containing ligands. RGD ligands include fibronectin and LAP among others. Adapted from Margadant *et al* (Margadant et al. 2011).

Integrins function as both cell anchoring and signalling molecules (Hynes 2002). Integrin activation occurs via two processes known as ‘inside-out’ and ‘outside-in’ signalling (Anthis & Campbell 2011). ‘Inside-out’ activation is when an intracellular signal causes increased affinity of the integrin ectodomain for its ECM ligand. This then allows among others, the adaptor protein Talin-1 to bind to the β-subunit of the cytoplasmic tail of the integrin, causing tail separation from the α-subunit and a distinct conformational change to the integrin ectodomain (Figure 1.4). ‘Outside-in’ activation on the other hand is brought about when binding to an ECM ligand leads to integrin conformational changes and integrin clustering, resulting in intracellular changes allowing Talin-1 to connect to the cell’s actin cytoskeleton allowing cell spreading, integrin clustering, and activation of signal transduction cascades (Figure 1.5) (Margadant et al. 2011). Talin-1 is therefore an important adaptor protein involved in ECM-cell signalling whose binding to the integrin β-chain and actin cytoskeleton induces a high-affinity state for subsequent ligand interactions (Kalli et al. 2011) resulting in signalling cascades to allow important cellular changes needed for growth as well as cell movement (Anthis & Campbell 2011).



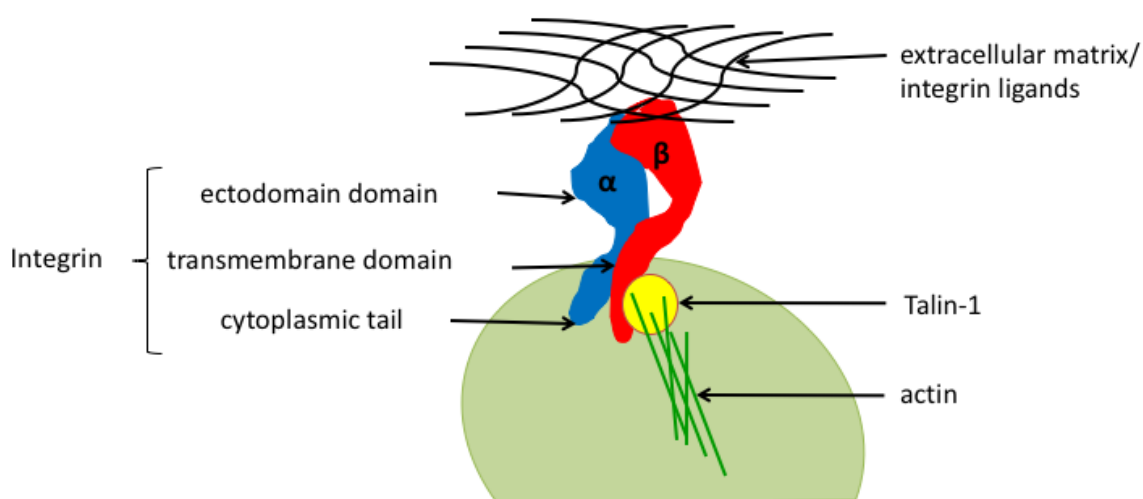


Figure 1.4 Integrin structure

Integrins are composed of an  $\alpha$  and  $\beta$  chain which compose the extracellular domain needed for ligand binding, a transmembrane domain and a cytoplasmic tail. Adaptor proteins, such as Talin-1, are then responsible for linking integrins to the cell cytoskeleton via the  $\beta$ -chain.

Cell migration is central to many normal and pathological processes including embryonic development, wound healing and tumour invasion and metastasis (Cox & Huttenlocher 1998). Attachment to the ECM and integrin activation is the crucial first step in cell movement and cell surface adhesion receptors play a critical role in mediating adhesive interactions between cells and the surrounding ECM (Cox & Huttenlocher 1998). Cell movement requires a continuous cycle of adhesion of protrusions known as lamellipodia at the 'leading edge' of the cell (Ridley 2011) and detachment of the 'trailing edge'. Lamellipodia are created by actin polymerisation pushing the plasma membrane of the cell forward. Activated integrins at the edge of the protrusion then contact the ECM causing 'outside-in' signalling to occur, consequent Talin-1 activation and a contraction of the actin cytoskeleton to promote a forward shift of the cell (Cox & Huttenlocher 1998).

Much of our understanding of cell migration comes from the investigation of cells within 2D assays. While these investigations can allow important understanding of cell-ECM and cell-cell interactions, it is important to remember that these interactions are invariably more complex within 3D microenvironments and that the physical microenvironment plays an equally important role in controlling cell migration (Doyle & Yamada 2016). The most important difference between 2D and 3D environments is dimensionality, with the simplest version of 3D assays consisting of two or more ECM surfaces in contact with a cell. In 3D microenvironments there are many added complications of ECM-dependent factors, such as ECM ligand density, fibril alignment, ECM pore size, and intra- and extra-fibril crosslinking that can influence matrix stiffness (Doyle & Yamada 2016). Matrix stiffness has been found to be important in an investigation by Doyle *et al* who found that, in 3D collagen gels of varying matrix microarchitectures, 3D adhesion dynamics are

locally regulated by ECM rigidity together with integrin-ECM association and myosin II contractility (Doyle et al. 2015). The authors discovered that unlike 2D migration, abrogating contractility stalls 3D migration regardless of ECM pore size and proposed that efficient 3D migration requires local balancing of contractility with ECM stiffness to stabilise adhesions, which facilitates the detachment of activated integrins from ECM fibrils (Doyle et al. 2015). The discussion of cell migration throughout this chapter is often discussed in the context of the 2D microenvironment to provide a simple explanation of cell migration but it's important to remember that *in vivo* these interactions would be much more complex and that the advancement of assays investigating 3D microenvironments will be pivotal in fully understanding cell motility.

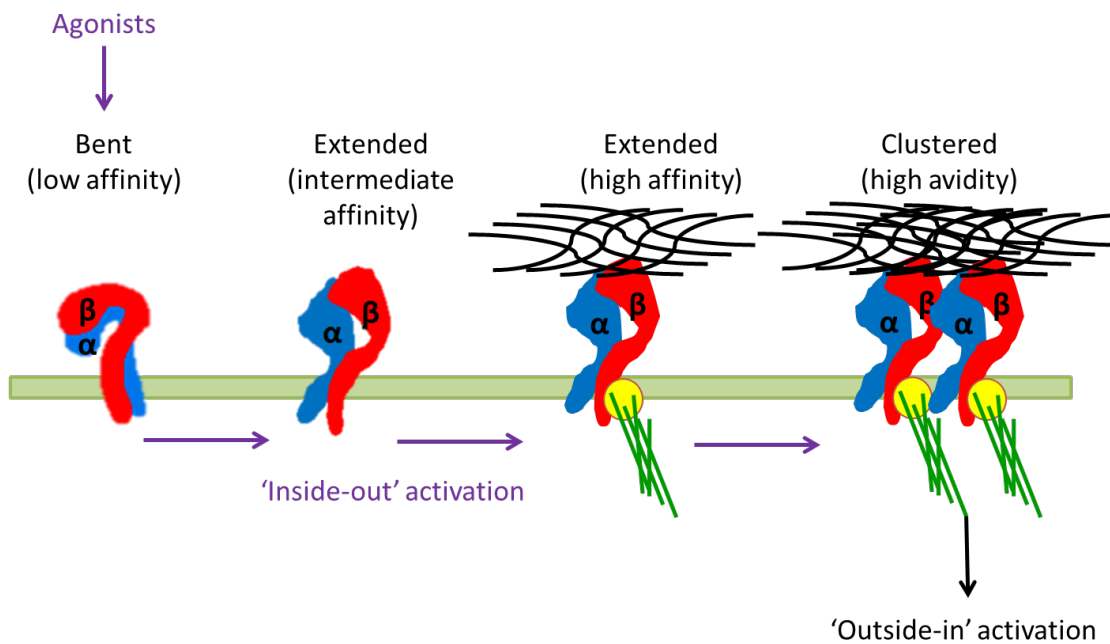


Figure 1.5 Summary of integrin and ECM interaction and activation

Cytoplasmic signalling allows integrins to change conformation ('inside-out' signalling) to allow binding to the ECM which clusters the bound integrins to increase the signalling capacity and trigger cell changes ('outside-in' signalling). Adapted from Margadant *et al* (Margadant et al. 2011).

Cell locomotion can become limited as a result of slow lamellipodia development, ineffective adhesion or ineffective detachment, therefore cell migration is a highly coordinated process between optimal adhesion and migration. Too strong or too weak an adhesion and cells cannot migrate effectively (Figure 1.6) (Cox & Huttenlocher 1998). Endocytosis and recycling of integrins plays an integral role in maintaining optimal migration (Caswell & Norman 2006) and adhesion and further evidence is emerging about the importance of the processes involved in this recycling. Additionally, actin organisation as a consequence of integrin downstream signalling is also an important step in cell motility and actin assembly plays an important role in integrin internalisation (Mooren et al. 2012). The role of integrin endocytosis and recycling and

consequent downstream signalling in cell motility will be discussed in detail in the subsequent sections.

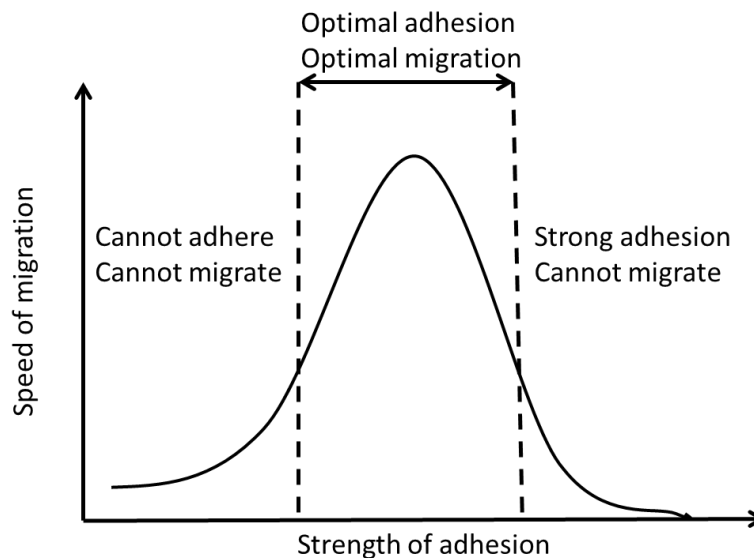


Figure 1.6 Biphasic relationship between adhesion and migration speed  
Cells require intermediate levels of substrate attachment to migrate optimally. At low adhesion levels, cells cannot form efficient attachments and at high adhesion levels cells cannot release attachments. Image adapted from paper by Cox and Huttenlocher (Cox & Huttenlocher 1998).

#### 1.4.1 Actin as a downstream 'target' of integrins

Actin organisation plays a crucial role in the maintenance of optimal cell motility following integrin 'outside-in' signalling from the ECM. In order to explore their environment cells must first project F-actin rich protrusions such as filopodia (finger-like projections) and lamellipodia (sheet-like projections) (Wehrle-Haller 2012). Initial filopodia contain  $\beta 1$  integrin subunits in order to initiate integrin-dependent adhesions (Galbraith et al. 2007). Integrin-ECM binding then allows the activation of many downstream signalling processes to occur which are vital for cell motility (Takenawa & Suetsugu 2007). Integrin binding to their ECM ligand induces integrin activation and the recruitment of intracellular adapter proteins, such as Talin-1, leading to integrin clustering. These clusters then stimulate a positive feedback loop, inducing further cell spreading and the recruitment of signalling proteins (Wehrle-Haller 2012) such as, Whiskott-Aldrich syndrome protein (WASP), Arp2/3, GTPases and their regulators GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs).

WASP and WASP-family verprolin-homologous protein (WAVE) family proteins are scaffold proteins linking upstream signals, such as those from GTP-Cdc42, to the activation of the Arp2/3 complex leading to a burst of actin polymerisation (Takenawa & Suetsugu 2007). Mammals have both WASP and WAVE proteins, while yeast contain just WASP. Two forms of WASP exist; WASP (expression restricted to haematopoietic cells) and N-WASP (so called due to its abundance in

neural tissue, but is found in other tissue types) (Takenawa & Suetsugu 2007). Both possess an important VCA domain required to activate the Arp2/3 complex. N-WASP activates Arp2/3 by binding to it at its VCA region along with an actin monomer. Actin polymerisation is initiated by the assembly of three actin monomers, the Arp2/3 complex has two-actin related molecules, so binding of a third initiated by N-WASP binding causes a burst of actin polymerisation (Takenawa & Suetsugu 2007). Actin polymerisation induced by WASP and WAVE proteins occurs during several biological functions such as the formation of filopodia and lamellipodia in cell migration, membrane trafficking and cell adhesion, among others. Rapid actin polymerisation at the leading edge is required for cells to migrate and these processes are highly governed by the Rho family of GTPases; specifically, filopodia formation is mediated by the Rho GTPase Cdc42, which is activated by the GEF Intersectin. Lamellipodia formation is mediated by Rac and retraction of the rear of the cell is mediated by RhoA (Takenawa & Suetsugu 2007). Actin reorganisation requires both the activation and inactivation of Rac1 and Cdc42 in the regulation of filopodia and lamellipodia production.

Cell motility is also regulated by interactions between integrins and growth factor receptor signalling such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF). Crosstalk between EGFR and integrin activation can affect invasion and proliferation of a gastric cancer cell line; SGC7901 (Dan et al. 2012). Inhibition of focal adhesion kinase (FAK) expression, which is increased following both EGF stimulation and fibronectin activation of integrins, reduced SGC7901 invasion suggesting crosstalk between the two signalling proteins (Dan et al. 2012). Additionally,  $\beta 1$  integrins are required both for fibroblast chemotaxis towards PDGF and growth factor-induced dorsal ruffling (King et al. 2011). King *et al* showed that  $\beta 1$  integrin stabilises and spatially regulates N-WASP to facilitate PDGF receptor traffic and directed motility. They showed that in intact cells, PDGF binding leads to rapid activation of  $\beta 1$  integrin within newly assembled actin-rich membrane ruffles. Active  $\beta 1$  in turn controls assembly of N-WASP complexes with both Cdc42 and WASP-interacting protein (WIP), the latter of which acts to stabilise the N-WASP and both of these protein complexes are required for PDGF internalisation and fibroblast chemotaxis downstream of  $\beta 1$  integrins. Their paper shows a mechanism by which integrins co-operate with growth factor receptors to promote localised signalling and directed cell motility (King et al. 2011).

## 1.5 Endocytosis pathways

The adhesion and detachment cycle necessary for cell motility requires integrin internalisation from the plasma membrane and subsequent redistribution to different cellular locations via endocytosis (A. Ramsay et al. 2007), of which there are several types. Two main endocytic routes

can be used independently or collectively; clathrin-independent endocytosis (CIE) and clathrin-mediated endocytosis (CME) (Polo & Di Fiore 2006).

Clathrin-independent endocytosis includes among others dynamin-dependent caveolar endocytosis and dynamin-independent macropinocytosis. Dynamin-dependent endocytosis utilises cholesterol-rich membrane domains, including lipid-rafts and caveolae (Nichols 2003). Lipid-rafts provide a platform for proteins that need to be transported to the same cellular destination to interact ready for internalisation, while caveolae are small (50-80 nm) (Pelkmans et al. 2001), uncoated-invaginations in the plasma membrane containing caveolin-1 protein (Nichols 2003). Lipid rafts and caveolae share certain characteristics and caveolae are currently considered as a subset of lipid rafts (Insel & Patel 2009). Raft/Caveolin-dependent endocytosis is partly responsible for the uptake of certain membrane components (e.g. glycosphingolipids (Sharma et al. 2004)); extracellular ligands (e.g. albumin (Li et al. 2013)), certain viruses (e.g. SV40 (Pelkmans et al. 2001)) and bacterial toxins (e.g. cholera toxin (Sandvig & van Deurs 2002)). The role of caveolae in the endocytosis of certain cell surface receptors, such as EGFR (Sigismund et al. 2005) and integrins (Ning et al. 2007) have also been suggested. Conversely, dynamin-independent macropinocytosis provides an efficient route for non-selective endocytosis of solute macromolecules (Swanson & Watts 1995) through large, heterogeneous vesicles called macropinosomes (Commisso et al. 2013) formed primarily at sites of ruffling at the margins of spread cells.

While the role of clathrin-independent pathways in receptor endocytosis is still under debate, the crucial role of clathrin-mediated endocytosis (CME) in receptor endocytosis is widely accepted. Cytoplasmic clathrin is recruited to the plasma membrane forming clathrin-coated pits which bud-off to form clathrin-coated vesicles. CME requires phosphorylation and ubiquitinylation of endocytic adaptor proteins to coordinate specific surface receptors into clathrin-vesicles (van Bergen En Henegouwen 2009). A number of different adaptor proteins have been shown to be involved in CME such as AP-2, Epsin and its partner protein EGFR pathway substrate 15 (Eps15) (McMahon & Boucrot 2011). It has been shown that AP-2 binds the COOH terminus of Eps15 (Benmerah et al. 1998) and this interaction has been suggested to be critical for CME (Benmerah et al. 1998). However, certain receptors can be differentially dependent on particular adaptor proteins. Endocytosis of EGFR for example has been shown to be more dependent on Epsin and Eps15 compared to other receptors such as the transferrin receptor (A. Ramsay et al. 2007). Eps15 contains a specific Eps15 homology domain (EH), which interacts with Epsin, Intersectin, and Synaptojanin, all of which are vital in clathrin-pit formation. Once formed, clathrin-vesicles shed and fuse with early-endosomes for receptor recycling back to the cell surface or degradation in a late-endosome/lysosome (A. Ramsay et al. 2007). Two receptor recycling routes are available

(Figure 1.7); Rab4-mediated 'short-loop' via early endosomes and Rab11-regulated 'long-loop' via the perinuclear recycling compartment (PNRC) (Caswell & Norman 2006).

Cell signalling is also affected by endocytosis and the protein Eps15 has been implicated in the regulation of intercellular communication between eukaryotic cells (Girão et al. 2009).

Involvement of Eps15 in targeting ubiquitin-conjugated cargo to clathrin-independent endocytosis has recently been demonstrated alongside clathrin-dependent pathways (Girão et al. 2009). Girão *et al* focussed on the protein Cx43; an integral membrane protein of the connexin family and suggested ubiquitination to serve as an internalisation signal for the protein. Connexins have an extremely short half-life in comparison to other plasma membrane proteins and studies have shown that docked connexins cannot be separated under physiological conditions thus entire gap junction (GJ) plaques are internalised by one of two adjacent cells as large double-membrane vesicular structures called 'annular GJ'. CME plays a critical role in internalisation, translocation and degradation of these 'annular GJ'. Girão *et al* used techniques including co-immunoprecipitation and immunofluorescence to show an interaction between Cx43 and Eps15, co-localisation occurring mostly at the plasma membrane. They found that depletion of Eps15 resulted in accumulation of Cx43 at the plasma membrane and use of an Eps15 mutant revealed that interaction of Eps15 with Cx43 requires the ubiquitin-interacting motif of Eps15 (UIM), allowing targeting of ubiquitinated Cx43 to the endocytic pathway (Girão et al. 2009).

Crosstalk between clathrin and caveolin-mediated pathways has also been suggested. In the case of EGFR it has been shown that stimulation with low doses of EGF results in exclusive clathrin-mediated endocytosis, while at high ligand concentration clathrin-independent mechanisms, possibly via caveolae, become more apparent (Sigismund et al. 2005). This suggestion has, however, been debated by others who found that EGF stimulation even at high doses does not trigger caveolae-mediated endocytosis (Kazazic et al. 2006). It has also been suggested that while EGFR internalises solely through clathrin-mediated pathways the inactive form of the receptor (in the absence of ligand or mutant, constitutively inactive EGFR) accumulates in caveolae and upon ligand stimulation it exits this membrane compartment, migrates in the plasma membrane and finally internalises via clathrin-coated pits (Mineo et al. 1999).

### 1.5.1 Actin in endocytosis

The actin cytoskeleton plays an essential role in several internalisation processes. It can create protrusions that encompass extracellular materials then support the processes of invagination of a membrane segment into the cytoplasm and elongation of the invagination. Scission of a new vesicle from the plasma membrane and movement of a vesicle away from the membrane are also reliant on actin organisation (Mooren et al. 2012). While actin is necessary for endocytosis in

yeast, its requirement in mammalian cells is more controversial as discussed in the review by Mooren *et al* (Mooren et al. 2012). Actin is thought to be required in yeast due to the relatively high turgor pressure of these cells relative to animal cells (Mooren et al. 2012). It is thought that because actin provides an increased level of force that actin would only be required in mammalian cell endocytosis of large cargo or endocytosis within cell areas that have dense actin filaments (Mooren et al. 2012). The authors discuss the high likelihood that actin is constitutively recruited to clathrin coated structures (CCSs) but that the amount of actin polymerisation needed in a given case is determined by the amount of force required to counteract membrane tension to allow endocytosis (Mooren et al. 2012). Actin networks at sites of endocytosis must be tightly regulated for efficient internalisation, which, as discussed, is imperative for effective cell signalling. The majority of research in the regulation of actin at sites of CME have been in budding and fission yeast and the phases of the endocytic process have been described as follows. Simply; during CME, clathrin and adapters are recruited at the very early stages and provide initial membrane curvature. Regulators of actin nucleation, such as WASP and Arp2/3, then follow, allowing actin polymerisation to proceed. Membrane curvature then increases and the invagination elongates. Lastly, amphiphysins and dynamin are recruited and membrane fission occurs (Mooren et al. 2012). The addition of membrane curvature proteins, such as those of the Bin-amphiphysin-Rvs (BAR) superfamily and BAR-domain proteins (BDPs), are important for both the initiation of membrane curvature as well as continued membrane curvature in the later stages of endocytosis (Anitei & Hoflack 2011). Some have even been shown to be important in activating actin polymerisation, thus spatial and temporal understanding of the proteins involved in actin assembly and endocytosis is complex. BAR proteins are also responsible for the opposite production of filopodia from the cell membrane. I-BAR proteins, bind to membranes and promote negative curvature, and can interact with several actin regulators such as; WASP (Mooren et al. 2012).

One question that is still not completely answered is; how can actin filaments exert the force required to push or pull endocytic vesicles away from the plasma membrane? Evidence suggests the possibility of several models (Anitei & Hoflack 2011; Mooren et al. 2012). One model suggests that force productions by myosins are required. Type I myosins localise to sites of CME in yeast and mammals and are functionally important for endocytosis. Evidence involving myosins have shown the pointed ends of actin filaments to be directed away from the membrane suggesting that myosins may walk along actin filaments and pull the CME vesicle away from the membrane. Another model requires membrane-bound nucleation promoting factors (NPFs). These NPFs nucleate actin filaments with barbed ends towards the membrane. NPFs remain on the

invaginating membrane while actin filament assembly pushes on the membrane at the initial curvature site to promote invagination, elongation and fission (Mooren et al. 2012).

### 1.5.2 $\beta 1$ and $\beta 6$ integrin endocytosis in cell migration and invasion

Accumulating evidence highlights the importance of good receptor endocytosis in appropriate cell motility. Integrin-mediated cell spreading and motility on immobilised substrates requires integrin engagement with the ECM at the cell's leading lamellae.  $\beta 1$  integrin recycling is mostly linked to the long-loop pathway (Caswell & Norman 2006) (Figure 1.7) and involves the EHD1 protein, containing a C-terminal Eps15 homology domain (EH) known to regulate clathrin-mediated internalisation and recycling. Cell spreading and migration on fibronectin is impaired in EHD1 knockout or depleted cells, suggesting a requirement for this molecule in integrin-recycling (Naslavsky & Caplan 2005). Dysregulation of integrins and their downstream effectors are well documented in many cancer types due to their roles in cell locomotion. Over-expression of the downstream effector Talin-1 (Lai et al. 2011) as well as aberrant activation of GTPases causes increased metastasis and poor patient prognosis.

Integrins contain two conserved sequence regions within their  $\beta$ -subunit (Cyto2 and Cyto3) which contain NXXY motifs similar to membrane receptor motifs that mediate clathrin-dependent endocytosis (Caswell et al. 2009). Furthermore,  $\alpha\beta 5$  integrin has been shown to co-localise with clathrin-coated pits via electron microscopy (De Deyne et al. 1998) and research has highlighted that CME has a dominant role at the leading edge of a motile cell (Rappoport 2003).

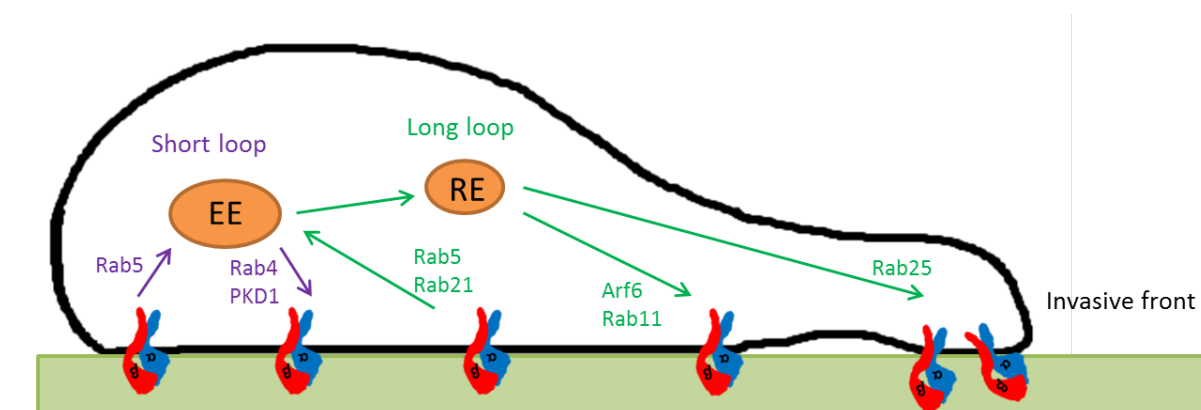


Figure 1.7  $\beta 1$  and  $\beta 6$  integrin endocytosis

Integrins containing a  $\beta 1$  subunit are often associated with the perinuclear recycling compartment long loop pathway in a Rab11 or Rab25 dependent manner, but can be organised into alternate pathways depending on their specific  $\alpha$  and  $\beta$  chain compositions. EE, early endosome; PKD1, protein kinase D1; RE, recycling endosome. Image adapted from a paper by Margadant *et al* (Margadant et al. 2011).

Integrins are highly involved in the progression of many cancer types due to their roles in cell locomotion as well as their downstream signalling targets and as such are potential therapeutic



targets for the development of new anti-cancer strategies. Integrins, including  $\beta 6$  and  $\beta 1$ , are associated with the progression of a range of cancers including colon, lung, oral, skin squamous cell carcinomas (SCC) and breast (Bandyopadhyay & Raghavan 2009; Lahlou & Muller 2011).  $\beta 1$  integrins constitute the largest subgroup of integrins as they can form heterodimers with the most different  $\alpha$ -subunits (Lahlou & Muller 2011). Due to their binding capabilities to a wide range of  $\alpha$ -subunits,  $\beta 1$  integrins are able to bind to a number of ECM proteins and are expressed in a wide variety of tissues throughout the body (Brakebusch & Fässler 2005). Due to their vast expression throughout the body,  $\beta 1$  integrins are involved in numerous signalling pathways and are critical for development, as seen through investigations with  $\beta 1$  knockout mice (Stephens *et al.* 1995; Fassler & Meyer 1995). Stephens *et al.* and Fassler *et al.* both generated mutant mice with a targeted disruption of the  $\beta 1$  integrin subunit gene and found that homozygous loss of  $\beta 1$  integrin expression was lethal during early post-implantation development, while heterozygous mutant mice were normal, suggesting the presence of  $\beta 1$  signalling to be crucial in early development (Stephens *et al.* 1995; Fassler & Meyer 1995). In relation to cancer progression, Yao *et al.* found a significant correlation between increased  $\beta 1$  expression and decreased overall survival and disease-free survival in invasive breast cancer (Yao *et al.* 2007). These results are supported by dos Santos *et al.* who discuss a negative correlation between increased  $\beta 1$  expression and decreased number of months survival following diagnosis with breast cancer (dos Santos *et al.* 2012).  $\beta 1$  expression is so important in breast cancer progression that its inhibition dramatically enhances the efficacy of radiotherapy in breast cancer models (Park *et al.* 2008). Park *et al.* used the  $\beta 1$  inhibitory antibody (AIB2) to inhibit  $\beta 1$  expression, consequently inhibiting the Akt signalling pathway too. Inhibition of  $\beta 1$  had considerable effect both *in vitro* and *in vivo*. *In vitro*, AIB2 increased apoptosis following ionizing radiation (IR) by down-regulating Akt in breast cancer colonies in 3D cultures and *in vivo* addition of AIB2 and IR enhanced tumour growth inhibition significantly compared with individual treatments in mouse xenographs (Park *et al.* 2008). Furthermore, the addition of AIB2 allowed a lower dose of IR to be added for the same effect on breast cancer growth inhibition in xenographs (Park *et al.* 2008).

$\beta 6$  integrin subunits only couple with the integrin subunit  $\alpha v$  forming a single heterodimer.  $\alpha v\beta 6$  is exclusively expressed in epithelial cells (Bandyopadhyay & Raghavan 2009) and not expressed in healthy adult epithelia, but is up-regulated during wound healing and in cancer (Breuss *et al.* 1995; Breuss *et al.* 1993) and increasing evidence suggests it may actually promote carcinoma progression (Bandyopadhyay & Raghavan 2009).  $\beta 6$  is important in cell migration; Huang *et al.* found  $\beta 6$  knockdown keratinocytes could not migrate as effectively on appropriate ECM as wild-type (WT) keratinocytes (Huang *et al.* 1998) but most importantly, integrin  $\alpha v\beta 6$  is found up-regulated in many carcinomas including breast, lung, oral and squamous cell carcinomas (SCC),

colon, stomach and others (Bandyopadhyay & Raghavan 2009) and the expression of  $\alpha\beta6$  is often associated with a poor prognosis (Thomas et al. 2006). It's been suggested that the role of  $\alpha\beta6$  in tumour progression could be down to the ability of the integrin to activate TGF- $\beta$  (Sheppard 2005) as well as metalloproteases (MMP) (Yang et al. 2008; Morgan et al. 2004; Impola et al. 2004).  $\alpha\beta6$  activates TGF- $\beta$  through initiating a conformational change in the latent TGF- $\beta$  complex (Sheppard 2005). TGF- $\beta$  is mostly secreted by cells in a latent form complexed with two polypeptides; latent TGF- $\beta$  binding protein (LTBP) and latency-associated peptide (LAP).  $\alpha\beta6$  is able to bind to an RGD motif present in LAP resulting in a conformational change and subsequent release of TGF- $\beta$  (Sheppard 2005). In regards to metalloproteases; Yang *et al* suggested that the high expression of  $\alpha\beta6$  found in colon cancer aids its progression through activation of MMP9. Yang *et al* found that the expression of  $\alpha\beta6$  in colon cancer cells correlated with increased MMP9 expression at the invading edge of the tumour (Yang et al. 2008). MMP9 can degrade collagen, thus Yang *et al* discuss that this could lead to disruption of the basement membrane and allow access to migratory cells. They found that patients who were  $\alpha\beta6$ -positive had significantly ( $p<0.01$ ) higher liver metastasis rates (17%, 21/122) than those who were  $\alpha\beta6$ -negative (3%, 7/236) (Yang et al. 2008). Morgan *et al* found that a specific 11 amino acid sequence of the  $\beta6$  tail activated MMP9 (Morgan et al. 2004) and Impola *et al* identified high levels of  $\alpha\beta6$  and MMP7, -9 and -12 as prognostic markers at the invasive fronts of squamous cell carcinomas (Impola et al. 2004).

The high expression of  $\alpha\beta6$  found at the invasive fronts of cancers and the important role of integrins in cell motility suggests the importance of appropriate integrin endocytosis in cancer progression. Ramsey *et al* investigated the relationship between increased clathrin-mediated  $\alpha\beta6$  integrin endocytosis and more aggressive tumours in oral carcinoma cells. The authors found that HS1-associated protein X-1 (HAX-1) regulated carcinoma cell migration via CME of integrin  $\alpha\beta6$ . Small interfering (si)RNA depletion of HAX-1 and competitive inhibition of the direct association between HAX-1 and the  $\beta6$  integrin subunit through Tat-linked blocking peptides (Tat-HAX-1) blocked  $\alpha\beta6$  internalisation and consequent  $\alpha\beta6$ -dependent carcinoma cell migration. Furthermore, a dominant-negative mutant of Eps15 (disrupting clathrin-pit formation) showed a 43% block of migration toward LAP (A. G. Ramsay et al. 2007). Thus, the authors concluded that integrin endocytosis is required for  $\alpha\beta6$ -dependent carcinoma cell motility and suggested this process to be an important mechanism in cancer progression.

## 1.6 Eps15

Epidermal growth factor receptor pathway substrate 15 (Eps15) was originally identified as a substrate for the kinase activity of the EGFR through application of an expression cloning method

which allowed direct isolation of cDNAs encoding substrates for tyrosine kinases (Fazioli et al. 1993). They found that EGFR is able to directly phosphorylate Eps15 *in vitro* and that over-expression of the gene *eps15* is sufficient to transform NIH-3T3 cells, suggesting that Eps15 is involved in the regulation of mitogenic signals (Fazioli et al. 1993). Eps15's alternative names/synonyms include AF-1P; ALL1 fused gene from chromosome 1 and MLLT5.

### 1.6.1 Regulation of Eps15 expression

Currently very little is known about the regulation of Eps15 expression but a limited number of papers have shown that Eps15 is a target of certain microRNA (miRs) such as miR-203 (Viticchie et al. 2011), miR-186 (Babenko et al. 2012) and miR-23b (Nicholls et al. 2011). Viticchie *et al* extracted RNA from DLD-1 cells (colorectal adenocarcinoma cells) following transfection with miR-203 precursor and found that 350 mRNAs were down-regulated in the presence of miR-203, 85 of which contained a conserved miR-203 target site in their 3' untranslated regions (UTRs). The author then performed Ingenuity Pathway analysis (IPA) to investigate the pathways most affected by miR-203 regulation of which Eps15 was present in 4 of the top 22 networks (Viticchie et al., 2011; Supplementary Material). Additionally, Eps15 was found to have a conserved site for miR-23b in Sertoli cells (Nicholls et al. 2011). Nicholls *et al* found that over-expression of miR-23b *in vitro* resulted in decreased translation of Eps15 protein and that Eps15 possesses a miR-23b target site in its 3'UTR (Nicholls et al. 2011). An additional study by Babenko *et al* investigating epigenetic responses to chronic stress also found a target site for miR-186 in the 3'UTR of the Eps15 gene (Babenko et al. 2012).

### 1.6.2 Structure

Eps15 is a ubiquitously expressed, 142 kDa protein formed of a tripartite structure. The structural features of the predicted *eps15* gene product were used to show that Eps15 can be subdivided into three domains; domain I contains regulatory domain signatures later described as Eps15 homology (EH) domains, containing multiple copies of the NPF amino acid motif (Asparagine-Proline-Phenylalanine) (Salcini et al. 1999), as well as a tyrosine phosphorylation site and EF-hand-type calcium-binding domains. Domain II comprises the characteristic heptad repeats of coiled-coil rod-like proteins and domain III contains a repeated DPF (Aspartate-Proline-Phenylalanine) motif, similar to the consensus sequence of several methylases (Fazioli et al. 1993) (Figure 1.8).

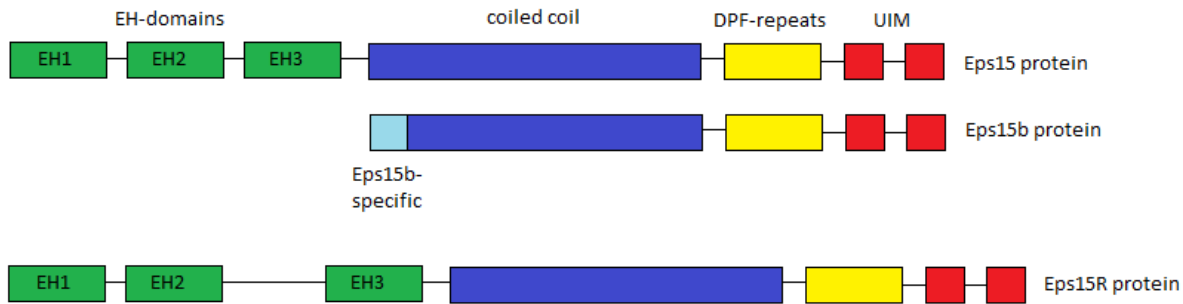


Figure 1.8 Schematic representation of Eps15 and its family members Eps15b and Eps15R. Eps15 contains three domains; domain I contains Eps15 homology domains, domain II contains the coiled coil region and domain III contains the DPF-repeats. Picture adapted from papers by Salcini *et al* (Salcini et al. 1999) and Roxrud *et al* (Roxrud et al. 2008).

The three Eps15 EH domains are also found in proteins required for the internalisation step of endocytosis in yeast and Benmerah *et al* found that loss of these EH domains in an Eps15 mutant inhibited correct coated pit targeting of Eps15 as well as a reduction in plasma membrane distribution of AP-2 and clathrin (Benmerah et al. 1999). They also found that the GTPase dynamin, found in coated pits, was homogeneously redistributed on the plasma membrane and endocytosis of transferrin was strongly inhibited (Benmerah et al. 1999). Carbone *et al* found similar results during experiments with dominant-negative mutants of Eps15 and micro-injection of anti-Eps15 and anti-Eps15R affinity-purified antibodies. Both caused inhibition of internalisation of EGF and transferrin indicating both Eps15 and Eps15R to be essential components of the endocytic machinery (Carbone et al. 1997).

Eps15 binds to proteins with the consensus amino acid sequence NPF, for example Epsin (Chen et al. 1999). Due to its binding capabilities Eps15 is shown to be associated with a number of proteins (Figure 1.9) including SH3BP4/TTP, FCHO1, FCHO2, ERBB2, SH3-containing Grb2-like protein 3-interacting protein 1 (SGIP1), Hrs; whose interaction bridges the interaction of STAM or STAM 2 with Eps15, STON2 and EPN1 and interacts via its SH3-binding sites with Crk.

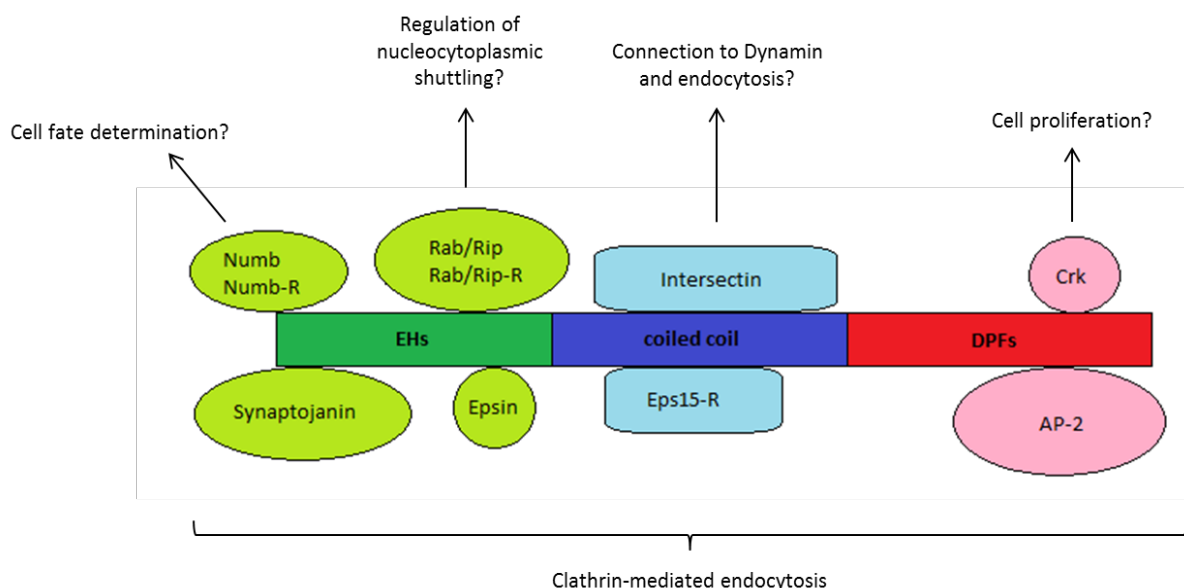


Figure 1.9 Potential binding partners of Eps15

Eps15 is able to bind to many proteins in order to function appropriately. Some binding partners are more represented in the literature than others. The role of Eps15 and Eps15R in endocytosis due to binding with proteins such as Epsin and AP-2 is well established while binding to other proteins is more speculative. Image adapted from a review by Salcini *et al* (Salcini et al. 1999).

Two isoforms of the human Eps15 protein are produced via alternative splicing; Eps15 and Eps15R, which undergo multiple post-translational modifications. Two of these modifications are triggered by activation of EGFR kinase: tyrosine phosphorylation and monoubiquitination (Salcini et al. 1999). These post-translational modifications are most important during Epsin and Eps15 roles in nerve terminals as Epsin and Eps15 expressed here undergo constitutive phosphorylation and depolarisation-dependent de-phosphorylation (Chen et al. 1999). Chen *et al* showed that both rat Epsin and Eps15 are mitotic phosphoproteins and that mitotic phosphorylation of these proteins inhibited binding to the  $\alpha$ -adaptin subunit of the adaptor protein AP-2, while de-phosphorylation enhanced their binding to AP-2 in brain extracts (Chen et al. 1999). The authors suggest that mitotic phosphorylation of these two proteins may be one of the mechanisms by which the invagination of clathrin-coated pits is blocked in mitosis; in mitotic cells clathrin coats are able to assemble but invagination is impaired. The authors also suggest that their stimulation-dependent de-phosphorylation at synapses may contribute to the compensatory burst of endocytosis after a secretory stimulus (Chen et al. 1999).

Furthermore, anti-eps15R sera in NIH-3T3 mouse embryonic fibroblast cells recognised three Eps15R proteins (Coda et al. 1998). The size of these proteins range in size at 76, 108 and 125 kDa. The 125 kDa species is a product of the *eps15R* gene, while the others are most likely products of alternative splicing events (Coda et al. 1998). Coda *et al* found that Eps15R proteins are tyrosine-phosphorylated following EGFR activation in NIH-3T3 cells. They suggest a role for Eps15R in CME due to its localisation in plasma membrane-coated pits and *in vivo* association to the coated pits

adaptor protein AP-2. They also demonstrated that Eps15R can exist as a complex with Eps15 and that the EH domains of Eps15R exhibit binding specificities that are partially distinct from those of Eps15. In this way the authors propose that Eps15 and Eps15R are multi-functional binding proteins that serve pleiotropic functions within the cell (Coda et al. 1998).

### 1.6.3 Eps15 binding proteins

As discussed, Eps15 is organised into 4 different domains, each with different binding capabilities. The first domain (Domain I) contains three different Eps15 homology (EH) domains, and enables the binding of Eps15 to a number of EH domain containing proteins (EHDs). Proteins containing EH domains are predominantly involved in intracellular trafficking and include the endocytic proteins Intersectin, Epsin1, STAM (EAST), among others (Yamabhai et al. 1998; Bache et al. 2003; Chen et al. 1998). The second Eps15 domain (Domain II) is a coiled-coiled region which has the capacity to dimerise and allow the formation of Eps15 dimers and tetramers (Cupers et al. 1997). Dimers can form parallel or anti-parallel and the authors suggest that changes in dimer conformation could allow further protein binding partners to attach, including Intersectin (Cupers et al. 1997; Sengar et al. 1999). The third domain (Domain III) contains several DPF (aspartate, proline, phenylalanine) motifs, which associate with the  $\alpha$ -adaptin component of the clathrin-adaptor AP-2 complex involved in clathrin-mediated endocytosis (Benmerah et al. 2000). DPF motifs can also act as ligands for the EH domains as the protein POB1 (Partner of RalBP1) was able to bind to the DPF region of Eps15 via its EH-domain (Santonico et al. 2007). The ability for Eps15 to dimerise as well as bind further EH domain containing proteins suggests the ability of Eps15 to produce large networks. The fourth domain (Domain IV) is the regulatory domain as it contains a number of regulatory sites including the tyrosine residue 850 (Tyr850) that becomes phosphorylated upon stimulation of the cell with EGF (Confalonieri et al. 2000) as well as ubiquitin interacting motifs (UIM) (Regan-Klapisz et al. 2005). Over-expression of an Eps15 mutant lacking Tyr850 inhibits EGFR endocytosis but not constitutive endocytosis of transferrin (Confalonieri et al. 2000). A review by van Bergen en Henegouwen summarised known Eps15 binding partners and their interacting domains. While the majority of these binding proteins function in endocytosis, others have much wider functions in the cell nucleus, endosome sorting and even actin organisation (Table 1.1) (van Bergen En Henegouwen 2009).

Table 1.1 Eps15 binding partners

Binding Partner	Interacting domains	Function	Reference
$\alpha$ -adaptin	DPF-motif	Endocytosis	
$\gamma$ -adaptin	Domain III	Secretion	
AP-2	DPF-motif	Endocytosis	(Salcini et al. 1999)
Crk	Domain IV	Unknown	(Schumacher et al. 1995)
Eps15/Eps15R	EH domain (Domain I)	Unknown	
Epsin	EH domain (Domain I)	Endocytosis	(Chen et al. 1998)
Grb2	Domain IV	Endocytosis	
Hrb	EH domain (Domain I)	Endocytosis/Nucleus	
Hrs	Not determined	Sorting	(Roxrud et al. 2008)
Intersectin	Coiled-coil domain (Domain II)	Endocytosis	(Yamabhai et al. 1998)
Numb	EH domain (Domain I)	Endocytosis	
Parkin	Domain IV	Mono-ubiquitination	
POB1	Domain IV	Endocytosis	(Santonico et al. 2007)
Phocein	Not determined	Trafficking	
Spartin	Not determined	Endocytosis	
STAM (EAST)	Not determined	Sorting	(Bache et al. 2003)
Stonin2	EH domain (Domain I)	Endocytosis	
Synaptojanin	EH domain (Domain I)	Endocytosis	
Ubiquilin/PLIC	Domain IV	Aggresome formation	(Regan-Klapisz et al. 2005)
Ubiquitin	Domain IV	Ubiquitination	(Mayers et al. 2013)

### 1.6.4 Function

Human Eps15 is mapped to chromosome 1p31-p32, a region that displays several non-random chromosomal abnormalities. The region includes deletions in neuroblastoma and translocation in acute lymphoblastic and myeloid leukemias (Salcini et al. 1999). Eps15 is also rearranged with the HRX/ALL/MLL gene in acute myelogenous leukemias implicating Eps15 to be involved in the abnormal cell proliferation in neoplasia (Salcini et al. 1999). Eps15 is involved in cell growth regulation and may also be involved in the regulation of mitogenic signals and control of cell proliferation (Fazioli et al. 1993). Eps15-null mice display a range of phenotypes depending on the Eps15 allele removed, including; increased body weight, decreased leukocyte number, increased circulating low density lipoprotein (LDL) circulation level and increased circulating high density lipoprotein (HDL) circulation level (IMPC 2016). These phenotypes could reveal more information on the functions of Eps15 *in vivo* but as of yet no papers have been published using these mice.

Most profoundly however, Eps15 is involved in the assembly of clathrin-coated pits in clathrin-mediated endocytosis (CME) for constitutive endocytosis (e.g. transferrin receptor) and the internalisation of ligand-inducible receptors of the receptor tyrosine kinase (RTK) type, in particular EGFR (Vieira et al. 1984). Through its role in EGFR internalisation CME is able to contribute to the down-regulation of EGFR signalling, crucial in cell function, as over-expression of EGFR has been linked to cancer progression (Normanno et al. 2006). It is now also accepted that CME of EGFR is crucial for the full activation of certain signalling proteins in the EGFR pathway (Vieira et al. 1984). Vieira *et al* utilised cells defective in clathrin-dependent receptor-mediated endocytosis (K44A cells) and found that endocytosis of inactive EGFR was not significantly affected in K44A cells suggesting CME to be crucial only in ligand-induced EGFR endocytosis (Vieira et al. 1984). Furthermore, Vieira *et al* found that following EGF stimulation, K44A cells contained many hyper-phosphorylated or hypo-phosphorylated proteins relative to un-mutated (WT) cells indicating normal EGFR endocytic pathways are required to trigger distinct signalling pathways, including mitogen-activated protein (MAP) kinase pathways (Vieira et al. 1984). Based on their observations Vieira *et al* suggest that EGFR ligands have evolved to regulate their signals by controlling EGFR trafficking and that in addition to initiating the clearance and down-regulation of the EGFR signalling complex, receptor trafficking plays a critical role in defining the signals transmitted by activated EGFR (Vieira et al. 1984). Miaczynska *et al* support these findings and go further to suggest that signalling machinery can exploit the compartmentalisation and functional specialisation of the endocytic pathway to achieve more complex regulation than just degradation of receptors as once thought (Miaczynska et al. 2004). Sorkin *et al* used fluorescence resonance energy transfer (FRET) and EGFR fused to cyan fluorescent protein (CFP) and Grb2 fused to yellow fluorescent protein (YFP). Stimulation by EGF resulted in the recruitment of Grb2–YFP to cellular



compartments that contained EGFR–CFP and a large increase in FRET signal amplitude, which should only occur if CFP and YFP are close enough to interact, suggesting interaction between EGFR–CFP and Grb2–YFP. These results indicate that activated EGFR–CFP interacted with Grb2–YFP in membrane ruffles and endosomes, supporting the theory that EGFR is able to signal in endosomes, once it is endocytosed (Sorkin et al. 2000). Compartmentalisation of inactive and active conformations of EGFR has been shown can provide another level of regulation (Burke et al. 2001). Burke *et al* found that adapter molecules, such as Shc, were associated with EGFR both at the cell surface and within endosomes, while other molecules, were found to be associated at one site primarily; for example, Grb2 molecules were primarily associated with surface EGFR, while Eps8 molecules were only found associated with intracellular receptors (Burke et al. 2001). The movement of endosomes can even direct activated signalling molecules to their target site (Verhey et al. 2001). Overall, clathrin-mediated endocytosis is needed for effective signalling as well as the necessary down-regulation of the signalling pathway following role completion and as a result, due to the important role of Eps15 in efficient CME, de-regulation of Eps15 could have devastating consequences on cell signalling.

#### 1.6.5 Eps15 function in receptor endocytosis

Endocytosis and degradation of active growth factor/receptor complexes is necessary to avoid over-expression of receptor tyrosine kinases, responsible for the development of a variety of malignancies. As mentioned previously, the trafficking of signalling receptor, such as EGFR, is extremely important in the regulation of cell signalling and consequent cell function (Verhey et al. 2001; Burke et al. 2001; Sorkin et al. 2000). As such, different isoforms of Eps15 could be extremely important in the distribution of specific endocytic compartments and their cargo for effective cell signalling. Chi *et al* discovered a novel short form of Eps15 (Eps15S), lacking 111 C-terminal amino acids present in the traditional Eps15 form, required for recycling of EGFR (Chi et al. 2011). Chi *et al* found that over-expression of Eps15S impaired EGFR recycling to the cell surface but had no effect on EGFR internalisation (Chi et al. 2011). Eps15S over-expression also substantially reduced cell proliferation, indicating EGFR recycling to be linked to downstream mitogenic effects (Chi et al. 2011). Eps15S was also found to be linked to the Rab11 long-loop recycling pathway (Figure 1.7) as EGFR accumulated in early endosomes upon expression of an Eps15S mutant. From their observations Chi *et al* suggested that EGFR is directed to an appropriate endocytic compartment depending on the distinct form of Eps15 available; traditional Eps15 directs EGFR to the late endosome/lysosome for degradation while Eps15S directs EGFR to the recycling endosome and back to the cell surface (Chi et al. 2011).

The protein Eps15 and its family members Eps15b and Eps15R have also been shown to be key in the endocytic machinery and sorting of the endocytosed receptors (Figure 1.10) (van Bergen En Henegouwen 2009). Eps15 is localised at clathrin-coated pits where it interacts with the clathrin assembly complex AP-2 and its binding protein Epsin (Salcini et al. 1999). Perturbation of Eps15 and Epsin inhibits receptor-mediated endocytosis of EGF and transferrin (an endocytosis indicator protein) indicating both proteins to be necessary in the endocytic machinery (Salcini et al. 1999). Additionally, Torrisi *et al* showed that upon activation of the EGFR kinase Eps15 undergoes dramatic re-localisation initially to the plasma membrane, then co-localises with the EGFR in various intracellular compartments of the endocytic pathway. They discuss that this re-localisation of Eps15 is independent of its binding to the EGFR or of binding to the receptor to AP-2 (Torrisi et al. 1999).

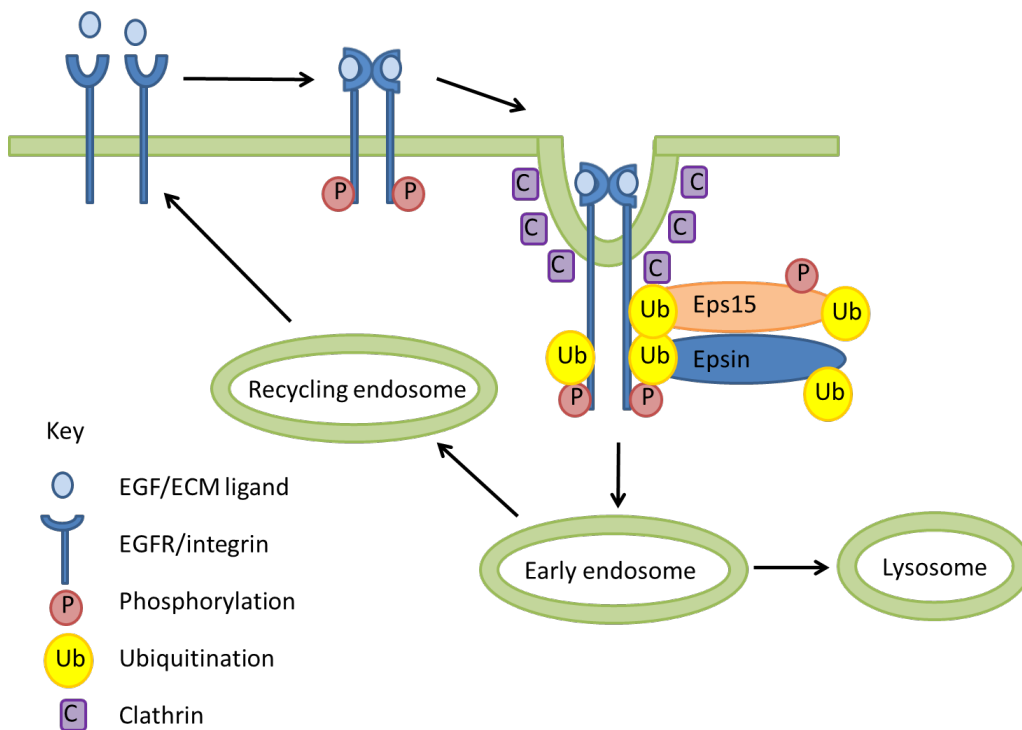


Figure 1.10 Possible role of Eps15 proteins in EGFR and integrin trafficking

Much literature is agreed on Eps15 playing a role in the endocytosis and trafficking of EGFR. Other proteins shown here are also mentioned in other literature such as Epsin and clathrin. Image adapted from a review by Paul MP van Bergen en Henegouwen (van Bergen En Henegouwen 2009).

Mayers *et al* concentrated on the regulation of ubiquitin-dependant cargo and found Eps15 to be one of the important adaptor proteins required for efficient sorting at the plasma membrane. The authors demonstrated that multiple plasma membrane endocytic adaptors function to regulate clathrin-mediated endocytosis and to recruit components of the endosomal sorting complex required for transport (ESCRT) machinery to the cell surface to direct the sorting of ubiquitin-modified substrates (Mayers et al. 2013). They found that in the absence of a hetero-oligomeric

adaptor complex composed of Fer/Cip4 homology domain-only proteins 1 and 2 (FCHO), Eps15 and Intersectin, ESCRT accumulation at the cell surface is diminished and degradation of the ubiquitin-modified cargo slows significantly (Mayers et al. 2013). Additionally, the authors discussed the idea that not all clathrin-coated pits are uniform in composition and suggested that different pits are strongly influenced by the presence of additional factors such as cargo molecules (Mayers et al. 2013).

Parachoniak and Park focused on the coiled-coil domain of Eps15 and found that it's required for efficient down-regulation of the Met Receptor Tyrosine Kinase (Parachoniak & Park 2009). Down-regulation of RTKs is modulated by receptor internalisation of which ligand-activated RTKs are mainly internalised via clathrin-dependant pathways for delivery to sorting endosomes. Efficient down-regulation of the Met RTK is necessary to avoid sustained signalling, cell transformation and in some cases even tumourigenesis (Parachoniak & Park 2009). Parachoniak and Park found that upon Met activation, Eps15 is recruited to the plasma membrane and becomes both tyrosine-phosphorylated and ubiquitinated (Parachoniak & Park 2009). Following Met activation, the recruitment of Eps15 to the plasma membrane requires domain II; the coiled-coil domain of Eps15 and the signalling adaptor molecule Grb2 (bound to a proline-rich motif in domain III). The authors found expression of the coiled-coil domain to be sufficient to displace a wild-type (WT) Eps15 protein complex from Met, resulting in a loss of tyrosine phosphorylation of Eps15. Furthermore, knockdown of Eps15 resulted in delayed Met degradation, which could be rescued by expression of Eps15 WT but not an Eps15 lacking the coiled-coil domain (Parachoniak & Park 2009).

Mediating the degradation of receptors following internalisation is another key process required in the regulation of receptor functions. EGFR degradation can occur following internalisation and has been found to be regulated by an endosomally localised isoform of Eps15; Eps15b (Roxrud et al. 2008). Roxrud *et al* found that although Eps15 mainly localises to clathrin-coated pits at the plasma membrane that Eps15b localises instead to Hrs-positive micro-domains on endosomes (Roxrud et al. 2008). Both Eps15 and Eps15b interact with the endosomal sorting protein Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) *in vitro*, *in vivo* Hrs specifically binds Eps15b (Roxrud et al. 2008). Roxrud *et al* found that over-expression of Eps15b or Hrs inhibited ligand-mediated degradation of EGFR, while Eps15 over-expression had no effect. Furthermore, depletion of Eps15b but not Eps15 caused a delay in degradation of EGFR and promoted its recycling instead. Additional research shows two further proteins to be involved in receptor sorting. Bache *et al* found that STAM 1 and STAM 2, regulators of receptor signalling and trafficking, interact directly with Hrs and propose that Hrs, Eps15 and STAM proteins form a

ternary complex and function in a multi-valent complex which sorts ubiquitinated proteins, such as EGFR, into the multi-vesicular body pathway (Bache et al. 2003).

### 1.6.6 Eps15 function independent of receptor endocytosis

A number of binding proteins of Eps15 are known to have roles independent of receptor-endocytosis such as Epsin, Intersectin and Crk. Epsin is an evolutionarily conserved endocytic clathrin adaptor however, Epsin knockout cells also display a dramatic cell division defect which correlates with a perturbation of the coupling between the clathrin coat and the actin cytoskeleton (Messa et al. 2014). Thus it's been proposed that Epsin plays a key role in coupling the endocytic machinery with the actin cytoskeleton. The actin cytoskeleton is thought to provide force for membrane invagination, and Epsin has been found to play a key role in mediating this process in yeast (*Saccharomyces cerevisiae*) (Skrzyny et al. 2012). Sla2; the HIP1R homolog in yeast, anchors Ent1; the yeast homolog of Epsin, to a stable endocytic coat by an interaction between Sla2's ANTH and Ent1's ENTH lipid-binding domains. The ANTH and ENTH domains bind each other in a ligand-dependent manner to provide critical anchoring of both proteins to the membrane. The C-terminal parts of Ent1 and Sla2 bind redundantly to actin filaments via phospho-regulated actin-binding domain in Ent1 and the THATCH domain in Sla2. By the synergistic binding to the membrane and redundant interaction with actin, Ent1 and Sla2 form an essential molecular linker that transmits the force generated by the actin cytoskeleton to the plasma membrane, leading to membrane invagination and vesicle budding (Skrzyny et al. 2012).

Intersectin (ITSN) is a multi-domain scaffold protein and a high throughput yeast two-hybrid screen by Wong *et al* found over 100 binding proteins across two mammalian ITSN genes; ITSN1 and ITSN2, including Eps15 (Wong et al. 2012; Yamabhai et al. 1998). ITSN1 has at least 24 splice variants and ITSN2 has at least 4 splice variants, which have altered interactions with specific targets. ITSN1 and ITSN2 share 59% identity and each encode a short and long isoform (Wong et al. 2012). Both ITSN short (ITSN-S) isoforms possess two amino-terminal Eps15 homology (EH) domains followed by a coiled-coil (CC) domain and five Src homology 3 (SH3 A-E) domains (Yamabhai et al. 1998). The ITSN long isoform (ITSN-L) contains all these domains in addition to an extended carboxy-terminus encoding a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, and a C2 domain. The DH and PH domains function together as a guanine nucleotide exchange factor (GEF) that regulates the activation of the Rho family GTPase Cdc42 (Wong et al. 2012), involved in actin reorganisation through its interaction with N-WASP (Hussain et al. 2001). By acting as a GEF of Cdc42, Intersectin is able to cause actin rearrangements specific for Cdc42. Hussain *et al* investigated a role for ITSN1 in a novel mechanism of N-WASP activation and in regulation of the actin cytoskeleton (Hussain et al. 2001). They found that N-WASP binds directly

to ITSN1, up-regulating its GEF activity, generating GTP-bound Cdc42 (GTP-Cdc42), a critical activator of N-WASP, in a positive feedback loop (Hussain et al. 2001).

Crk is another binding protein of Eps15 which is also involved in the activation of WASP (Sasahara et al. 2002). Crk proteins are thought to transduce signals from tyrosine kinases to downstream effectors and an expression library screened for Crk binding partners revealed that both Eps15 and Eps15R could bind to the amino-terminal of the SH3 domain of Crk (Schumacher et al. 1995). Furthermore, both c-Crk and v-Crk co-precipitated equivalently with Eps15 and Eps15R (Schumacher et al. 1995). Crk is involved in the activation of WASP in activated T-cells by forming a complex with WIP following release of WASP inhibition from a WIP-WASP complex (Schumacher et al. 1995). Known associations with these proteins suggest that Eps15 could play a role in actin organisation independent of its role in endocytosis indirectly. Additionally, the yeast Eps15-like protein; Pan1p, has also been shown to have a direct association with the actin cytoskeleton (Toshima et al. 2016), so perhaps even a direct association of Eps15 with the actin cytoskeleton is possible.

## 1.7 Hypothesis

Results generated in our group have shown that CtBPs regulate integrin-dependent cell motility in various cancer types. In a gene array, performed on the squamous cell carcinoma cell line, SCC25, it has also been found that Eps15 is down-regulated upon knockdown of CtBP1 (Figure 1.11) (Chrzan 2014). Endocytosis and trafficking of integrins is crucial for cell motility and it has been shown that inhibition of integrin endocytosis inhibits cell motility (A. G. Ramsay et al. 2007; Riggs et al. 2012). The role of Eps15 in integrin endocytosis has also been demonstrated (A. G. Ramsay et al. 2007). Based on these previous findings, we therefore hypothesise that CtBPs regulate cell motility by up-regulation of Eps15 in cancer cells to allow increased integrin endocytosis and therefore increased cell motility.

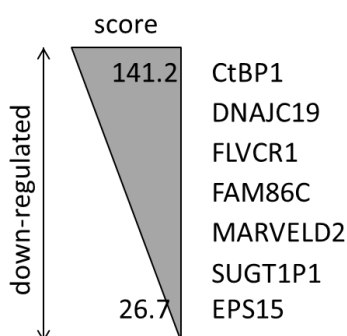


Figure 1.11 Microarray score of top seven genes down-regulated upon CtBP1 siRNA knockdown. Microarray data performed in our group previously on the squamous cell carcinoma cell line, SCC25 (Chrzan 2014), revealed Eps15 to be one of the genes down-regulated upon CtBP1

knockdown (KD), suggesting a link between the two genes. Upon CtBP1 KD, CtBP1 showed a fold change of 3.9 and Eps15 showed a fold change of 2.4. Due to its roles in endocytosis, this gene was picked to research further.

### 1.8 Aims and objectives

- To investigate the role of CtBP1 and CtBP2 in regulation of Eps15 expression using CtBP siRNA and Western blotting techniques in a variety of cancer cell lines
- To investigate the role of Eps15 in the regulation of  $\beta 6$  and  $\beta 1$  integrin endocytosis using Eps15 RNA interference and a functional endocytosis assay
- To investigate the role of CtBP1 in the regulation of  $\beta 6$  and  $\beta 1$  integrin endocytosis using RNA interference and a functional endocytosis assay
- To determine the role of Eps15 in integrin-dependent tumour cell motility through functional assays including adhesion, cell spreading, Transwell® migration and Matrigel® invasion along with organotypic models

## Chapter 2: Materials and Methods

### 2.1 Cell culture

#### 2.1.1 Cell culture techniques

Routine cell culture was carried out in a mycoplasma-clean laminar flow hood and cell lines (Table 2.1) were cultured in their preferred medium (Table 2.2 - Table 2.3). The absence of mycoplasma contamination in the cell lines was regularly confirmed via mycoplasma PCR (Section 2.1.5).

Table 2.1 Cell lines investigated in this study and their culture medium

Cell line name	Origin	Growth medium
BICR6	Head and neck cancer cell line (Edington et al. 1995)	KGM-CTX
H357	Oral (tongue) squamous cell carcinoma (Prime et al. 1990)	KGM-CTX
HEK293-T	Human embryonic kidney cells	10% DMEM
HFFF2	Human foetal foreskin fibroblast	10% DMEM
MCF7	Breast cancer cell line (Soule et al. 1973)	10% DMEM
SCC25	Oral (tongue) squamous cell carcinoma (Rheinwald & Beckett 1981)	10% HAM's F12: DMEM (1:1)
SKHEP1	Human liver adenocarcinoma (Fogh et al. 1977)	10% DMEM
SW620	Metastatic colorectal cell line (Leibovltz et al. 1976)	10% DMEM
VB6	Cell line derived from H357 by transfection with $\alpha$ v and $\beta$ 6 cDNA (Thomas et al. 2001)	KGM-CTX

Table 2.2 Growth medium compositions

Growth medium	Reagent	Supplier
KGM-CTX	$\alpha$ -MEM	GIBCO
	10% gamma-irradiated FCS	Biosera
	Adenine ( $1.8 \times 10^4$ M)	Sigma-Aldrich
	Hydrocortisone (5 mg/ml)	Sigma-Aldrich
	Epidermal Growth Factor (10 ng/ml)	Sigma-Aldrich
	L-glutamine (2 mM)	PAA
AF-KGM	$\alpha$ -MEM	GIBCO
	Adenine ( $1.8 \times 10^4$ M)	Sigma-Aldrich

Growth medium	Reagent	Supplier
	L-glutamine (2 mM)	PAA
	10% heat-inactivated BSA	GE Healthcare
KGM for Organotypics	$\alpha$ -MEM	GIBCO
	Adenine ( $1.8 \times 10^{-4}$ M)	Sigma-Aldrich
	L-glutamine (2 mM)	PAA
	10% gamma-irradiated FCS	Biosera
10% DMEM	DMEM	PAA
	10% gamma-irradiated FCS	Biosera
	L-glutamine (2 mM)	PAA
Migration DMEM	DMEM	PAA
	L-glutamine (2 mM)	PAA
	10% heat-inactivated BSA	GE Healthcare
10% glucose-free DMEM	Glucose and pyruvate-free DMEM powder	Sigma-Aldrich
	L-glutamine (2 mM)	PAA
	Sodium Pyruvate (1 mM)	Sigma-Aldrich
	10% gamma-irradiated FCS	Biosera
SCC25 medium	HAMS-F12	PAA
	DMEM	PAA
	10% gamma-irradiated FCS	Biosera
	L-glutamine (2 mM)	PAA
SCC25 migration buffer	HAMS-F12	PAA
	DMEM	PAA
	10% heat-inactivated BSA	GE Healthcare
	L-glutamine (2 mM)	PAA



Table 2.3 Routinely used trypsin and medium volumes

Flask	Trypsin (ml)	Media (ml)
T25	1	8
T75	3	20
T125	5	30

### 2.1.2 Cell counting

For completion of functional assays and optimal transfection cells were plated at specific densities. Cells were counted using a CASY cell counter (Roche-Innovatis, Germany). Cells were harvested as usual and a 20 µl sample was diluted in 10 ml filtered CASYton, an electrolyte buffer. A 400 µl diluted cell suspension was passed through a precision measuring capillary (150 µm in size) in triplicate by the CASY cell counter (the final cell count was expressed as the mean of triplicates), which passes the cells through a low voltage field between two platinum electrodes. The principle of the CASY counter relies on the integrity of the plasma membrane of the cells to be counted. When exposed to the low voltage field, the electric current cannot go through an intact plasma membrane of living cells but can go through those of injured or dead cells. This way the CASY counter is able to measure cell concentration, viability and volume of cells as the electrical signals passing across them measures amplitude, pulse width, course of time and resulting pulse area (RJMsales 2014). To count cells following migration or invasion assays cells were diluted 20X only due to much smaller numbers to improve accuracy.

### 2.1.3 Freezing cells

Cells were frozen down for long term storage and care was taken in order to ensure that cells stayed intact so that the majority were viable when needed for future culture. To preserve cell integrity cells were frozen down in medium-containing 10% of the cryoprotectant dimethyl sulfoxide (DMSO) (Pegg 2007). Once cells reached 70-80% confluency they were trypsinised as usual. Once detached the trypsin was neutralised with normal growth media and the cells were span at 1250 rpm, for 3 minutes. Cells were then re-suspended in 10% DMSO-containing medium and transferred into labelled cryovials in a container encased with isopropanol (Mr Frosty, Nalgene), which provides an environment with a rate of cooling very close to -1°C/minute. Cells were put into a -80°C freezer for 24 hours after which the vials were transferred into liquid nitrogen for long-term storage.

### 2.1.4 Thawing cells for culture

Thawing cell lines for culture was carried out as quickly as possible to avoid stress to the cells to protect them as much as possible during the process. To facilitate this, cells were thawed in a 37°C water bath. Once thawed, the 1 ml of cell suspension from each cryovial was added into 10 ml pre-warmed medium and span at 1250 rpm for 3 minutes to remove the DMSO. The supernatant was then removed, the cell pellet was re-suspended in pre-warmed medium and cells were plated into a T25 tissue culture flask (Corning).

### 2.1.5 Mycoplasma PCR protocol

All cells used were confirmed mycoplasma free before use to avoid changes in cellular behaviour associated with mycoplasma contamination. Cells were grown in antibiotic-free medium for minimum two weeks before testing. 5-10 ml of cell culture supernatant was collected from a confluent culture and centrifuged at high speed (4500 rpm; 5 minutes). The excess medium was discarded and the debris re-suspended in ~500 µl medium for use in the first round of the Polymerase Chain Reaction (PCR), which amplified the 16S-23S spacer region in rRNA operons, common to 14 different species of Mycoplasma. The following primers were used for amplification:

*Fwd1* ACT CCT ACG GGA GGC AGC AGT A

*Fwd2* CTT AAA GGA ATT GAC GGG AAC CCG

*Rev* TGC ACC ATC TGT CAC TCT GTT AAC CTC

Each reaction was prepared on ice with a negative control (RNase free water) and a positive control (5PT cell supernatant previously confirmed positive for mycoplasma) run alongside. Two rounds of PCR were set up with 20 µl reaction mixture for each sample (

Table 2.4) and the PCR was performed at the cycling conditions described in Table 2.5. Following the second round of PCR, 10 µl of each reaction was run on a 1% agarose gel with SYBER safe Red (10 000X diluted; Life Technologies) at 120V for 1 hour in 1X TAE buffer (Table 2.6). Samples were run next to Quick Load 100 base pair (bp) marker (Promega) and bands were visualised using a GelDoc imager (UVP) (Figure 2.1).

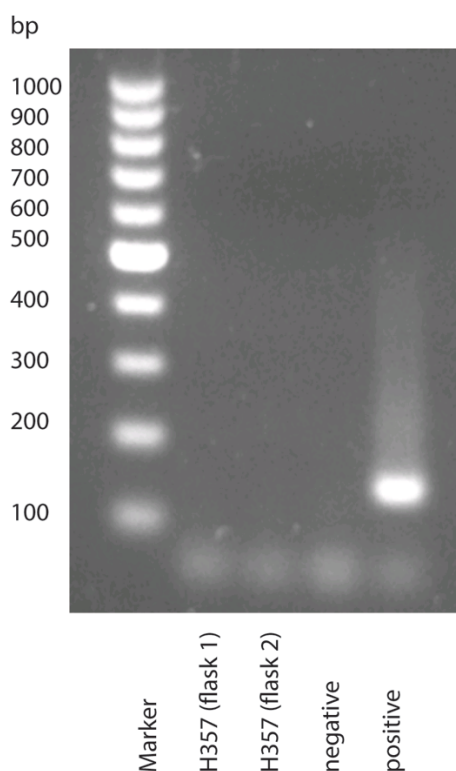


Figure 2.1 Example of mycoplasma PCR results

Following PCR amplification of cell supernatant debris, each solution was run on a 1% agarose gel to visualise any positive mycoplasma bands. Cell culture supernatant from a mycoplasma positive cell line was run alongside as our positive control; a clear band between 100-200bp can be seen. Clean media (not plated on any cell culture) was also used as a negative control so no band is present. No bands are present in either lane labelled H357 indicating that both flasks of cultured H357 are mycoplasma-free.

Table 2.4 Round 1 and Round 2 of Mycoplasma PCR test

1 <sup>st</sup> Round		2 <sup>nd</sup> Round	
Sample DNA/Supernatant (1µl)	1µl	1 <sup>st</sup> Round PCR Product	1µl
Forward Primer 1 (100pmol/µl)(F1)	1µl	Forward Primer 2 (100pmol/µl)(F2)	1µl
Reverse Primer (100pmol/µl) (R)	1µl	Reverse Primer (100pmol/µl)	1 µl
Formamide (Form)	0.3µl	Master Mix	17µl
Master Mix	16.7µl		

Table 2.5 Mycoplasma test PCR cycling conditions

Cycling Conditions
95°C x 30s
35 cycles:
95°C x 30s
55°C x 30s
72°C x 1 min
72°C x 1 min

Table 2.6 50X TAE buffer composition

Solution was diluted 50X in dH<sub>2</sub>O for a 1X final concentration.

50X TAE buffer for 1L final volume
242 g Tris-Base
57.1 ml Glacial Acetic Acid
18.6 g EDTA

## 2.2 Protein and mRNA analysis

### 2.2.1 SDS-PAGE and Western blotting

To analyse protein expression in cells following specific treatments cells were lysed on ice with an appropriate amount of NP40 lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P40, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 5 mM NaF) with 1X protease inhibitor cocktail mix (Calbiochem, Merk Chemicals). Cells were incubated in lysis buffer on ice for 5 minutes then centrifuged at 4°C for 5 minutes. Total protein content of the supernatant was analysed using DC-Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer's instructions.

20-40 µg of protein and 5X Laemmli buffer were diluted to equal volumes with NP40 lysis buffer and heated at 95°C for 8 minutes. Equal amount of protein from each sample was loaded onto an SDS-PAGE gel (Table 2.7 and Table 2.8). Gels were run in a Bio-Rad Mini-PROTEAN 3 Cell, at 150V for 90 minutes. The protein was then transferred onto polyvinylidene difluoride (PVDF; Millipore) membrane using a BioRad Mini-Trans Blot Cell at 100V for 90 minutes (Table 2.9 and Table 2.10).

Table 2.7 Summary of resolving gel solutions

Resolving gel reagent (15 ml)	8%	10%
30% Acrylamide/ 0.8% Bis (Protogel)	4ml	5ml
4X Tris-HCL pH 8.8	3.8ml	3.8ml
dH <sub>2</sub> O	6.9ml	4.9ml
10% SDS	0.15µl	0.15µl
10% APS	0.15µl	0.15µl
TEMED	0.009µl	0.006µl

Table 2.8 Summary of stacking gel solutions

Stacking gel reagent (4 ml)	
30% Acrylamide/ 0.8% Bis (Protogel)	0.67ml

4X Tris-HCL pH 6.8	0.5ml
dH <sub>2</sub> O	2.78ml
10% SDS	0.04μl
10% APS	0.04μl
TEMED	0.004μl

Table 2.9 5X running buffer solution

Reagents were mixed with 2 L of dH<sub>2</sub>O and stored at 4°C. Before use the stock was diluted to 1X with dH<sub>2</sub>O.

Running buffer reagent (5X)	Amount (g)
Trizma-Base (Sigma-Aldrich)	30
Glycine (Sigma-Aldrich)	140
SDS	10

Table 2.10 10X transfer buffer solution

Before use 100 ml stock was diluted to 1X with 700 ml H<sub>2</sub>O and 200 ml ethanol.

Transfer buffer reagent (10X SDS-free)	Amount (unit)
Trizma-Base (Sigma-Aldrich)	14 (g)
Glycine (Sigma-Aldrich)	72 (g)
H <sub>2</sub> O	1000 (ml)

Table 2.11 Antibodies used in this study for protein detection

Protein detection was used in many protocols including Western blotting (WB), immunofluorescence (IF), immunohistochemistry (IHC) and flow cytometry (FC).

Antibody	Supplier	Application (final concentration)
Anti-mouse Alexa 488	Life technologies	IF (1:250), FC (20 μg/μl)
Anti-mouse Alexa 546	Life technologies	IF (1:250)
Anti-rabbit Alexa 488	Life technologies	IF (1:250)
Anti-rabbit Alexa 546	Life technologies	IF (1:250)
Anti-rat Alexa 488	Life technologies	IF (1:250), FC (20 μg/μl)
Clathrin	BD Transduction Laboratories	WB (1:5000),
CtBP1 (E12)	Santa Cruz	WB (1:500), IF (1:500), IHC (1:100)
CtBP (H440)	Santa Cruz	ChIP (5 μg/μl)
CtBP2 (E16)	BD Sciences	WB (1:500),

Antibody	Supplier	Application (final concentration)
Eps15 (ab84810)	Abcam	IHC (1:250)
Eps15 (G-3)	Santa Cruz	WB (1:500), IF (1:250)
Eps15 (#8855)	Cell Signalling Technology	WB (1:500), IF (1:250)
Giantin	Novus biologicals	IF (1:100)
HSC70	Santa Cruz	WB (1:1000),
Kindlin-1 (MAB2616)	Millipore	WB (1:1000)
Kindlin-2 (K3269)	Sigma-Aldrich	WB (1:1000)
Mouse secondary	Dako UK Ltd.	WB (1:1000)
Phalloidin-FITC	Sigma-Aldrich	IF (1:200)
Phalloidin-TRITC	Millipore	IF (1:5000)
Rabbit secondary	Dako UK Ltd.	WB (1:1000)
Talin-1	Sigma-Aldrich	WB (1:1000),
Total integrin $\beta$ 1 (12G10)	Abcam	FC (10 $\mu$ g/ $\mu$ l)
Total integrin $\beta$ 6 (620W)	In-house	FC (10 $\mu$ g/ $\mu$ l)
Vinculin	Millipore	WB (1:1000)

The blots were blocked with 5% milk (dried non-fat Marvel) made in 0.1% PBS-Tween (PBS-TW) for 1 hour at room temperature (RT) then washed 3 x 5 minutes in 0.1% PBS-TW before incubating with the appropriate primary antibody (Table 2.11) diluted in 1% bovine serum albumin (BSA) and PBS-TW overnight at 4°C. The blot was then washed and incubated with appropriate secondary antibody (Table 2.11) for 1 hour/RT and washed again 3 x 5 minutes. To visualise; 750  $\mu$ l stable peroxide solution and 750  $\mu$ l luminol enhancer were mixed to form Supersignal West Pico Chemilluminescent substrate reagent (Thermo Fisher Scientific Inc., Rockford, IL), applied to the membrane and the signal was detected using Chemidoc imager and software (UVP). Densitometric analysis was performed, when required, on bands using Fiji imaging software (Schindelin et al. 2012) and appropriate plug-ins. Bands of interest were adjusted for loading control (HSC70).

### 2.2.2 RNA interference (RNAi)

RNA interference (RNAi) involves the use of small interfering RNA (siRNA) transfected into cells in order to disrupt the expression of specific genes with complementary nucleotide sequences. siRNA is composed of double-stranded RNA molecules, 20-25 base pairs in length. Due to the

siRNA's complementary sequence to the gene of interest, binding of the complementary nucleotides during transcription causes a disruption at the transcription stage of the required gene so that protein translation cannot occur, stopping the expression of the protein required.

Cell lines were transfected with appropriate siRNA using Oligofectamine reagent (Invitrogen). Cells were seeded at a density of  $5 \times 10^4$ /well in a 6-well plate 16 hours prior to transfection. Several siRNAs were used throughout the project; a control non-targeting siRNA (Silencer Negative Control #1, Ambion) and targeting siRNA against clathrin heavy-chain, CtBP1, CtBP2 and Eps15 (Table 2.12). For the most significant knockdown short-term functional assays (Transwell® migration assays, xCELLigence RTCA adhesion assays) were set up 48 hours post-transfection while long term organotypic culture and Matrigel® invasion assays were set up 24 hours post-transfection.

Table 2.12 siRNAs used for RNA interference

Product name	Target sequence	Supplier	Concentration (mM)
Silencer Negative Control #1	NA	Ambion	10
Clathrin heavy-chain (CHC)	SMART pool: GAGAAUGGCUGUACGUAAU UGAGAAAUGUAAUGCGAAU GCAGAAGAAUCAACGUUUAU CGUAAGAAGGCUCGAGAGU	Thermo Scientific/ Dharmacon	30
CtBP1	AAACGACTTCACCGTCAAGCA	Qiagen	10
CtBP1_5	CTGGATGTGCACGAGTCGGAA	Qiagen Gene Solution siRNA (gene pool)	30
CtBP1_6	ATGAACGTTCTTGTCTGTGTA		
CtBP1_7	CACCGTCAAGCAGATGAGACA		
CtBP1_8	CTCGTTAAGCAGAAGAAGTCA		
CtBP2	AAGCGCCTTGGTCAGTAATAG	Qiagen	10
CtBP2_7	TCCCATGAGTTAAGAGCTTGA	Qiagen Gene Solution siRNA (gene pool)	30
CtBP2_8	AAGACCGCTGACAATTAATTA		
CtBP2_9	TGGGTTAGTGCTAAATCAATT		
CtBP2_10	AGGGAGGGAAGTGAACCTTGA		
Eps15_1	GTGGACCAACATAATATTA	Qiagen	10
Eps15_5	CAGGAAATTAGTTCAATGCAA		
Eps15_7	TAGCCTATAAATAAATTCCAA		

### 2.2.3 Fugene Transfection

Eps15 over-expression analysis required transfection of vectors using Fugene HD transfection reagents (Promega). Cells were plated at a density of  $2 \times 10^5$  in each well of a 6-well plate and left to adhere overnight before transfection was performed as per manufacturer's instructions. 2  $\mu$ g DNA; either Eps15-pmCherryN1 (addgene) or empty pmCherryN1 (made in house) was diluted in 97  $\mu$ l Opti-MEM<sup>®</sup>. Optimised amounts of Fugene transfection reagent was then added. The mixture was then vortexed and left at room temperature for 15 minutes before adding to appropriate wells of a 6-well plate. Transfected cells were then used 48 hours post-transfection for functional assays.

#### 2.2.3.1 Expanding stocks of over-expression DNA (heat shock transformation)

When stocks of over-expression vectors were running low, plasmids were transformed into competent XL1 *E.coli* cells for amplification before being isolated using Qiagen maxi prep kit as per manufacturer's instructions. Firstly, 1  $\mu$ l DNA was added to 50  $\mu$ l competent XL1 *E.coli* cells and left on ice for 20 minutes to facilitate attachment of the DNA to the host cell wall. The cells were then heat shocked by suspending them in a water bath at 42°C for 45 seconds. This rapid increase in temperature opens pores within the cell membrane causing the DNA stuck on the cell surface to enter the bacteria. The cells are then put back onto ice for 2 minutes to close the pores and stabilise the cell membrane again. Once the DNA is successfully taken up by the cells, cells are put into 250  $\mu$ l LB broth and left to shake for 1.5 hours at 37°C. Following incubation, cells were plated onto agar plates containing appropriate antibiotics and left to colonise over night at 37°C. After overnight incubation isolated bacterial colonies were picked with a pipette tip and grown in a universal with 5 ml LB broth throughout the day. After approximately 8 hours, the 5 ml bacterial broth was transferred into a larger flask of LB broth and bacteria was left to amplify overnight. Following overnight incubation bacteria were pelleted by centrifugation at 4500 rpm at 4°C for 30 minutes and the plasmids were then extracted from bacteria pellets using Maxi Prep Kits (Qiagen) according to manufacturer's instructions. Isolated plasmids were then re-suspended in water containing 10% TE buffer and quantified using a nano-drop.

### 2.2.4 Flow cytometry

Flow cytometry was used to measure the total cell surface levels of integrins  $\beta 6$  and  $\beta 1$ . Cell surface integrins were bound to fluorescent labelled antibodies (Table 2.11) which were excited by a laser when run through a flow cytometer. When the antibodies were excited a signal was produced, the stronger the signal, the greater the amount of protein of interest available.



Integrin levels were measured on cells 48 hours post-transfection following Eps15 knockdown. Cells were transfected as usual at confluency of  $5 \times 10^4$  cells per well of a 6-well plate. After 48 hours, transfected cells were collected with trypsin, as trypsin does not cleave off the integrins from the cell surface. Experiments were always carried out alongside control treated cells too; one half of which were treated with secondary antibody only, to confirm that antibodies have worked in the assay. Once cells were detached they were neutralised with E4 (DMEM containing 0.1% BSA).  $1 \times 10^5$  cells were added to each FACS tube, spun down and the media removed. The cells were then re-suspended in 50  $\mu$ l of primary antibody diluted in E4 (10  $\mu$ g/ml) in each FACS tube on ice. The tubes were always kept on ice following this step. The tubes were then vortexed and the primary antibodies incubated on ice for 60 minutes (Table 2.11). Just E4 medium was added to the unstained control cells. Following 60-minute incubation cells were washed 2X with 2 ml E4 by centrifuging for 3 minutes at 1200 rpm and 4°C. After the second wash around 50  $\mu$ l of cell suspension was left in the FACS tube and the secondary antibody (20  $\mu$ g/ml) was added (Table 2.11). Cells were incubated for a further 60 minutes in the dark in secondary antibody, then kept in the dark following this step. Cells were washed twice more with E4, then washed once with FACS wash (buffer; 10 g BSA, 1 g 0.1% sodium azide dissolved in PBS up to 1 L). After this final spin around 200  $\mu$ l of cell suspension was left per tube and cells were then scanned on a FACS Canto I (BD Bioscience) by acquiring  $1 \times 10^4$  events. Data was then plotted as mean fluorescence (arbitrary units (AU)).

### 2.2.5 Immunofluorescence

In order to image cells via immunofluorescence first cells needed to be fixed to coverslips (13 mm diameter) and antibody labelled. First, coverslips were sterilised with 70% ethanol for 15 minutes at room temperature (RT). They were then washed 1X with PBS and coated with either TGF- $\beta$ 1 latency-associated peptide (LAP, ligand of integrin  $\alpha$ v $\beta$ 6; Sigma-Aldrich) = 0.5  $\mu$ g/ml or rat-tail collagen I (COLI; Millipore) = 10  $\mu$ g/ml. Coverslips were washed once more with PBS before the cells were plated. Cells were plated  $2.5 \times 10^4$  or  $1.5 \times 10^5$  for scratch assays. Once cells had adhered for an appropriate length of time they were washed 1X PBS then fixed in 0.5 ml of 4% Formaldehyde for 20 minutes at RT on rocker. Cells were then washed once more in PBS and stained with appropriate antibodies. Before staining cells were permeabilised in 300  $\mu$ l 0.2% Triton-X + 100 mM Glycine (in PBS) for 15 minutes at RT on rocker. Coverslips were then washed 3 x 5 minutes with PBS and then blocked with 300  $\mu$ l 3% BSA in PBS for 30 minutes. Required primary antibodies in 300  $\mu$ l 0.6% BSA in PBS were then added for 60 minutes (Table 2.11). Coverslips were then washed 3 x 5 minutes with 500  $\mu$ l PBS before adding appropriate secondary antibodies in 0.6% BSA in PBS (1:250 for Alexa antibodies) for 60 minutes. Coverslips were then washed 2 x 5 minutes with PBS before adding phalloidin diluted in PBS in the dark for 15 minutes

(Table 2.11). Coverslips were then washed 2 x 5 minutes more with PBS then rinsed in deionised water. Excess water was blotted from the coverslips which were mounted using ProLong<sup>®</sup> Gold Antifade Mountant with DAPI (Thermofisher Scientific; P36931). All cells were visualised using a Zeiss Axiovert 200 fluorescence microscope with a 40X objective, and images were collected using an Orca-ER digital camera (Hamamatsu) and processed using Openlab 3.5.1 Software (Improvision). Identical exposure times were applied for different images within the same experiment and analysis was completed using Fiji imaging software (Schindelin et al. 2012) and appropriate plug-ins.

### 2.2.6 Immunohistochemical staining and analysis

Immunohistochemistry (IHC) is a technique used to detect specific proteins in tissue sections embedded in paraffin blocks. These tissue sections can then be cut and mounted to make tissue microarrays (TMAs) which can be stained for analysis. Tissue sections used in this study were stained by Pathology services (Cellular Pathology Department, University of Southampton) using Eps15 antibody (abcam, ab84810; 1:250) and CtBP1 antibody (E12; 1:100) (Table 2.11) and scored according to expression patterns by a trained pathologist.

The analysis of Eps15 and CtBP1 staining was performed on a cohort of 80 different human tumours (OSCC n=10, lung n=9, colon n=25, breast n=26, ovary n=6, pancreatic n=2 and prostate n=2). Three different areas from a single slide were then analysed and scored. TMA scores ranged from 0-3; samples with no expression were scored 0, low levels of protein expression were scored as 1; moderate expression was labelled as 2, while high levels were scored a 3. Correlation analysis was then performed using SPSS statistical analysis software. University of Southampton is a Cancer Research UK Centre and holds ethical approval for the use of human tissues and animals for research purposes.

### 2.2.7 TaqMan<sup>®</sup> real time polymerase chain reaction (RT-PCR)

TaqMan<sup>®</sup> real time quantitative polymerase chain reaction (RT-PCR) uses dual labelled hydrolysis probes along with a light source able to measure fluorescence emitted as a consequence of fluorescence energy resonance transfer (FRET) to quantify the amount of a particular gene sequence in a sample. TaqMan<sup>®</sup> probes consist of an 18-22 base pair oligonucleotide probe labelled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end. Prior to PCR cycling, the close proximity of the reporter and quencher fluorophore (separated only by the length of the probe) cause the fluorescence of the reporter dye to be mostly quenched (although some background fluorescence is still available). During PCR however, the probe anneals specifically between the forward and reverse primer attached to the DNA region of

interest about to be replicated via PCR. During the replication stage of PCR the polymerase carries out the extension of the primer and replicates the template to which the TaqMan® is bound, cleaving the probe in the process due to the 5' exonuclease activity of the polymerase. Cleaving the probe releases the reporter molecule, stopping the quencher fluorophore from blocking the reporter fluorescence. As a result, the amount of fluorescence detected directly relates to the amount of DNA template present in the PCR.

TaqMan® RT-PCR was used as a means to measure the change in Eps15 mRNA expression as a consequence of CtBP knockdown (KD). Prior to PCR amplification, RNA was extracted from samples to be analysed using RNeasy Kit (Qiagen) according to manufacturer instructions before re-suspending in RNase-free water, quantifying via Nanodrop and storing at -80°C until use. cDNA was then synthesised from collected RNA using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to manufacturer instructions. The TaqMan® assay was then performed by adding 5 µl of cDNA (10 ng/µl) in duplicates to a 96-well PCR plate (Applied Biosystems) to 15 µl of reaction mix on ice. The reaction mix contained TaqMan® Universal Master PCR Mix (Applied Biosystems), 20X TaqMan® MGB probes (Applied Biosystems) and nuclease-free water. Once combined, the 96-well plate was covered with a film lid and centrifuged at 2000 rpm for 2 minutes at 4°C to ensure the reaction mix and cDNA were at the bottom of each well. The plate was then placed in a 7900HT fast-real time PCR System (Applied Biosciences). The PCR program derives relative CT values by normalising the amount of target mRNA to a housekeeping control (beta-actin or ubiquitin). The cycle threshold (CT) is the number of cycles required for the fluorescent signal to exceed background level; the lower the CT level the greater the amount of target nucleic acid in the sample. The target CT can then be compared to the housekeeping CT to calculate the relative mRNA available in each sample.

#### **2.2.7.1 Roche Universal Probe Library (RT-PCR)**

In order to validate our genes of interest found following RNA sequencing we performed RT-PCR with *unvalidated* primer sequences obtained from Roche's Universal Probe Library Assay design centre and ordered from Eurofins. Each primer correlated to a specific fluorophore within the Roche Universal Probe library. This technique is much like TaqMan® RT-PCR but the probes and primers are not conjugated and so were added separately.

#### **2.2.8 Chromatin Immunoprecipitation (ChIP) assay**

Chromatin immunoprecipitation (ChIP) is a technique used to study the association of certain proteins with specific regions of the genome. We used the MAGnify™ Chromatin Immunoprecipitation System (Invitrogen) to analyse our protein of interest; Eps15, to analyse its

## Chapter 2

association with the region of the genome encoding CtBP. In a standard ChIP assay, a cell is fixed with formaldehyde treatment and the chromatin is sheared and immune-precipitated via a highly specific antibody. The DNA is then analysed to identify the genomic regions where the chromatin-associated proteins bind to the chromatin *in vivo*.

Before ChIP can be carried out, the cells must first be cultured and sonicated to break up the chromatin for efficient pull down. Cells were cultured in 10 cm culture dishes at 1 million cells. The media was changed 16 hours and 1 hour before collection with normal media and at 48 hours, the cells were collected as follows. The media was aspirated and the cells were collected after trypsinisation as per usual. Once the cells had detached, 10 ml room temperature PBS was added and the cells were pipet up and down to mix. Cells were then transferred to a 15 ml falcon and spun at 1250 rpm for 5 minutes to pellet. The pellet was then re-suspended in 500  $\mu$ l of PBS. 13.5  $\mu$ l of 37% formaldehyde was then added to the 500  $\mu$ l of sample, for a final concentration of 1%. The tube was then inverted and left to incubate for 10 minutes at room temperature. To stop the reaction, 57  $\mu$ l of room-temperature 1.25 M glycine was added to the sample, inverted and incubated for 5 minutes at room temperature. Following this the crosslinked cells are kept on ice and then spun 3 x 4000 rpm for 10 minutes at 4°C. After the final wash the crosslinked cells are lysed and sonicated. Cells are prepared at a concentration of 4 million per 200  $\mu$ l lysis buffer. Cells are lysed as described in the MAGnify™ Chromatin Immunoprecipitation System protocol from Invitrogen. Once lysed, cells are sonicated using power setting 3.5 with the program lasting 75 seconds; 5 x 15 second burst with 20 seconds rest in between each burst. Samples were then snap frozen in liquid nitrogen and stored at -80°C until the beads were labelled and ready to be used for pull-down.

To perform the pull-down first, the antibodies were coupled to the dynabeads, all reagents and the magnet used in this step are kept on ice. A mix of 100  $\mu$ l cold dilution buffer and 10  $\mu$ l fully re-suspended Dynabeads® Protein A/G are added to individual 0.2 ml PCR tubes. The tubes are then placed into the DynaMag™-PCR Magnet so the beads form a tight pellet. The beads are then washed once with 100  $\mu$ l cold dilution buffer before adding 25  $\mu$ l of the antibody of interest (Table 2.11) and a further 100  $\mu$ l dilution buffer to the appropriate experimental tubes. 1  $\mu$ l of control antibody supplied in the kit is added to appropriate tubes for a final concentration of 1  $\mu$ g/ $\mu$ l. The beads were then rotated end to end for 1 hour at 4°C.

Once the beads and chromatin were prepared the ChIP assay was performed as described in the MAGnify™ Chromatin Immunoprecipitation System protocol (Invitrogen). The protocol described the steps required for ChIP including diluting the chromatin, binding chromatin to the beads, washing the bound chromatin to remove chromatin not bound to the beads, reversing the

crosslinking to separate the DNA pulled down from the bead bound proteins and finally washing and eluting the DNA ready for PCR amplification.

In order to analyse the DNA pulled down PCR amplification was performed similar to that of the mycoplasma PCR protocol using the cycling conditions shown in Table 2.13. As the potential target gene for CtBP1 transcriptional regulation was Eps15 the primers used were designed to span the Eps15 promoter region. Based on previous ChIP attempts we chose 6 primer pairs ranging in size no larger than 250 bp and with an overlap between them to ensure that all fragments of the promoter are amplified.

Table 2.13 PCR cycling conditions for ChIP

Cycling Conditions
95°C x 30s
40 cycles:
95°C x 30s
55°C x 30s
72°C x 30s
72°C x 1 min

## 2.3 Next generation sequencing of RNA

### 2.3.1 Collection of RNA samples

For completion of RNA sequencing we isolated RNA from cells treated with control siRNA and Eps15 siRNA from 3 independent transfections. Cell pellets were collected at 48 hours post-transfection following a change of media at 24 hours post-transfection and 1 hour prior to cell pellet collection. RNA was isolated using Qiagen RNeasy extraction kit according to manufacturing instructions and resulting RNA was re-suspended in RNase-free water, quantified using a Nanodrop and RNA quality was determined using Bioanalyzer analysis (Agilent Technologies Inc.) to obtain RNA integrity numbers prior to downstream processing by Expression Analysis (EA) Genomic Services (Durham, USA).

### 2.3.2 RNA sequencing analysis

Following quality control analysis, 250-300 ng of total RNA at a minimum concentration of 25 ng/ $\mu$ l was sent to EA genomics laboratories who performed next generation sequencing using Illumina Truseq Standard Protocol, with a coverage of 25 million (5bp PE) in both directions. Obtained Reads were mapped to the human genome with Tophat 2.0.13 (Trapnell et al. 2012), indexed and sorted with Samtools -1.2 (Li et al. 2009) and counted using HTseq (Anders et al. 2013) to allow normalisation and differential gene expression analysis with help from Jason

Fleming . Finally, analysis of the raw data using 'R' software was aided by Steve Thirdborough and network analysis was performed using Ingenuity Pathway Analysis (IPA).

## 2.4 Analysis of cell metabolism

### 2.4.1 Effect of glucose on Eps15 expression

Cell function can alter dramatically in response to changes in cell metabolism. One way of altering cell metabolism was to manipulate the amount of glucose available within the cell culture medium. To examine the effect of altered metabolism on Eps15 expression, cells were plated at  $8 \times 10^5$  cells/100 mm dish in 10 ml glucose-free 10% DMEM (Table 2.2). Glucose was then added at 0, 2, 5 and 10 mM in 10 ml of medium/dish. Cells were collected after 48 and 72 hours incubation by trypsinisation and analysed for protein or mRNA expression using Western blotting and RT-PCR respectively. Media was changed every 24 hours in order to ensure that glucose concentration remained consistent as based on previous results cells utilise glucose quickly.

### 2.4.2 Hypoxia treatment of cells

A second method of analysing metabolism changes in cells was to expose cells to hypoxic conditions (1% oxygen). SCC25 cells were grown until confluent in paired T25 flasks. Once confluent, one flask was subjected to hypoxic conditions by placing in a chamber that could be filled then sealed with 1% oxygen for 16 hours. Air within the chamber was replaced by running air from a BOC gas canister at 25 L/min for 4 minutes. This was then repeated an hour later to ensure cells were at 1% oxygen. This chamber was then sealed and placed back into a standard cell incubator where the paired T25 flask was also kept overnight, this flask acted as our normoxic control (20% oxygen). Following 16 hours incubation both cell populations were trypsinised then collected in PBS to be analysed by Taqman<sup>®</sup> RT-PCR technique.

## 2.5 TGF- $\beta$ luciferase assay

To quantify and compare the amount of TGF- $\beta$  activated by each cell line an MLEC (mink lung epithelial cell) assay was performed. The MLECs used in this assay are stably transfected with an expression construct containing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene. These promoter elements contain DNA response sequences which are positively regulated by TGF- $\beta$  signalling, therefore, increased TGF- $\beta$  signalling causes an increase in luciferase expression. For this assay our cells of interest were plated on top of the MLECs, and the amount of luciferase produced was measured to provide quantitative analysis of the amount of TGF- $\beta$  secreted by each cell line.

$5 \times 10^4$  MLECs were plated into each well of a 96-well plate and incubated overnight at  $37^\circ\text{C}$ . In the morning the MLECs were serum starved for 4 hours, before plating our cells of interest on top, to get rid of the effect of TGF- $\beta$  in the serum or secreted by the cells during incubation;  $4 \times 10^4$  cells/well in 100  $\mu\text{l}$  of our cells were then plated on top with 6 parallels/sample and incubated for a minimum of 16 hours. The media was then removed and the cells were washed with PBS before adding 25  $\mu\text{l}$  of lysis buffer (Promega) and freezing at  $-80^\circ\text{C}$  for at least 30 minutes. Following freezing the plate was defrosted and the cells scraped and spun down in the plate at 2600 rpm for 10 minutes. The recovered lysate was then transferred to a luciferase plate, luciferase assay substrate (Promega) was added and luminescence was measured using a plate reader (Varioscan).

## 2.6 Functional assays

### 2.6.1 Transwell® migration assay

Cell migration assays were performed through ECM-coated polycarbonate filters (8  $\mu\text{m}$  pore size, Transwell®, Corning) in a 24-well plate. 200  $\mu\text{l}$  of appropriate ECM solution (fibronectin (FN, ligand of integrin  $\alpha 5\beta 1$ ; Sigma-Aldrich) = 10  $\mu\text{g}/\text{ml}$ , TGF- $\beta 1$  latency-associated peptide (LAP, ligand of integrin  $\alpha \nu \beta 6$ ; Sigma-Aldrich) = 0.5  $\mu\text{g}/\text{ml}$ , rat-tail collagen I (COLI; Millipore) = 10  $\mu\text{g}/\text{ml}$ ) was added to the lower chamber, incubated for 1 hour at  $37^\circ\text{C}$  then removed and replaced with 200  $\mu\text{l}$  of migration buffer for 30 minutes at  $37^\circ\text{C}$  to block. Cells were plated in the upper chamber of triplicate wells 48 hours post-transfection at a density of  $5 \times 10^4$  in 100  $\mu\text{l}$  migration buffer for 24 hours (Figure 2.2). After 24 hours, the cells in the lower chamber were trypsinised (0.5 ml) and counted on a CasyCounter (Roche-Innovatis, Germany).

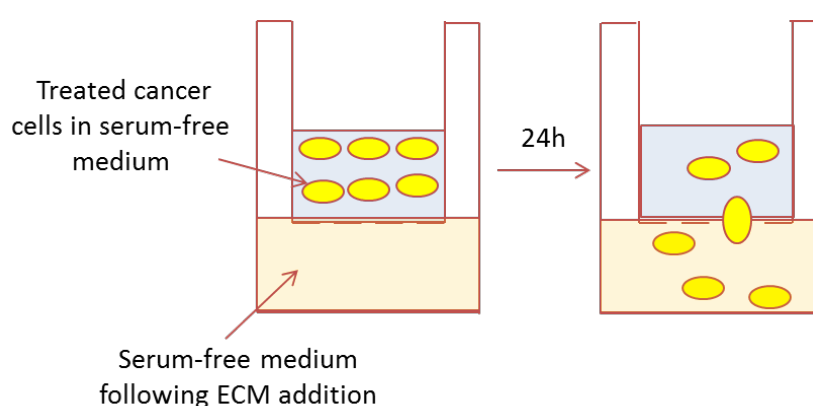


Figure 2.2 Transwell® migration assay structure

The underside of the Transwell® is coated with an ECM solution, which is then replaced with serum-free migration medium following 1 hour incubation at  $37^\circ\text{C}$ . Cell counts of migrated cells were then measured with a CasyCounter (Roche-Innovatis, Germany) and the results analysed on GraphPad Prism software.

### 2.6.2 Matrigel® invasion assay

Matrigel® invasion assays were performed through the same polycarbonate filters (8 µm pore size, Transwell®, Corning) as migration assays (Figure 2.2) however, the top surface of the membranes were coated with Matrigel® to support cell invasion (Figure 2.3). 70 µl Matrigel (diluted 1:2 in sterile DMEM) was added to the upper chamber of the Transwells® and incubated for 1 hour at 37°C. In the lower chamber of the 24-well plate containing the Transwells® cell specific growth medium (containing serum and - depending on the cell line studied - growth factors) was added as a chemoattractant. Treated tumour cells were then plated in the upper chamber of quadruplicate wells 24 hours post-transfection at a density of  $5 \times 10^4$  in 200 µl of cell specific migration buffer and incubated at 37°C for 72 hours. After 72 hours the cells in the lower chamber were trypsinised and counted on a CasyCounter (Roche-Innovatis, Germany).

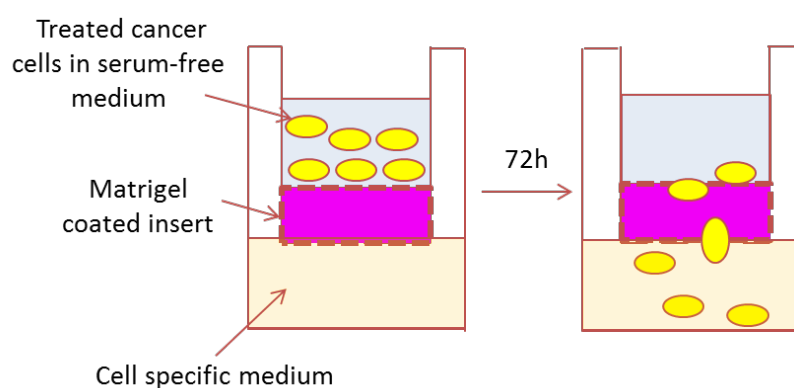


Figure 2.3 Matrigel® invasion assay

Transwells® were coated with 70 µl of Matrigel® diluted 1:2 in sterile DMEM to support cell invasion. Cell specific growth medium was put into the bottom of the chamber as a chemoattractant. The number of invaded cells was then measured with a CasyCounter (Roche-Innovatis, Germany) and the results analysed on GraphPad Prism software.

### 2.6.3 Organotypic culture

Organotypic cultures were carried out to observe cell invasion in a more physiologically relevant 3D setting and were prepared as previously described (Nyström et al. 2005). In an organotypic culture cancer cell invasion is studied in the presence of fibroblasts modelling the *in vivo* tumour micro-environment. On day 1,  $2.5 \times 10^5$  human foetal foreskin fibroblasts (HFFF2) per gel were embedded into a rat-tail collagen I:Matrigel® mixture (Table 2.14) and left to polymerise at 37°C for 1 hour. After complete polymerisation, 1 ml 10% DMEM was added on top of each gel in growth-factor-free 10% serum-containing medium (Table 2.2) and they were incubated at 37°C overnight. On day 2, cancer cells (24 hours post-transfection with siRNA) were mixed 2:1 ( $5 \times 10^5$  :  $2.5 \times 10^5$  /gel) with untreated HFFF2 fibroblasts and plated on top of the gels and incubated overnight at 37°C. On the same day sterile nylon sheets were coated with rat-tail collagen I (Table



2.15) for 30 minutes at 37°C. Following collagen polymerisation, the collagen-coated nylon sheets were fixed in 1% glutaraldehyde (Sigma-Aldrich) in PBS for 1 hour at 4°C, then washed 3X with PBS, once with 10% DMEM and incubated in 10% DMEM overnight at 4°C. On day 3, gels were raised on top of sterile steel grids covered with a collagen-coated nylon sheet in a 6-well plate and the well was filled with standard keratinocyte growth medium without cholera toxin (KGM-CTX; Table 2.2) up to under-surface of the grid (Figure 2.4). Medium was replaced every 2 days until the gels were harvested 7 days post-transfection at which point gels were bisected, fixed in 10% formaldehyde overnight, then 70% ethanol overnight before sending for sectioning and haematoxylin-eosin (H&E) and cytokeratin staining at the University Hospital Trust Histopathology Department.

Table 2.14 Organotypic gel mix

1 ml was added to each well of a 24-well plate, incubated at 37°C for 1 hour then 1 ml of complete DMEM was placed on top before the gel was incubated overnight at 37°C

Organotypic gel reagent	Volume
Collagen : matrigel (1:1)	7 volumes (3.5:3.5 volumes)
10X DMEM	1 volume
FCS	1 volume
Fibroblasts	1 volume

Table 2.15 Nylon sheet solution

Solution was neutralised if required with 0.1 M NaOH then 250 µl of mixture was added to each nylon sheet, fixed with 1% glutaraldehyde, washed with PBS and 10% DMEM then stored in 10% DMEM at 4°C for 24 hours until use

Nylon sheet reagent	Volume
Collagen	7 volumes
10X DMEM	1 volume
FCS	1 volume
Complete DMEM	1 volume

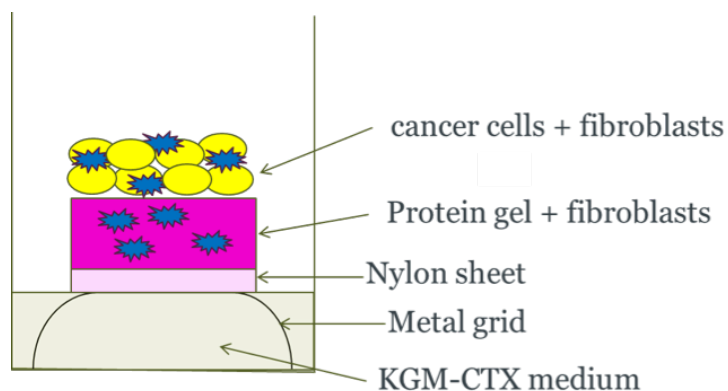


Figure 2.4 Organotypic protocol

Treated tumour cells were plated on top of a collagen I:Matrigel gel containing fibroblasts and left to invade for 7 days. Following 7 days of culture gels were bisected, treated with formaldehyde overnight, then 70% ethanol overnight before sending for sectioning and haematoxylin-eosin (H&E) and cytokeratin staining at the University Hospital Trust Histopathology Department.

#### 2.6.4 Scratch wound assay

Scratch wound assays were used for two types of analysis; firstly, the assay was used to analyse the effect of Eps15 knockdown on wound healing and secondly, cells were scratched and then fixed and stained to analyse cell polarity following Eps15 knockdown. SCC25 cells were transfected with control (non-targeting) siRNA or Eps15 siRNA and used 48 hours post-transfection in wound healing assays. Wells of a 24-well plate and coverslips were coated with COLI (10 µg/ml) before cells were plated. Cells were then plated at  $1.5 \times 10^5$  in each well of a 24-well plate or onto each coverslip.

For standard scratch assay protocol cells were plated and left to form a confluent monolayer overnight. A scratch was made the next day along the centre of the well with a sterile P200 pipette tip, all wells were washed with buffer to remove any detached cells, and finally 1 ml of buffer was added to each well. The 12-well plate was then placed on a heated stage (37°C) in an environmental chamber connected to Olympus IX81 microscope (located in the Bio-imaging Unit, Southampton General Hospital). The CO<sub>2</sub> flow was connected to the 24-well plate using a 0.8mm needle. The Cell<sup>^</sup>P software was set up to image three different fields within each well over 48 hours, taking a picture every 15 minutes using a 20X objective of Zeiss AxioCam MRm camera. Collected images were then processed using Fiji software.

To analyse cell polarity cells were treated as discussed in the immunofluorescence section of this chapter (Section 2.2.5). A scratch was made onto the coverslip once a confluent monolayer had adhered overnight and the cells were fixed at intervals until wound closure. Cells were then stained for the Golgi, Eps15 and phalloidin to assess the effect of Eps15 knockdown on cell polarity. All cells were visualised using a Zeiss Axiovert 200 fluorescence microscope with a 40X objective, and images were collected using an Orca-ER digital camera (Hamamatsu) and processed

using Openlab 3.5.1 Software (Improvision). Identical exposure times were applied for different images within the same experiment and analysis was completed using Fiji imaging software (Schindelin et al. 2012) and appropriate plug-ins.

### 2.6.5 xCELLigence real time analysis of cell adhesion

The xCELLigence real-time cell analyser (RTCA) system provides quantitative information about the biological status of cells; including cell adhesion/spreading, viability, and morphology using gold-coated E-Plate VIEW plates on which cells were plated. Upon cell plating the local ionic environment at the electrode/solution interface becomes affected, leading to an increase in electrode impedance. The more cells attached on the electrodes or the greater the cell adhesion/spreading, the larger the increase in electrode impedance; displayed as cell index (CI) values (Figure 2.5) (ACEA 2013).

E-Plates were coated either with 80  $\mu$ l ECM protein-solution (LAP to study  $\alpha$ v $\beta$ 6-specific adhesion or FN to study  $\alpha$ 5 $\beta$ 1-integrin-specific adhesion) or migration buffer (as a negative control) and incubated (37°C/10% CO<sub>2</sub>) for 1 hour. The protein solution was then replaced with 100  $\mu$ l migration buffer and left at room temperature for 30 minutes. siRNA treated cells were then plated onto corresponding wells (each condition was plated on both BSA- and ECM-coated wells) of the E-Plate 48 hours post-transfection at a density of  $2 \times 10^4$  /100  $\mu$ l migration media. Cells were allowed to settle for 5 minutes at room temperature before placing the entire plate in the xCELLigence real time analyser to measure cell adhesion over 48 hours.

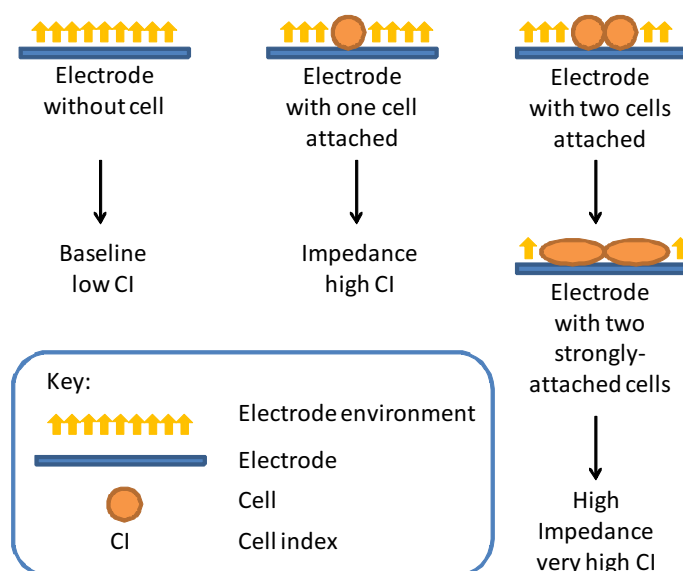


Figure 2.5 Schematic of the interdigitated microelectrodes on the well bottom of an E-Plate  $2 \times 10^4$  siRNA treated cells in 100  $\mu$ l migration medium were plated onto each well of an E-Plate 72 hours post-transfection. As the cells began to adhere and spread the impedance of the electrodes increased resulted in increasing the value of the cell index (CI) for analysis.

### 2.6.6 96-well adhesion assay

In contrast to the xCELLigence RTCA measurement of speed of adhesion, the 96-well adhesion method gives quantitative measurement of the strength of cell adhesion. To enable adhesion, 40 µl of appropriate ECM solution; fibronectin (FN, ligand of integrin  $\alpha 5\beta 1$ ; Sigma-Aldrich) = 10 µg/ml or TGF- $\beta 1$  latency-associated peptide (LAP, ligand of integrin  $\alpha v\beta 6$ ; Sigma-Aldrich) = 0.5 µg/ml was added to an appropriate number of wells of a 96-well plate. Control wells were plated with 40 µl/well migration buffer containing 0.1% heat-inactivated BSA (Table 2.2) and the whole plate was incubated for 1 hour at 37°C in a CO<sub>2</sub> incubator. After 1 hour, ECM-coated wells were washed 1X with PBS then blocked with 40 µl/well migration 0.1% heat-inactivated BSA for 30 minutes at 37°C in a CO<sub>2</sub> incubator. After 30 minutes, the migration buffer was removed and cells were plated 4 x 10<sup>4</sup> cells/well in 50 µl of appropriate cell migration buffer (Table 2.2). Cells were detached and counted as previously described and plated on ice to avoid premature cell adherence. Once all the wells were filled, the plate was left to stand for 5 minutes to allow cells to settle before putting the plate into a 37°C CO<sub>2</sub> incubator for approximately 30 minutes, or until cells had adhered to their ECM substrate but before non-specific adherence to BSA had begun. Non-adherent cells were then washed 2X with PBS before fixing with 40 µl/well 1% glutaraldehyde (diluted in PBS) for 10 minutes. Cells were then washed 1X with PBS and stained with crystal violet in methanol (MeOH) for 15 minutes. Following staining, cells were washed 1X with PBS and the crystal violet stain was dissolved by adding 40 µl 50% acetic acid into each well. Absorbance of the wells, corresponding to cell adhesion values, was then read on a multi-plate reader at 540 nm.

### 2.6.7 24-well proliferation assay

A proliferation assay was carried out to assess if Eps15 siRNA transfection caused a significant change to the ability of tumour cell lines to proliferate. Cells were transfected as described in Section 2.2.2 with control and Eps15 siRNA. After 48 hours transfection cells were re-plated onto a 24-well plate at 2.5 x 10<sup>4</sup> in 500 µl media. Cells were then trypsinised and counted at 24, 48 and 72 hours post-plating. Cells were counted on a CasyCounter (Roche-Innovatis, Germany). The data was then analysed for significance using GraphPad Prism software.

## 2.7 Integrin endocytosis assay

### 2.7.1 Ligand-dependent internalisation assay

Endocytosis assays were carried out as previously described by Roberts *et al* (Roberts et al. 2001) with minor modifications (Figure 2.6). Briefly, cell surface proteins are biotinylated and cells are stimulated with warm migration media at 37°C, as endocytosis is temperature dependent and

more efficient in adherent cells (Arjonen et al. 2012). This can be carried out over several time-points in order to see the amount of endocytosis occurring over time. Following biotinylation the remaining cell-surface biotin is stripped off the cells, which are then lysed and the amount of biotin (representing the amount of total surface or intracellular integrins) is detected using Capture-ELISA.

During optimisation, untreated cells were used before moving onto cells treated with 10 nM Eps15 and CtBP1 siRNA to test the effect of Eps15/CtBP1 knockdown on levels of endocytosis. 30 mm tissue culture plates were coated with 10 µg/ml collagen I diluted in PBS (Collagen, Type I, rat tail (100 mg/25 ml); Cat # 08-115; Upstate) for 1 hour at 37°C before washing 1X with sterile PBS. Enough plates to account for 1 time-point (15-20 minutes), a control to measure the integrins, which were already internalised at the start of the stimulation (0) and total surface integrin (T) were coated. Cells were serum-starved overnight and  $1.5 \times 10^5$  cells/1 ml migration medium was plated in 30 mm dish for 1.5 hours or until cells were fully spread. Subsequently, adherent cells were treated with primaquine (Sigma-Aldrich) for 30 minutes at 37°C then washed twice with ice-cold PBS on ice then labelled with 500 µl/30 mm dish ice-cold biotin (EZ-Link Sulfo-NHS-SS-Biotin (100 mg/vial); Cat. # 21331; Perbio Science (UK) Ltd.) dissolved in cold PBS (0.2 mg/ml). Biotinylation was carried out for 1 hour in cold room on rotator gently shaking. Biotin is a thiol-cleavable amine-reactive reagent and as such is able to covalently bind to proteins. Biotin only binds cell surface proteins as its negative charge does not allow the reagent to permeate cell membranes, once bound to proteins, however, biotin can be internalised and once inside the cell biotin binding is stable.

Following 1 hour incubation, any unbound biotin was washed off 2X with ice-cold PBS before stimulation with pre-warmed (37°C) migration buffer and used to stimulate cells for 15-20 minutes. Plates were then put back on ice and washed 2X with ice-cold PBS before adding 500 µl MesNa reagent (Sodium 2-mercaptoethanesulfonate (10 g/vial); Fluka product Cat. #63705-10G; Sigma-Aldrich) for 15 minutes at 4°C, except the total surface samples. MesNa is a membrane impermeant reducing agent able to remove the biotin from any proteins on the cells' surface which have not been internalised. The reducing potential of MesNa is then quenched with iodoacetimide (IAA) so as not to affect the internal biotin labelling upon cell lysis.

IAA=iodoacetamide (Iodoacetamide (25 g/vial); Fluka product Cat. # 57670-25G-F; Sigma-Aldrich) (0.37g in 10 ml water) was added 50 µl/500 µl MesNa for 10 minutes at 4°C on a shaker. Cells were then lysed in 100 µl NP40 lysis buffer following 2X wash with PBS, incubated on ice for minimum 10 minutes, spun at full speed for 5 minutes at 4°C before collection of lysates for capture ELISA.

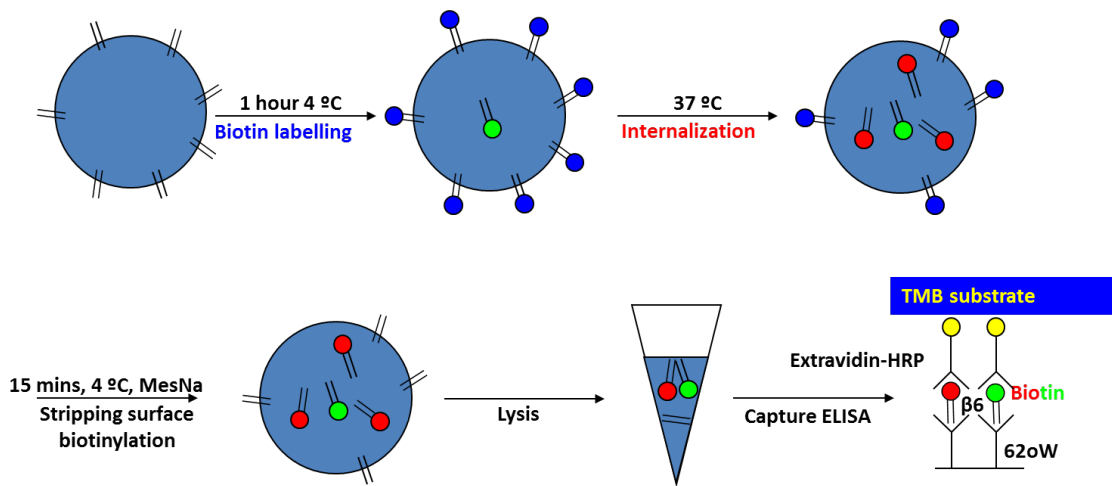


Figure 2.6 Biotinylation assay

Endocytosis assays require four key steps; firstly, labelling of cell surface integrins with Biotin, carried out at 4°C to inhibit integrin internalisation. Secondly; cell stimulation with specific extracellular matrix proteins at 37°C to support integrin internalisation. Thirdly, stripping of external Biotin on the cell surface so that just internalised proteins are left labelled and finally, lysis of cells and analysis of internalised biotin labelled proteins to quantify internalisation rates.

### 2.7.2 Capture ELISA

24 hours prior to performing the internalisation assay, Medisorp 96-well plates (MW 96F Medisorp, straight; Cat. #467320; NUNC) were coated with 5 µg/ml appropriate anti-integrin antibodies (62ow (home-made) for β6 internalisation assay and P5D2 (abcam) for β1 internalisation assay) in PBS and incubated overnight at 4°C (100 µl antibody dilution/well). After 24 hours incubation any unbound antibody was removed and the plate washed 3X with PBS and blocked with PBS containing 0.05% Tween-20 (PBS-T) and 5% BSA for 1 hour at room temperature (200 µl/well) then washed 3X in PBS-T. 50 µl of the cell lysate collected from the internalisation assay was then incubated on the plate at 4°C overnight. Following 24 hours incubation with primary antibody the cell lysate was removed from the plate and the plate washed 3X with PBS-T before incubating wells with streptavidin-conjugated horseradish peroxidase antibody (ExtrAvidin, peroxidase conjugate (1 ml/vial); Sigma-Aldrich Cat. #E-2886) diluted 1:1000 in PBS-T containing 1% BSA for 1 hour at 4°C (100 µl/well). Biotin binds to Streptavidin with extremely high affinity and once bound is extremely stable. The plate was washed again 3X with PBS-T then 1X with PBS. For detection, 100 µl TMB+ one-step substrate system (1 litre/bottle; Cat. #S1599; Dakocytomation) reagent was added per well for up to 15 minutes and read at 650 nm using a multi-plate reader.

## 2.8 Generation of a stable knockdown

For long-term *in vivo* experiments we produced a cell line with a stable Eps15 knockdown. Knockdown via transient transfection with siRNA lasts for approximately 7 days while transfection

with an shRNA produces a stable knockdown of your protein of interest by incorporating a hairpin shRNA into the cell's genome so that the knockdown is permanent. Generation of a stable knockdown required several steps. Briefly, we ordered 2 validated Eps15 MISSION shRNA Bacterial Glycerol Stock (Sigma-Aldrich) (Table 2.16) for which we had a paired control non-targeting MISSION vector (Sigma-Aldrich) and grew up these stocks in 125 ml LB broth with ampicillin (100 µg/ml) and grew up overnight. We then isolated the DNA using Qiagen MAXI-prep kit according to manufacturer's instructions. We then, transfected our expressed vectors into HEK239-T cells along with viral packaging system using Lipofectamine 3000. Conditioned media from cultured HEK239-T cells was then used to infect our cell line of choice; a metastatic liver adenoma cell line, SKHEP1, which were then cultured in puromycin (2 µg/ml) to isolate cells which had been successfully infected with the Eps15 shRNA so that they could be cultured and used in *in vivo* experiments.

Table 2.16 Eps15 MISSION shRNA Bacterial Glycerol Stock

shRNA	Sequence	Mean knockdown level
TRCN0000007980 (aka 7980)	CCGGCCCAGAATGGATTGGAAGTTTCTC GAGAAACTTCCAATCCATTCTGGGTTTTT	86%
TRCN0000007978 (aka 7978)	CCGGGCAGTGAAACAGCCAACCTTACTC GAGTAAGGTTGGCTGTTTCACTGCTTTTT	79%

### 2.8.1 Transfection of HEK239-T cells

HEK239-T cells were plated in 6-well plates at 50-70% confluency for efficient transfection with our shRNA. Cells were transfected with 7980 or 7978 Eps15 shRNA using Lipofectamine 3000 reagent according to manufacturer's instructions. Each shRNA was transfected with 2 mg of lentiviral packaging mix and 5 µl p3000 reagent. 24 hours post-transfection HEK239-T cells were re-plated into T75 flasks for culture and the supernatant was collected at day 3 and day 6 post-transfection to be used as a source of infection capable lentivirus. The virus were aliquoted and stored at -80°C in a leak proof plastic bag.

### 2.8.2 Infection of SKHEP1 cell line with Eps15 shRNA

SKHEP1 cells were plated in a 6-well plate at 50-70% for efficient infection. Following plating, cells were infected with the viral supernatant collected from HEK239-T cells previously (Section 2.8.1). Cells were infected with control vector; UT (Sigma-Aldrich; SHC012 MISSION pLKO.1-puro-CMV-TagRFP<sup>TM</sup> positive control), and the same control vector with inserts 7980, 7978 or a mix of both shRNAs (Sigma-Aldrich). Following 24 hours incubation the media on these cells was changed and at 48 hours post-infection cells were split into culture in 6 cm dishes. The cells were cultured with

2 µg/ml puromycin in order to kill any cells which did not contain the virus. Once all cells without the virus were killed, cells were cultured according to normal cell practice.

### 2.8.3 Infection of SKHEP1 clones with red vector

Eps15 knockdown efficiency was tested in all cells by Western blotting. Once knockdown was confirmed cell lines were infected with another lentivirus containing TagFP585 so that the cells would fluoresce red. The BD LSR Fortessa™ cell analyser was used first to detect TagFP585 expression in the PE-Texas Red channel. The Canto I cell analyser was next used to detect TagFP585 cells using the PE channel. This channel is the same as that used by the Aria II cell sorter and so it was necessary to confirm that the positive populations could be detected by the lasers found in this machine. Two clear populations could be seen and so cells were sorted with the FACS Aria II cell sorter. We were left with a control (UT) and Eps15 knockdown (7980) SKHEP1 populations that were ready for use *in vivo*.

### 2.8.4 In vivo experiments with stable Eps15 shRNA SKHEP1 cells

#### 2.8.4.1 Animal care

All animals were kept in the biomedical research facility at Southampton General Hospital (SGH) under Dr. Ruth French's project licence. For subcutaneous injections  $1 \times 10^6$  SKHEP1 cells were injected into each flank by Richard Reid.  $1.5 \times 10^6$  cells were suspended in 1:1 PBS:matrigel mix and injected into immunocompromised mice (CD1-nude strain). Nude mice were used so that no immune response would be mounted against the injected human cancer cells. These mice also lack hair, reducing the auto-fluorescence seen with the IVIS® imaging technique and Living Image® software. We chose SKHEP1 cell line with a view to performing orthotopic surgery on immune-compromised CD1 nude mice to analyse subsequent metastatic potential of control cells versus stable Eps15 knockdown cells. Unfortunately, the license which needed to perform orthotopic surgery was not renewed in time and so sub-cutaneous injection was carried out instead. Length and width of tumour sizes were made using an electronic calliper (kindly performed by Hollie Robinson). Tumour volume was calculated from these values using the formula  $V = ((W^2 \times L)/2)$  as described previously (Faustino-Rocha et al. 2013).

#### 2.8.4.2 Sample preparation

After 6 weeks growth the animals were culled (prior to reaching ethical limits of tumour burden). First the mice were imaged using the IVIS® imaging technique and Living Image® software to assess if any metastasis had occurred. For optimal and unbiased comparison, the unit of signal is set to average radiant efficiency (Fluorescence emission radiance per incident excitation power)



as recommended by the manufacturer. Additionally, the tumours were excised and fixed in 10% formaldehyde and processed to paraffin ready for any future staining (IHC).

## 2.9 Statistics

GraphPad Prism 6.0 software was used for the production of all graphs and statistical analysis including the calculation of mean and standard deviation of the mean of all repeats carried out. The number of repeats for each experiment is given in the description of the results. The analysis of two samples required an un-paired two-tailed t-test while the analysis of multiple samples required a one-way or two-way ANOVA. For analysis of a control sample against multiple other samples an ordinary one-way ANOVA was applied using Dunnett's multiple comparison's test while the comparison of multiple samples compared against each other required a two-way ANOVA using Tukey's multiple comparisons test. Significant statistical results are labelled with asterisks on each graph; P values less than 0.0001 are labelled with four asterisks (\*\*\*\*), P values less than 0.001 are labelled with three asterisks (\*\*\*), P values less than 0.01 are marked with two asterisks (\*\*) and one asterisk for P values less than 0.05 (\*). Results which did not differ significantly from the control group were described as non-significant and labelled 'ns'. For correlation analysis between CtBP1 and Eps15 expression in our human tissue microarrays (TMAs) a Chi squared test was performed on SPSS statistical software. A significant correlation is seen by linear-by-linear association of  $p < 0.01$ .



## Chapter 3: CtBPs regulate Eps15 expression

Cancer cells commonly metabolise glucose via aerobic glycolysis which is known to activate the metabolic sensors C-terminal binding proteins (CtBPs) (Kumar et al. 2002; Fjeld et al. 2003). A previous project demonstrated that CtBPs can promote integrin-dependent cell motility. This was modulated through suppression of the integrin adapter protein Talin-1, which inhibited integrin activation and cell adhesion (Chrzan 2014). Besides Talin-1, a gene array also found a number of other proteins regulated by CtBPs. One of the genes down-regulated upon CtBP1 knockdown was the epidermal growth factor receptor substrate 15 (*EPS15*). Eps15 is a crucial scaffold protein involved in clathrin-mediated endocytosis of cell surface receptors, including epidermal growth factor receptor (EGFR), and integrins (van Bergen En Henegouwen 2009; Benmerah et al. 1998; Salcini et al. 1999), therefore this preliminary data proposed a potential link between metabolism and clathrin-dependent integrin endocytosis (Chrzan 2014). Furthermore, due to the known link between Eps15 and clathrin-mediated endocytosis of receptors, we hypothesised that Eps15-dependent integrin endocytosis could play a role in the regulation of cell motility. Indeed, the importance of clathrin-mediated endocytosis in cell adhesion and consequent migration has been highlighted in the literature (A. G. Ramsay et al. 2007; Rappoport 2003). While the clathrin-mediated endocytosis of  $\beta 1$  integrin (Jović et al. 2007; Ezratty et al. 2009) is the most researched, a study by Ramsay *et al* has found that the integrin  $\alpha v\beta 6$  is also internalised via a clathrin-mediated route (A. G. Ramsay et al. 2007). Ramsey *et al* observed that inhibition of clathrin-mediated endocytosis of  $\alpha v\beta 6$  significantly decreased oral squamous cell carcinoma migration (A. G. Ramsay et al. 2007). Other authors also found that impaired clathrin-mediated endocytosis of  $\beta 1$  integrins led to stronger cell adhesion and subsequently a reduction in cell migration (Ezratty et al. 2009; Jović et al. 2007). Therefore, we hypothesise that CtBPs could regulate cell motility by up-regulation of Eps15 in cancer cells thereby allowing increased integrin endocytosis and therefore increased tumour cell motility.

### 3.1 Eps15 expression in a selection of cell lines investigated

Firstly, we wanted to confirm that CtBPs regulate Eps15 expression in cancer cells both at the mRNA and at the protein level, therefore we screened a range of cancer cell lines of different origin for Eps15 expression. Six cell lines were investigated; four oral squamous cell carcinoma (H357, VB6, BICR6 and SCC25), a metastatic colorectal adenocarcinoma (SW620) and a breast cancer cell line (MCF7).

All cell lines examined expressed Eps15 at varying levels, with H357, VB6 and BICR6 having the highest basal expression (Figure 3.1). Antibodies recognising the Eps15 gene product have been shown previously to detect a 142 kDa and an additional 155 kDa version of the protein and Fazioli and co-workers suggested that the 155 kDa component is a result of EGF-induced post-translational modification, including phosphorylation at a higher stoichiometry compared to the 142 kDa component (Fazioli et al. 1993). All cell lines expressed 142 kDa Eps15, with a 155 kDa band present in BICR6, H357 and VB6 cells (Figure 3.1). The cell lines all expressed CtBP1 and CtBP2; CtBP1 expression appeared more uniform in its expression across the cell lines, with more variable CtBP2 expression (Figure 3.1). CtBP1 appears as a single band despite the availability of two isoforms; CtBP1-S and CtBP1-L. The band seen is CtBP1-S as it is the predominantly expressed isoform of the two and is usually the only isoform able to be distinguished via Western blotting techniques as CtBP1-L is so minimally expressed and the two differ by just 11 amino acids in size. On the other hand, CtBP2 appears as two distinct bands, representing both isoforms of CtBP2; CtBP2-S and CtBP2-L (Birts et al. 2010).

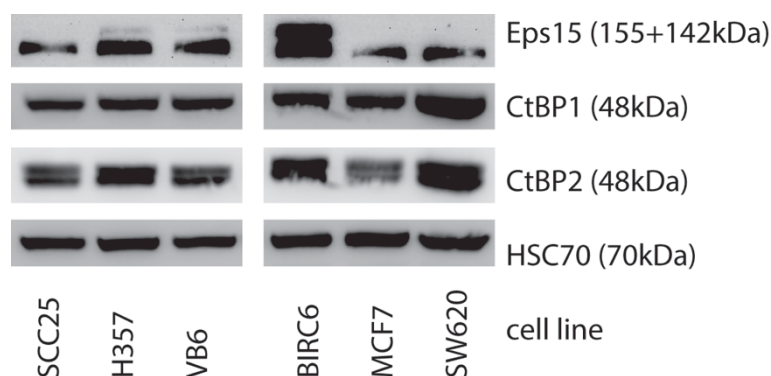


Figure 3.1 Endogenous Eps15 expression varies between cell lines

Eps15 expression was analysed using whole cell lysates from confluent cultures and running 40µg protein of each cell line on SDS-PAGE followed by Western blotting. Eps15 is expressed in all six cell lines investigated. H357, VB6 and BICR6 cell lines appear to have the highest basal levels of Eps15. All cell lines expressed CtBP1 and 2. HSC70 was used as a loading control.

### 3.2 CtBP1 and CtBP2 siRNA optimisation

We had previously used and optimised siRNAs from Qiagen for both CtBP1 and CtBP2 (Chrzan 2014). We began by optimising these siRNAs again in the squamous carcinoma cell line; SCC25, to confirm that set-up for these siRNAs is still the same (Figure 3.2A). The siRNAs for CtBP1 and CtBP2 were tested at increasing concentrations, over three time-points 24, 48 and 72 hours (Figure 3.2A). Both siRNAs showed good knockdown at 48 hours post-transfection with a similar level of knockdown maintained at 72 hours (Figure 3.2A). The knockdown effect was observed even at the lower concentration of 10 nM. Although the 72 hour time-point appeared best for the CtBP2 siRNA, most functional assays were performed at the 48 hour time-point and terminated at

72 hours. Both siRNAs were optimal at the lower 10 nM concentration so transfections were carried out using this concentration.

To exclude off-target effects of our chosen siRNA we purchased validated gene pool siRNAs from Qiagen and tested their effect on knockdown of our target proteins; CtBP1 and CtBP2. Two important aspects of the siRNA were studied; knockdown efficiency of the target CtBP protein and the consequent effect on Eps15 expression. The siRNAs labelled CtBP1 and CtBP2 are those used previously by our group and optimised in Figure 3.2A (Chrzan 2014) and the siRNAs labelled CtBP1\_5-8 and CtBP2\_7-10 were our validated gene pool siRNAs (Table 2.12). All CtBP1 siRNAs produced an efficient knockdown of CtBP1 protein which resulted in a consequent decrease in Eps15 expression compared to control (non-targeting) siRNA treated cells, with the most prominent effect occurring with the previously used CtBP1 siRNA sequence (Figure 3.2B) (Chrzan 2014). Statistical analysis revealed a significant decrease in relative Eps15 mRNA expression following treatment with CtBP1. A decrease was also seen following treatment with some of the other CtBP1 siRNA sequences but the effect was not significant (Figure 3.2B). The most efficient knockdown of CtBP2 protein occurred with the previously used CtBP2 siRNA sequence (Figure 3.2C) (Chrzan 2014). The effect on Eps15 expression was not consistent and no effect was observed using the previously used CtBP2 siRNA sequence (Figure 3.2B), which corresponds with the original gene array results (Chrzan 2014), where only CtBP1 knockdown showed down-regulation of Eps15. CtBP2, CtBP2\_7, CtBP2\_9 and CtBP2\_10 had no significant effect on Eps15 relative mRNA expression compared to control siRNA while CtBP2\_8 decreased Eps15 mRNA expression significantly compared to control siRNA. Given these results the original CtBP1 and CtBP2 siRNA from Qiagen were used for all RNAi experiments to follow as these siRNA sequences produced the most efficient knockdown of their retrospective target proteins and showed a consistent effect on Eps15.

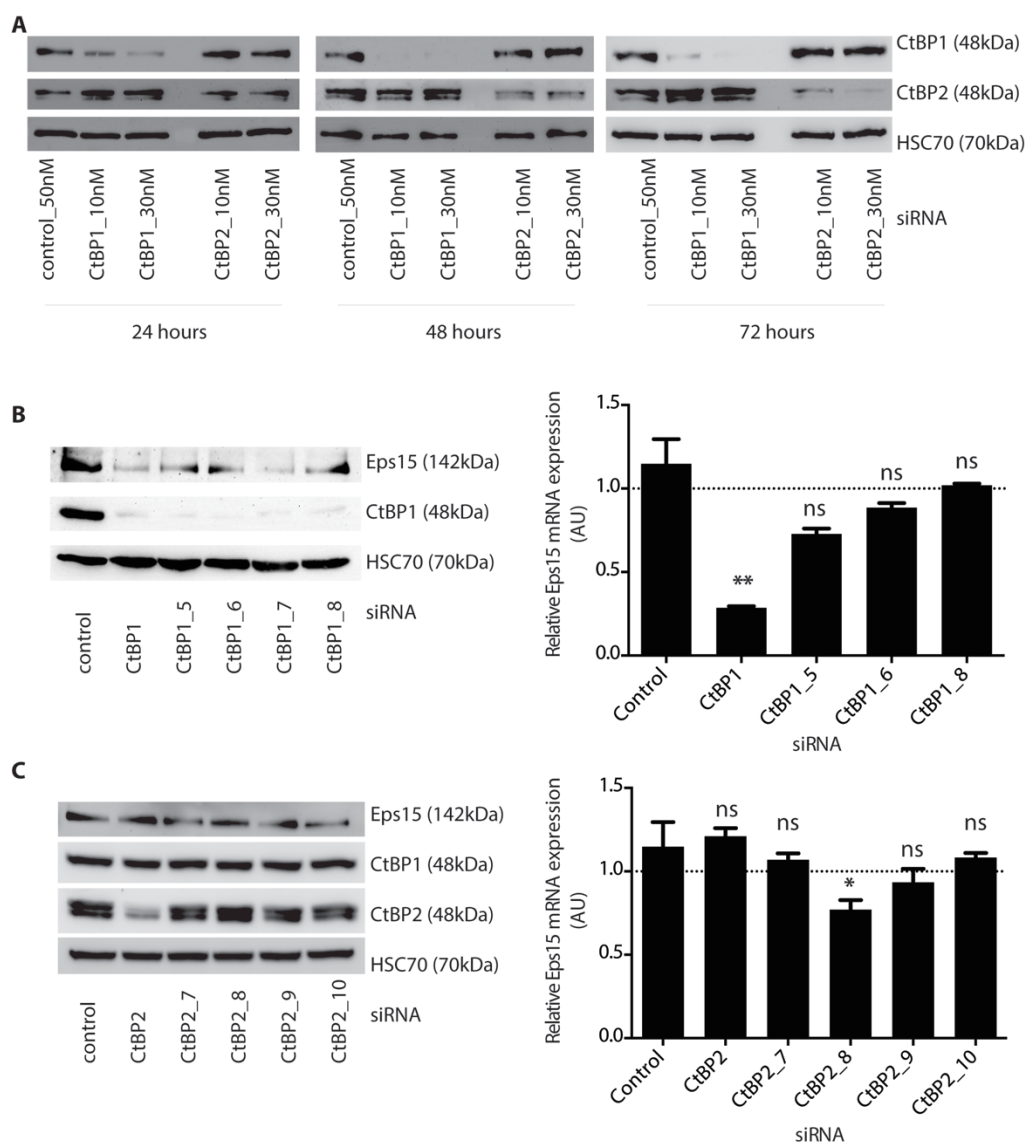


Figure 3.2 CtBP1 and CtBP2 siRNA optimisation

(A) CtBP1 and CtBP2 expression was analysed using whole cell lysates from SCC25 cell cultures transfected with siRNA (Qiagen). Equal amounts of protein were run on SDS-PAGE followed by Western blotting. Both CtBP1 and CtBP2 siRNA work optimally at a concentration of 10 nM, with maximal knockdown occurring at 48 hours post-transfection and continued effect at 72 hours. HSC70 was used as a loading control (B-C) SCC25 cells were transfected with one siRNA sequence and cells were collected 48 hours post-transfection for analysis and 40 µg of protein was electrophoresed. For mRNA analysis; RNA extraction, cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit and TaqMan® RT-PCR were carried out to quantify Eps15 mRNA in each condition. Graphs show mean and standard error of the mean (SEM) of two technical repeats (n=2). Data is normalised to beta-actin and to Eps15 mRNA expression of control lysates (AU = arbitrary unit) (B) CtBP1 siRNAs all showed efficient knockdown of CtBP1 protein while the previously used CtBP1 siRNA showed the most prominent effect on consequent Eps15 expression. mRNA data supports the conclusions of the protein data. One-way ANOVA statistical analysis revealed a decrease in relative Eps15 mRNA expression following treatment with CtBP1 siRNA in comparison to control siRNA ( $p < 0.01$ ). CtBP1\_5, CtBP1\_6 and CtBP1\_8 also produce a decrease in Eps15 mRNA expression but the effect is not significant. (B) The most efficient CtBP2 knockdown was seen with the previously used CtBP2 siRNA but effect on Eps15 expression was inconsistent. Equal loading was confirmed by HSC70. CtBP2, CtBP2\_7, CtBP2\_9 and CtBP2\_10 have no significant effect on Eps15 relative mRNA expression compared to control siRNA while CtBP2\_8 shows a significant decrease in Eps15 mRNA expression compared to control siRNA ( $p < 0.05$ ).

### 3.3 CtBP1 knockdown decreases Eps15 expression

#### 3.3.1 CtBP1 knockdown decreases Eps15 protein expression of cancer cell lines of different origin

Our data showed that CtBP1 knockdown caused a reduction in the transcription of Eps15, shown via microarray (Figure 1.10) (Chrzan 2014). Results obtained during the optimisation of the CtBP siRNA transfection in SCC25 cells confirmed this finding and showed that a similar decrease also occurs at the protein level (Figure 3.2). To examine whether the decrease in Eps15 expression following down-regulation of CtBP1 was cell line specific or a general phenomenon we examined the effect of CtBP1, CtBP2 and CtBP1+2 knockdown on Eps15 expression in a range of cancer cell lines of different origin. Upon CtBP1 and CtBP1+2 RNAi, the protein expression of Eps15 decreased over time, most significantly at 48 and 72 hours post-transfection in SCC25, H357, SW620, BICR6 and MCF7 cell lines (Figure 3.3). The cell line VB6 (Figure 3.4) showed the same prominent decrease of Eps15 following CtBP1 and CtBP1+2 RNAi but only at 96 hours post-transfection. This could be due to differences in the speed of protein turnover between different cell lines. Talin-1 was used as a positive control for some cell lines as previous data in our group had shown Talin-1 levels increase following CtBP1 and CtBP2 knockdown (Chrzan 2014). In summary, these results show that CtBP1 regulates Eps15 protein expression in all six cell lines tested therefore the effect of CtBP1 on Eps15 expression appears to be a general phenomenon across different tumour types.

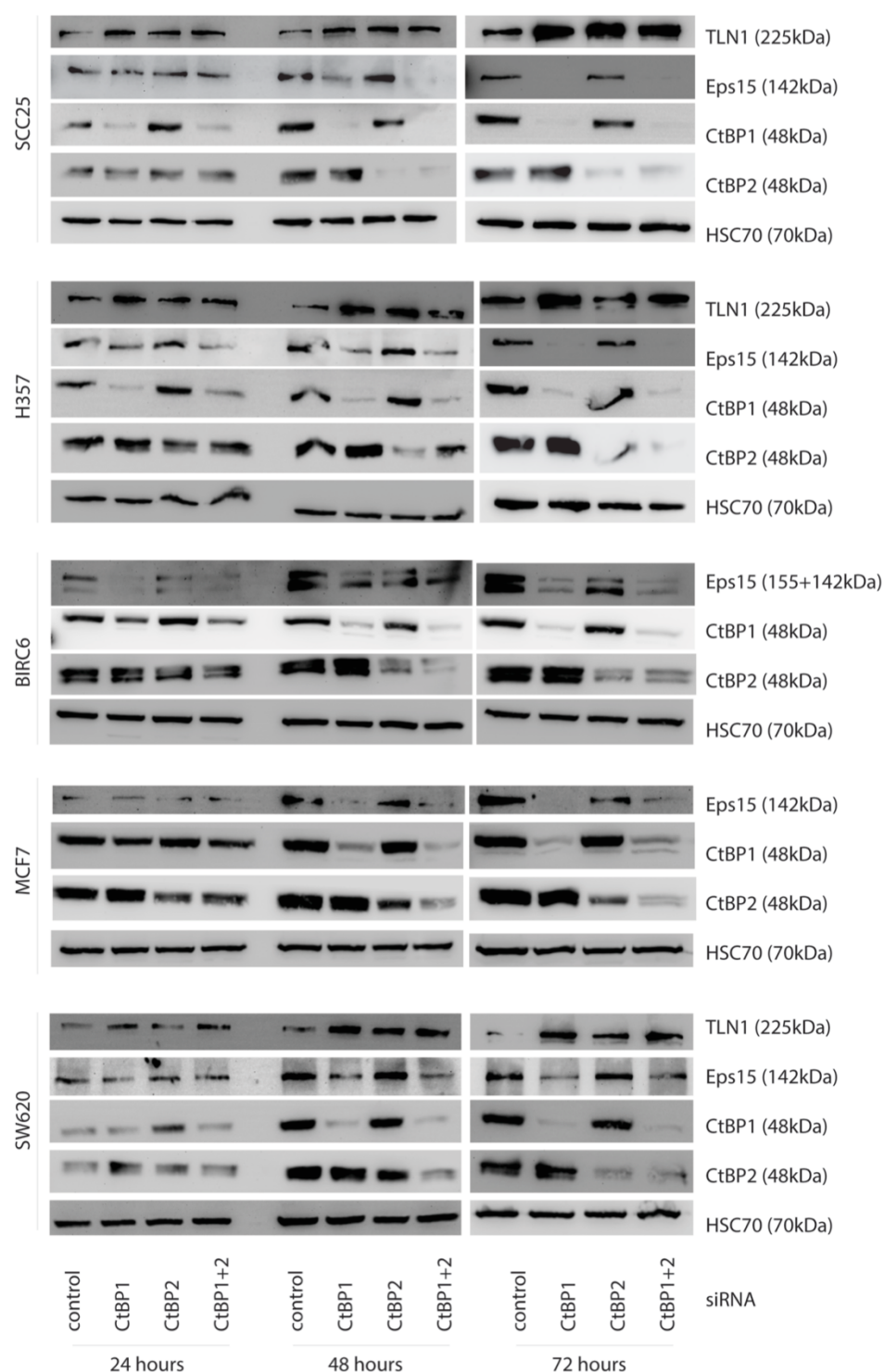


Figure 3.3 CtBP1 knockdown decreases Eps15 protein expression up to 72 hours post-transfection. Eps15 expression was analysed using whole cell lysates collected at 24 hours, 48 hours and 72 hours post-transfection with CtBP1, CtBP2 and CtBP1+2 siRNA then running 40µg of protein on SDS-PAGE followed by Western blotting. CtBP1 and CtBP1+2 knockdown decreased basal Eps15 protein expression at 48 hours post-transfection and most dramatically at 72 hours post-transfection in SCC25, H357, SW620, BICR6 and MCF7 cell lines. HSC70 was used as a loading control while Talin-1 (TLN1) was used as a positive control as previous research within our group showed that CtBP knockdown increases Talin-1 protein expression. It is confirmed here that with CtBP knockdown Talin-1 protein expression increases, most prominently with CtBP1 knockdown.



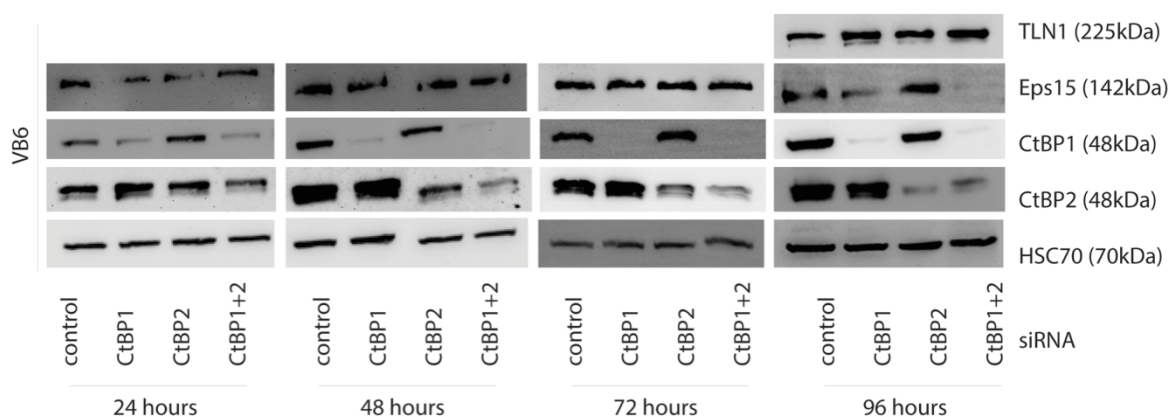


Figure 3.4 CtBP1 knockdown decreases Eps15 protein expression 96 hours post-transfection. Eps15 expression was analysed using whole cell lysates collected at 24 hours, 48 hours, 72 hours and 96 hours post-transfection with CtBP1, CtBP2 and CtBP1+2 siRNA then running 40µg of protein on SDS-PAGE followed by Western blotting. CtBP1 and CtBP1+2 knockdown decreased basal Eps15 protein expression but the effect is noticed at the later time-point of 96 hours post-transfection in VB6 cell line. HSC70 was used as a loading control while Talin-1 (TLN1) was used as a positive control as previous research within our group showed that CtBP knockdown increases Talin-1 protein expression. It is confirmed here that with CtBP knockdown Talin-1 protein expression increases, most prominently with CtBP1 knockdown.

### 3.3.2 CtBP1 knockdown decreases Eps15 mRNA transcription, possibly directly

To investigate whether the effect of CtBP1 down-regulation on Eps15 protein expression was direct or indirect, as a result of growth arrest or similar, we performed a quantitative TaqMan® RT-PCR time-course to measure the expression of Eps15 mRNA following CtBP1, CtBP2 and CtBP1+2 knockdown over time. Transfection with CtBP1 and combined CtBP1+2 siRNA produced the same negative effect on the transcription of Eps15 (Figure 3.5) as on the translation of Eps15 (Figure 3.3-Figure 3.4). Treatment with CtBP1 and CtBP1+2 siRNA caused approximately -0.5 fold overall change in Eps15 mRNA expression in the three cell lines tested; H357 (Figure 3.5A), SW620 (Figure 3.5B) and SCC25 (Figure 3.5C-D). Overall, Eps15 mRNA expression appears to have a +1 fold change as a consequence of CtBP2 knockdown with a possible decrease at earlier time-points. This data shows that the metabolic sensors CtBP1 and CtBP2 have an effect on Eps15 mRNA transcription and that the effect occurs after a relatively short timescale, suggesting the effect is direct. To further confirm these results and to exclude potential off-target effects a second set of CtBP siRNA from Ambion were also used to transfect cells (Figure 3.5D). Ambion siRNA showed the same negative effect on Eps15 as treatment with Qiagen siRNAs (Figure 3.5A-C). This result strengthens our conclusion that CtBP1 knockdown causes a decrease in Eps15 expression at the level of transcription and that this effect is most likely direct, while the effect seen by CtBP2 is less consistent and less prominent. Following these observations, all subsequent investigations focused on the effect of CtBP1 on Eps15.

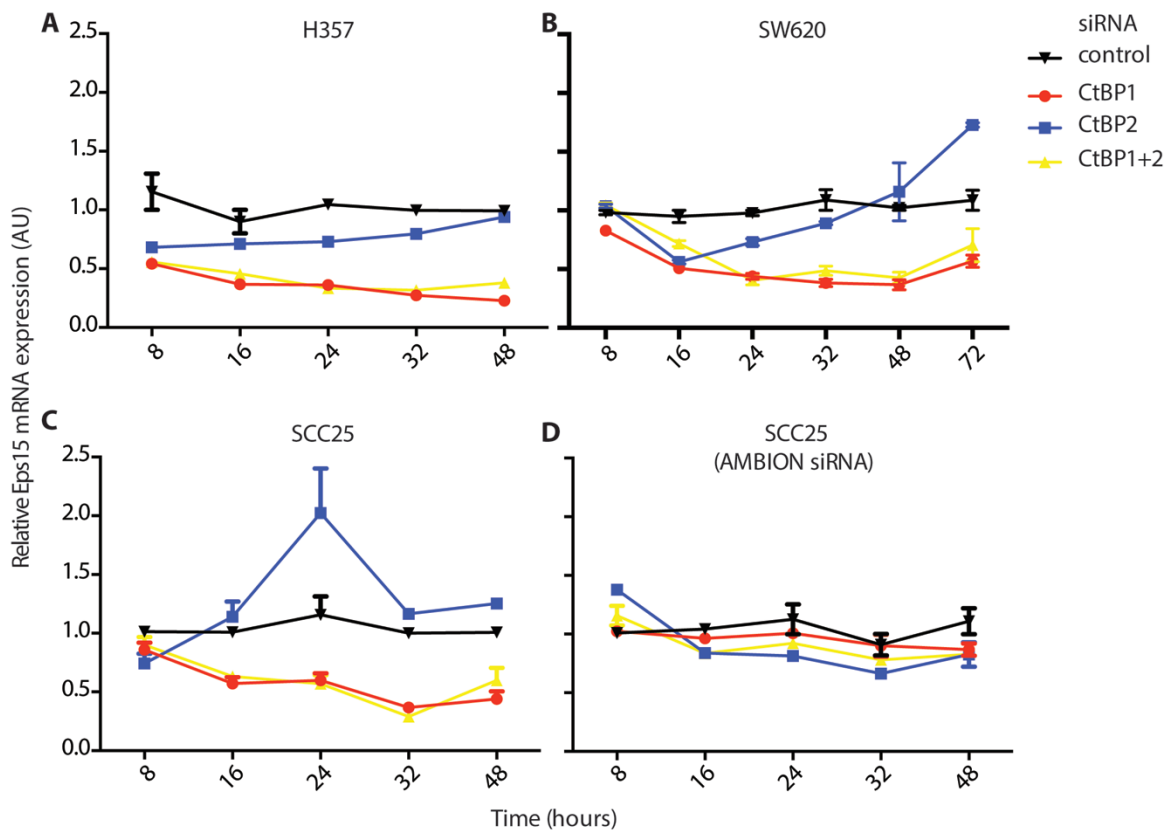


Figure 3.5 CtBP1 knockdown decreases Eps15 mRNA expression

Cells were transfected with CtBP1 and CtBP2 siRNA individually and combined and collected over a 48 hour period post-transfection at 8, 16, 24, 32, 48 and 72 hours. Following RNA extraction and cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit, TaqMan® RT-PCR was carried out to quantify Eps15 mRNA expression in each condition. Graphs show mean and standard error of the mean (SEM) of two technical repeats (n=2). Data is normalised to beta-actin and to Eps15 mRNA expression of control lysates (AU = arbitrary unit). CtBP1 and CtBP1+2 treatment decreased Eps15 mRNA expression while CtBP2 treatment appeared to increase Eps15 expression. The maximum effect occurred relatively quickly after just 16 hours.

### 3.4 CtBP1 has a direct effect on Eps15 expression

CtBP1 is a known transcriptional regulator (Nardini et al. 2003; Kumar et al. 2002; Kim et al. 2005) and the speed at which CtBP1 knockdown affects Eps15 expression suggests a direct positive effect by CtBP1 on the promoter region of Eps15, rather than an indirect effect. Notably, CtBP1 is most well-known for being a transcriptional repressor while here, the negative effect of CtBP1 knockdown on Eps15 expression suggests that CtBP1 is acting as a transcriptional activator of Eps15. In a small number of studies, CtBPs have previously been shown to be involved in transcriptional activation of a number of other proteins (Phippen et al. 2000; Paliwal et al. 2012). In order to assess whether the positive effect of CtBP1 on Eps15 expression is direct a Chromatin Immunoprecipitation (ChIP) assay was carried out (Figure 3.7).

ChIP is a technique used to study the association of certain proteins with specific regions of the genome, such as the association of CtBP1 protein with the promoter region of Eps15.

Immunoprecipitation of sheared chromatin was performed using polyclonal anti-CtBP antibody (H440; Santa Cruz) and in total six primer pairs spanning the entire promoter region of Eps15 were used to amplify any Eps15 DNA potentially bound to CtBP1. A schematic of the Eps15 promoter region showing the location of all primer pairs used from E1-E6 is given in Figure 3.6. E2 is not shown in the ChIP PCR results (Figure 3.7) as this primer pair did not produce any bands, even with input control samples. ChIP analysis revealed that the effect of CtBP1 on Eps15 expression was in fact direct (Figure 3.7). SCC25 cell lysates were used and CtBP1 protein was pulled down attached to at least three points on the Eps15 promoter region (Figure 3.7). A clear band is seen with primer regions E3, E4, and E6. A faint band can also be seen with E5. These bands suggest that CtBP1 binds the Eps15 promoter at the E3-E6 region.

The E-cadherin primer pair (ECAD) was used as a positive control as a protein known to be associated with CtBPs (Paliwal et al. 2012). Beads coated with CtBP antibody (H440) consistently pulled down this region of E-cadherin. Two concentrations of input control (IC) were investigated; IC 1/30 and IC 1/90 as a marker of PCR saturation. Additionally, these concentrations allow crude quantification of our pull-down bands.

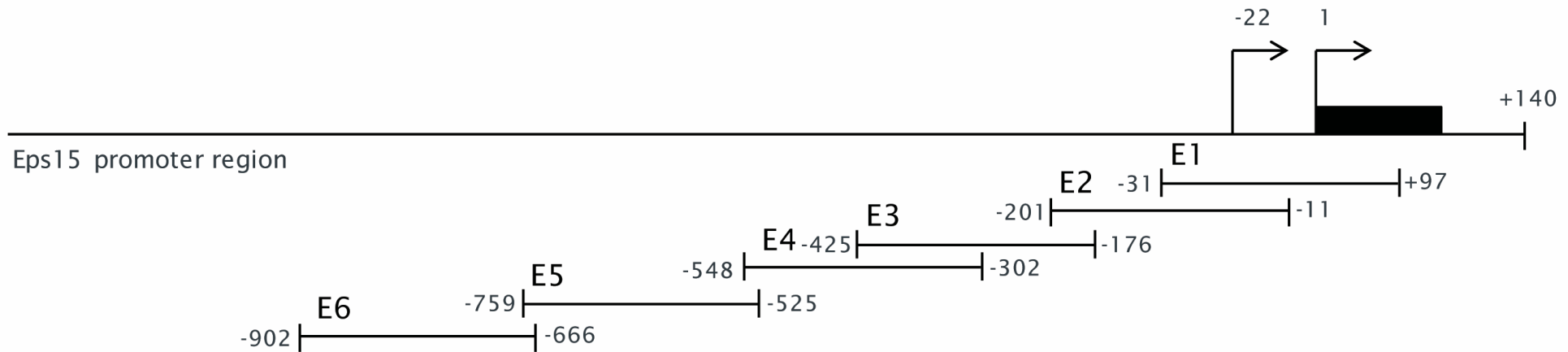


Figure 3.6 Schematic of the Eps15 promoter region

Primer pairs spanning the Eps15 promoter region were used to amplify the DNA pulled down via ChIP analysis. In total six primer pairs ranging between 129-250bp in length were used to account for the entire promoter region. Each pair had a short overlapping section with the next primer pair to ensure the entire promoter region was accounted for.

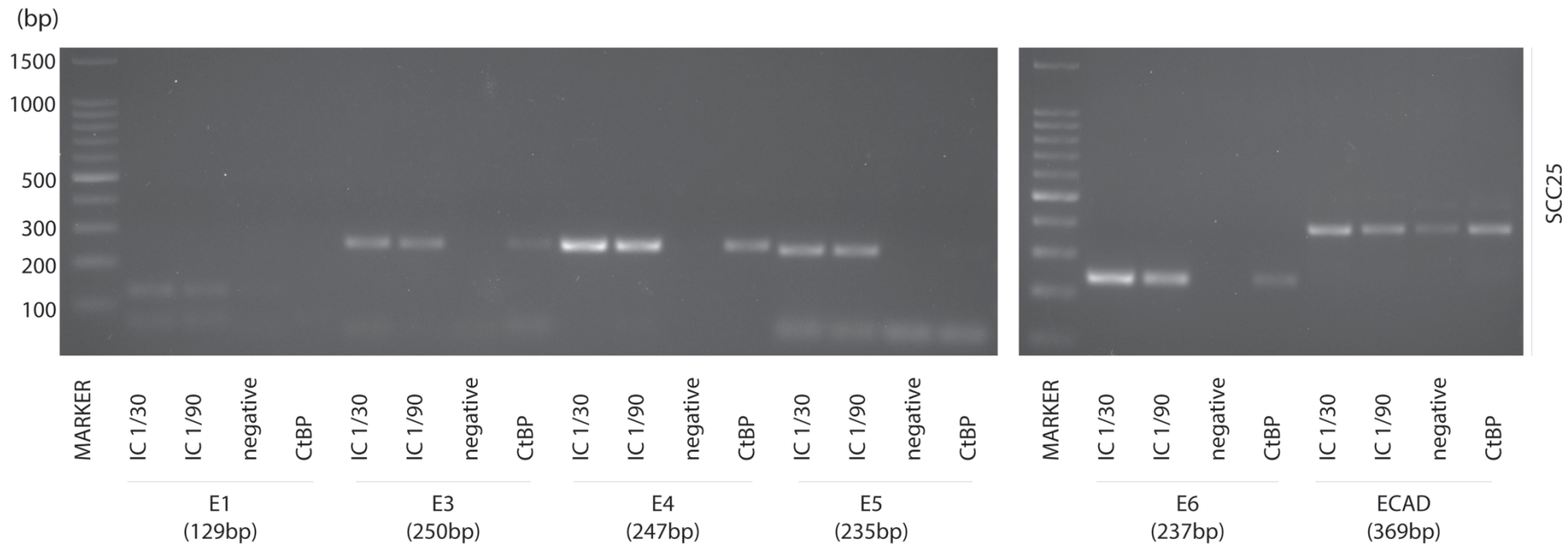


Figure 3.7 CtBPs directly affect Eps15 expression

Chromatin immunoprecipitation (ChIP) was carried out using SCC25 cell lysates in which first the protein:DNA interactions were crosslinked with formaldehyde. Next, the chromatin was sheared into smaller fragments and incubated with A/G agarose beads labelled with either IgG control antibody or our antibody of interest; polyclonal CtBP (H440). Following incubation, we un-crosslinked the pulled protein from the DNA attached to remove the DNA for subsequent PCR amplification. PCR amplification of the DNA was performed with primer pairs corresponding to six sections spanning the Eps15 promoter and results were run on an agarose gel. Results show a clear pulldown in at least three sites on the promoter region; E3, E4 and E6, suggesting a direct positive effect of CtBP on Eps15 transcription. Final cell counts for each IP reaction was approximately 100,000 cells. Input control 1/30 (IC 1/30) was therefore approximately 3333 cells per reaction while input control 1/90 (IC 1/90) was approximately 1111 cells per reaction. All samples were run on one gel but were run on two lines as each comb used to create sample wells had a maximum 20 wells. Thus, the gel was cut in the middle; E1-E5 were run on the top half of the agarose gel while E6 and ECAD were run on the bottom half of the gel.

3.5 Eps15 expression does not affect CtBP1 expression

Eps15 is almost exclusively known as an adapter protein in clathrin-mediated endocytosis. However, recently a few papers have suggested the possibility that certain endocytic proteins can also regulate transcription. Miaczynska *et al* discuss the role of compartmentalisation in control of signalling pathways in the cell, and discuss that the transcription of some proteins might be inhibited by increased endocytosis of signalling receptors that activate their transcription (Miaczynska *et al.* 2004). Additionally, Vecchi *et al* suggested that Eps15 itself could act as a positive modulator of transcription (Vecchi *et al.* 2001). In the previous chapters we have shown that CtBP1 regulates expression of Eps15 in cancer cells (Figure 3.3-Figure 3.4). As positive or negative feedback loops can exist between two proteins we wanted to investigate whether down-regulation of Eps15 using RNA interference could also affect expression of CtBP1.

To this end we optimised transfection with three unrelated Eps15 siRNA sequences (Figure 3.8). It's clear that transfection of our cells with Eps15 sequence 5 and 7 results in a more efficient knockdown of the target protein than that of sequence 1 (Figure 3.8). Eps15\_5 and Eps15\_7 siRNAs showed inhibition of Eps15 expression as early as 24 hours post-transfection and a complete knockdown was achieved at 48 hours, which was still evident 72 hours post-transfection. The lower concentration of 10 nM siRNA was as effective at knocking down Eps15 as the 30 and 50 nM concentrations, therefore 10 nM siRNA was chosen to be used in all functional assays (such as Transwell<sup>®</sup> migration, Matrigel<sup>®</sup> invasion assays and xCELLigence RTCA adhesion), which were set up 48 hours post-transfection.

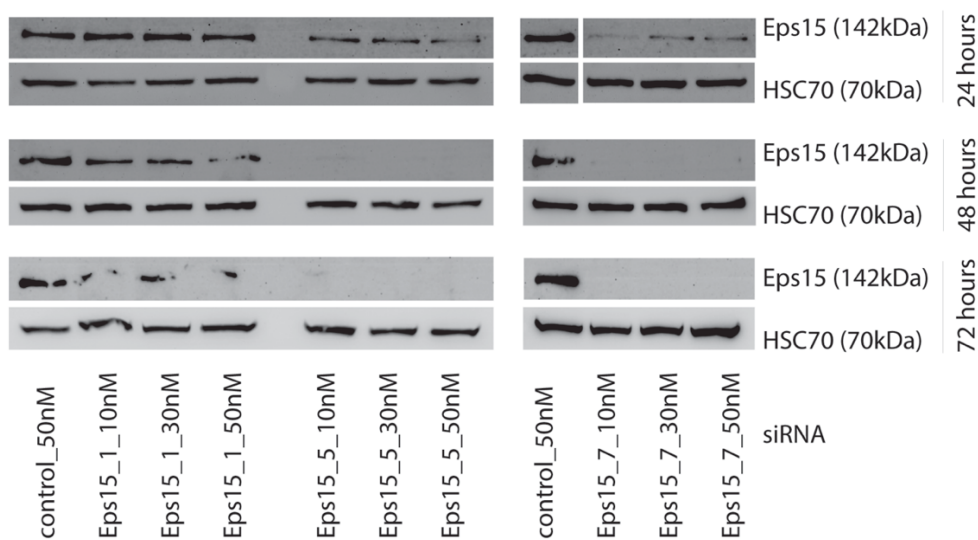
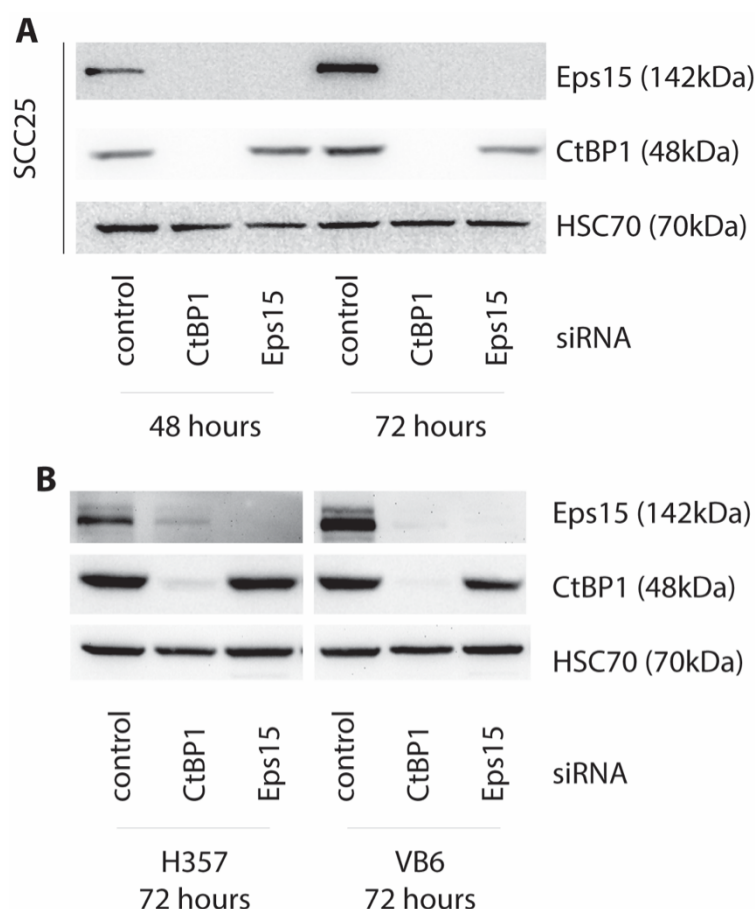


Figure 3.8 Eps15 siRNA optimisation  
Eps15 expression was analysed using whole cell lysates from SCC25 cell cultures transfected with different sequences of Eps15 siRNA and running 40µg of protein on SDS-PAGE followed by Western blotting. Eps15\_5 and Eps15\_7 show a more efficient knockdown than that of Eps15\_1

sequence. Sequence 5 and 7 also work optimally at just 10 nM concentration with optimal knockdown occurring at 48 hours post-transfection and continuing even at 72 hours post-transfection. HSC70 was used as a loading control.

### 3.5.1 Eps15 does not affect CtBP1 protein expression

Following siRNA optimisation Eps15\_5 siRNA was used to test the effect of Eps15 knockdown on CtBP1 protein expression (Figure 3.9). Figure 3.9 shows the effect of Eps15 knockdown on CtBP1 protein levels in SCC25 cell line at both 48 hours and 72 hours post-transfection and H357 and VB6 cells at 72 hours post-transfection. No consistent effect of Eps15 knockdown on CtBP1 protein expression was observed in any cell line, at either time-point.



**Figure 3.9 Eps15 does not affect CtBP1 protein expression**  
Eps15 and CtBP1 protein expression was analysed using whole cell lysates collected from (A) SCC25 cells at 48 hours and 72 hours post-transfection and (B) H357 and VB6 cells at 72 hours post-transfection with CtBP1 and Eps15 siRNA then running 40µg of protein on SDS-PAGE followed by Western blotting. CtBP1 knockdown decreased basal CtBP1 levels as expected as well as Eps15 protein expression, as seen previously (Figure 3.3). Eps15 knockdown also decreased basal Eps15 levels as expected while no obvious change in CtBP1 basal levels is seen. HSC70 was used as a loading control.

### 3.5.2 Eps15 knockdown does not affect CtBP1 mRNA expression

While it is clear that Eps15 has no effect on CtBP1 protein expression (Figure 3.9), we also looked at the effect of Eps15 knockdown on CtBP1 mRNA transcription. Previously, the housekeeping gene used for RT-PCR analysis had been beta-actin however, we observed that Eps15 knockdown had a negative effect on beta-actin levels (Figure 3.10). Eps15 knockdown causes a prominent decrease in beta-actin levels shown in both H357 and VB6 (Figure 3.10) cell lines. Optimisation of a new housekeeping gene for RT-PCR quantification was necessary and so the effects of Eps15 on ubiquitin were assessed. The CT values obtained for ubiquitin were less affected by Eps15 knockdown than beta-actin and so was chosen as the housekeeping gene for all future RT-PCR involving Eps15 knockdown.

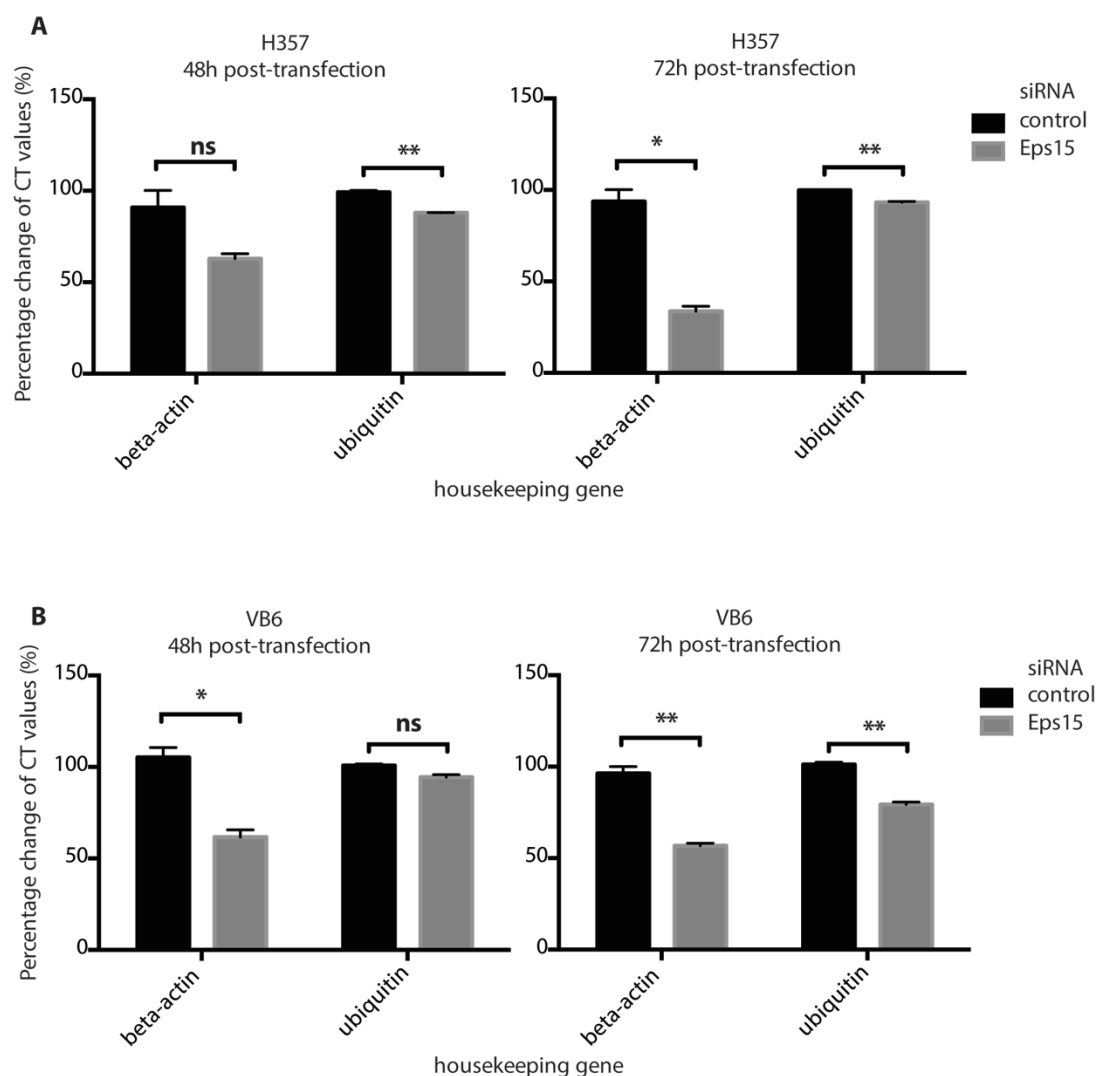


Figure 3.10 Eps15 knockdown affects beta-actin housekeeping gene

Cells were transfected with control and Eps15 siRNA and collected at 48 hours and 72 hours post-transfection. Following RNA extraction and cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit, TaqMan® RT-PCR was carried out to quantify beta-actin and ubiquitin mRNA expression in each condition. Change in housekeeping CT values as a result of Eps15 siRNA are



normalised to control CT values. Eps15 caused prominent and consistent reduction in beta-actin levels at both 48 hours and 72 hours post-transfection in both (A) H357 (48 hours, beta-actin;  $p=ns$ , ubiquitin;  $p<0.01$ , 72 hours, beta-actin;  $p<0.05$ , ubiquitin;  $p<0.01$ ) and (B) VB6 cells 48 hours, beta-actin;  $p<0.05$ , ubiquitin;  $p=ns$ , 72 hours, beta-actin;  $p<0.01$ , ubiquitin;  $p<0.01$ ). Ubiquitin was less affected by Eps15 siRNA treatment, despite significance values. Graphs showing the effect of control and Eps15 siRNA on beta-actin and ubiquitin CT values shown as percentage change (%). Values are calculated from two technical repeats in each experiment ( $n=2$ ).

Using ubiquitin as the housekeeping gene ensured that any differences in relative CT values seen with RT-PCR were a result of Eps15 knockdown on our gene of interest and not due to changes in housekeeping gene. There is no consistent significant effect of Eps15 knockdown on CtBP1 at the mRNA level across three cell lines (Figure 3.11). A time-course of early time-points revealed no significant changes in CtBP1 expression as a consequence of Eps15 knockdown compared with control cells until 48 hours post-transfection (Figure 3.11A). Because of this significant increase in CtBP1 seen at 48 hours we decided to look at longer time-points post-transfection. Once again we saw no consistent, significant difference of Eps15 knockdown on CtBP1 expression (Figure 3.11B-D). From these results we concluded that while CtBP1 knockdown consistently significantly decreases Eps15 expression, this effect does not create a positive feedback loop. Eps15 knockdown does not consistently, significantly effect CtBP1 expression.

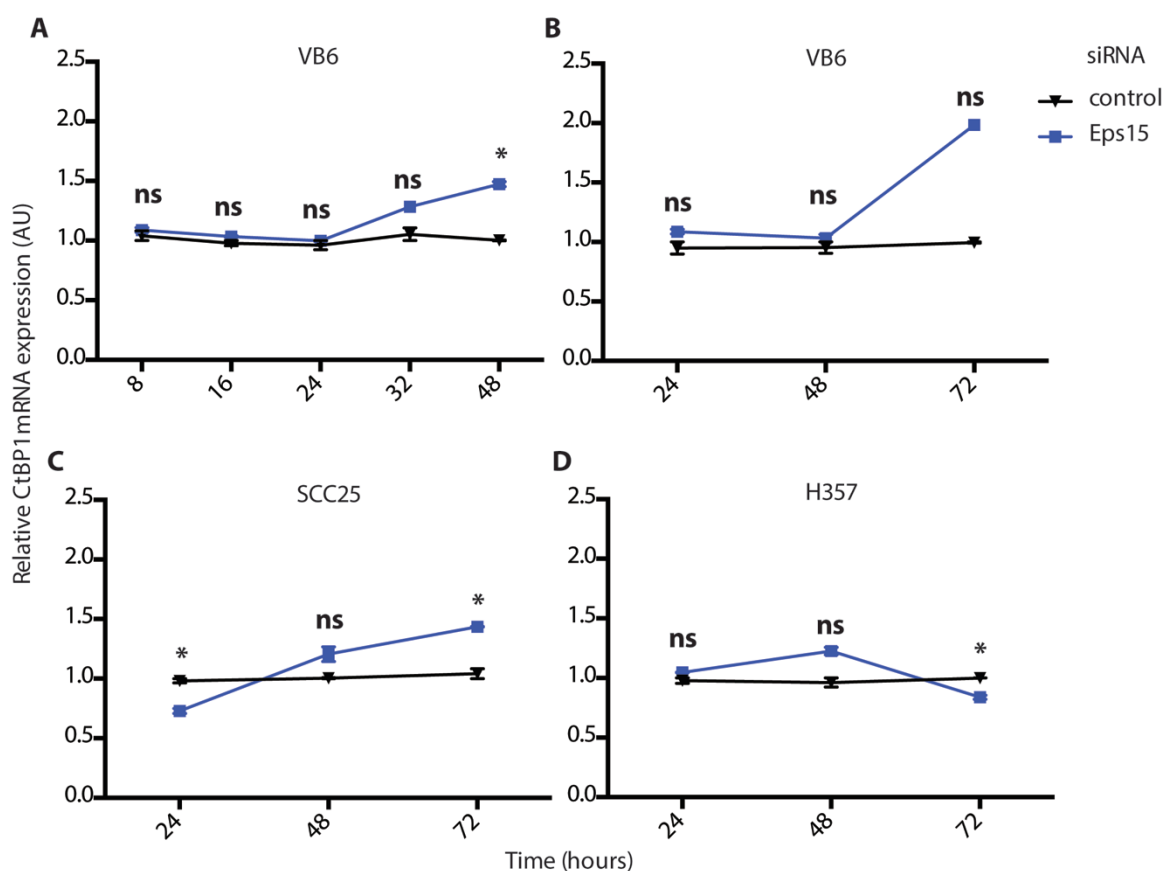


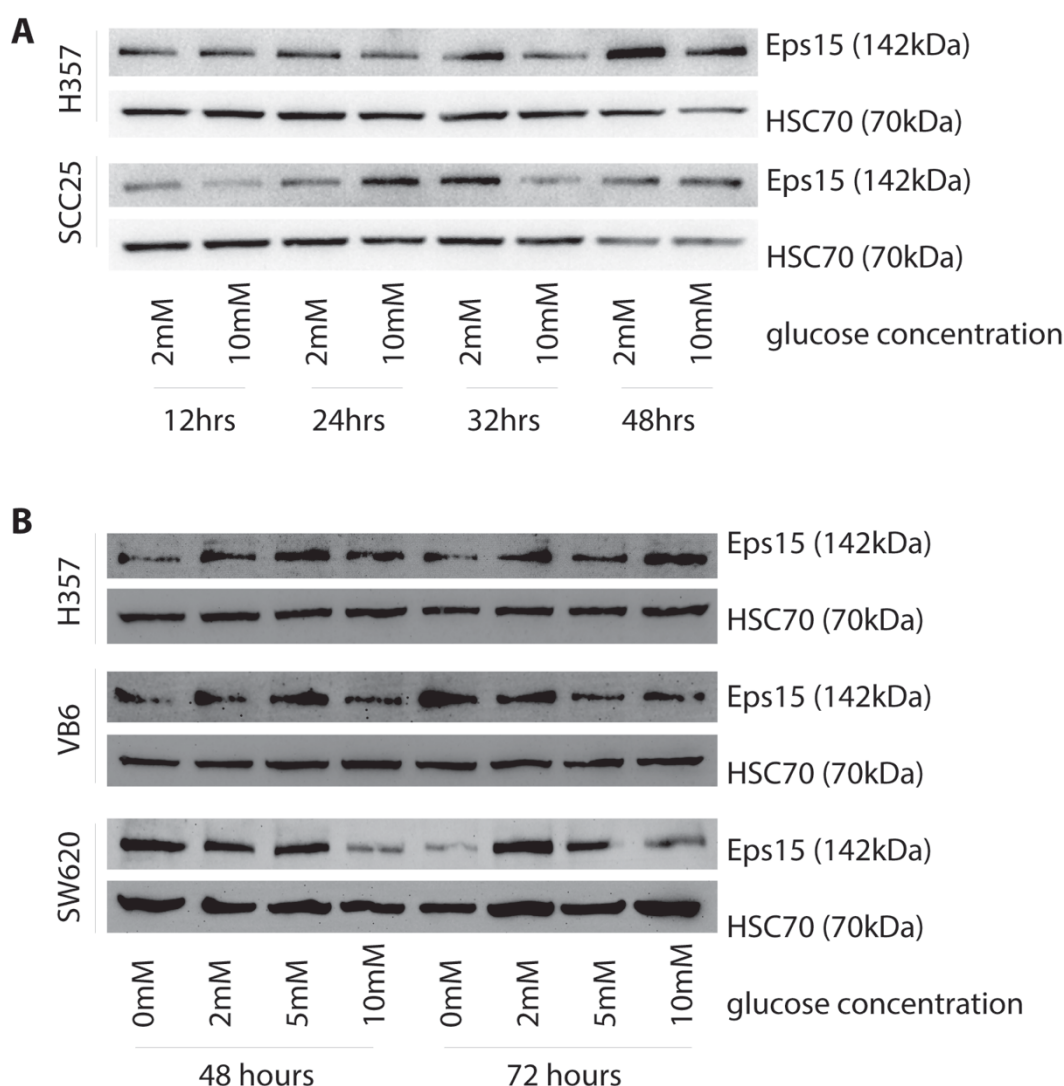
Figure 3.11 Eps15 knockdown does not consistently affect CtBP1 mRNA expression  
Cells were transfected with control or Eps15 siRNA and collected at 8, 16, 24, 32, 48 hours and 72 hours post-transfection. Following RNA extraction and cDNA synthesis using a High Capacity cDNA

Reverse Transcriptase Kit, TaqMan® RT-PCR was carried out to quantify CtBP1 mRNA in each condition. Graphs show mean and standard error of the mean (SEM) of two technical repeats (n=2). Data is normalised to ubiquitin and to CtBP1 mRNA expression of control lysates (AU = arbitrary unit). No consistent effect on CtBP1 expression was seen at the mRNA level in (A) VB6 cells at early time-points (8 -32 hours, p=ns; 48 hours post-transfection p<0.05) (B) VB6 cells at longer time-points (p=ns) (C) SCC25 cells (24 hours p<0.05; 48 hours p=ns; 72 hours p<0.05) and (D) H357 cells (24 hours p=ns; 48 hours p=ns; 72 hours p<0.05).

### 3.6 Altered metabolism does not affect Eps15 expression

#### 3.6.1 Altered glucose concentration does not affect Eps15 protein expression

To metabolise glucose tumour cells utilise aerobic glycolysis rather than oxidative phosphorylation (Hanahan & Weinberg 2011; Upadhyay et al. 2013) leading to the dimerisation of the family of glycolytic sensors; CtBPs (Chrzan 2014; Kumar et al. 2002; Zhang et al. 2006), allowing them to act as transcriptional co-regulators. Following observations that CtBP1 inhibition causes decreased Eps15 expression, we hypothesised that altered metabolism could potentially also regulate Eps15 expression, as increased glucose availability and consequent metabolism leads to CtBP dimerisation and activation in tumour cells (Fjeld et al. 2003). Based on our previous observations, this increase in CtBP dimerisation could then lead to an increase in Eps15 expression. To test this hypothesis, cells were grown in environments with increasing glucose concentration and Eps15 expression was tested at both the protein (Figure 3.12) and mRNA levels (Figure 3.13). Increasing glucose availability had no or minimal effect on Eps15 protein expression in any of the cell lines tested (Figure 3.12).



**Figure 3.12 Altered glucose does not affect Eps15 protein expression**  
Cells were grown for up to 72 hours in either 10% glucose-free DMEM or 10% DMEM containing 2, 5 or 10 mM glucose. Media was changed every 24 hours to ensure glucose levels remained constant. At 48 and 72 hours cells were collected and lysed and 40µg of protein was then run on SDS-PAGE followed by Western blotting. Eps15 expression shows minimal changes with increasing glucose availability in all cell lines tested (A) SCC25 and H357 and (B) H357, VB6 and SW620. Representative results of two independent repeats.

### 3.6.2 Altered glucose concentration does not affect Eps15 mRNA expression

The effect of increased glucose on Eps15 mRNA expression was similarly inconsistent. Increased glucose had no consistent significant effect on Eps15 mRNA expression at two time-points and in three cell lines (Figure 3.13) Two-way ANOVA analysis revealed that only two significant differences occurred; a significant decrease occurred at 48 hours in the SCC25 cell line between 2 mM and 10 mM concentration of glucose and a significant increase occurred in the H357 cell line between 2 mM and 10 mM at 72 hours but overall no consistent difference was observed similarly to Eps15 protein levels.

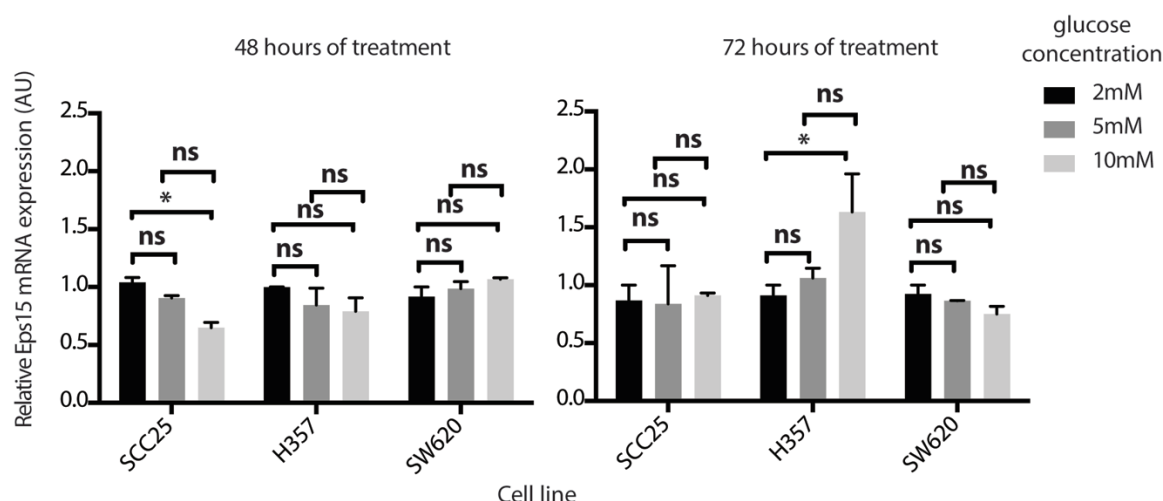


Figure 3.13 Altered glucose does not affect Eps15 mRNA expression

Cells were grown for up to 72 hours in 10% glucose-free DMEM with the addition of glucose to the required concentration. Media was changed every 24 hours to ensure glucose levels remained constant. At 48 hours and 72 hours cells were collected, followed by RNA extraction and cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit, TaqMan® RT-PCR was then carried out to quantify Eps15 mRNA expression in each condition. Graphs show mean and standard error of the mean (SEM) of two technical repeats (n=2). Data is normalised to beta-actin and to Eps15 mRNA expression of 2 mM treated lysates (AU = arbitrary unit). Two-way ANOVA revealed a significant decrease in Eps15 mRNA expression following treatment with 10 mM glucose concentration compared to 2 mM glucose concentration in the SCC25 cell line ( $p < 0.05$ ) and a significant increase in the H357 cell line at 72 hours between the 2 mM and 10 mM glucose concentration ( $p < 0.05$ ). No significant difference in Eps15 mRNA expression was observed with any other glucose concentrations at either time-point and in any other cell line.

### 3.6.3 Hypoxia does not affect Eps15 mRNA expression

Changes in glucose levels did not show a significant change in Eps15 expression. This could be due to the wide range of cell functions, which are affected by changes in cell metabolism. An increase or decrease in glucose levels from physiological levels (5 mM glucose concentration) could lead to a substantial change in cell metabolism, consequently affecting the expression of a range of genes, which could potentially affect an individual gene both positively and negatively, thereby leading to no detectable change overall. Another method to induce changes in gene expression caused by a cancer environment is to put cells under hypoxic conditions. Low oxygen conditions (hypoxia) is often seen in solid tumour micro-environments forcing the cells into a more glycolytic state and interest is increasing in the literature into how this environment affects specific genes that can play a role in cancer progression (Dachs & Tozer 2000).

One gene highly up-regulated under hypoxic conditions is the carbonic anhydrase IX (CA9) gene (Shin et al. 2011). CA9 expression is notably induced by hypoxic conditions and is under tight regulation by the hypoxia-inducible factor 1 alpha (HIF1- $\alpha$ ) (Shin et al. 2011). For this reason, it was used as a positive control during our hypoxia experiments and indeed CA9 mRNA expression

was significantly induced in hypoxic conditions compared with normoxic conditions, but no significant change in Eps15 mRNA expression was seen (Figure 3.14). As previously discussed, cancer cells metabolise via glycolysis even when adequate oxygen supplies are available. Therefore, cancer cells have adapted to survive in hypoxic conditions by changing their metabolism and by observing the effects of hypoxia on cancer cell gene expression we can see how an effect of cell metabolism can be driving cancer cell progression (Bartrons & Caro 2007). These results suggest that changes in metabolism are possibly not the driving force for changes in Eps15 expression seen after down-regulation of the metabolic sensor CtBP1 (Figure 3.4). Other factors may regulate Eps15 expression but these would need further investigation.

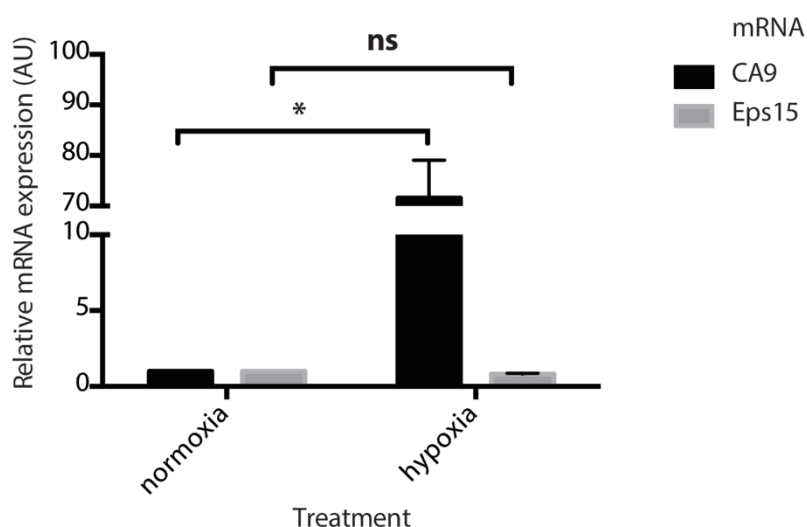


Figure 3.14 Hypoxia has no effect on Eps15 mRNA levels

Cells were grown in either normoxic conditions (20% oxygen) or hypoxic conditions (1% oxygen). After 24 hours cells were collected, followed by RNA extraction and cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit, TaqMan® RT-PCR was then carried out to quantify CA9 and Eps15 mRNA expression in each condition. Data is normalised to ubiquitin and normoxia (AU = arbitrary unit). Hypoxia caused no significant change in Eps15 mRNA expression but a significant increase in CA9 mRNA expression ( $p < 0.05$ ). CA9 was used as a positive control as it is highly activated during hypoxic conditions. Graphs show mean and standard error of the mean (SEM) of three biological repeats ( $n=3$ ).

### 3.7 CtBP1 and Eps15 are positively associated in human tissues

To examine the correlation between CtBP1 and Eps15 in human tumour tissue we examined CtBP1 and Eps15 expression using immunohistochemistry. A tissue microarray (TMA) of 80 different human tumours (OSCC  $n=10$ , lung  $n=9$ , colon  $n=25$ , breast  $n=26$ , ovary  $n=6$ , pancreatic  $n=2$  and prostate  $n=2$ ) previously generated in our group was stained for CtBP1 and Eps15 and scored according to expression patterns by a trained pathologist. TMA scores ranged from 0 to 3 indicating negative (0), low (1), moderate (2) or high (3) staining. Chi squared analysis of our TMAs showed a strong positive correlation between CtBP1 and Eps15 expression ( $p < 0.01$ ) across the

different cancer types. This analysis is given in Figure 3.15 alongside an example of two cases sequentially stained for CtBP1 and Eps15. These findings further strengthen our *in vitro* findings, which suggest a direct relationship between CtBP1 expression and Eps15 expression (Figure 3.3- Figure 3.7).

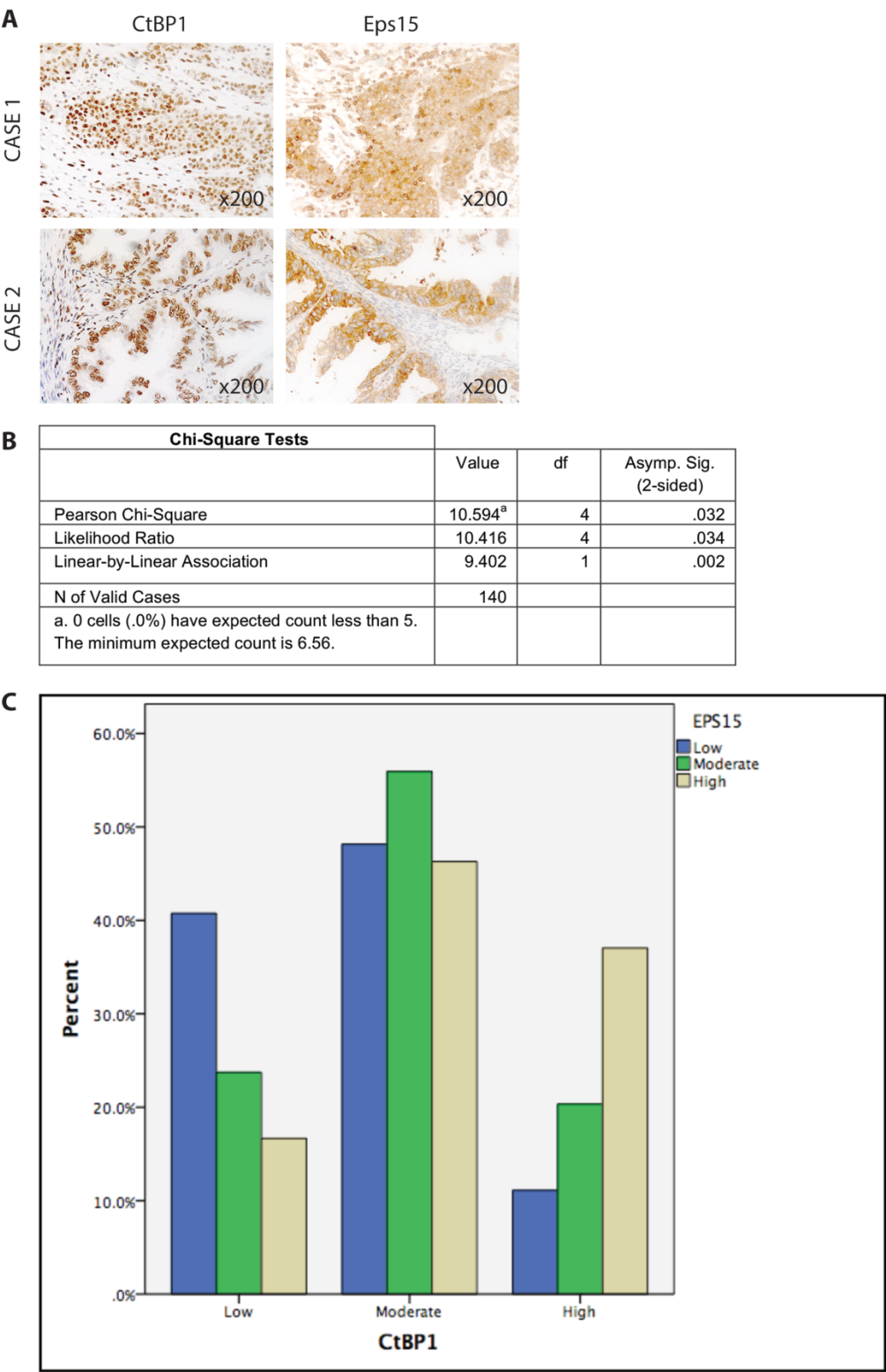


Figure 3.15 Immunohistochemical analysis of human tissue show CtBP1 expression correlates with Eps15 expression

(A) Sequential sections of human tissue microarrays (TMAs) were stained for CtBP1 and Eps15 and scored for expression by a trained pathologist from negative to high expression. Here are shown an example of two cases out of the 80 tumours analysed. (B) Chi squared analysis revealed that there is a significant positive correlation between CtBP1 and Eps15 expression in human tumour tissues ( $p < 0.01$ ). (C) A bar graph shows the correlation between CtBP1 and Eps15 expression. Cases with low CtBP1 expression also have low Eps15 expression as is the same for cases with high expression of each protein.

### 3.8 Discussion

Previous research has shown that CtBPs can play an important role in motility of tumour cells in response to hypoxic conditions (Zhang et al. 2006) but their exact role in the regulation of cell motility and the molecular mechanisms they use have yet to be identified. Previous unpublished research by Marta Chrzan suggested a strong, previously undescribed, link between the metabolic sensors CtBP1 and 2 and integrin-dependent motility (Chrzan 2014). It has been shown that CtBP down-regulation inhibits cell migration and invasion by increasing the level of integrin activator protein Talin-1, which then leads to increased integrin activation and increased cell adhesion (Chrzan 2014). Optimal adhesion of cells is critical for migration to occur (Cox & Huttenlocher 1998) and increased adhesion can make cells adhere too strongly to the substrate to be able to move.

Preliminary gene array data from our group showed that upon CtBP1 knockdown the protein Eps15, essential for clathrin-mediated endocytosis, becomes down-regulated (Figure 1.10) (Chrzan 2014). Clathrin-mediated endocytosis of integrins has been shown to be critical for cell motility (Rappoport 2003; A. G. Ramsay et al. 2007; Caswell & Norman 2006) therefore, we hypothesised that reduced integrin endocytosis due to the reduction of Eps15 levels could be another contributing factor for the decreased invasive properties of tumour cells following CtBP1 knockdown.

The first step in confirming our hypothesis required investigation into the link between CtBP expression and consequent Eps15 expression in more detail. This investigation was carried out both at the protein and mRNA level. A decrease in both Eps15 protein expression (Figure 3.3) and mRNA expression following CtBP1 knockdown was found (Figure 3.5) and this was confirmed in a number of cancer cell lines of different origin, suggesting that the mechanism contributing to this effect is a general phenomenon in various cancer types. Our results showed a consistent link between CtBP1 and Eps15 expression, however, the results following CtBP2 knockdown was less consistent and requires further investigation. Therefore, CtBP1 remained the focus of investigation due to its prominent and consistent effect on Eps15 expression both at the translational and transcriptional levels.

An explanation for the difference in the two CtBP isoforms has been discussed in the literature and it was shown that the two variants of CtBPs have distinct roles *in vivo*; while CtBP1-deficient mice are able to survive but are very small and die young, CtBP2-deficient mice die *in utero* (Chinnadurai 2003). The two genes are also mapped to different chromosomes in vertebrates; CtBP1 is mapped to the human chromosome 4p16, while CtBP2 is mapped at 21q21.3 (Chinnadurai 2002) so it would make sense that the proteins were able to function independently and therefore affect other proteins, including Eps15, differently. Other research has also suggested that the two isoforms function differently during therapeutic interventions (Birts et al. 2010). Birts *et al* used RNAi to reduce expression of CtBP1 and CtBP2 both individually and combined in the breast cancer cell line MCF7 and then treated cells with a range of chemotherapy agents including 5-fluorouracil, Taxol, etoposide and cisplatin. They then measured the half maximal inhibitory concentration (IC<sub>50</sub>) of each drug and thus the sensitivity of cells to each chemotherapeutic agent following RNAi treatment. Down-regulation of CtBP1 and CtBP2 individually increased sensitivity of cells to all chemotherapeutic drugs tested, with down-regulation of CtBP2 having a greater effect on cell sensitivity than CtBP1 and the greatest effect seen following combined knockdown (Birts et al. 2010). These results suggest different roles for each CtBP in chemoresistance but more research is needed to fully elucidate their individual roles in regulation of cell functions.

It is also possible that CtBP hetero- and homodimers are capable of interacting differently with proteins. CtBP2 contains a nuclear localisation signal (NLS) not available on CtBP1, CtBP1 therefore needs to dimerise with CtBP2 or interact with certain transcription factors which facilitate translocation into the nucleus, which could affect their interaction with protein targets (Verger et al. 2006). Additionally, CtBP/BARS is a CtBP1 homodimer and has been discussed as having a dual-function; capable of both transcriptional co-repression and Golgi membrane fission depending on its structural conformation (Nardini et al. 2003). Nardini *et al* discuss that CtBP/BARS co-repressor activity is regulated by NAD(H) binding, as binding to NADH triggers dimer formation allowing histone deacetylases to also bind. Contrary to this dimer formation CtBP/BARS can remain in an open conformation when not bound to NADH allowing acyl-CoA-dependent acyltransferase activity required for membrane fission, additionally, the closed NAD(H)-bound structure shields the acyl-CoA-binding site. Thus the different conformations of CtBP/BARS allows CtBP/BARS to be a dual-function protein involved in both Golgi membrane fission and transcriptional co-repression (Nardini et al. 2003). Thus perhaps different conformations allow different functions of CtBPs. Perhaps we see different effects of CtBP1 and CtBP2 on Eps15 expression due to their slightly different structures. Additionally, Bhambhani *et al* investigated CtBP<sup>MONO</sup> constructs (unable to form dimers) and CtBP<sup>WT</sup> constructs (able to form



dimers) in the context of the small eye phenotype (occurring as a result of Wg signalling (Wingless signalling pathway in *Drosophila*)). Bhambhani *et al* found that expression of CtBP<sup>MONO</sup> was able to significantly enhance the small eye phenotype, suggesting Wg signalling activation, in comparison to CtBP<sup>WT</sup> which greatly suppressed the small eye phenotype, suggesting Wg signalling repression (Bhambhani *et al.* 2011). These results suggest that CtBPs can have markedly different functions depending on their dimerisation state in the cell, altering the activation or repression of proteins in response.

Down-regulation of CtBP1 led to a consistent decrease in Eps15 expression, which was an unexpected result as CtBPs mostly function as transcriptional repressors (Chinnadurai 2002). Ordinarily, one would expect that down-regulation of a transcriptional repressor would lead to an increase in expression of another protein, as was the case with the integrin activator, Talin-1 (Chrzan 2014). A paper by Phippen *et al* suggests that CtBPs are able to activate the expression of certain genes by repressing the activity of other repressors (Phippen *et al.* 2000). Phippen *et al* found that alternate domains of *Drosophila* CtBP (dCtBP) were required for the production of different multi-protein complexes required for transcriptional activation or repression (Phippen *et al.* 2000). It was proposed that the activator regions of dCtBP were able to interact with co-repressor histone deacetylase complexes, attenuating transcriptional repression (Phippen *et al.* 2000).

Our ChIP data (Figure 3.7) however, suggests that the effect of CtBP1 on Eps15 is direct and a direct co-activator role of CtBPs is possible. Investigations into the role of human CtBPs as transcriptional activators has been carried out (Paliwal *et al.* 2012). Paliwal *et al* found CtBP2 is able to regulate human cancer cell migration by transcriptional activation of T-cell lymphoma invasion and metastasis 1 (Tiam1) (Paliwal *et al.* 2012). CtBP2 activates Tiam1 following recruitment to the Tiam1 promoter site by CtBP-interacting Kruppel-like factor 8 (KLF8) and down-regulation of CtBP2 by RNAi decreased protein and mRNA Tiam1 expression by approximately 40% (Paliwal *et al.* 2012). CtBP1 has also been found to be involved in the transcriptional activation of multidrug resistance gene 1 (MDR1) in human multidrug resistant cancer cells (Jin *et al.* 2007). Drug resistance caused by over-expression of P-glycoprotein (P-gp), the *MDR1* gene product, limits the therapeutic outcome. Jin *et al* investigated human multidrug resistant (MDR) cell lines and found that the expression of CtBP1 was increased ~4 fold in them as compared to their sensitive counterparts and that silencing of CtBP1 expression by RNAi decreased the MDR1 mRNA and P-gp. The authors performed ChIP analysis and found that CtBP1 was physically bound to the promoter region of the MDR1 gene suggesting CtBP1 can contribute to the activation of MDR1 transcription through directly interacting with the MDR1 promoter, just as we have seen with CtBP1 and the Eps15 promoter. Furthermore, in a reporter gene assay, co-

transfection of MDR1 promoter constructs with a CtBP1 expression vector resulted in approximately 2–4-fold induction of MDR1 promoter activity. The authors concluded that CtBP1 might be one of the key transcription factors involved in the induction of MDR1 gene. Thus CtBPs can act as transcriptional activators, while they are mostly known as co-repressors. These results are consistent with our findings and while it is possible that CtBP1 could further affect Eps15 expression indirectly, by regulating other proteins, our ChIP analysis (Figure 3.7) suggests a direct effect of CtBP1 on Eps15 expression.

We also investigated the possible role of Eps15 as a transcriptional regulator by measuring the effect of Eps15 knockdown on CtBP1 expression (Figure 3.9 and Figure 3.11). A form of endocytosis has long been established in the nucleocytoplasmic shuttling of proteins between the cytoplasm and the nucleus but new research suggests that maybe their role goes further than this and that endocytic proteins are involved in regulating gene expression in the nucleus (Pilecka et al. 2007; Pyrzynska et al. 2009). For example, Enari *et al* found that expression of clathrin heavy-chain (CHC) is a requirement for p53-mediated transcription (Enari et al. 2006). This discovery was particularly interesting given that mutations in the p53 gene have been found in >50% of human cancers, as this gene encodes a protein important in preventing tumorigenesis (Olivier et al. 2010). Enari *et al* discovered that the CHC was not only available in the cytosol, where it plays a vital role in endocytosis, but was also available in the cell nuclei. Here, CHC was required for transactivation of p53-responsive promoters, in fact transactivation of p53AIP1 promoter was enhanced fivefold when cells were transfected with CHC and p53 compared with p53 alone. Furthermore, reduction of CHC by RNAi attenuated p53 transcriptional activity (Enari et al. 2006). Eps15 has also been found to play a role in regulation of gene transcription (Vecchi et al. 2001; Croce et al. 1997; Meyer et al. 2009). Croce *et al* investigated the localisation of HRX/ALL1 gene in acute leukemias and found that fusion with Eps15 was able to alter the cellular compartmentalisation of HRX/ALL1 to be consistently found in the cell nucleus providing a putative mechanism for activation of HRX/ALL1 (Croce et al. 1997). Additionally, binding of Eps15, among other genes, has been found to play a role in activation of MLL in acute leukemias (So et al. 2003; Meyer et al. 2009). MLL is a histone methyltransferase that can be converted into an oncoprotein following fusion with a number of proteins in acute leukemias, one of which has been shown to be Eps15 (So et al. 2003; Meyer et al. 2009). Vecchi *et al* provided a possible explanation for Eps15's role in transcriptional regulation as they found that Eps15 and CALM (clathrin assembly lymphoid myeloid leukemia) acted as positive modulators of transcription in a GAL4-based transactivation assay, suggesting that Eps15 itself could play a direct or indirect role in transcriptional regulation (Vecchi et al. 2001). While the role of endocytic proteins such as Eps15 have been discussed in the literature as possible regulators of gene transcription (Vecchi et

al. 2001; Pyrzynska et al. 2009), we saw no effect of Eps15 knockdown on CtBP1 mRNA expression. While this does not rule out a possible function of Eps15 as a transcriptional regulator it does show that a feedback loop between Eps15 and CtBP1 does not exist in the cells we examined, suggesting a linear mechanism between CtBP1 expression and consequent Eps15 expression leading to an effect on cell function.

The effect of altered metabolism was also investigated by altering the amount of glucose available in the surrounding cell environment. We found that neither changing the amount of glucose available to cells, nor placing the cells under hypoxic conditions, affected Eps15 expression, either at the protein level or mRNA level. Altering environmental glucose is perhaps not the most appropriate method of measuring altered metabolism due to its non-specific affects. Altering glucose concentration alters the expression and function of various proteins, which can potentially effect Eps15 expression. For example, high glucose concentration has been shown to up-regulate nitric oxide synthase (NOS) expression as well as superoxide anion generation ( $O_2^-$ ) and COX2 expression in human aortic endothelial cells (Cosentino et al. 1997; Cosentino et al. 2003), which could both put cells under stress, this would affect a number of cell processes and so we might not see a direct effect on individual proteins, such as, Eps15.

Interestingly, a link between Eps15 and clathrin-mediated distribution of the glucose transporter GLUT4 has been suggested by Guilherme and colleagues who showed that efficient insulin-stimulated redistribution to the plasma membrane of the glucose transporter GLUT4 requires an Eps15 homology (EH) domain-containing protein, EHD1 (Guilherme et al. 2004). Guilherme *et al* discovered that EHD1 controls the peri-nuclear localisation of glucose transporting GLUT4-containing membranes. Normally in muscle and adipose tissues insulin stimulates glucose transport by recruiting the glucose transporter GLUT4 to the plasma membrane and the authors concluded that EHD1 is necessary for insulin-stimulated recycling of these GLUT4-containing membranes in cultured adipocytes (Guilherme et al. 2004). They expressed a dominant-negative construct of EHD1 (missing the EH domain) and performed EHD1 RNAi and found GLUT4 translocation to the plasma membrane to be inhibited leading to GLUT4-containing membranes to be dispersed throughout the cytoplasm (Guilherme et al. 2004). These observations are also discussed in the review by Ishiki and Klip (Ishiki & Klip 2005). These authors discuss that the mechanisms leading to GLUT4 distribution are still not fully understood. They discuss that GLUT4 distribution can be disrupted through both cellular and protein manipulations but that the manipulation of EHD1 really stands out in correct regulation of GLUT4 distribution (Ishiki & Klip 2005). These observations would suggest that when cells are plated in an environment with a large amount of glucose, that the availability of EHD1 proteins at the membrane might increase in order to match the increasing demands of GLUT4 distribution. This could then increase the

amount of Eps15 also localised to the membrane but might not regulate its expression, but these are all hypotheses. Indeed, we showed that altering the glucose concentration in media of cells did not change the expression of Eps15 at the protein or mRNA level.

Similar inconclusive results were seen when the cells were treated with hypoxic conditions of 1% oxygen levels. Hypoxic conditions mimic the low oxygen conditions seen in many solid tumours (Dachs & Tozer 2000) and can lead to the activation of CtBP repressor activity. Hypoxia increases free NADH levels which promotes CtBP recruitment to the E-cadherin promoter and the repression of E-cadherin gene expression (Zhang et al. 2006). Given the effect of hypoxia on CtBPs ability to regulate its target proteins we hypothesised that perhaps CtBP regulation of Eps15 expression could also be antagonised by hypoxic conditions, however this was not the case as we observed no consistent effect of hypoxia on Eps15 expression (Figure 3.12-Figure 3.13). The prolyl hydroxylase PHD3 is regulated by hypoxia and plays an important role in tumour progression through regulation of epidermal growth factor receptor (EGFR) activity through the control of EGFR internalisation (Garvalov et al. 2014). Garvalov *et al* found that PHD3 controls EGFR activity by acting as a scaffolding protein that associates with Eps15 to promote the internalisation of EGFR. During hypoxia, PHD3 expression is highly induced, leading to a reduction in EGFR signalling, while loss of PHD3 in tumour cells suppressed EGFR internalisation, hyper-activating EGFR signalling, enhancing cell proliferation and survival. Thus suggesting a role for Eps15 in the regulation of EGFR endocytosis, even in hypoxic conditions. Although this paper suggests a role for Eps15 in the regulation of receptor endocytosis even under hypoxic conditions, there are no papers showing that hypoxia could affect Eps15 expression. We certainly did not see any and while we cannot conclusively say that metabolism does not regulate Eps15 expression, using the above treatments we could not show such a link.

Finally, our *in vitro* observations were supported by analysis of CtBP1 and Eps15 expression in human tissues using tissue microarrays (TMAs) from a range of cancer types. We showed a strong positive correlation between CtBP1 expression and Eps15 expression across 80 different human tumours from OSCC, lung, colon, breast, ovary, pancreatic and prostate tumour types (Figure 3.15). While their association is new, their individual role in tumours has been investigated (Deng et al. 2013; Meng et al. 2015). Deng *et al* performed CtBP1 immunohistochemistry on melanoma tissue arrays which contained; 21 cases of melanocyte-derived nevi, 56 cases of malignant melanoma lesions, and 20 cases of metastasis. Positive nuclear CtBP1 staining was found in a large percentage of nevi, malignant melanoma, and metastasis cases but was rarely found in normal skin. Additionally, CtBP1 over-expression was detected in 11/21 (52%) of benign nevocellular nevi and 39/49 (80%) of stage I-II malignant melanoma cases, suggesting CtBP1 over-expression is an early event in melanoma development (Deng et al. 2013). Meng *et al* examined

Eps15 homology domain 1 (EHD1) protein expression in paraffin sections of 85 resected small cell lung cancer (SCLC) tissues, metastatic lymph nodes and normal bronchial epithelial tissues. They used immunohistochemistry to study the correlation between EHD1 expression and patient clinicopathological features and found that EHD1 protein was significantly increased in SCLC tissues compared with normal tissues and that EHD1 expression was positively correlated with tumour size. They conclude that their data demonstrates a correlation between the cytoplasmic expression of EHD1 protein and adverse prognosis in patients receiving early-stage cisplatin treatment for resected SCLC. These investigations show that expression of CtBP1 and EHD1 are both positive markers in tumours. These data are intriguing as we have found that in our own cohort of 80 different human tumours that CtBP1 and Eps15 expression are significantly positively correlated suggesting that CtBP1 and perhaps Eps15 could be good prognostic makers of tumour progression.

Following our observations on the effect of CtBP1 inhibition on Eps15 expression we next investigated the effect of Eps15 on cell function; first on cell migration and invasion.

### 3.9 Summary

1. CtBP1 knockdown reduces Eps15 protein and mRNA expression
2. CtBP1 directly associates with the *EPS15* promoter
3. CtBP1 can act as a transcriptional activator as well as a transcriptional repressor
4. The relationship between CtBP1 and Eps15 is one-way; Eps15 knockdown does not affect CtBP1 expression at either the protein or mRNA level
5. Changes in metabolism by altering glucose concentrations or hypoxic conditions does not change Eps15 expression
6. CtBP1 expression is significantly positively correlated to Eps15 expression levels *in vivo*



## Chapter 4: Eps15 regulates tumour cell motility

In the previous chapter we showed that CtBP1 down-regulation inhibits Eps15 expression and from previous results in our laboratory (Chrzan 2014) we know that CtBP1 knockdown inhibits integrin-dependent cell motility. We hypothesised that down-regulation of Eps15 by CtBP1 knockdown might contribute to the anti-migratory effect of CtBP1 down-regulation. If our hypothesis is correct Eps15 knockdown, similarly to CtBP1 down-regulation, should inhibit cell motility.

### 4.1 Eps15 regulates tumour cell migration

#### 4.1.1 Eps15 regulates tumour cell migration in Transwell® migration assays

Eps15 plays an integral role in clathrin-mediated integrin endocytosis, which has been shown to be crucial for optimal cell motility (A. G. Ramsay et al. 2007). Ramsay and co-workers showed that inhibition of Eps15 by over-expression of the dominant-negative mutant (EΔ95/295 or DIII) of the protein in OSCC cell lines significantly reduces  $\alpha\text{v}\beta\text{6}$ -integrin dependent cell migration in Transwell® assays (Ramsay et al. 2007, Supplementary Material). To confirm the role of Eps15 in migration of our OSCC cell lines they were transfected with Eps15\_5 and Eps15\_7 siRNAs and their migration measured using Transwell® assays. The cell lines chosen have previously been characterised for integrin expression; H357 cells express integrin  $\alpha\text{5}\beta\text{1}$  and this is the primary integrin they utilise to migrate towards fibronectin (Yap et al. 2009). The VB6 cell line was generated from H357 cells by over-expressing the  $\alpha\text{v}$  and  $\beta\text{6}$  integrin subunits (Thomas et al. 2001). We have previously established that SCC25 cells over-express endogenous  $\alpha\text{v}\beta\text{6}$  and they migrate towards the latency associated peptide of TGF- $\beta\text{1}$  (LAP) using solely  $\alpha\text{v}\beta\text{6}$  (Chrzan 2014). Thus, cell migration was examined either towards LAP (VB6 and SCC25,  $\alpha\text{v}\beta\text{6}$ -dependent), or fibronectin (H357,  $\alpha\text{5}\beta\text{1}$ -dependent) (Yap et al. 2009). These cell lines therefore provide a suitable model to study integrin-specific functions and therefore we focused on these cell lines in the remainder of our study.

Transfection of cells with Eps15 siRNA resulted in a significant inhibition of both  $\alpha\text{v}\beta\text{6}$ - (VB6, SCC25) and  $\alpha\text{5}\beta\text{1}$ -dependent (H357) cell migration compared to cells treated with control siRNA and this was consistent with both siRNA sequences (Figure 4.1A). Western blotting was used to confirm knockdown of Eps15 protein following siRNA treatment (Figure 4.1B). These results confirm that Eps15 is indeed crucial for integrin-specific cell migration. It is also consistent with

our proposed hypothesis that CtBP1 knockdown could potentially inhibit integrin-specific cell migration by down-regulating Eps15.

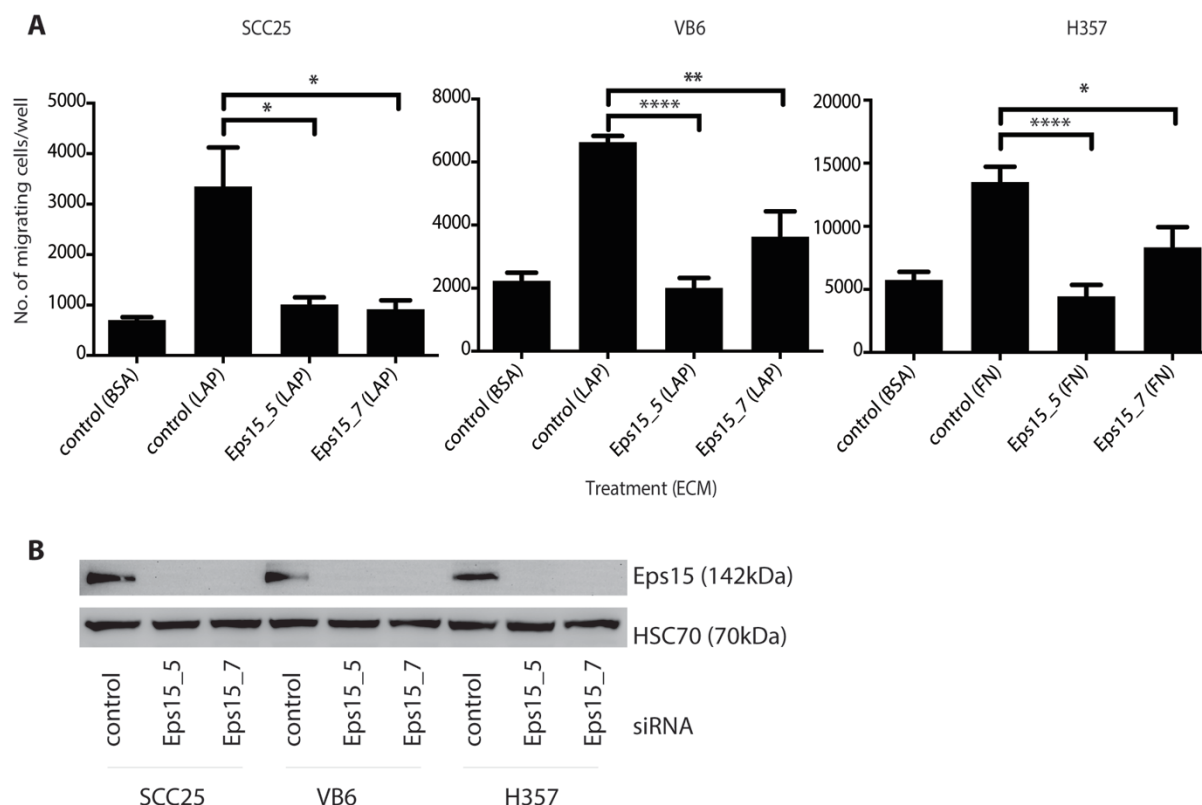


Figure 4.1 Eps15 knockdown inhibits tumour cell migration

(A) Cell migration of OSCC cell lines was studied 72 hours post-transfection with control or Eps15 siRNA using Transwell® migration assays. LAP was used as an attractant for VB6 and SCC25 cells to study  $\alpha\beta6$ -specific migration, while FN was used in H357 cells to measure  $\beta1$ -specific migration. Conditions were set up in triplicates and graphs show an accumulation of all repeats (SCC25: n=3, VB6: n=3, H357: n=4). Eps15 knockdown resulted in significant inhibition of cell migration compared to cells treated with control siRNA in all three cell lines (SCC25: Eps15\_5  $p<0.05$ ; Eps15\_7  $p<0.05$ ; VB6: Eps15\_5  $p<0.0001$ ; Eps15\_7  $p<0.01$ ; H357: Eps15\_5  $p<0.0001$ ; Eps15\_7  $p<0.05$ ). Significance was calculated by unpaired t-test and each graph shows mean with error bars indicative of standard error of the mean (SEM) (B) Cells were collected after the migration assay was set up at 24 hours post-transfection and Eps15 protein knockdown efficiency was confirmed by Western blotting. 40 $\mu$ g of protein was loaded for each sample and HSC70 was used as a loading control.

## 4.2 Eps15 regulates tumour cell invasion

### 4.2.1 Eps15 regulates tumour cell invasion in Matrigel® invasion assays

We next examined the effect of Eps15 knockdown on tumour cell invasion through a 3D matrix using Matrigel®-coated Transwell® invasion assays. These were performed 24 hours post-transfection with Eps15 siRNA and the number of invading cells were counted following 72 hour incubation.



Similarly to Transwell® migration, Eps15 knockdown significantly inhibited 3D invasion compared to cells transfected with non-targeting siRNA in all three cell lines tested (Figure 4.2A). This was also consistent with the effect of CtBP1 down-regulation, which was used as a positive control and resulted in equal inhibition of invasion in all the cell lines studied. Furthermore, integrin-specific invasion was blocked with an appropriate antibody in order to confirm that 3D invasion was also integrin-dependent. Blocking of integrin  $\beta 6$  with antibody 63G9 caused significant reduction of invasion of both SCC25 and VB6 cells, while treatment with antibody P5D2 blocked  $\beta 1$  integrins, significantly reducing  $\beta 1$ -dependent cell invasion of H357 cells (Figure 4.2B). Western blotting was used to confirm the CtBP1 and Eps15 protein knockdown following siRNA treatment (Figure 4.2C).

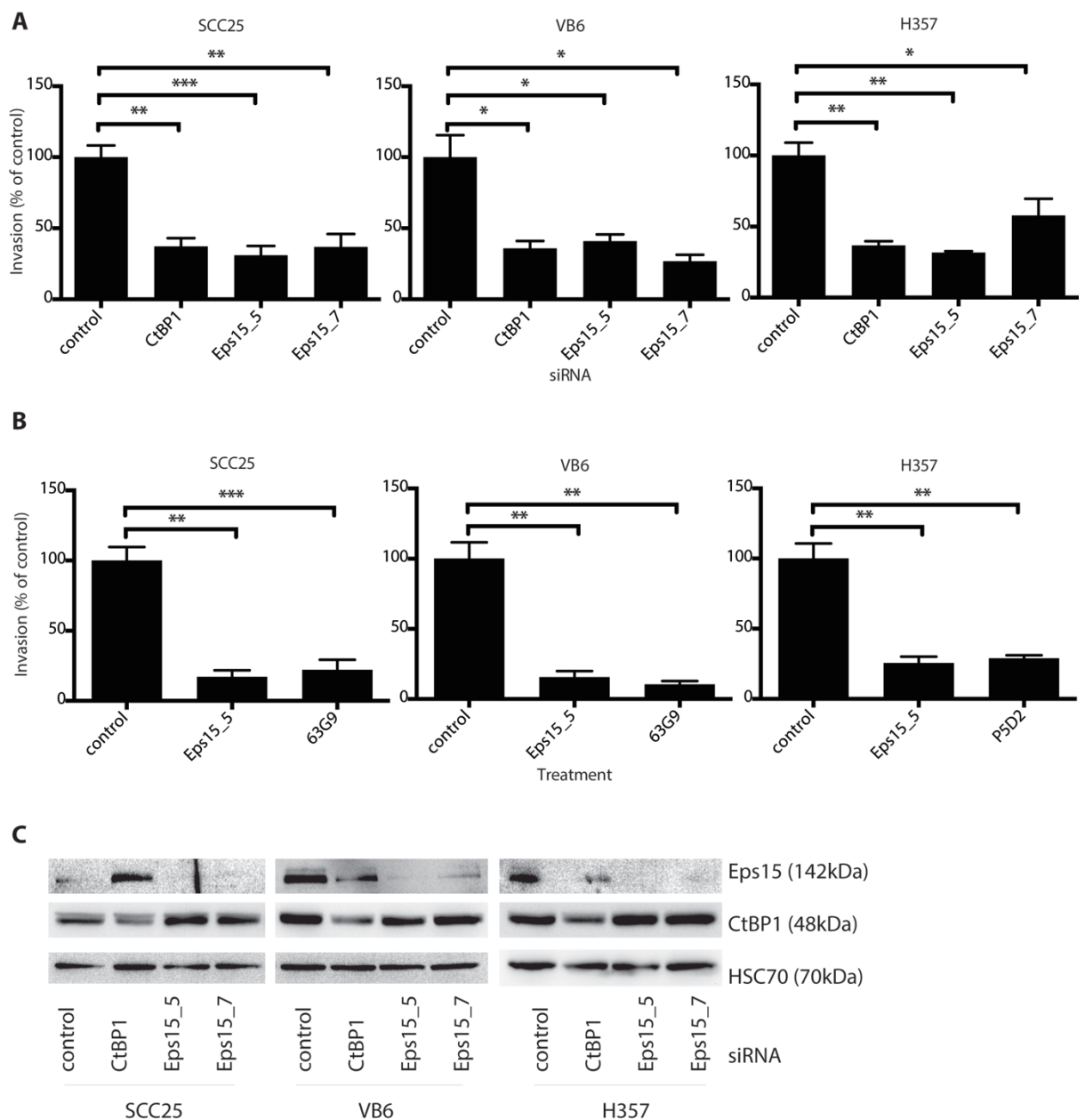


Figure 4.2 Eps15 knockdown inhibits tumour cell invasion

(A) Matrigel® invasion assays were set up in quadruplicates 24 hours post-transfection with control, CtBP1, Eps15\_5 and Eps15\_7 siRNA and cells were allowed to invade for 72 hours. Graphs

are a representative of three independent experiments. Experiments could not be combined due to large shifts in control invasion between experiments. Graphs show mean and error bars indicate standard error of the mean (SEM) of 4 technical repeats. Eps15 knockdown resulted in significant inhibition of cell invasion in all three cell lines to similar levels as seen with CtBP1 inhibition compared to cells treated with control siRNA (SCC25: CtBP1  $p < 0.01$ ; Eps15\_5  $p < 0.001$ ; Eps15\_7  $p < 0.001$ ; VB6: CtBP1  $p < 0.05$ ; Eps15\_5  $p < 0.05$ ; Eps15\_7  $p < 0.05$ ; H357: CtBP1  $p < 0.01$ ; Eps15\_5  $p < 0.01$ ; Eps15\_7  $p < 0.05$ ) (B) A significant decrease in invasion was also seen with integrin blocking treatment. Blocking of integrin  $\beta 6$  with antibody 63G9 caused significant reduction in invasion in SCC25 and VB6 cells (63G9: SCC25  $p < 0.001$ ; VB6  $p < 0.01$ ), while treatment with  $\beta 1$  blocking antibody, P5D2, significantly reduced  $\beta 1$ -dependent cell invasion of H357 cells (P5D2; H357  $p < 0.01$ ). Once again, Eps15 knockdown significantly reduced cell invasion (Eps15\_5: SCC25  $p < 0.01$ ; VB6  $p < 0.01$ , H357  $p < 0.01$ ). Significance was calculated by unpaired t-test and each graph shows mean with error bars indicative of standard error of the mean (SEM) Graphs are representative of two independent experiments (C) Cells were collected and lysed following invasion assay set up at 24 hours post-transfection to confirm CtBP1 and Eps15 knockdown efficiency by Western blotting. 40  $\mu$ g protein was loaded for each sample and HSC70 was used as a loading control.

These results show that Eps15 is not only crucial for 2D cell migration but also plays an important role in the more physiologically relevant 3D invasion of OSCC cells. Eps15\_5 siRNA was used for all further investigation, unless otherwise stated, due to its consistent effects in all functional assays performed.

#### 4.2.2 Eps15 knockdown does not affect cell proliferation

In order to ensure that the invasion results were not due to effects on cell growth, we performed a proliferation assay using a 24-well plate. We plated  $2.5 \times 10^4$  cells into each well and counted the number of cells which grew over three days. Eps15 knockdown did not consistently significantly affect cell proliferation across three time-points and three cell lines (Figure 4.3).

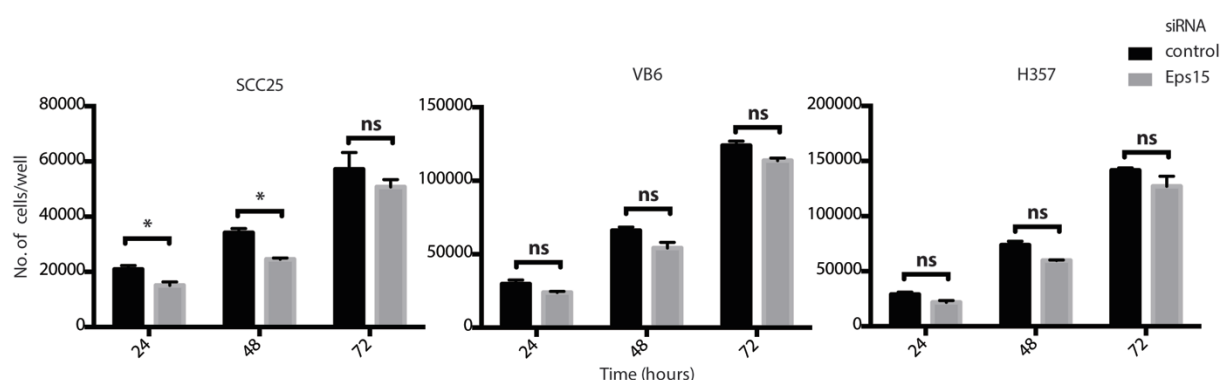


Figure 4.3 Eps15 knockdown does not significantly affect tumour cell proliferation

Using a 24-well plate proliferation was measured across three time-points and three cell lines.  $2.5 \times 10^4$  cells were initially plated and then counted every 24 hours up to 72 hours later. Eps15 siRNA treatment did not significantly affect cell proliferation in VB6 and H357 at any time-point. SCC25 showed small significance at 24 and 48 hours but this difference disappeared at 72 hours (24 hours;  $p < 0.05$ , 48 hours;  $p < 0.05$ , 72 hours;  $p = ns$ ).

#### 4.2.3 Eps15 regulates tumour cell invasion in organotypic culture

In order to analyse invasion in a more physiologically relevant setting, organotypic cultures were set up with CtBP1 or Eps15 siRNA-treated cells. Organotypic gels are a more physiological way to study cell motility as invasion occurs in 3D in the presence of fibroblasts, recapitulating the tumour micro-environment.

Two cell lines were investigated in organotypic cultures; SCC25 and VB6 with siRNA targeting CtBP1 or Eps15. Significant invasion was observed with both SCC25 and VB6 cells treated with control siRNA (Figure 4.4A). These cell lines are highly invasive, a trait which is thought to be due to their high  $\alpha\beta6$  expression. The invasiveness is due, at least in part, to increased  $\alpha\beta6$ -dependent TGF- $\beta$  activation, which transdifferentiates fibroblasts into invasion-promoting myofibroblasts (Sheppard 2005; Gaggioli et al. 2007; Marsh et al. 2011; Moutasim et al. 2011). Initially we stained organotypics with hematoxylin and eosin (H&E) (Figure 4.4A). However, in order to highlight the cancer cells for quantitative analysis, we stained sections with cytokeratin, which is an epithelial-specific marker, and thereby clearly identifies cancer cells without recognising the stroma (Figure 4.4B). Our results showed that individual knockdown of CtBP1 expression and Eps15 expression produced significant inhibition of invasion in both SCC25 and VB6 cell lines compared with control cells (Figure 4.4C). CtBP1 and Eps15 knockdown efficiency was confirmed by Western blotting and HSC70 was used as a loading control (Figure 4.4D).

Following organotypic analysis a TGF- $\beta$  activation assay was performed using a co-culture system with Mink Lung Epithelial Cells (MLEC) expressing a luciferase construct containing a TGF- $\beta$ -responsive promoter of the plasminogen activator inhibitor-1 (PAI; Abe et al., 1994) in order to analyse the levels of TGF- $\beta$  activation of each cell line (Figure 4.5). Both VB6 and SCC25 cells significantly activate TGF- $\beta$ 1, while H357 cells (lacking  $\alpha\beta6$ ) show relatively low activation of the cytokine in comparison (Figure 4.5). As invasion in organotypic cultures is highly dependent on the activation of the stromal-component, which is dependent on TGF- $\beta$ 1 this may explain why H357 cells did not produce significant basal invasion in this model.

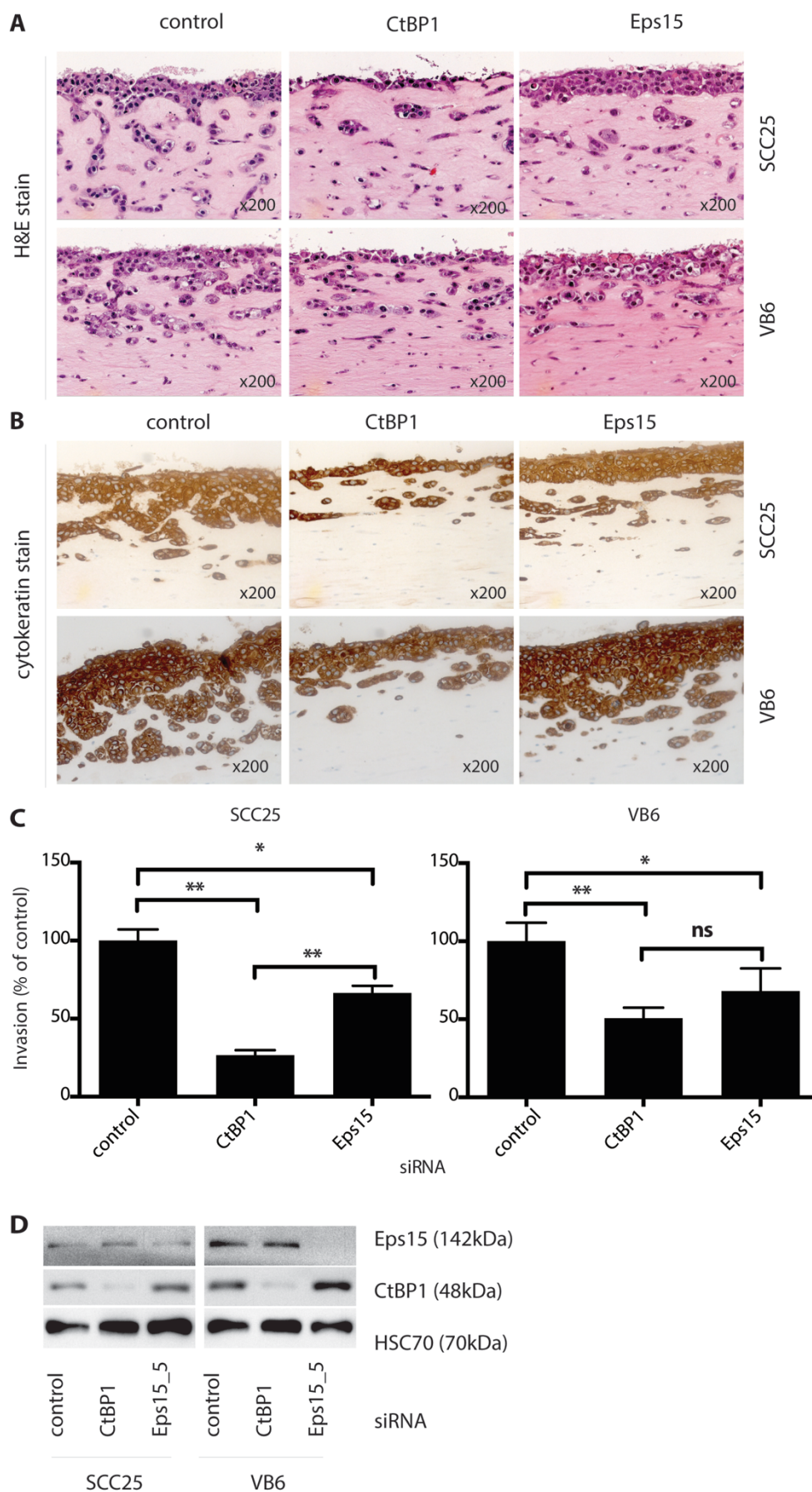


Figure 4.4 CtBP1 and Eps15 regulate invasion in organotypic culture  
Organotypic invasion of SCC25 and VB6 cells transfected with control, CtBP1 or Eps15 siRNA. (A) Hematoxylin and eosin stain (H&E) stain of organotypics mounted onto microscope slides, difficult

to analyse invasion (B) Cytokeratin staining shows high basal levels of invasion in SCC25 and VB6 cells treated with control siRNA. Knockdown of CtBP1 results in a greater reduction of cell invasion than that seen with Eps15 siRNA but a reduction in invasion with Eps15 siRNA is still significant compared to control siRNA-treated cells. (C) Quantitative analysis of depth of invasion shows significant inhibition of invasion upon CtBP1 knockdown in both cell lines; SCC25,  $p < 0.01$  and VB6,  $p < 0.01$ . A significant reduction in cell invasion is also seen with Eps15 siRNA but this effect is less significant than that of CtBP1 siRNA; SCC25,  $p < 0.05$  and VB6,  $p < 0.05$ . A significant difference is also seen between the invasion of CtBP1 and Eps15 siRNA treated SCC25 cells,  $p < 0.01$  but not of VB6 cells,  $p = \text{ns}$ . Significance was calculated by unpaired t-test and each graph shows mean with error bars indicative of standard error of the mean (SEM) (D) CtBP1 and Eps15 knockdown was confirmed by Western blotting in lysates collected upon organotypic harvest; 7 days post-transfection. 40 $\mu\text{g}$  protein was loaded per sample and HSC70 was used as a loading control.

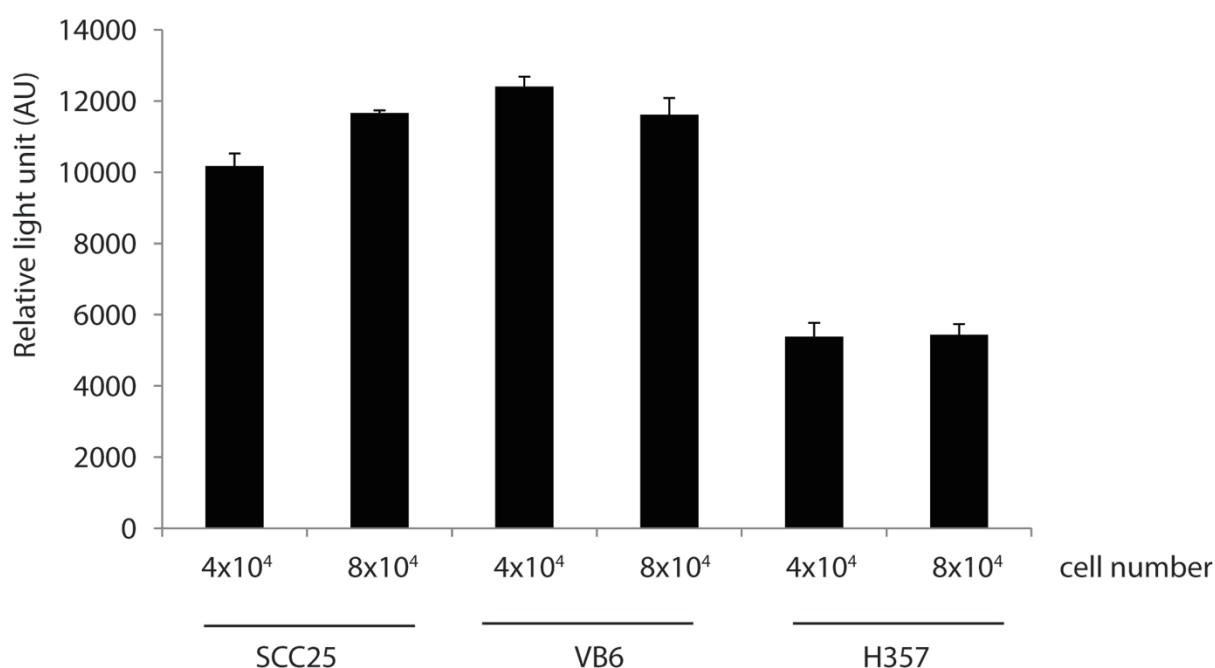


Figure 4.5 SCC25 and VB6 activate high levels of TGF- $\beta$  compared to H357 cells  
An MLEC assay measuring TGF- $\beta$  activation of each cell line reveals the expected trend of TGF- $\beta$  activation. VB6 cells activate the most TGF- $\beta$ ; SCC25 cells activate similarly high levels, while H357 cells activate a relatively low amount of TGF- $\beta$  in comparison. Graph shows mean with error bars indicative of standard deviation (SD). AU represents arbitrary unit.

### 4.3 Eps15 knockdown does not affect sheet cell motility

Our previous observations indicate that Eps15 is involved in the regulation of single cell motility; both single cell migration (Figure 4.1) and single cell invasion (Figure 4.2). However, cells are also able to migrate together in a group as a sheet, best observed in wound repair, tissue morphogenesis and also cancer (Friedl et al. 2004; Friedl et al. 1995).

We therefore analysed another read-out of cell motility by utilising a wound-healing (scratch) assay. Cells were plated and left to adhere as a confluent monolayer and subsequently, a wound was made on the monolayer by scraping along the middle of the well. Cell migration into the



wound was then analysed via time-lapse microscopy using an Olympus IX81 microscope (Figure 4.6A). The size of the wound was analysed at four time-points; 0, 5, 10 and 15 hours post-scratch. SCC25 cells treated with control siRNA were able to close the wound in just under 16 hours. No significant difference was observed in the size of the wound at any time-point between control and Eps15 siRNA treated cells (Figure 4.6B), suggesting that Eps15 does not play a role in this form of cell migration but is an important factor in the regulation of single-cell motility.

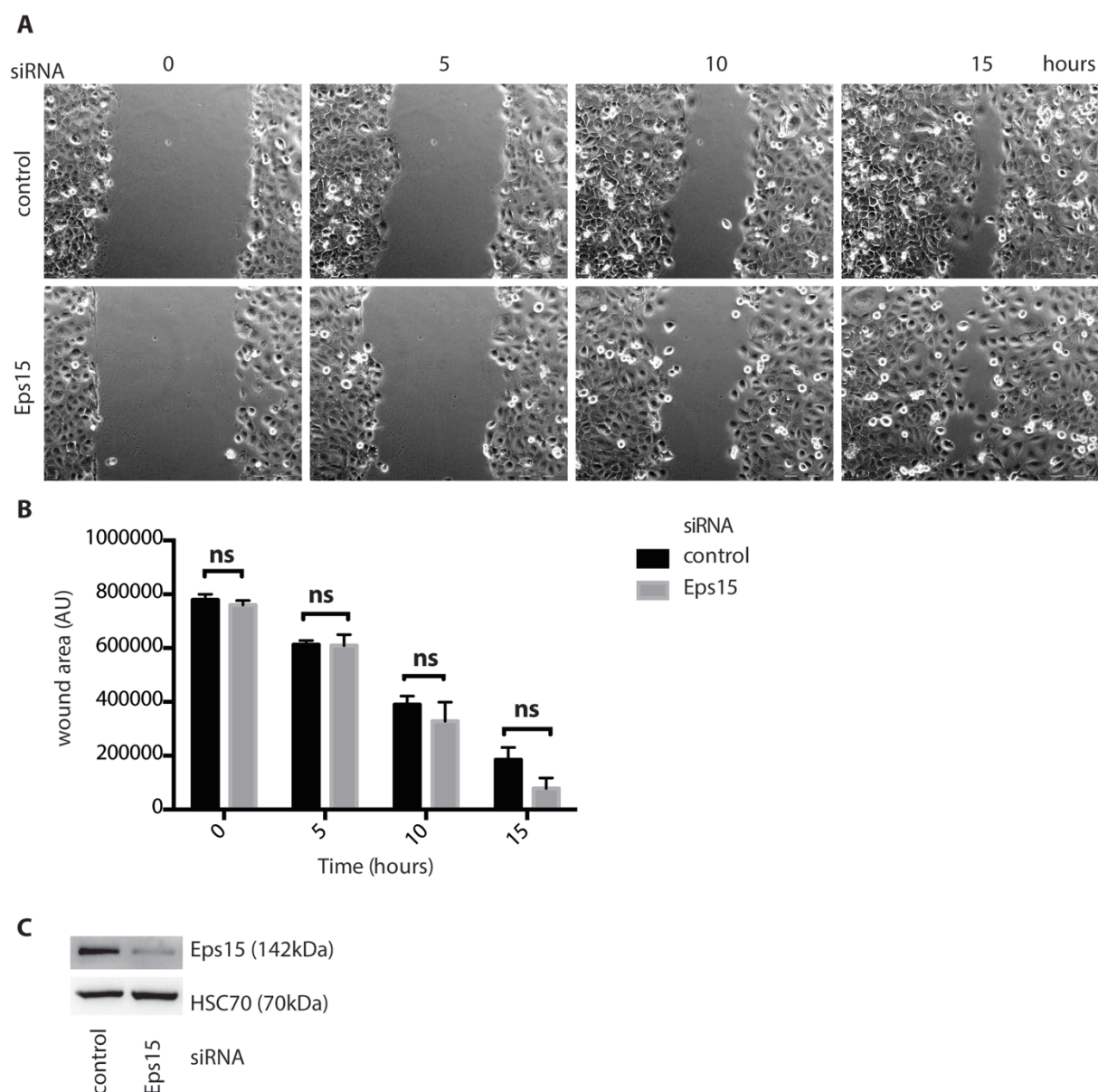


Figure 4.6 Eps15 knockdown does not regulate sheet cell motility

(A)  $1.5 \times 10^5$  cells were plated into one well of a 24-well plate 24 hours post-transfection, with control siRNA or Eps15 siRNA, and left to adhere overnight to form a confluent monolayer. Following overnight incubation, a wound was induced along the middle of the well with a P200 pipette tip and was analysed via time-lapse microscopy using an Olympus IX81 microscope. Both control and Eps15 knockdown cells closed the wound at 16 hours post-scratch. (B) Quantitative analysis was made at four time-points; 0, 5, 10 and 15 hours post-scratch. At each time-point an average wound area was calculated from 3 pictures along the wound of each condition. The wound area was measured using Fiji software and statistical significance was calculated by multiple t-test of each time-point; control vs Eps15 knockdown. The graph shows mean with error

bars indicative of standard error of the mean (SEM). There was no significant difference between sheet cell motility of control and Eps15 knockdown cells at any of the time points (AU = arbitrary unit). (C) Eps15 knockdown efficiency was confirmed by Western blotting in lysates collected after plating the confluent monolayer; 24 hours post-transfection. 40µg protein was loaded per sample and HSC70 was used as a loading control.

A crucial first step for migration of cell populations in contexts such as tissue morphogenesis and wound healing is polarisation of the cell-motility apparatus (Desai et al. 2009). During migratory polarisation, many structures, including Golgi complex, organise towards the leading edge of the cell and it has been found that disrupted Golgi architecture can cause an inhibition of cell migration (Millarte & Farhan 2012). We therefore wanted to determine if Eps15 knockdown could affect cell migration by modulating cell polarity and decided to investigate Golgi localisation as cells polarise at the leading edge of the migrating sheet. In order to examine the effect of Eps15 knockdown on Golgi orientation and cell polarity during wound healing, cells were plated on coverslips and Golgi orientation was analysed using immunofluorescence (Figure 4.7). The actin cytoskeleton and the Golgi network were identified by staining with phalloidin and the Golgi-marker, Giantin to observe differences in Golgi orientation following Eps15 siRNA treatment. Cells alongside the edge of the wound treated with control siRNA showed polarised Golgi organisation with Giantin staining orientated towards the edge of the wound (Figure 4.7A), while cells treated with Eps15 siRNA showed no organisation of the Golgi with staining seen surrounding the nucleus and not orientated in any particular direction (Figure 4.7B). Additionally, the monolayer produced by cells with Eps15 knockdown appeared less robust than that produced by control treated cells. Interestingly, in Eps15 knockdown cell monolayers we consistently observed that the cells had more gaps between them and appeared to be less tightly interacting compared to control cells. Although this could have a number of potential explanations it cannot be excluded that Eps15 could potentially affect cell-cell adhesion as well as cell polarity, however this suggestion would need further investigation (Figure 4.7). These results suggest that Eps15 knockdown could be affecting Golgi polarisation and possibly cell–cell contacts. While disruption to these structures do not appear to be affecting sheet cell motility they could explain the inhibition of migration seen in our functional assays measuring single cell motility, but further investigation is needed to confirm that this is the mechanism by which Eps15 is involved in cell motility.

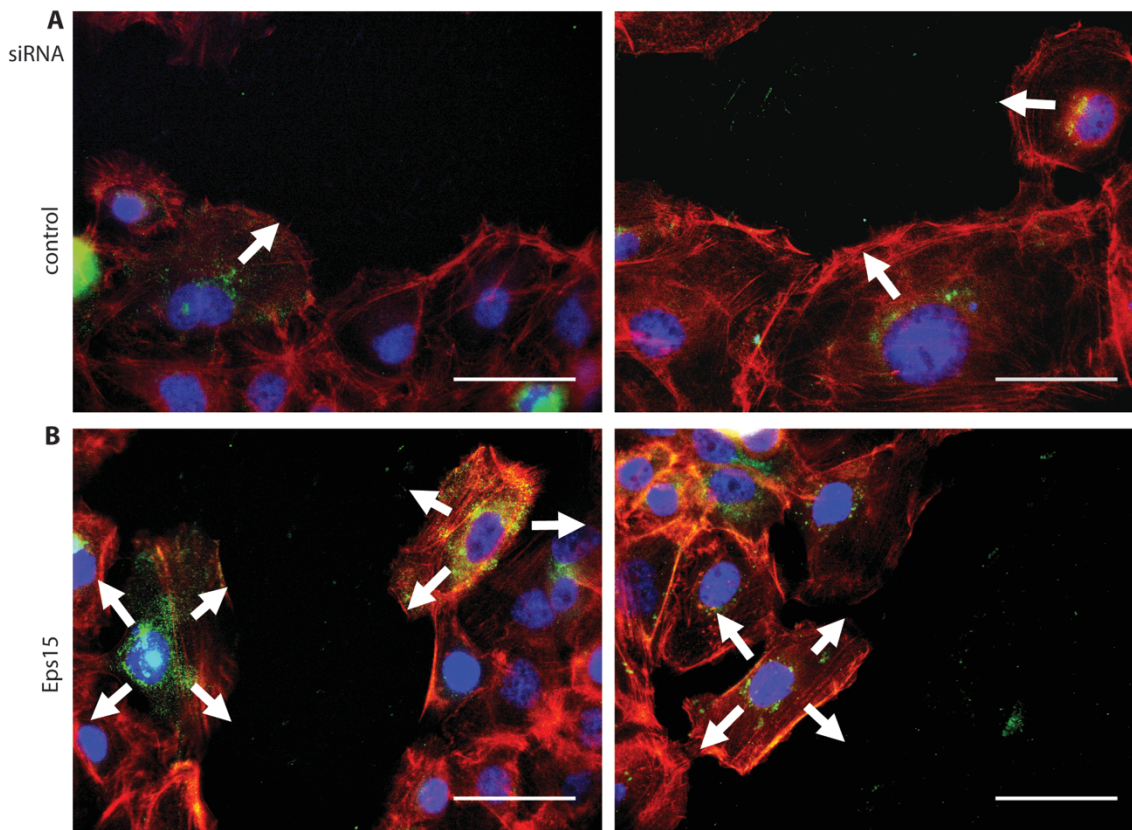


Figure 4.7 Eps15 knockdown affects Golgi polarisation

Representative immunofluorescence pictures fixed alongside time-lapse microscopy. Pictures show cells 24-hours post-scratch. Nucleus stained with DAPI (blue), Golgi apparatus stained with Giantin antibody (green) and actin stained with Phalloidin-TRITC (red) (A) SCC25 cells transfected with control siRNA show polarised Golgi orientation towards the wound (B) SCC25 cells transfected with Eps15 siRNA show non-polarised Golgi network orientation with staining shown around the nucleus. Scale bars represent 50μm.

#### 4.4 Eps15 reduces tumour size *in vivo*

Our observations indicate that Eps15 has a significant effect on cell motility *in vitro* (Figure 4.1- Figure 4.4) but for true physiological relevance the effect of Eps15 knockdown was also analysed *in vivo* by measuring tumour growth in mice (CD1-nude strain), which were subcutaneously injected with a liver metastatic cell line in which Eps15 expression was stably down-regulated using an Eps15 shRNA construct.

The liver cell line SKHEP1 was chosen as it is known to produce metastatic tumours in immune-compromised mice in just 5 weeks (Fogh et al. 1977). For stable cell line production, we purchased two validated Eps15 shRNAs (Sigma-Aldrich); 7980 and 7978, respectively. SKHEP1 cells were transduced with control shRNA (UT) and either Eps15 shRNA 7980, Eps15 shRNA 7978 or a combination of both Eps15 shRNAs; 7980 + 7978 (MIX).



The individual shRNA 7980 population and the combined MIX population grew well in *in vitro* culture and proliferated at a similar rate to control cells (UT) so were used for our *in vivo* experiments. Following generation of stable cell lines, migration assays were set up towards collagen IV (COLIV) (Giannelli et al. 2001) to confirm that, similarly to transient down-regulation of Eps15 using siRNA, cell migration is inhibited by Eps15 knockdown in the stably transduced cells. The stable cell line SKHEP1 7980 had significantly reduced cell migration compared to control SKHEP1 UT cells. Migration of the MIX population was not statistically different compared to control cells (Figure 4.8). The amount of migration seen was much more than that seen previously with cell lines SCC25, VB6 and H357 (Figure 4.1) due to the extremely metastatic nature of the SKHEP1 cell line. As a consequence of these migration assays, the 7980 cell population were used for *in vivo* experiments as they showed cell function most similar to our Eps15 siRNA treated cell lines in a migration assay.

#### 4.4.1 SKHEP1 cells expressing Eps15 stable knockdown reduce tumour cell migration in Transwell® migration assays

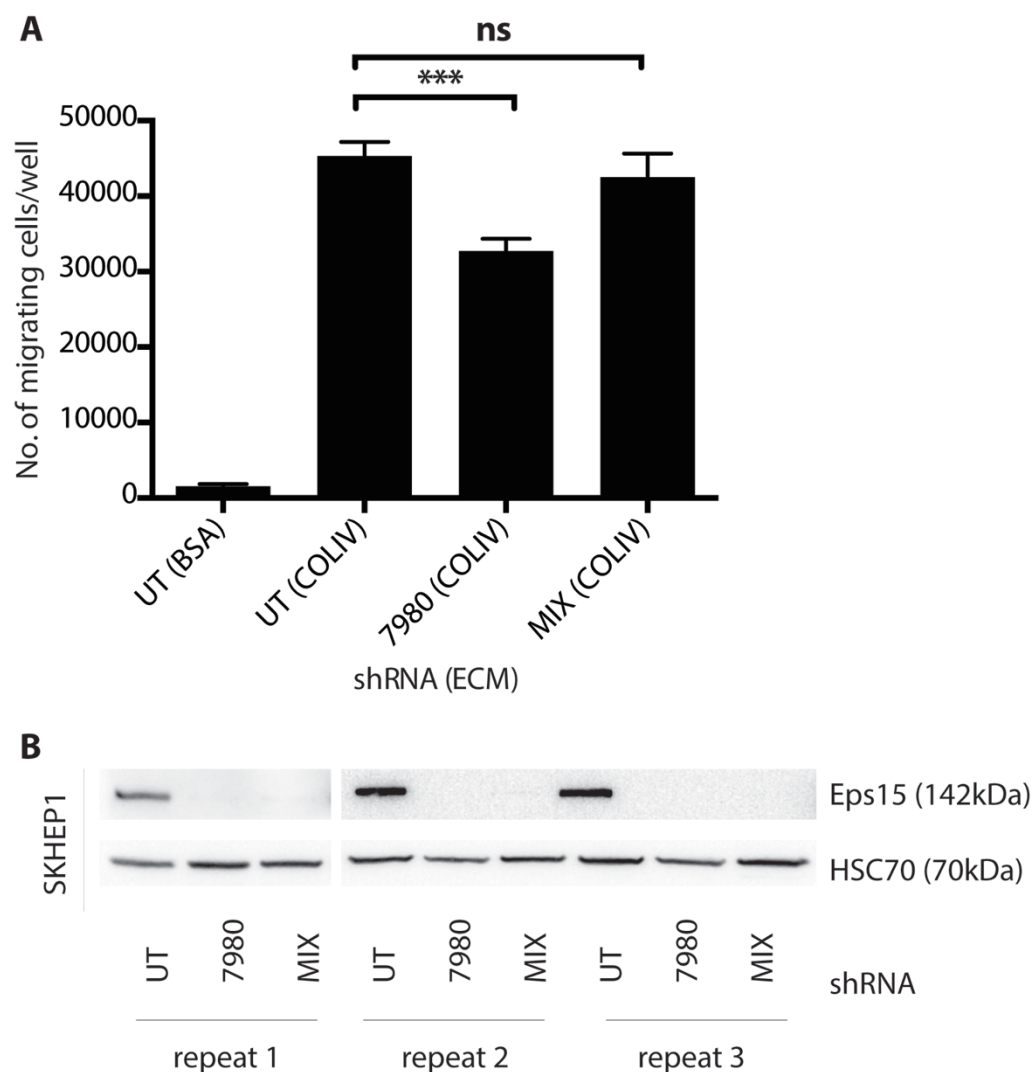


Figure 4.8 SKHEP1 cells expressing Eps15 stable knockdown reduce tumour cell migration in Transwell® migration assays

(A) Cell migration was studied with SKHEP1 clones; SKHEP1 with control shRNA (UT), SKHEP1 with 7980 Eps15 shRNA (7980) and SKHEP1 with combined 7980 + 7978 Eps15 shRNA (MIX). Transwells® were coated with BSA or the ECM protein collagen IV (COLIV) to encourage cell migration. Conditions were set up in triplicates and graphs show the accumulated results of three independent migration assays (n=3). MIX cells show no significant decrease in tumour cell migration while 7980 cells show a significant inhibition of cell migration compared to UT cells (7980:  $p < 0.001$ ; MIX:  $p = ns$ ). Significance was tested by unpaired t-tests and the graph shows mean with error bars indicative of standard error of the mean (SEM) (B) Cells were collected after the migration assay was set up and Eps15 protein knockdown efficiency was confirmed by Western blotting. 40µg of protein was loaded for each sample and HSC70 was used as a loading control.

#### 4.4.2 SKHEP1 TagFP585 vector infection for use in IVIS® imaging

Following sub-cutaneous injection of SKHEP1 cells we wanted to analyse the growth of primary-tumours and observe any consequent metastases using the IVIS® animal imager. IVIS® is able to

image pre-labelled bioluminescent or fluorescent tumour-cells in 3D so can identify which mice develop tumours, including the presence and location of metastases.

To be able to use IVIS® for analysing tumour growth, stable SKHEP1 cells (UT and 7980) were infected with another lentivirus containing TagFP585 vector. Following infection, cells were tested for red fluorescence using the Fortessa™ and Canto I cell analysers (Figure 4.9). There was clear infection of TagFP585 cells in both cell lines. However, while UT cells were ~97% positive, the 7980 cell line was only ~18% positive suggesting the need to sort these cells before use *in vivo*. Both flow cytometers were required because the Fortessa™ has a yellow laser which is more accurate for detecting TagFP585 using the PE-Texas Red channel, while the Canto I can only detect TagFP585 using the PE channel and so the shift in positive population is reduced. The FACS Aria™ II cell sorter used to sort these cells has the same lasers as the Canto I and so we wanted to ensure that there was enough of a shift in positive cells for clear detection on the FACS Aria™ II cell sorter.

Gating during cell sorting was set up to ensure that only cells expressing similarly high levels of red fluorescence, indicating high TagFP585 expression, were collected from each cell population, resulting in >90% of cells in both populations expressing the same high level of TagFP585 post-sort (Figure 4.10A). Western blotting was used to confirm that the sorted SKHEP1 cells were still expressing the Eps15 shRNA vector and showing an efficient knockdown of the Eps15 protein (Figure 4.10B). SKHEP1 UT and SKHEP1 7980 populations now expressing the red vector were ready to be used *in vivo*.

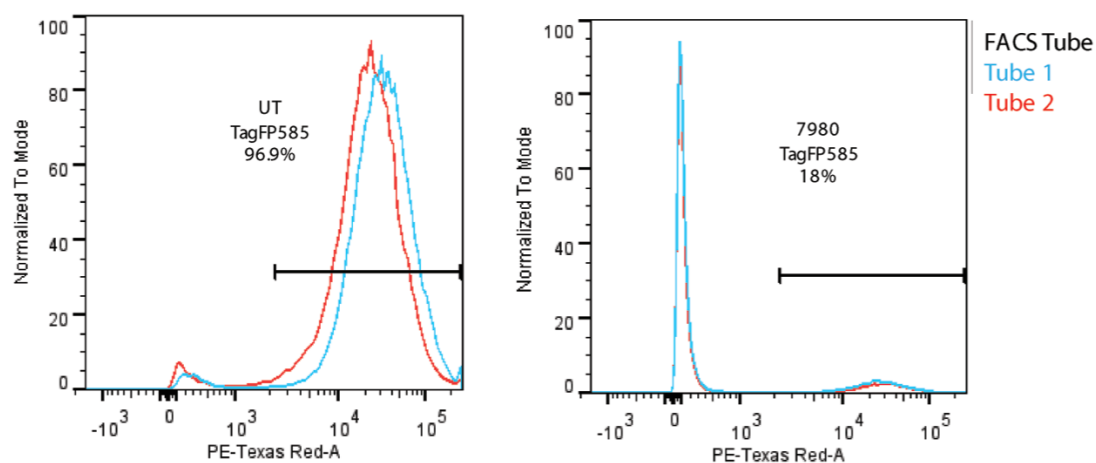
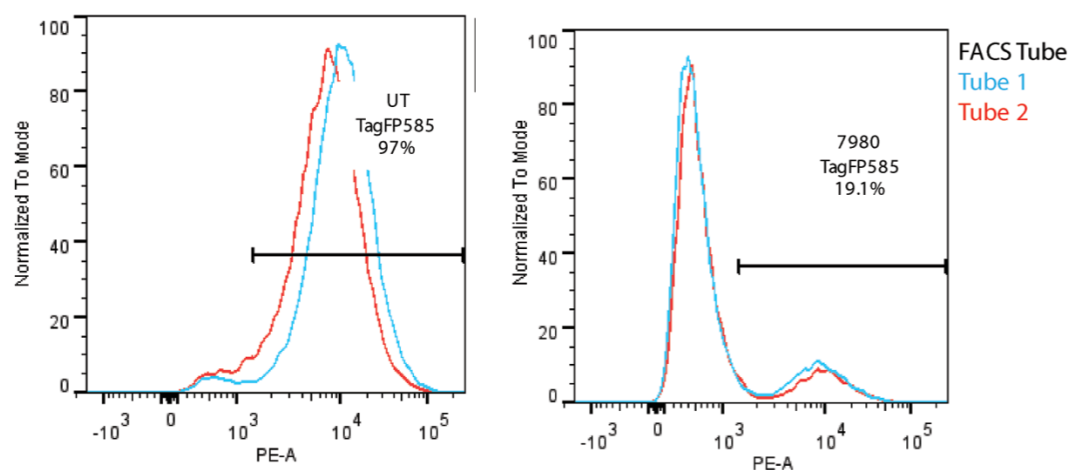
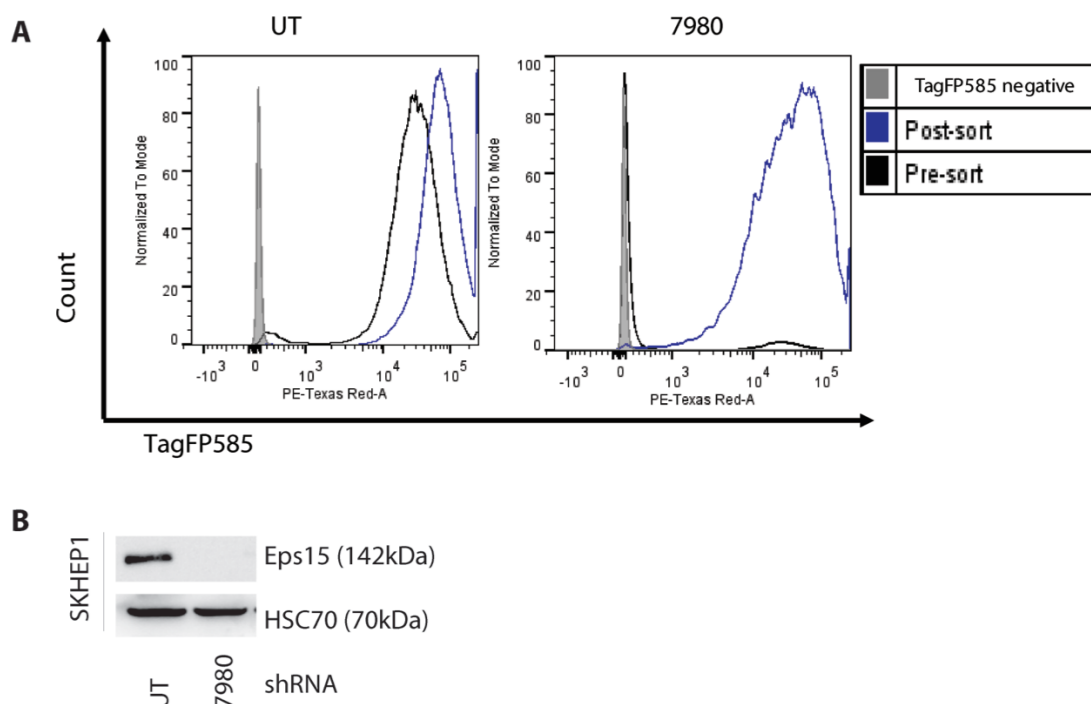
**A** Fortessa X-20 – TagFP585 read on PE-Texas Red channel**B** Canto I – TagFP585 read on PE channel

Figure 4.9 SKHEP1 7980 cells expressing TagFP585 (pre-sort)

SKHEP1 UT and 7980 shRNA expressing cells were infected with TagFP585 vector to express red fluorescence for IVIS® imaging. (A) The Fortessa™ cell analyser was used first to detect TagFP585 expression in the PE-Texas Red channel. The UT cell line showed ~97% infection while the 7980 cell line showed ~18%. (B) The Canto I cell analyser was next used to detect TagFP585 cells using the PE channel. This channel is the same as that used by the FACS Aria™ II cell sorter and so it was necessary to confirm that the positive populations could be detected by the lasers found in this machine. The percentages of positively infected cells remained the same as with the Fortessa™ but the shift in TagFP585 expression was reduced. However, two clear populations could be seen suggesting that cell sorting with the FACS Aria™ II would be possible. Two FACS tubes of each population were analysed, labelled Tube 1 and Tube 2 respectively.

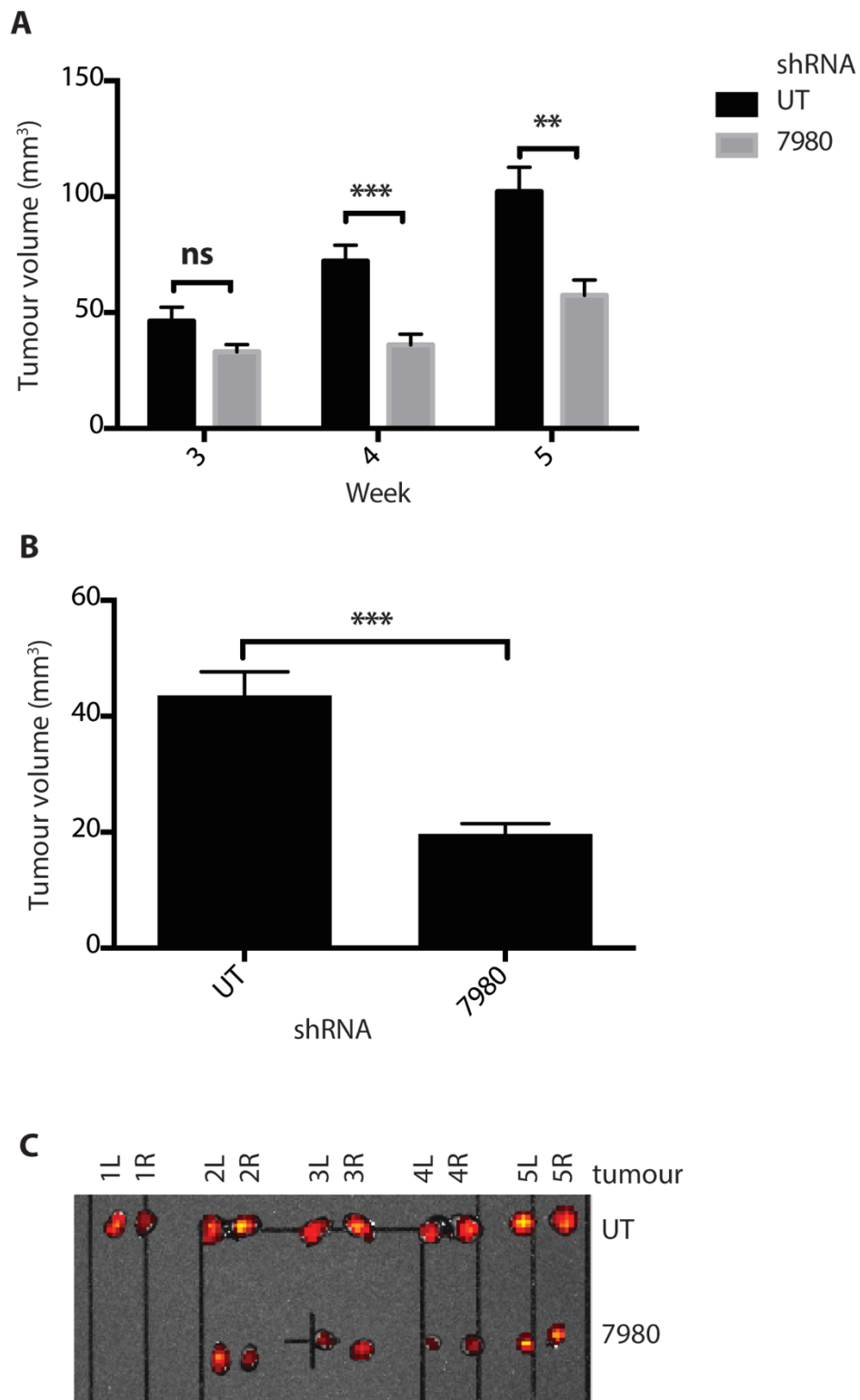


**Figure 4.10 SKHEP1 cells expressing Eps15 shRNA and TagFP585 (post-sort)**  
SKHEP1 UT and 7980 shRNA expressing cells were infected with TagFP585 vector to express red fluorescence for IVIS® imaging. Following infection, cells were sorted using FACS Aria™ II cell sorter to isolate cells which had high TagFP585 expression. (A) Following cell sorting the cells were run on the Fortessa™ cell analyser and compared to pre-sorted cells. The grey peak shows a separate TagFP585 negative control population of SKHEP1 cells. The black line indicates cell fluorescence pre-sorting; UT cells are >80% TagFP585 expressing while <20% of 7980 cells are TagFP585 expressing shown by very small peaks. The blue line indicates cells post-sorting with both populations showing a large peak of red fluorescence above  $10^4$  in the PE-Texas Red channel indicating high TagFP585 expression in >90% of cells. (B) Post-sort Eps15 shRNA knockdown efficiency was confirmed by Western blotting. 40µg of protein was loaded for each sample and HSC70 was used as a loading control.

#### 4.4.3 Eps15 knockdown significantly reduces tumour volume *in vivo*

Ten CD1-nude mice were subcutaneously injected with tumour cells into both flanks; five mice were injected with SKHEP1 UT cells and five mice were injected with SKHEP1 7980 cells, producing ten tumours in total per condition. Due to the highly metastatic nature of the SKHEP1 cell line (Eun et al. 2014) it was important to consider the potential formation of metastases when injecting the mice. If we had injected mice with SKHEP1 UT cells in one flank while SKHEP1 7980 cells on the other, if distant metastases had developed it would have been difficult to determine from which primary tumour the metastases had originated. By injecting mice with only one condition we would be able to compare, on a per mouse basis, whether control or Eps15 knockdown cells were more metastatic. Additionally, injection of control or Eps15 knockdown cells could induce different stromal and immune responses, which could potentially affect overall tumour growth in the same animal. By separating the conditions, we can remove this variation as

well as remove any bias which could come as a result of injecting into the left or the right flank, one of which could experience favourable growth. Lumps created by tumours were measured using an electronic calliper each week (length and width) until the mice were to be culled after 6 weeks growth. Tumour volume was then calculated from these measurements and we saw that 7980 cell population tumours were smaller each week than UT cell population. This difference in size was significant after 4 weeks growth (Figure 4.11A). We measured the tumours at week 6 following extraction and confirmed that the 7980 cell population produced tumours which were significantly smaller than UT cell population tumours (Figure 4.11B). Extracted tumours were imaged using the IVIS<sup>®</sup> imager and Living Image<sup>®</sup> software to confirm that our tumours were composed of red-expressing tumour cells (Figure 4.11C). Tumours composed of the 7980 cell population were visually smaller than their UT counterparts and all tumours expressed TagFP585 vector as they show red fluorescence (Figure 4.11C). One of the mice injected with 7980 cells only produced one tumour in the left flank and this tumour was much larger than all other tumours produced by this group of mice. This outlier was removed from the analysis as it was not representative of this group of tumours (Appendix 1 – Figure S1.1).



**Figure 4.11 Eps15 knockdown significantly reduced tumour growth**  
 Injected mice were housed for 6 weeks to allow tumour growth. Volume ( $\text{mm}^3$ ) was calculated from length and width measurements of each tumour ( $V = ((W^2 \times L)/2)$ ) (UT;  $n=10$ , 7980;  $n=8$ ) (A) Tumours produced by the 7980 cell population were smaller than UT cell population tumours and this difference was significant following 4 weeks growth (week 4;  $p < 0.001$ , week 5;  $p < 0.01$ ). (B) This difference in tumour size was still significant following tumour extraction ( $p < 0.001$ ). (C) Extracted tumours imaged using IVIS<sup>®</sup> imaging technique and Living Image<sup>®</sup> software. 7980 tumours are visually smaller than UT tumours. All tumours measured are shown from mice 1-5 left (L) and right (R) flanks.

Imaging the mice on IVIS<sup>®</sup> we saw no obvious metastasis. Red fluorescence was localised to the sites of subcutaneous injection where tumour lumps had formed (Figure 4.12A). Pictures were taken of all 5 UT mice and all 5 7980 mice and max and min fluorescence was set to the same parameters for analysis and the same size region of interest (ROI) was used for each tumour area (Figure 4.12B). Living Image<sup>®</sup> software takes 6 pictures of sequential spectrum, to allow us to determine the background fluorescence from that of the tumour (Appendix 1 – Figure S1.2). Relative fluorescence unit (RFU) parameters were then set to appropriate max and mins by assessing these images and all images were analysed at the same parameters. The amount of light shown with Living Image<sup>®</sup> software is proportional to the number of cells thus calculation of RFU using Living Image<sup>®</sup> software showed significantly fewer cells expressing red fluorescence in 7980 mice tumours compared with UT mice tumours (Figure 4.12C), indicating that Eps15 knockdown inhibits the growth of tumour cells *in vivo*.

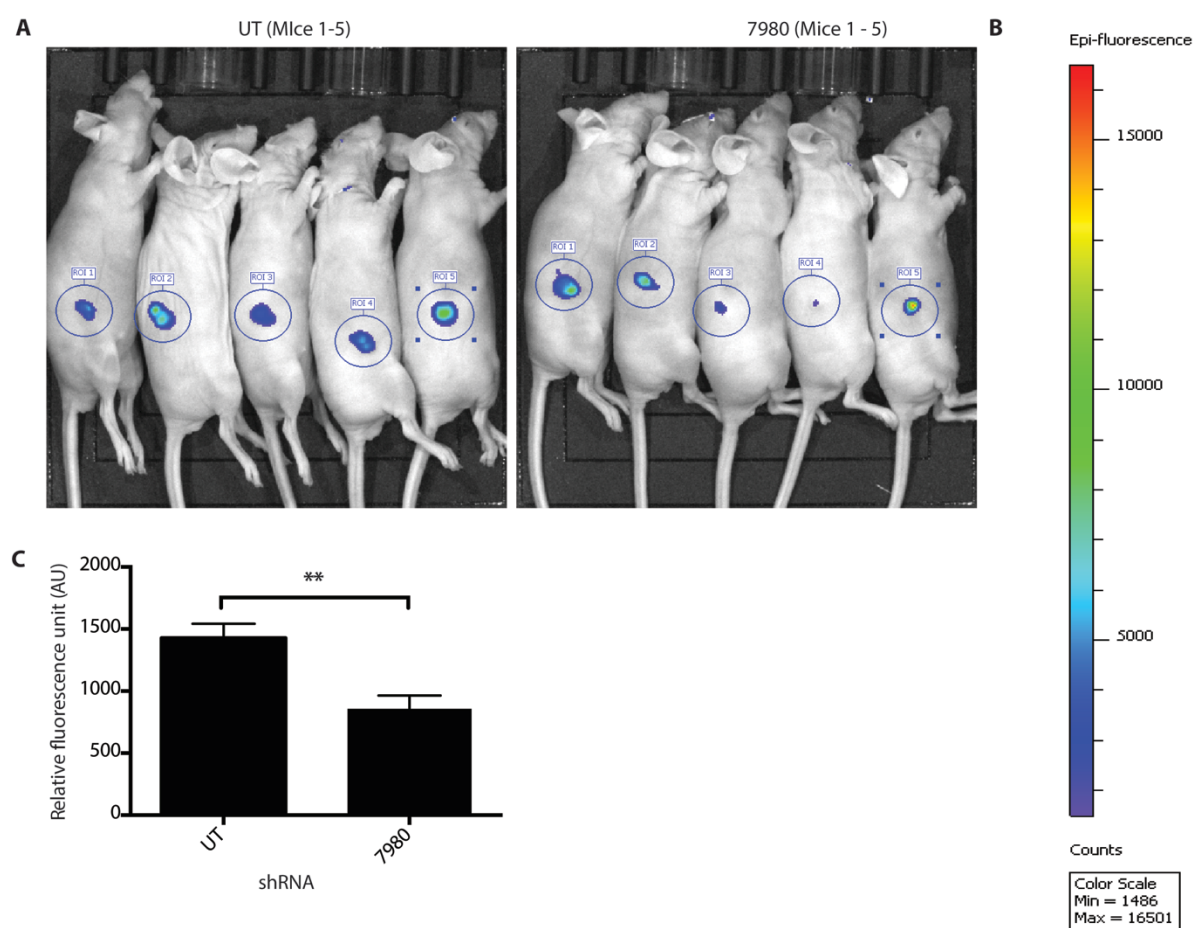


Figure 4.12 Eps15 knockdown significantly reduces tumour size *in vivo*

(A) Mice were imaged in two groups; UT and 7980, using IVIS<sup>®</sup> Lumina series and Living Image<sup>®</sup> software once culled. Max and min fluorescence was set to the same parameters for UT mice and 7980 mice (B) Living Image<sup>®</sup> software measures fluorescence of the tumour cells *in vivo* of a defined region of interest (ROI) (C) Relative Fluorescence Unit (AU = arbitrary unit) correlates to size of tumour; 7980 tumours show significantly less fluorescence than UT tumours indicating smaller size compared to UT tumours ( $p < 0.01$ ).



Once all the mice had been imaged we imaged a representative pair of mice for an appropriate comparison of the two conditions. Once again 7980 tumours showed significantly less RFU than UT tumours indicating that they are smaller (Figure 4.13).

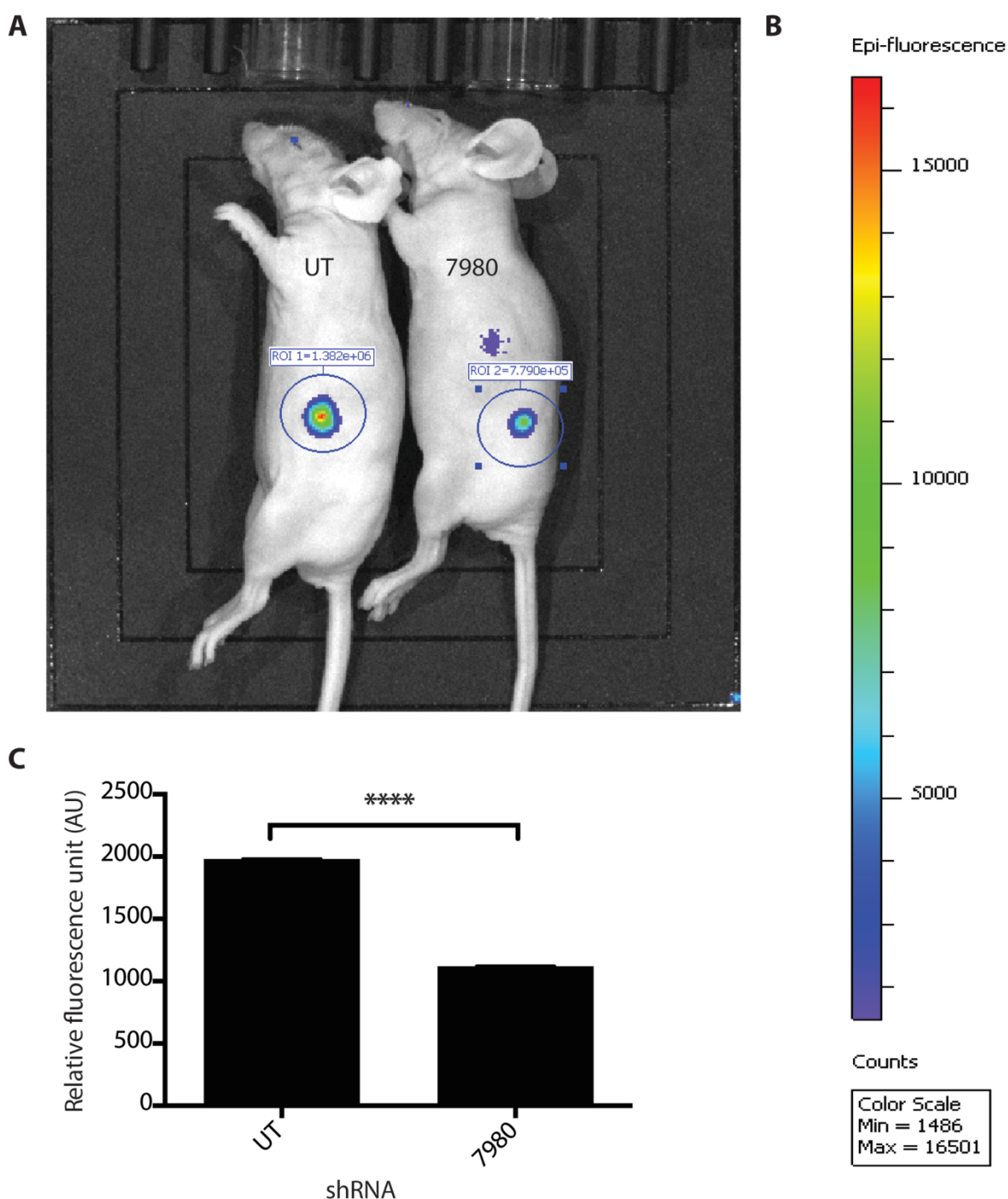


Figure 4.13 Eps15 knockdown significantly reduces tumour size *in vivo* (representative pair) (A) Mice were imaged using IVIS<sup>®</sup> Lumina series and Living Image<sup>®</sup> software once culled. A representative mouse from each group; UT and 7980 were chosen for separate analysis. Max and min fluorescence was set to the same parameters for UT mice and 7980 mice. (B) Living Image<sup>®</sup> software measures fluorescence of the tumour cells *in vivo* of a defined region of interest (ROI) (C) Relative fluorescence unit (AU = arbitrary unit) correlates to size of tumour; 7980 tumours show significantly less fluorescence than UT tumours indicating smaller size compared to UT tumours ( $p < 0.0001$ ).

## 4.5 Discussion

Previous research by Ramsey *et al* highlighted a role for clathrin-mediated integrin endocytosis in tumour cell migration and invasion (A. G. Ramsay *et al.* 2007). Although the authors did not investigate the role of Eps15 in cell motility in detail, they showed that inhibition of  $\alpha\text{v}\beta 6$  integrin endocytosis either by HAX-1 knockdown or by down-regulation of clathrin heavy-chain significantly inhibits  $\alpha\text{v}\beta 6$ -specific cell migration and invasion. Ramsay and co-workers only tested the role of Eps15 in Transwell® migration of VB6 cells and concluded that over-expression of the dominant-negative mutant of Eps15 inhibits  $\alpha\text{v}\beta 6$ -specific motility (Ramsay *et al.* 2007, Supplementary Material). Our results are consistent with Ramsey and co-workers' findings suggesting an important role of Eps15 in clathrin-mediated endocytosis and its regulation of cell motility. While the role of clathrin-mediated endocytosis in the internalisation of integrins has previously been investigated (Caswell & Norman 2006; Caswell *et al.* 2009), there is very limited information in the literature on the role of Eps15 in this process. Although, it is well described that Eps15 is a crucial component of clathrin-mediated endocytosis of other cell surface receptors (Benmerah *et al.* 1998; Salcini *et al.* 1999), there are currently no studies according to our knowledge, which show the direct role of Eps15 in integrin endocytosis or integrin-related functions apart from the Transwell® migration assay presented by Ramsay *et al* (A. G. Ramsay *et al.* 2007).

Jović *et al* investigated the effects of Eps15-homology domain-containing protein (EHD1) on  $\beta 1$  endosomal transport. The EHD domain was originally identified in the NH2 terminal of Eps15 and Eps15R (Wong *et al.* 1995; Fazioli *et al.* 1993) and was later found to be conserved in a number of other proteins (including EHD1), many of which play a role in endocytic pathways. Jović and co-workers showed that EHD1 is required for integrin-mediated downstream functions including focal adhesions, cell spreading and migration. The authors demonstrated that the control of  $\beta 1$  integrin function depends upon its subcellular localisation. Upon *Ehd1* RNAi-knockdown,  $\beta 1$  integrins were found to accumulate in transferrin-containing endocytic recycling compartments due to impaired recycling of  $\beta 1$  integrins. Furthermore, the authors found *Ehd1*<sup>-/-</sup> mouse embryonic fibroblast (MEF) cells had lower overall levels of  $\beta 1$  integrins on the plasma membrane but higher cell surface-expressed activated  $\beta 1$  integrins, producing larger and more prominent focal adhesions resulting in impaired migration (Jović *et al.* 2007). These larger focal adhesions were the result of slower focal adhesion disassembly of *Ehd1*<sup>-/-</sup> MEF cells, results also supported by Ezratty *et al* who investigated the role of clathrin in focal adhesion (FA) disassembly (Ezratty *et al.* 2009). Ezratty *et al* found that clathrin mediates integrin endocytosis for FA disassembly in migrating cells, as depletion of clathrin led to the accumulation of cell surface  $\alpha 5\beta 1$  integrin

usually lost during FA disassembly (Ezratty et al. 2009). Based on these findings it is possible that perhaps Eps15 is similarly playing a role in FA disassembly and that Eps15 down-regulation could be causing de-regulation of FA disassembly, leading to decrease in cell motility. However, this theory was opposed by Ezratty *et al* when they inhibited Eps15 protein function through use of a dominant-negative Eps15 construct, Eps15 $\Delta$ 95-295. Transfection with the dominant-negative version of Eps15 caused inhibition of transferrin internalisation but had no effect on FA disassembly (Ezratty et al. 2009). However, due to many other endocytic routes that cells are able to utilise, as well as the complexity of FA disassembly, it is perhaps too simplistic to think that inhibition of just Eps15 could affect FA disassembly so drastically. Therefore, more research into the link between Eps15 knockdown and decreased cell motility is required.

As a mediator of receptor endocytosis, Eps15 has also been shown to be involved in the internalisation of other receptors including Epidermal Growth Factor Receptor (EGFR) and Receptor Tyrosine Kinases (RTK) and disruption to the internalisation of both has been shown to affect tumour cell motility (Parachoniak & Park 2009; Li et al. 2014). It is therefore possible that Eps15 regulates cell migration by affecting endocytosis of other receptors, not just integrins and that by inhibiting Eps15 expression we are affecting the internalisation of these receptors too. Impaired down-regulation of the Met hepatocyte growth factor RTK (also known as Met) leads to sustained signalling, cell transformation, and tumorigenesis and Parachoniak and Park showed that, following Met activation, the endocytic adaptor protein, Eps15, is recruited to the plasma membrane and becomes both tyrosine-phosphorylated and ubiquitinated (Parachoniak & Park 2009). Recruitment of Eps15 requires Met receptor kinase activity and involves the coiled-coil domain of Eps15 and the signalling adaptor molecule, Grb2, which binds through a proline-rich motif in the third domain of Eps15. The authors found that knockdown of Eps15 with siRNA results in delayed Met degradation. While the authors did not discuss the effect of this delayed Met degradation, we know that impaired RTK down-regulation severely affects cell function and often increases tumorigenesis. We found that Eps15 knockdown causes a decrease in cell migration and while these data show that Eps15 knockdown could possibly increase tumorigenesis it is clear that Eps15 knockdown could be affecting cell function through regulation of RTK degradation and we cannot exclude this as a possible mechanism for our effect on cell motility.

Additionally, over-expression of EGFR has been shown to cause increased tumour cell migration of human breast cancer cells (Verbeek et al. 1998) and regulation of the phosphorylation state of Eps15 regulates EGFR internalisation and signalling (Li et al. 2014). Upon EGFR stimulation with EGF, Eps15 becomes phosphorylated allowing downstream signalling of EGFR and internalisation of the receptor. Regulation of this phosphorylation has been shown by the PDZ domain-

containing protein tyrosine phosphatase, dPtpmeg in *Drosophila* but also the human homolog PTPN3 (Li et al. 2014). PTPN3-mediated tyrosine de-phosphorylation of Eps15 promotes EGFR for lipid raft-mediated endocytosis and lysosomal degradation attenuating its signalling. The authors found that ectopic expression of PTPN3 or Eps15-Y850F-expressing cells (Eps15 that cannot be phosphorylated following EGF stimulation) reduced cell migration in Transwell® migration assays and that tumours produced *in vivo* by these cells were smaller. The authors suggest that results indicate that PTPN3 and Eps15-Y850F possess the potential for tumour suppression as depletion of PTPN3 impairs the degradation of EGFR and enhances proliferation and tumorigenicity of lung cancer cells. Taken together, these results indicate that PTPN3 may act as a tumour suppressor in lung cancer as PTPN3-mediated Eps15 de-phosphorylation promotes the ligand-bound EGFR for lysosomal degradation and down-regulates EGFR signalling (Li et al. 2014). While these authors did not knockdown Eps15 protein expression, they have shown that by reducing the ability of Eps15 to internalise EGFR that tumour cell function can be drastically affected. We cannot exclude therefore that Eps15 could be affecting cell motility in our assays by affecting the regulation of EGFR but further investigation would be needed to confirm this in our cell lines.

While most studies focus on the role of Eps15 in receptor trafficking, a previous study by Vecchi *et al* has shown that Eps15 and other proteins involved in the regulation of endocytosis, such as clathrin assembly lymphoid myeloid leukemia (CALM) and  $\alpha$ -adaptin, shuttle between the cytoplasm and nucleus and these two processes appeared to be independent of each other. They have shown that when in the nucleus, Eps15, Epsin1 and CALM act as positive regulators of transcription in a luciferase reporter assay (Vecchi et al. 2001). While this study did not examine the role of Eps15 in the transcription of any gene in particular we cannot exclude that Eps15 might regulate cell motility by affecting the transcription of certain cell signalling molecules regulating cell motility.

Eps15 homology domain-containing 2 (EHD2) is a tumour suppressor gene, over-expressed in several solid tumours, including ovarian cancer and oesophageal squamous cell carcinoma (Yang et al. 2015). Using immunohistochemistry (IHC) of 80 breast cancer and paired non-cancerous breast tissues it was found that a notably lower level of EHD2 expression was found in breast cancer tissues. Furthermore, over-expression of EHD2 suppressed, while elimination of EHD2 promoted, the migration and invasion of breast cancer cells in Transwell® migration assays. Molecular data showed that EHD2 inhibited breast cancer migration and invasion probably by dampening the expression of Ras-related C3 botulinum toxin substrate 1 (Rac1) (Yang et al. 2015). This data shows that knockdown of Eps15 could be affecting other genes which are involved in tumour suppression. Our data shows that knockdown of Eps15 reduces cell migration, while here suppression of EHD2 promotes cell migration. The relationship of Eps15 and cell motility is

perhaps more complicated than first thought and would need further investigation to elucidate how Eps15 could be affecting cell motility.

Our research, along with above literature examples have discussed the effects of single cell motility, while other forms of cell motility are just as important in tissue morphogenesis, wound healing and cancer (Malet-Engra et al. 2015; Weijer 2009; Friedl et al. 2004). Collective cell motility requires the maintenance of cell-cell junctions to move the cell cluster as a whole and stresses the importance of 'leader' cells at the front of the migrating group (Mayor & Etienne-Manneville 2016). This mode of locomotion differs to single cell motility, which requires a single cell to break cell-cell junctions, polarise and move as a single unit. By maintaining cell-cell junctions, collective cell motility allows both active and passive translocation of both motile and non-motile cells (Friedl et al. 2004) and can even contribute to resistance to chemo-repulsion, whereby a group of cancer cells does not migrate away from a chemotherapeutic substance like individual cancer cells would (Malet-Engra et al. 2015). While collective cell migration requires 3D context, we measured sheet cell motility which has some parallels with collective cell motility (Figure 4.6). Differences between single and collective modes of motility suggest that they are regulated differently so perhaps our results, which suggest Eps15 can regulate single cell motility (Figure 4.1-Figure 4.4) and not sheet cell motility (Figure 4.6), are not so surprising. Eps15 knockdown may reduce single cell motility by disrupting receptor endocytosis, but perhaps cell-cell junctions are not affected, allowing cells to still migrate as a collective layer. Cell motility is reduced by Eps15 knockdown but this knockdown is by no means 100% efficient. Therefore, perhaps passive motility, as a consequence of intact cell-cell junctions, is occurring if 'leader' cells at the wound edge are able to migrate (Friedl et al. 2004). Furthermore, in our Transwell<sup>®</sup> migration assay we were looking at  $\alpha v\beta 6$  and  $\alpha 5\beta 1$  integrin-specific motility, while in our wound healing assay, other integrins might play a role. This could make a large difference on the amount of cells which migrate, as based on our results we cannot say with certainty that Eps15 affects every single integrin.

Our observations that the Golgi apparatus was not polarised was an unusual observation given that cells were able to close the wound as quickly as control treated cells (Figure 4.7). This observation was unusual given that organisation of cell polarity is considered the first step in directional cell motility (Mayor & Etienne-Manneville 2016; Weijer 2009; Ridley et al. 2003). During both single cell and collective cell motility cells must first polarise in order to direct appropriate cell motility. Cell polarisation requires the organisation of many structures either to the front or rear of the cell to initiate appropriate migration. The Golgi complex, lamellipodia and microtubules all organise towards the leading edge of the cell, whereas other structures localise towards the cell rear; including the nucleus, stress fibres and mature focal adhesions (Ridley et al.

2003). During collective cell motility the same polarisation is observed but polarity extends across the whole cell cluster (Weijer 2009). In wound-healing assays, the cells at the wound-edge orient their centrosomes towards the wound and many papers suggest that this polarisation is triggered by the formation of new cell-ECM adhesions as cells spread into the wound (Etienne-Manneville & Hall 2001). A paper by Desai *et al* discuss that although cell-ECM contacts can play a role in cell polarity that cell-cell contact can also correlate with good polarisation, membrane ruffling and consequent cell migration (Desai et al. 2009). Desai used cells infected with a truncated, dominant-negative mutant of E-cadherin (Ad-EΔ) during their investigations to show that E-cadherin at cell-cell contacts is required for mediating and maintaining cell polarity as cells lacking E-cadherin were unable to orient the Golgi network to face the wound. Interestingly, these cells were still able to migrate directionally to close the wound with a speed comparable to control cells (Desai et al. 2009), similar to our own results (Figure 4.6 and Figure 4.7). The cell monolayer produced with Eps15 knockdown cells in our own assay appeared less stable than that of control treated cells and Golgi orientation was disrupted (Figure 4.7), but these cells were still able to close the wound at speeds similar to control treated cells. Our observations indicate that Eps15 could potentially be affecting cell-cell contacts as well as cell polarity and perhaps this disruption occurs through E-cadherin. Interestingly, the E3 ubiquitin-protein ligase also known as mouse double minute 2 homolog (MDM2) promotes cell motility and invasiveness by regulating E-cadherin degradation through a mechanism that involves endocytosis (Yang et al. 2006). Yang *et al* found that MDM2 directly binds and down-regulates E-cadherin and that expression of ectopic MDM2 not only reduced levels of E-cadherin but also enhanced cell dissociation, motility and invasive activity of breast cancer cells (Yang et al. 2006). The authors found that MDM2 and E-cadherin co-localise in the early endosome and that endocytosis is necessary for MDM2-mediated regulation of E-cadherin function (Yang et al. 2006). Yang *et al* transfected MCF7 cells with a dominant-negative mutant of dynamin (a general endocytosis inhibitor) and found that this led to significant increase in E-cadherin protein and interfered with the interaction between E-cadherin and MDM2. Cells that expressed both MDM2 and dominant-negative dynamin had a greater degree of attachment to other cells than cells which expressed MDM2 and wild-type dynamin, indicating that MDM2 facilitates cell motility by degrading E-cadherin through a mechanism that involves endocytosis (Yang et al. 2006). A dominant-negative mutant of Eps15 also disrupted the interaction between MDM2 and E-cadherin further confirming a role for endocytosis in facilitating the interaction of MDM2 and E-cadherin and supporting a role for both dynamin and Eps15 in the regulation of E-cadherin function by MDM2 (Yang et al. 2006). The authors suggest that MDM2 is capable of ubiquitinating E-cadherin, which may serve as a sorting signal for endocytosis, leading to E-cadherin cycling to the early endosome. Therefore, Eps15 is potentially involved in the regulation of E-cadherin expression and could affect cell motility as a consequence, but further

research would need to occur to establish a link between Eps15 and E-cadherin. To conclude, our observations alongside those by Desai *et al* and Yang *et al* highlight the complexity of cell migration and show how cell-cell as well as cell-ECM cues are interlinked but can also have very separate effects on a cell (Desai *et al.* 2009; Yang *et al.* 2006).

Our *in vivo* work also suggests a role for Eps15 in tumour growth. We found that cells expressing a stable knockdown of Eps15 produced significantly smaller tumours compared with control SKHEP1 cells (Figure 4.12-Figure 4.13). Eps15 knockdown did not significantly affect cell proliferation *in vitro* (Figure 4.3) suggesting that loss of Eps15 is able to affect tumour growth *in vivo* without affecting cell proliferation. We have only found one study, which examined the role of Eps15 in tumour progression in a mouse model. Li *et al* showed that expression of a mutant of Eps15 that could no longer phosphorylate following EGF stimulation (Eps15-Y850F) produced markedly smaller tumours than control cells (Li *et al.* 2014). Over-expression of the protein tyrosine phosphatase PTPN3 was able to markedly reduce tumour size *in vivo* by de-phosphorylating Eps15 and targeting EGFR for degradation, drastically affecting EGFR signalling (Li *et al.* 2014). These data suggest that by inhibiting Eps15 expression we could be inhibiting tumour growth by affecting the regulation of EGFR. Li *et al* subcutaneously injected H1975 cells stably expressing PTPN3, Eps15-Y850F or vector only controls into athymic nude mice and measured tumour volume over time (Li *et al.* 2014). Over-expression of PTPN3 or Eps15-Y850F caused a marked decrease in the tumour growth rate, similar to that seen in our own data. In addition to this Li *et al* performed immunohistochemical staining of their excised tumours (4 weeks after injection) and saw that over-expression of PTPN3 and Eps15-Y850F drastically reduced the amount of EGFR and phosphorylated Eps15 in excised tumours (Li *et al.* 2014). These results suggest that PTPN3-mediated Eps15 de-phosphorylation inhibits lung cancer formation and progression *in vivo*. Our own investigations inhibited Eps15 protein expression but we observed the same effect on tumour size. Further research would be needed to see if this decrease in tumour size was as a result of impaired EGFR signalling or another mechanism entirely, but this is an interesting discussion.

We originally set up our *in vivo* model to investigate the effect of Eps15 knockdown on tumour metastasis. It must be noted however, that our model used was with a liver metastatic cell line and that orthotopic surgery would have been a better model to assess whether Eps15 knockdown could have an effect on metastasis. However, we used the model available to us and we were able to observe an effect of Eps15 on tumour size as a result of this model. Further investigation would involve optimisation of an appropriate metastatic model to see if Eps15 knockdown is able to have an effect on tumour metastasis. Additionally, while we observed no significant effect on cell proliferation with Eps15 knockdown *in vitro* using siRNA, we cannot rule out that Eps15 could

have different effects *in vivo* and to test this Ki67 (a proliferation marker) staining of the tumour sections is going to be performed during follow-up investigations. The effects of the tumour cell micro-environment could be affecting our cells here, but further investigation would be needed to assess the interaction of Eps15 and the tumour environment. To my knowledge no one has injected Eps15 knockdown cells *in vivo* before and so investigating how Eps15 could be reducing tumour size would require further investigation, but it is promising that we have observed a significant decrease in tumour growth as a result of Eps15 knockdown compared to control tumour growth.

In summary, our results show that Eps15 plays a crucial role in regulating single-cell integrin-dependent motility consistently across a number of OSCC cell lines. We found that the role of Eps15 is not specific to  $\alpha v\beta 6$  but also detectable in  $\alpha 5\beta 1$ -dependent cell functions, which is consistent with the potential role of Eps15 in clathrin-mediated endocytosis of integrins. Based on these observations, in the next chapter we investigated the role of Eps15 in  $\beta 1$  and  $\beta 6$  integrin endocytosis as a possible explanation for the effect of Eps15 RNAi on tumour cell motility.

### 4.6 Summary

1. Eps15 knockdown significantly reduces tumour cell migration in 2D Transwell<sup>®</sup> migration assays
2. Eps15 knockdown significantly reduces tumour cell invasion in 3D Matrigel<sup>®</sup> invasion assays
3. Eps15 knockdown significantly reduces tumour cell invasion in 3D organotypic models
4. Eps15 knockdown does not significantly affect sheet cell motility in a wound healing assay
5. Eps15 knockdown significantly reduces tumour size *in vivo*



## Chapter 5: Eps15 does not regulate $\beta 6$ and $\beta 1$ integrin endocytosis

Our previous results indicate a link between the glycolytic sensor CtBP1 and the endocytic adaptor protein Eps15. We have found that knockdown of both CtBP1 and Eps15 individually cause a significant decrease in tumour cell migration and invasion. Due to its known association with endocytosis we hypothesised that Eps15 knockdown might inhibit motility by inhibiting clathrin-mediated endocytosis (CME). A link between endocytosis and oral cancer cell motility has been discussed previously by Ramsay *et al* who found that knockdown of HAX-1, another known endocytic protein decreased cell motility and invasion via an endocytosis-dependent mechanism (A. G. Ramsay et al. 2007). They also found that transfecting oral squamous cell carcinoma cells with the dominant-negative mutant of Eps15 inhibited  $\beta 6$  integrin-dependent cell migration. Regulation of  $\beta 1$  integrin recycling has also been shown to affect cell motility. RNAi knockdown of Eps15-homology (EH) domain-containing protein (EHD1) results in impaired recycling of  $\beta 1$  integrins and mouse embryonic fibroblast cells derived from EHD1-knockout mice (*Ehd1*<sup>-/-</sup> MEF) exhibit impaired migration (Jović et al. 2007). This chapter therefore examines the role of Eps15 in  $\beta 1$  and  $\beta 6$  integrin endocytosis.

### 5.1 Eps15 and CtBP1 do not regulate $\beta 6$ integrin endocytosis

#### 5.1.1 Eps15 and CtBP1 do not regulate $\beta 6$ integrin endocytosis

Endocytosis assays were carried out using cells transfected with Eps15 siRNA or CtBP1 siRNA. SCC25 cells were used to test endocytosis of  $\beta 6$ , while H357 cells were used to test  $\beta 1$  endocytosis. Based on the known role of Eps15 in clathrin-mediated endocytosis (CME) one would expect that knockdown of Eps15 expression would lead to a significant reduction in integrin endocytosis. Furthermore, given the link established between CtBP1 and Eps15, we would hypothesise that knockdown of CtBP1 would also lead to a reduction in endocytosis through its effect on Eps15 expression.

Six independent endocytosis assays were carried out in total on SCC25 cells treated with either Eps15 or CtBP1 siRNA. Each of these experiments contained three technical repeats for zero, TOTAL and stimulated cells. Cells were stimulated between 10 and 20 minutes at 37°C with SCC25 migration media. We found no consistent significant trend for an effect of Eps15 siRNA or CtBP1 siRNA on the amount of  $\beta 6$  endocytosis (Figure 5.1A). In addition, we also saw no consistent significant trend of the amount of total cell surface integrins on cells, which had undergone Eps15

or CtBP1 knockdown compared with control siRNA treated cells (Figure 5.1B). All experimental data is shown as individual experiments as it was not possible to combine the experiments due to big day-to-day variations. The percentage of  $\beta 6$  integrins internalised in control cells varied from 10% - 50% and percentages of  $\beta 6$  integrins internalised by Eps15 siRNA treated cells or CtBP1 siRNA treated cells was not consistently more or less than control siRNA treated cells (Figure 5.1A). These results indicate that  $\beta 6$  integrin endocytosis is not consistently regulated by Eps15 expression or CtBP1 expression.

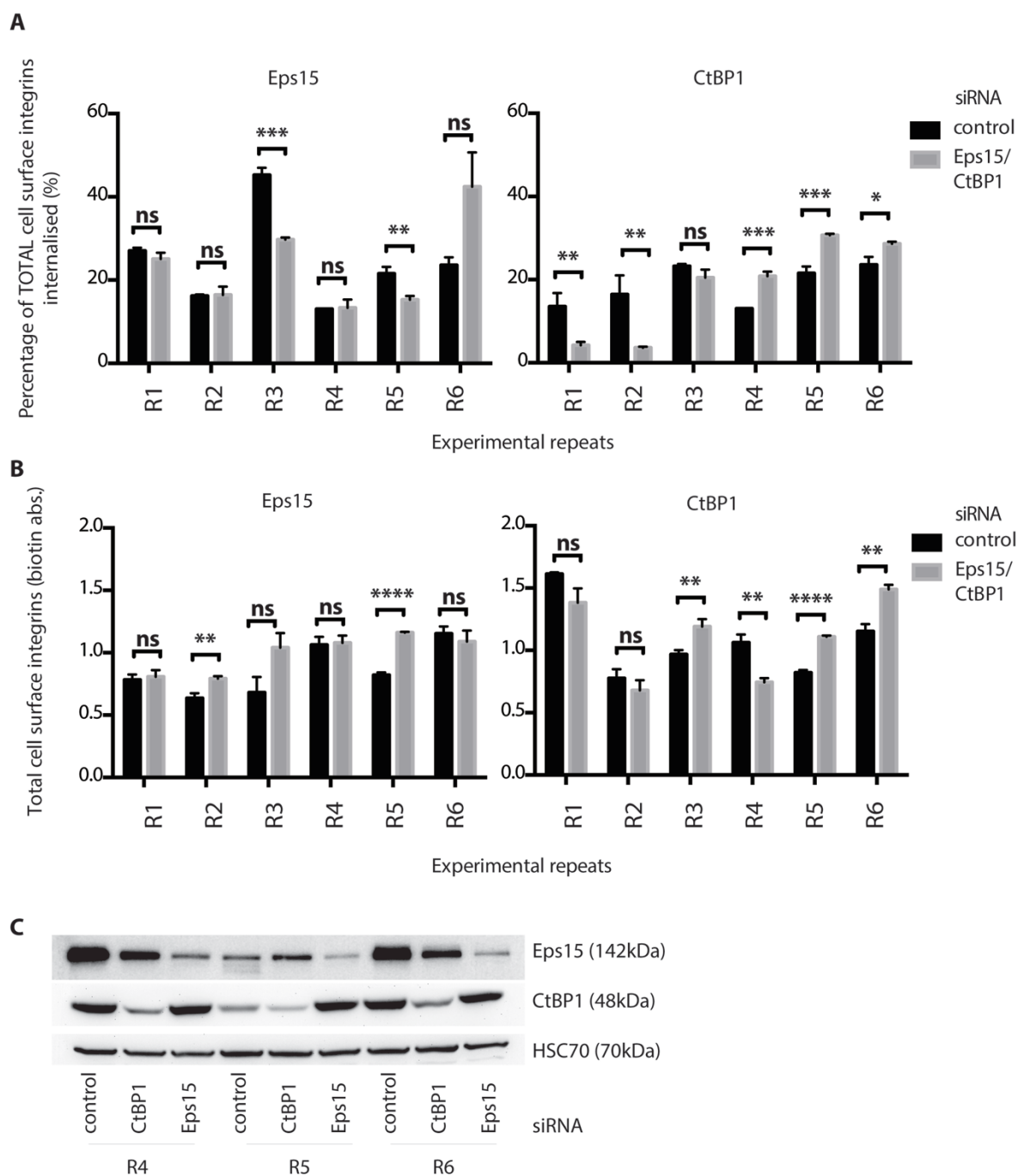


Figure 5.1 Eps15 and CtBP1 do not regulate  $\beta 6$  integrin endocytosis

Internalisation of  $\beta 6$  integrin was tested through utilisation of a biotinylation assay combined with a capture ELISA. Six repeat endocytosis assays (denoted R1-R6) using cells treated with Eps15

siRNA or CtBP1 siRNA were carried out to analyse the effect of Eps15 or CtBP1 knockdown on  $\beta 6$  endocytosis (A) Neither Eps15 knockdown, nor CtBP1 knockdown caused a consistent change in levels of  $\beta 6$  endocytosis. Multiple t-tests reveal that only experiments R3 and R5 were significant following Eps15 siRNA treatment (R1 p=ns, R2 p=ns, R3 p<0.001, R4 p=ns, R5 p<0.01, R6 p=ns). All experiments showed significant changes in  $\beta 6$  endocytosis following CtBP1 siRNA treatment except R3 (R1 p<0.01, R2 p<0.01, R3 p=ns, R4 p<0.001, R5 p<0.001, R6 p<0.05) but these experiments do not consistently show an increase or decrease in  $\beta 6$  endocytosis. All graphs show mean with standard deviation (SD) of three technical replicates in each experiment (B) No consistent change in the total of cell surface level of  $\beta 6$  integrins was also seen following Eps15 knockdown or CtBP1 knockdown. All graphs show mean with standard deviation (SD) of three technical replicates in each experiment. (Eps15; R1 p=ns, R2 p<0.01, R3 p=ns, R4 p=ns, R5 p<0.0001, R6 p=ns CtBP1; R1 p=ns, R2 p=ns, R3 p<0.01, R4 p<0.01, R5 p<0.0001, R6 p<0.01) (C) Eps15 and CtBP1 knockdown was confirmed via Western blotting. Cells were collected when siRNA treated cells were plated on COLI-coated plates for endocytosis analysis. 40 $\mu$ g of each protein was added and HSC70 was used as a loading control. A representative Western from three experiments are shown.

### 5.1.2 Eps15 and CtBP1 do not regulate cell surface $\beta 6$ integrin levels

As an additional control, total cell surface levels of  $\beta 6$  integrins were also examined via fluorescence-activated cell sorting (FACS) analysis using a Canto I flow cytometer. SCC25 cells were treated with Eps15 or CtBP1 siRNA individually and the amount of  $\beta 6$  integrins on the cell surface were analysed by FACS 48 hours post-transfection. Prior to FACS analysis cells were treated with Primaquine for 30 minutes to ensure that conditions were close to those during an endocytosis assay. Consistent with our previous results (Figure 5.1B) we saw no significant difference in total cell surface  $\beta 6$  integrins in Eps15 or CtBP1 siRNA treated cells compared to control siRNA treated cells (Figure 5.2A). In summary, these data support the conclusion that  $\beta 6$  integrin endocytosis is not consistently regulated by Eps15 expression or CtBP1 expression.

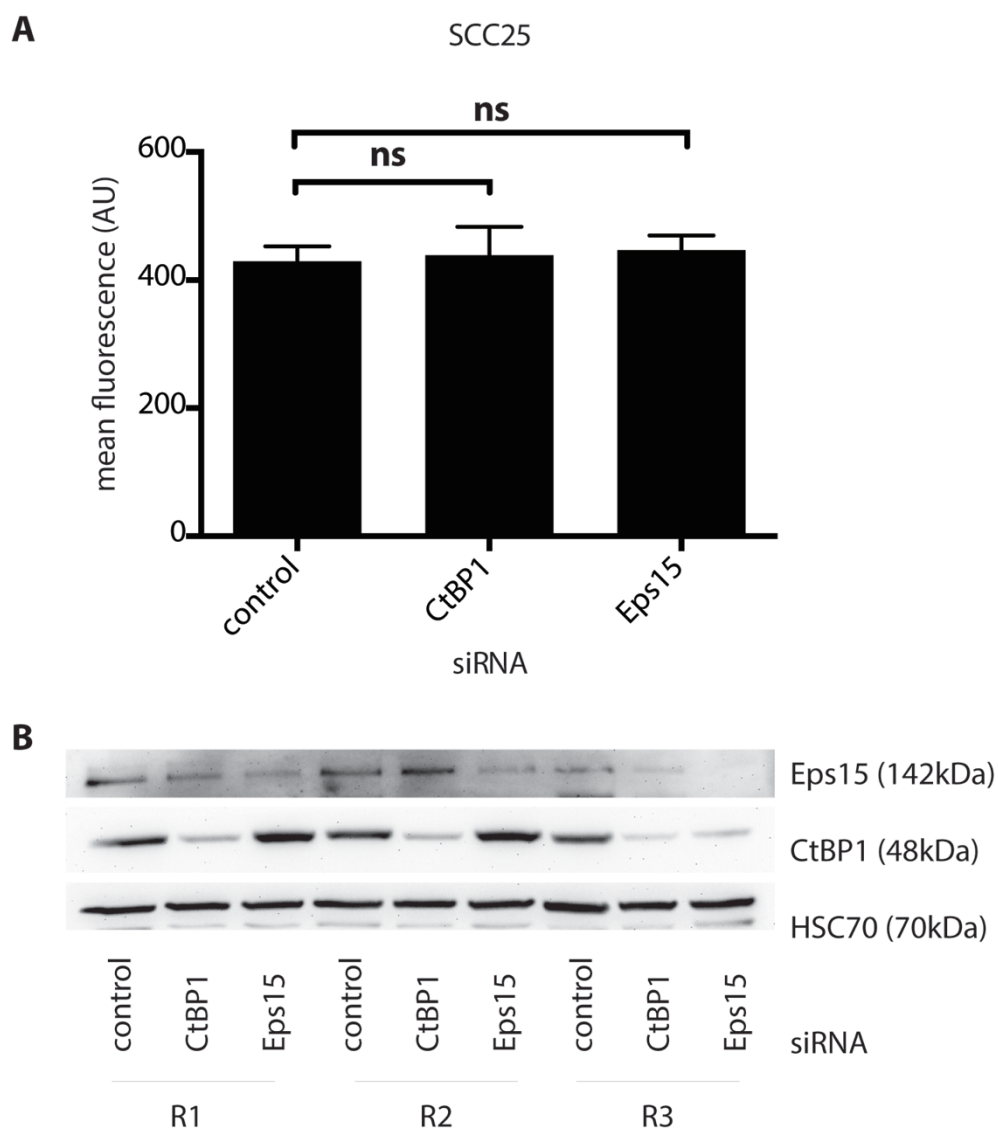


Figure 5.2 Eps15 and CtBP1 do not regulate cell surface  $\beta 6$  levels

Fluorescence-activated cell sorting (FACS) analysis was performed on a Canto I flow cytometer to analyse the total cell surface levels of  $\beta 6$  integrins expressed in SCC25 cells. Cells were treated with control, Eps15 or CtBP1 siRNA and analysed via FACS 48 hours post-transfection. Prior to analysis cells were treated with Primaquine for 30 minutes to mimic conditions of the endocytosis assay used previously (Figure 5.1) (A) FACS analysis revealed no significant difference in total  $\beta 6$  cell surface integrins seen with either Eps15 siRNA or CtBP1 siRNA compared to control siRNA. Graphs show mean and standard error of the mean (SEM) of three biological repeats for each siRNA (AU = arbitrary units) (B) Eps15 and CtBP1 knockdown was confirmed by Western blotting for each of the three experiments (R1-R3). Cells were collected 48 hours post-transfection when siRNA treated cells were collected for FACS analysis. 40 $\mu$ g of each protein was added and HSC70 was used as a loading control.

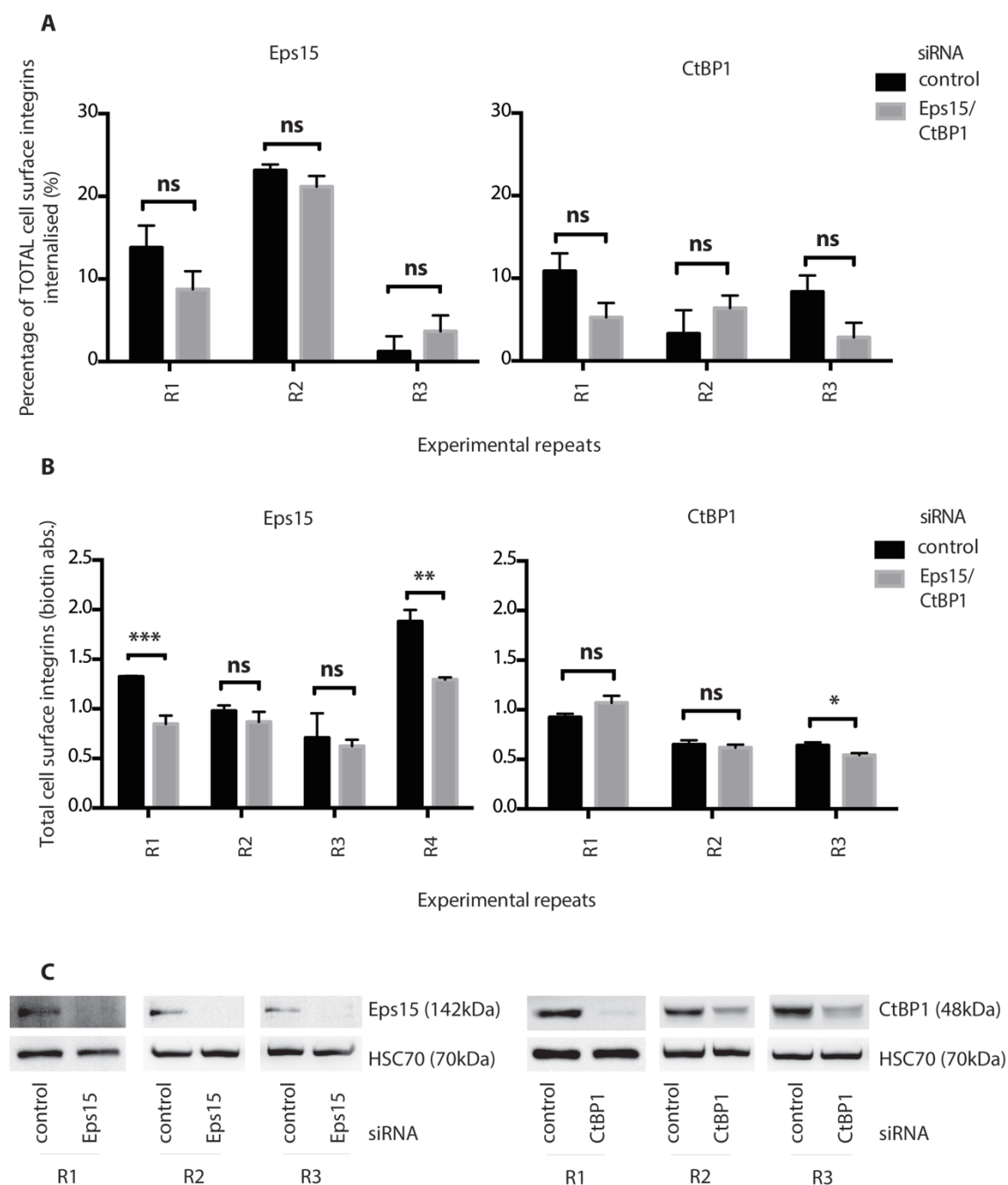
## 5.2 Eps15 and CtBP1 do not regulate $\beta$ 1 integrin endocytosis

### 5.2.1 Eps15 and CtBP1 do not regulate $\beta$ 1 endocytosis

We have established that  $\beta$ 6 integrin endocytosis is not affected by Eps15 expression or CtBP1 expression (Figure 5.1). In order to see if this observation on  $\beta$ 6 integrin internalisation can be generalised across integrins, a second cell line, H357, which expresses  $\beta$ 1 integrin, was examined.

No significant change in the levels of internalised  $\beta$ 1 integrins was observed following treatment of H357 cells with either Eps15 or CtBP1 siRNA (Figure 5.3). Two experiments showed a non-significant trend of decreased endocytosis following Eps15 knockdown, while a third showed a non-significant increase. The same inconsistent change in endocytosis was also seen with CtBP1 siRNA treated cells (Figure 5.3A).

Similarly, there was no consistent effect of either Eps15 or CtBP1 knockdown on the total cell surface levels of  $\beta$ 1 integrin either (Figure 5.3B). While in two experiments Eps15 knockdown caused a statistically significant decrease in total  $\beta$ 1 surface integrins, in two other experiments no significant effect was detected. Similarly, CtBP1 knockdown had no consistent effect on total cell surface levels of  $\beta$ 1 integrins; while it caused a statistically significant decrease in cell surface levels of  $\beta$ 1 integrins in one experiment, this effect could not be reproduced in two more repeats (Figure 5.3B). The change in pattern seen with both Eps15 and CtBP1 siRNA treated cells suggest that neither knockdown consistently significantly affects either  $\beta$ 1 integrin or  $\beta$ 6 endocytosis.

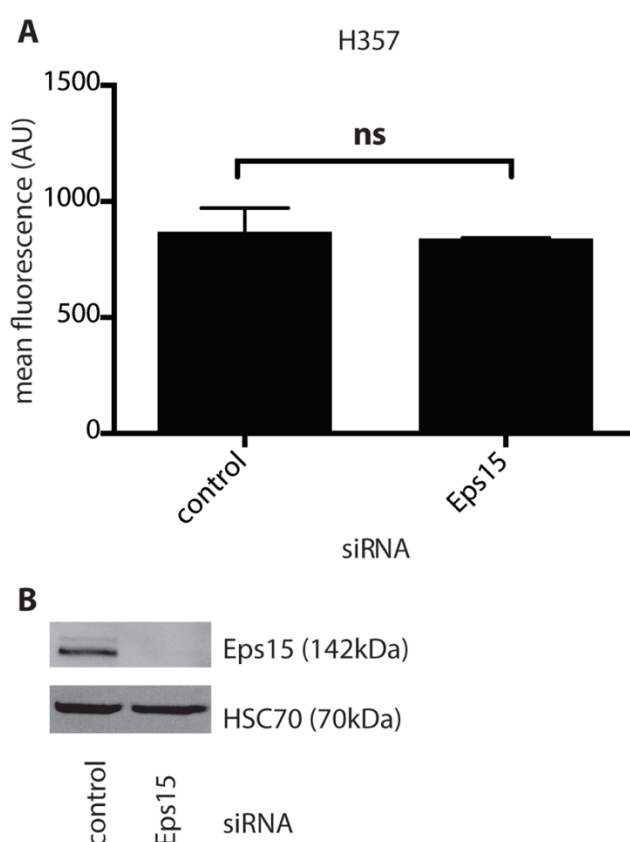


**Figure 5.3 Eps15 and CtBP1 do not regulate  $\beta$ 1 endocytosis**

Internalisation of  $\beta$ 1 integrin was tested through utilisation of a biotinylation assay combined with a capture ELISA. (A) Three repeat endocytosis assays (R1-R3) using cells treated with siRNA were carried out to analyse the effect of Eps15 and CtBP1 knockdown on  $\beta$ 1 endocytosis. Multiple t-tests reveal that neither Eps15, nor CtBP1 knockdown caused a significant change in the percentage of  $\beta$ 1 integrins internalised in any of the three experiments carried out. All graphs show mean with standard deviation (SD) of three technical replicates in each experiment (B) No consistent significant change was seen in the total cell surface levels of  $\beta$ 1 integrins following Eps15 or CtBP1 knockdown. (Eps15; R1<0.001, R2 p=ns, R3 p=ns, R4 p<0.01 CtBP1; R1 p=ns, R2 p=ns, R3 p<0.05). All graphs show mean with standard deviation (SD) of three technical replicates in each experiment (C) Eps15 and CtBP1 knockdown was confirmed via Western blotting. All Westerns (R1-R3) correspond to endocytosis experiments performed (R1-R3). A Western for R4 is not shown as not enough cells were left over to perform the Western. Cells were collected 48 hours post-transfection when siRNA treated cells were plated on COLI-coated plates for endocytosis analysis. 40 $\mu$ g of each protein was added and HSC70 was used as a loading control.

### 5.2.2 Eps15 does not regulate cell surface levels of $\beta 1$ integrin

Total cell surface levels of  $\beta 1$  integrins were also examined by FACS analysis to confirm our results using Capture ELISA. The amount of  $\beta 1$  integrins on the cell surface of H357 cells were analysed 48 hours post-transfection and prior to FACS analysis cells were treated with Primaquine for 30 minutes to ensure that conditions were similar to those during the endocytosis assay. Just as previously presented for integrin  $\beta 6$ , we saw no significant difference in total cell surface  $\beta 1$  integrins in Eps15 siRNA treated cells compared to control cells (Figure 5.4A). These data give some support to the observations made in our endocytosis assays, which show that Eps15 was unable to produce a consistent, significant effect on total cell surface  $\beta 1$  integrin levels (Figure 5.3B).



**Figure 5.4 Eps15 does not regulate cell surface levels of  $\beta 1$  integrin**

Fluorescence-activated cell sorting (FACS) analysis was performed on a Canto I flow cytometer to analyse the level of total cell surface  $\beta 1$  integrins expressed on H357 cells. Cells were treated with control or Eps15 siRNA and analysed via FACS 48 hours post-transfection. Prior to analysis cells were treated with Primaquine for 30 minutes to mimic conditions of the endocytosis assay used previously (Figure 5.3). (A) FACS analysis revealed no significant difference in the level of total cell surface  $\beta 1$  integrins in cells treated with Eps15 siRNA compared to control siRNA. Graph shows mean and standard error of the mean (SEM) of three biological repeats (AU = arbitrary units) (B) Eps15 knockdown was confirmed by Western blotting (blot shown is representative of all 3 experiments). Cells were collected 48 hours post-transfection, when siRNA treated cells were collected for FACS analysis. 40 $\mu$ g of each protein was added and HSC70 was used as a loading control.

### 5.3 Clathrin regulates $\beta 6$ integrin endocytosis

The fact that we have found no consistent effect of Eps15 down-regulation on either  $\beta 6$  or  $\beta 1$  integrin endocytosis or cell surface levels is surprising as the majority of the literature characterises Eps15 as an important clathrin-mediated endocytosis adaptor protein. Therefore, to confirm that clathrin-mediated endocytosis indeed plays a role in  $\beta 6$  or  $\beta 1$  integrin endocytosis, we examined the effect of clathrin heavy-chain (CHC) knockdown on the endocytosis of  $\beta 6$  integrin (Figure 5.5) in two cell lines over-expressing the integrin  $\alpha \beta 6$ .

Consistent with previous reports (A. Ramsay et al. 2007; A. G. Ramsay et al. 2007) knockdown of CHC in VB6 cells caused a consistent decrease in the endocytosis of  $\beta 6$  integrins suggesting that  $\beta 6$  internalisation is indeed dependent on clathrin-mediated endocytosis (Figure 5.5). Stimulation with serum-free medium resulted in a maximum 15% endocytosis of total cell surface  $\beta 6$ , while RNAi down-regulation of clathrin reduced the level of internalised integrins to just 5%.

Unfortunately, due to large experimental drift, combination of these experiments was just below statistical significance however, a consistent trend was seen across experiments, which was not previously seen with Eps15 or CtBP1 knockdown (Figure 5.2). Inhibition of clathrin-mediated endocytosis with CHC knockdown showed a consistent 10% reduction of  $\beta 6$  internalisation in all three experiments, which suggests that  $\beta 6$  internalisation is mediated by clathrin-dependent endocytosis pathways (Figure 5.5).

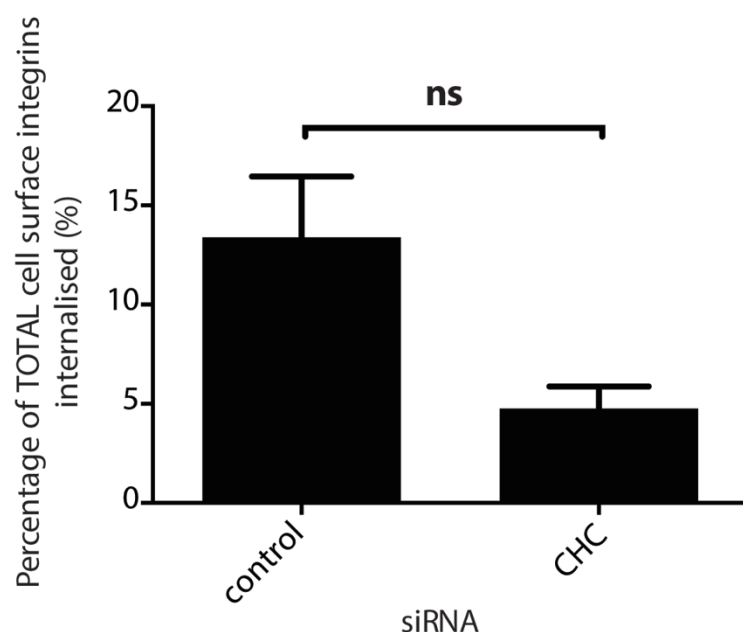


Figure 5.5 Clathrin regulates  $\beta 6$  integrin endocytosis

$\beta 6$  internalisation was tested using VB6 cells plated on human COL1 then stimulated with media for 30 minutes.  $\beta 6$  internalisation was tested through utilisation of a functional endocytosis assay and results were analysed via a capture ELISA which was read at 650 nm using a multi-plate reader. The effect of clathrin-mediated endocytosis of  $\beta 6$  was carried out using a functional



endocytosis assay with siRNA treated cells. Clathrin heavy-chain (CHC) knockdown caused consistent but non-significant decrease in  $\beta 6$  endocytosis in three independent experiments ( $p=ns$ ). Graph shows mean and standard error of the mean (SEM) of three independent experiments ( $n=3$ ). Statistical significance was determined by unpaired t-test.

To confirm our previous results with Eps15 knockdown, SCC25 cells were treated with either Eps15 or clathrin heavy-chain (CHC) siRNA. Cells were treated for 30 minutes with Primaquine as before and three technical repeats were performed for each RNAi condition. Following stimulation with SCC25 media at 37°C for 20 minutes we saw no significant effect on  $\beta 6$  internalisation with Eps15 siRNA but a significant decrease was detected in cells treated with clathrin heavy-chain siRNA (Figure 5.6A). The non-significant change in the percentage of  $\beta 6$  integrins internalised in Eps15 knockdown cells compared with control siRNA treated cells was consistent with our previous observations (Figure 5.1A). The significant decrease in  $\beta 6$  internalisation seen with clathrin knockdown cells in comparison to control siRNA treated cells is also consistent with previous reports (A. G. Ramsay et al. 2007).

Clathrin knockdown cells also exhibited a significant increase in total cell surface  $\beta 6$  integrin levels (Figure 5.6B) correlating with the decrease in  $\beta 6$  endocytosis. Eps15 siRNA treated cells also show a significant increase in total cell surface  $\beta 6$  integrin levels (Figure 5.6B). We have seen this trend previously (Figure 5.1), however these results were less consistent than those seen following clathrin knockdown.

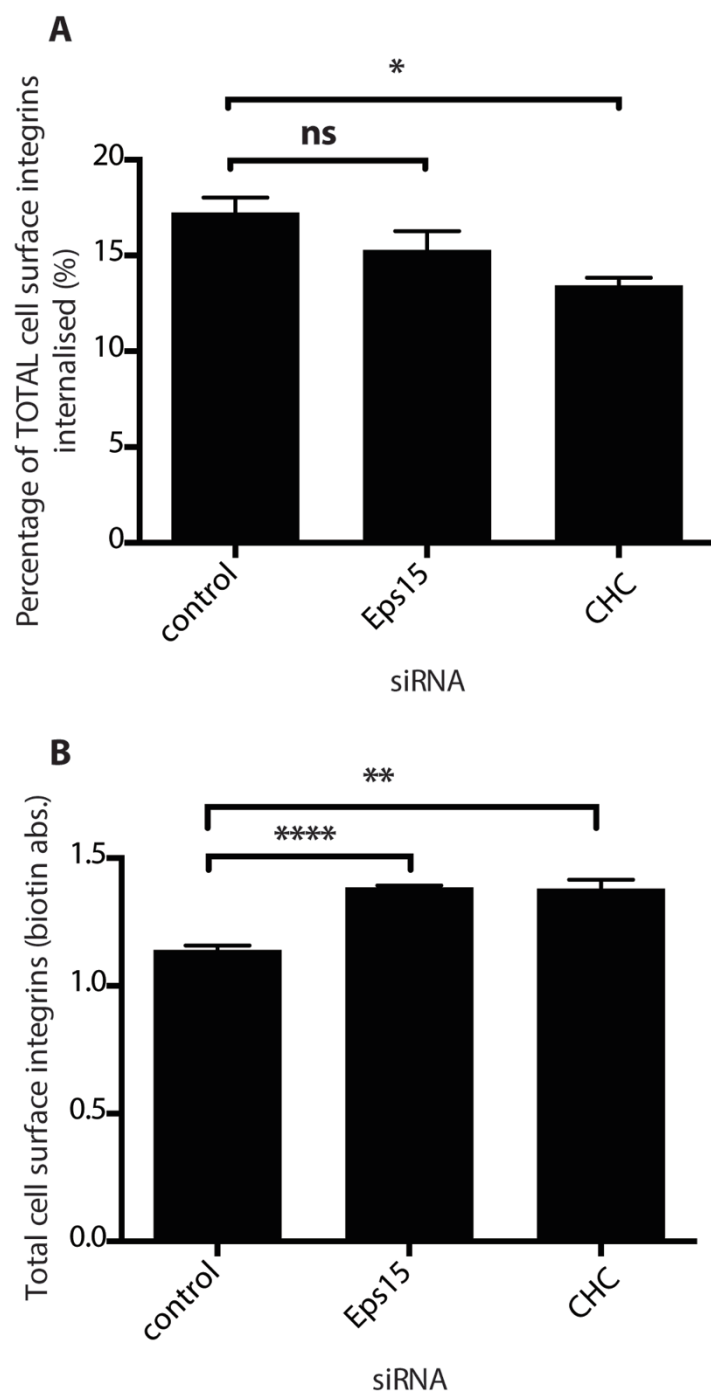


Figure 5.6  $\beta 6$  integrin endocytosis is regulated by clathrin but not Eps15

$\beta 6$  internalisation was tested using SCC25 cells plated on human COL1 then stimulated with media for 20 minutes.  $\beta 6$  internalisation was tested through utilisation of a functional endocytosis assay and results were analysed via a capture ELISA which was read at 650 nm using a multi-plate reader. (A) Cells treated with clathrin heavy-chain (CHC) siRNA show a significant decrease in the percentage of  $\beta 6$  integrins endocytosed ( $p < 0.05$ ) while Eps15 knockdown cells show a non-significant decrease in the percentage of  $\beta 6$  integrins that are endocytosed. Graphs show mean and standard error of the mean (SEM) of three technical repeats during one experiment. Statistical significance was determined by unpaired t-test. (B) Total cell surface integrins were also determined using a  $\beta 6$  internalisation assay. Both clathrin and Eps15 knockdown resulted in significant increase in cell surface  $\beta 6$  levels (clathrin  $p < 0.01$ , Eps15  $p < 0.0001$ ). Graphs show mean and standard error of the mean (SEM) of three technical repeats during one experiment. Statistical significance was determined by unpaired t-test.

In conclusion, Eps15 knockdown does not have a consistent effect on the endocytosis or the total cell surface levels of  $\beta 6$  and  $\beta 1$  integrins. On the other hand, clathrin knockdown, another known endocytic protein, caused a reduction in the percentage of  $\beta 6$  integrins internalised as well as an increase in cell surface  $\beta 6$  integrin levels as a consequence. These results suggest that while  $\beta 6$  integrin endocytosis is dependent on clathrin expression it is not regulated by Eps15.

## 5.4 Discussion

Clathrin-mediated endocytosis (CME) is required for the internalisation of a wide range of cell surface receptors including epidermal growth factor receptor (EGFR), transferrin and integrins (van Bergen En Henegouwen 2009; Benmerah et al. 1998). While clathrin has an important role in cargo and receptor internalisation from the membrane, it is unable to bind directly to the membrane or to cargo receptors and thus relies on adaptor proteins and complexes (such as adaptor protein 2 (AP-2)) and accessory proteins (such as AP180 and Epsin) to be recruited to the plasma membrane (McMahon & Boucrot 2011). Eps15 is another adaptor protein shown to be involved in clathrin-mediated endocytosis of growth factor receptors and constitutive endocytosis of transferrin (Benmerah et al. 1998). We therefore wanted to examine the potential link between Eps15 and its role in integrin internalisation.

Integrins can be internalised via both clathrin-independent and clathrin-dependent pathways (Caswell et al. 2009). Clathrin-mediated endocytosis (CME) relies on a number of adapter molecules for integrin internalisation including HAX-1 for  $\alpha v\beta 6$  internalisation (A. G. Ramsay et al. 2007) and DAB2 and AP-2 for a number of  $\beta 1$  containing integrins (Caswell et al. 2009; Teckchandani et al. 2009). While there are papers discussing the adaptor proteins required for CME of integrins (Caswell et al. 2009) the role of the adaptor protein Eps15 has yet to be investigated. One paper by Jovic *et al* discusses the role of the C-terminal Eps15-homology (EH) domain-containing protein; EHD1, in the regulation of  $\beta 1$  integrin transport (Jović et al. 2007) but to the best of my knowledge none have ever directly investigated the role of the adaptor protein Eps15 on integrin internalisation. Jovic *et al* demonstrated that *Ehd1* knockdown with siRNA impaired recycling of  $\beta 1$  integrins and that fibroblast cells derived from EHD1-knockout mice (*Ehd1*<sup>-/-</sup> MEF) had lower overall levels of  $\beta 1$  integrins on their plasma membrane but higher cell surface-expressed activated  $\beta 1$  integrins (Jović et al. 2007). Additionally, Jovic *et al* found that migration and spreading on fibronectin was impaired in *Ehd1*<sup>-/-</sup> MEF cells and that this defect could be induced in wild-type MEF cells by EHD RNAi (Jović et al. 2007). These data correspond to our own observations that Eps15 knockdown is able to impair tumour cell migration and suggests that if Eps15 is a key adaptor protein required for  $\beta 1$  integrin endocytosis that by knocking down

Eps15 in our own experiments we would see an effect on surface integrin levels. While this paper only focuses on the role of EHD1 another paper has shown a direct link between Eps15 and the integrin  $\beta 1$  tail (Humphries et al., 2009; Supplementary Material) but their investigation went no further than proteomic analysis which showed that  $\beta 1$  and Eps15 did interact. Eps15 was also found to be a regulator of  $\beta 1$  integrin activity (Pellinen et al. 2012). Pellinen *et al* used a cell spot microarray (CSMA) and found that silencing of *EPS15* resulted in the most frequent  $\beta 1$  integrin inactivation among the different cell lines that they tested (Pellinen et al. 2012). While this does not give us information on how Eps15 could be affecting integrin internalisation it does show that Eps15 can affect integrin activation which could consequently affect integrin internalisation (Margadant et al. 2011). Given the role of integrin activation in the regulation of cellular downstream processes, including cell motility, it is possible that the activation status of the integrin could affect their rate of endocytosis. However, little work has been carried out discussing the role of active versus inactive integrins and their subsequent endocytosis but new evidence is emerging, which suggests that integrins in active and inactive conformation have distinct recycling routes (Arjonen et al. 2012). The relationship between integrin activity conformations and their endocytic fate is not completely understood, and so far research has only described a possible mechanism involving active and inactive  $\beta 1$  integrins. Arjonen *et al* found that both active and inactive conformations of  $\beta 1$  are endocytosed in a clathrin and dynamin-dependent manner but the net endocytosis rate of the active  $\beta 1$  is higher. The authors concluded that inactive  $\beta 1$  integrin undergoes rapid recycling back to the plasma membrane, causing localisation mainly at the plasma membrane when cells are in a steady state, while the active conformation is predominantly intracellular (Arjonen et al. 2012). The fact that Pellinen *et al* found that Eps15 knockdown resulted in integrin inactivation is interesting as even if Eps15 is not involved directly in integrin internalisation, its effect on integrin activation could affect integrin internalisation. These papers suggested the possibility of a role for Eps15 in  $\beta 1$  integrin internalisation, however our results showed that knockdown of Eps15 did not affect the endocytosis or the cell surface levels of either  $\beta 6$  or  $\beta 1$  integrins.

The fact that we showed that Eps15 knockdown did not inhibit internalisation of either  $\beta 6$  or  $\beta 1$  integrins is interesting as it is possible that integrins are internalised via different pathways depending on their heterodimer (which  $\alpha$ - or  $\beta$ -subunits they are made of) and as a consequence which adaptor proteins are available (Caswell & Norman 2006; Margadant et al. 2011). The majority of the literature focuses on the method of  $\beta 1$  internalisation (discussed above) but it may not be possible to extrapolate these conclusions across all integrins. This could explain why despite the fact that the literature suggests that Eps15 could play a role in  $\beta 1$  integrin endocytosis that we did not see this to be the case in our investigations as integrin internalisation can be

selective depending on the  $\alpha$ -subunit available (Bretscher 1992). Bretscher *et al* found that certain heterodimers ( $\alpha 5\beta 1$  and  $\alpha 6\beta 4$ ) could be recycled rapidly while others remained at the plasma membrane ( $\alpha 3\beta 1$  and  $\alpha 4\beta 1$ ) (Bretscher 1992). Their investigation suggested that it was the  $\alpha$ -subunit which determined the endocytic route of the integrin. Additionally, De Franceschi *et al* recently found that a subset of  $\alpha$ -subunits contain an evolutionarily conserved and functional YXX $\phi$  motif (X is any amino acid and  $\phi$  a bulky hydrophobic amino acid) which selects these integrins for internalisation by the clathrin adaptor, AP-2 (De Franceschi *et al.* 2016). The authors found that site-directed mutagenesis of this motif impaired selective heterodimer endocytosis and attenuated integrin-mediated cell migration and proposed that their results highlight a mechanism by which integrins have evolved to enable selective integrin-receptor turnover in response to changing matrix conditions (De Franceschi *et al.* 2016). While we only investigated the effect of Eps15 on  $\beta$ -subunit internalisation, it is possible that  $\alpha$ -chain interactions are able to allow internalisation to continue if their interaction with another adaptor protein is strong enough, but investigations looking at the  $\alpha$ -chain would be needed to confirm this theory.

Growth-factor activated signalling kinases can also confer selectivity on integrin recycling (Roberts *et al.* 2001). Roberts *et al* studied the endo/exocytic cycle of  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  using mouse 3T3 fibroblast cell lines. They found that in serum-starved cells, internalised integrins were transported through Rab4-positive, early endosomes and arrived at the Rab11-positive, perinuclear recycling compartment approximately 30 minutes after endocytosis. From the recycling compartment, integrins were then recycled to the plasma membrane in a Rab11-dependent fashion. Following treatment with platelet derived growth factor (PDGF) however,  $\alpha v\beta 3$  but not  $\alpha 5\beta 1$ , was rapidly recycled directly back to the plasma membrane from the early endosomes via a Rab4-dependent mechanism without the involvement of Rab11 (Roberts *et al.* 2001). Such selective distribution was even shown to affect integrin function as inhibition of PDGF-stimulated  $\alpha v\beta 3$  recycling using dominant-negative Rab4 mutants compromised cell adhesion and spreading on vitronectin (a ligand for  $\alpha v\beta 3$ ), but adhesion to fibronectin (a ligand for  $\alpha v\beta 3$  and  $\alpha 5\beta 1$ ) was unchanged (Roberts *et al.* 2001). This observation could be important given our own data. We saw that Eps15 knockdown did not affect  $\beta 6$  or  $\beta 1$  integrin internalisation but, despite using serum-depleted conditions, we cannot rule out that loss of Eps15 is not having an effect on other receptors, such as epidermal growth factor receptor (EGFR) (Benmerah *et al.* 1998) which could be affecting cell motility directly or disrupting integrin signalling, causing the decrease in cell migration that occurs as a result of Eps15 knockdown.

Integrins can be regulated by growth factor receptor signalling, so it could be possible that knockdown of Eps15 affects EGFR endocytosis and integrin function as a consequence of this. Thus, while we have not seen an effect of Eps15 knockdown on integrin internalisation, the effect

of Eps15 knockdown on cell motility could be as a consequence of growth factor signalling on integrin function. EGFR has been shown to directly bind integrin  $\alpha 2$  (Yu et al. 2000) and focal adhesion kinase (FAK) has been shown to mediate the crosstalk between EGFR and integrin to affect tumour cell motility (Sieg et al. 2000). Understanding of the crosstalk between growth factor receptors and integrins is increasing. Interactions between integrins and growth factor receptors can be direct or indirect and can be collaborative with growth factor receptor (GFR) ligands or individual regulation by integrins can occur (Yamada & Even-ram 2002). Interactions do not just occur at the cell substratum but have also been investigated at sites of cell-cell contact (Yu et al. 2000). Yu *et al* found that EGFR was phosphorylated at sites of cell-cell contact even in serum-free medium, while EGFR at other sites were de-phosphorylated in the same conditions (Yu et al. 2000). This phosphorylation could be abrogated with a function-blocking  $\alpha 2$ -antibody but not a function-blocking  $\alpha 3$ -antibody. Additionally, this phosphorylation of EGFR was not seen in suspended cells or sparsely plated cells indicating cell-cell contact was required (Yu et al. 2000). This could explain our opposing results seen in different assays. Eps15 knockdown caused significant decrease in single cell motility and invasion (Figure 4.1 and Figure 4.2) but did not affect collective cell motility in a scratch wound assay (Figure 4.6). If Eps15 is affecting EGFR function to affect single cell motility than perhaps this same effect is not so pronounced in an assay measuring collective cell motility as integrin-EGFR association could be compensating for loss of Eps15 regulation of EGFR activation, but further investigation would be needed to confirm Eps15 knockdown effect on EGFR function.

Such an effect on epidermal growth factor receptor (EGFR) internalisation could be important due to the effect of EGFR signalling on cell function. Eps15b interacts with hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) to mediate degradation of EGFR (Roxrud et al. 2008) and over-expression of EGFR has been shown to increase tumour cell migration in breast cancer cell lines (Verbeek et al. 1998). This would suggest that disruption of EGFR by Eps15 knockdown could cause an increase in cell invasion, while we have observed the opposite, although this could be a consequence of using a different cell line. Thus, the effect of Eps15 down-regulation on cell migration as a consequence of regulation of this receptor would need further investigation. Eps15 is also involved in the regulation of c-Met receptor internalisation (Parachoniak & Park 2009). Over-expression of the Met receptor can also lead to changes within the cell and de-regulation of it causes increased tumour invasion and tumorigenesis (Parachoniak & Park 2009). Once again, over-expression of this receptor, possibly as a result of loss of Eps15, could result in increased cell invasion and not decreased like we have observed. However, Met receptor signalling can be regulated via other mechanisms (Lefebvre et al. 2012). Perhaps loss of Eps15 causes Met receptor

signalling to be attenuated by another mechanism, which could over compensate for the loss of Eps15, leading to reduced cell migration.

Endocytosis is a highly dynamic process and as a result there are massive redundancies built into the endocytic machinery. A review by Traub discusses clathrin-mediated endocytosis (CME) and the different adaptor proteins involved, which interact with AP-2 and clathrin, referred to as clathrin-associated sorting proteins (CLASPs) (Traub 2009). This review highlights the variety of adaptors available for CME and discusses the possibility that different adaptors could be required for selective signalling. For example; Dab2 and ARH selectively enhance the uptake of [FY]XNPX[YF]-containing but not YXX $\phi$ -containing cargos (X is any amino acid and  $\phi$  a bulky hydrophobic amino acid), while Eps15 preferentially binds UIM on ubiquitinated cargo; where EGFR is often used as an example of a protein that undergoes ubiquitin-dependent endocytosis and we know this to be possible (Benmerah et al. 1998). Simply; this review suggests the possibility that different receptors and cargo have unique motifs recognised by distinct adaptor proteins for selective internalisation into clathrin-coated pits where AP-2 and clathrin are already present (Traub 2009). However, the authors discuss that this explanation is too simple due to the multiple binding sites of adaptor proteins and the ability of receptors to bind to multiple adaptors in some cases. For example; the  $\beta$ -chain of integrins contain an activating NPX[YF] sequence that binds to the FERM domain of Talin-1 or the CLASPs Dab2, NUMB and AP-2. The NPX[YF] signal can alternate between binding Talin-1 to induce focal adhesions and binding CLASPs to mobilise integrins in a polarised manner (Traub 2009). It could be hypothesised therefore that even if Eps15 were to bind to the  $\beta$ -chain of integrins, that loss of Eps15 could merely cause the  $\beta$ -chain to bind to another CLASP for continued internalisation. While this shift could allow the continued internalisation of integrins, downstream signalling could still be disrupted enough to cause the inhibition of cell motility which we observe following Eps15 knockdown.

It is even possible that the very initiation of receptor endocytosis is highly flexible (Brach et al. 2014). Brach *et al* deleted seven genes encoding early endocytic proteins in yeast, including *Ede1* (the yeast homologue of Eps15), to try to elucidate which was responsible for endocytosis initiation. However, they found that even in the deleterious strain;  $\Delta$ , 97% of observed Sla-GFP patches were still internalised, suggesting that vesicle budding was still able to occur (Brach et al. 2014). The authors suggest that endocytosis is not as linear as was once thought and such a modular design could easily adapt and evolve to respond to different cellular requirements. The authors tested the ability of different early endocytic proteins (FRB tagged) to recruit other endocytic proteins (GFP tagged) to the membrane. Apl1-FRB, Syp1-FRB or Yap1802-FRB were all able to recruit Ede1-GFP. Conversely, Ede1-FRB was able to recruit Apl1-GFP, Syp1-GFP as well as Yap1802-GFP. These interactions show that early endocytic proteins are able to recruit each other

suggesting cooperation and flexibility in endocytosis initiation (Brach et al. 2014). The authors demonstrated that the initiation mechanism of endocytosis is highly flexible as they found that regulated cargo recruitment was defective and suggested that perhaps cargo or lipid gradients favour the assembly of the initiating adaptor proteins in the bud (Brach et al. 2014).

Alternatively, perhaps integrins could even utilise a different endocytic pathway such as clathrin-independent pathways if their CME route became perturbed. EGFR is able to alternate between methods of internalisation depending on the concentration of its substrate (Ning et al. 2007; Sigismund et al. 2008). It is therefore possible that when Eps15 knockdown occurs that the cell utilises a clathrin-independent mechanism to continue receptor internalisation, including integrins.  $\alpha 5 \beta 1$  has been shown to localise to both clathrin-coated structures as well as alternative cholesterol-sensitive caveolar routes (Shi & Sottile 2008).  $\alpha 5 \beta 1$  internalisation normally depends on NXXY motifs and clathrin but can be internalised independent of clathrin and NXXY motifs by over-expressing Rab21 (Pellinen et al. 2008). Perhaps knockdown of Eps15 is able to disrupt CME of  $\beta 1$  integrins but they are still able to internalise via a different mechanism if possible, ultimately revealing no effect of Eps15 knockdown on  $\beta 1$  internalisation.

While it is possible that  $\beta 1$ -containing integrins could utilise CME or clathrin-independent endocytosis there is no evidence to suggest that the integrin  $\alpha v \beta 6$  can enter the cell by any means other than clathrin-mediated endocytosis (A. G. Ramsay et al. 2007). Ramsay *et al* performed experiments using HAX-1 siRNA to investigate the role of HS1-associated protein X-1 (HAX-1) on clathrin-mediated endocytosis of integrin  $\alpha v \beta 6$  in head and neck cancer cell motility (A. G. Ramsay et al. 2007). The authors found that HAX-1 regulated carcinoma cell migration via clathrin-mediated endocytosis (CME) of integrin  $\alpha v \beta 6$ . Small interfering (si)RNA depletion of HAX-1 and competitive inhibition of the direct association between HAX-1 and the  $\beta 6$  integrin subunit through Tat-linked blocking peptides (Tat-HAX-1) blocked  $\alpha v \beta 6$  internalisation and consequent  $\alpha v \beta 6$ -dependent carcinoma cell migration (A. G. Ramsay et al. 2007). The authors treated VB6 and H400 cells with clathrin heavy-chain (CHC) siRNA and 48 hours post-transfection found that siRNA treatment reduced CHC levels by more than 70% and the internalisation rate of  $\alpha v \beta 6$  quantified by capture ELISA was reduced in both cell lines (A. G. Ramsay et al., 2007; Supplementary Material). Our own data (Figure 5.5-Figure 5.6) supports these observations by Ramsay *et al*, indicating  $\alpha v \beta 6$  endocytosis is dependent on clathrin expression. Additionally, Ramsay *et al* found that expression of  $\alpha v \beta 6$  was unaffected in both cell lines treated with CHC siRNA but that these cells had moderately increased levels of biotinylated  $\alpha v \beta 6$  surface expression. While we did not investigate levels of  $\alpha v \beta 6$  expression we did observe a significant increase in  $\beta 6$  surface expression following CHC siRNA treatment (Figure 5.6). While we did not investigate the effect of CHC knockdown on  $\beta 1$ -integrin internalisation the fact that CHC knockdown consistently



decreased  $\beta 6$ -integrin endocytosis suggests that this integrin at least was unable to utilise another form of endocytosis when clathrin was inhibited. While we hypothesise that integrins containing  $\beta 1$  could switch to clathrin-independent endocytosis for internalisation we would hypothesise that this is not the case for  $\beta 6$ -containing integrins. Therefore, we would suggest that either Eps15 does not affect  $\beta 6$ -integrin internalisation or internalisation is occurring in a clathrin-dependent but Eps15-independent manner.

Clathrin-dependent endocytosis can occur independent of Eps15 (De Melker et al. 2004; Pu & Zhang 2008). In fact, it has been shown that only clathrin, AP-2 and dynamin, is needed to initiate the budding of clathrin-coated vesicles *in vitro* (Dannhauser & Ungewickell 2012). Pu and Zhang investigated the mouse hepatitis virus type 2 (MHV-2) and showed that MHV-2 was able to infect cells via the clathrin-mediated pathway independent of Eps15 expression. Infection by MHV-2 was significantly inhibited in cells where the clathrin-mediated pathway was blocked and viral gene expression was significantly inhibited when cells were transfected with CHC siRNA. Over-expression of a dominant-negative mutant of caveolin-1 did not have any effect on MHV-2 infection suggesting internalisation to be reliant on clathrin-mediated endocytosis. Additionally, over-expression of a dominant-negative form of Eps15 also had no effect on viral gene expression or infectivity suggesting MHV-2 entry is mediated through clathrin-dependent but Eps15-independent endocytosis (Pu & Zhang 2008). In the context of our own results it is possible that while clathrin-knockdown consistently inhibits integrin internalisation, integrins in Eps15 knockdown cells are still able to undergo clathrin-mediated endocytosis. While it is possible for CME to occur independently of Eps15 I have found no papers which have investigated the role of Eps15 in integrin internalisation or whether Eps15 is involved in the CME of integrins. Our data suggests that Eps15 is not required for CME of integrins as loss of Eps15 showed no consistent effect on integrin internalisation or total integrin surface levels.

Eps15 expression, both protein and mRNA, is regulated by CtBP1 expression as CtBP1 knockdown causes a decrease in Eps15 expression (Figure 3.3 – Figure 3.5). Therefore, we wanted to see if CtBP1 down-regulation had the same inconsistent effect on  $\beta 6$  and  $\beta 1$  internalisation as Eps15 down-regulation. CtBPs have been implicated in integrin-internalisation through clathrin- and dynamin-independent mechanisms (Bonazzi et al. 2005; Hansen & Nichols 2009). CtBP1/BARS were thought to function during membrane fission by acylating lysophosphatidic acid resulting in membrane curvature (Weigert et al. 1999), but this theory was quashed by Gallop *et al* who showed that CtBPs/BARS are not acyl transferases in endocytosis or Golgi fission, as this activity was actually a co-purification artefact (Gallop et al. 2005). More recently CtBP1 has been shown to be recruited to macropinosomes that are induced by high concentrations of EGF and loss of CtBP1 results in reduced production of these structures (Liberali et al. 2008). A study by Gu *et al*

investigated whether the mechanisms of integrin trafficking from the trailing edge of the cell to the leading edge were the same as in basal cell migration (Gu et al. 2011). The authors showed that growth factor-stimulated migration, such as that by platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), utilises a special circular dorsal ruffle (CDR) macropinocytosis mechanism that recruits, internalises and recycles integrins. CDRs are massive actin cytoskeletal remodelling structures and have been thought to initiate massive macropinocytosis. Gu *et al* discuss that when focal adhesions (FAs) disassemble integrins are trafficked to CDRs, internalised by macropinocytosis and then redistributed to FAs at the leading edge during stimulated cell migration, a pathway which is independent from basal cell migration (Gu et al. 2011). While Gu *et al* did not discuss whether CtBP1 was involved in regulating macropinocytosis in this context, the fact that CtBP1 has been shown to be recruited to macropinosomes that are induced by high concentrations of EGF could suggest that CtBP1 is involved in macropinocytosis of migrating cells. However, we found the same inconsistent effect of CtBP1 knockdown on  $\beta 6$  and  $\beta 1$  internalisation and cell surface levels of integrin compared with control siRNA treated cells, as well as the same significant decrease in cell migration as seen with Eps15 knockdown. If integrin internalisation mechanisms differ between migrating cells and basal integrin internalisation, then perhaps the different conditions of the assays we are using are causing different methods of integrin internalisation. Our migration assays could be initiating pathways of integrin internalisation in the context of motile cells, which may be affected by CtBP1 knockdown, while our biotinylation assay measures integrin internalisation of stable cells, which may not be affected by CtBP1 knockdown, resulting in our inconsistent data. Perhaps Eps15 knockdown effect on integrin internalisation is also affected by the motile state of cells. CtBP1 and Eps15 knockdown decrease cell motility which could be as a result of inhibited integrin internalisation but when we try to measure this internalisation by a biotinylation assay we get inconsistent results as integrin internalisation mechanisms change from moving cells to stationary cells which is why we see no difference in integrins on the cell surface as these pathways are not affected in stationary cells.

It is also important to consider that CtBP1 knockdown affects more than one gene, therefore it is possible that CtBP1 knockdown is inhibiting endocytosis but speeding up recycling, leading to inconsistent surface levels of biotin labelled integrins seen following CtBP1 knockdown. Similarly Eps15 has been suggested to be a positive regulator of transcription (Vecchi et al. 2001), however it remains to be elucidated, which genes could be affected by this process. One can hypothesise therefore that Eps15 knockdown might have significant effects on transcription of genes, which could impact endocytosis and recycling of integrins in either positive or negative manner and therefore such an indirect effect of Eps15 could influence our results. For example, if Eps15 knockdown inhibits the transcription and therefore the expression of a protein or proteins, which

normally inhibit integrin endocytosis that could potentially lead to increased endocytosis and decreased cell surface levels of integrins.

In summary, we can conclude that Eps15 and CtBP1 knockdown do not consistently, significantly affect  $\beta 6$  or  $\beta 1$  integrin endocytosis or the consequent cell surface expression of  $\beta 6$  or  $\beta 1$  integrins (Figure 5.1 -Figure 5.4). Inhibition of the clathrin-heavy chain on the other hand consistently, decreased  $\beta 6$  endocytosis and consistently, significantly increased cell surface expression of  $\beta 6$  integrin (Figure 5.5-Figure 5.6). We have discussed various explanations for why these inconsistencies occur but in order to better understand how Eps15 knockdown is affecting cell motility we must first investigate its effect on other cell processes, such as cell adhesion. The role of integrins in the regulation of cell motility is entwined with their regulation of cell adhesion, so perhaps by investigating the effect of Eps15 knockdown on cell adhesion we can begin to better understand the cell processes affected by Eps15 and how they are regulated. Additionally, previous data in our group found that CtBP1 knockdown increases adhesion in order to decrease motility and so our next chapter investigates the effect of Eps15 knockdown on cell adhesion.

## 5.5 Summary

1. Eps15 and CtBP1 do not regulate  $\beta 6$  integrin endocytosis or  $\beta 6$  cell surface integrin levels
2. Eps15 and CtBP1 do not regulate  $\beta 1$  integrin endocytosis or  $\beta 1$  cell surface integrin levels
3.  $\beta 6$  integrin endocytosis is dependent on clathrin expression but not Eps15 expression



## Chapter 6: Eps15 regulates tumour cell adhesion

Our previous results show that Eps15 and CtBP1 both promote tumour cell motility but probably not through the regulation of integrin endocytosis. Our original hypothesis suggested that Eps15, a protein known to be involved in clathrin-dependent receptor endocytosis (Jović et al. 2007; Teckchandani et al. 2012), regulates tumour cell motility through this mechanism. We hypothesised that Eps15 down-regulation could lead to reduced integrin endocytosis causing an increase in the number of integrins on the cell surface, therefore increasing adhesion to the ECM, and suppressing motility. Indeed we have previously found that down-regulation of CtBP1 leads to increased adhesion due to increased integrin activation, which partly explains its inhibitory effect on cell motility (Chrzan 2014). While our previous results did not show a consistent effect of Eps15 or CtBP1 on integrin endocytosis, we could not exclude that Eps15 might regulate integrin function via other mechanisms (e.g. effect on integrin activation), therefore we examined the effect of Eps15 expression on cell adhesion. Cell adhesion is an indirect measure of integrin function (although integrin function is not necessarily the sole cause of changes in cell adhesion) and optimal cell adhesion is crucial for migration to occur (Cox & Huttenlocher 1998). As CtBP1 has previously been shown to inhibit cell adhesion, Eps15 might contribute to this effect. If CtBP1 is affecting cell motility and cell adhesion through regulation of Eps15 expression, then Eps15 knockdown should produce the same consistent increase in cell adhesion as CtBP1 knockdown.

### 6.1 Eps15 knockdown inhibits tumour cell adhesion

Initially, the effect of CtBP1 knockdown on cell adhesion was confirmed using the xCELLigence Real Time Cell Analyser (RTCA), which measures the kinetics of cell adhesion (Figure 6.1). The xCELLigence RTCA measures impedance of electrodes by cells on specialised E-Plates. The more impedance, the higher the cell index (CI) measured. SCC25; cells which endogenously express high levels of  $\alpha v \beta 6$  integrins and H357; cells which adhere to fibronectin in an  $\alpha 5 \beta 1$  integrin-dependent manner, were transfected with non-targeting or CtBP1-targeting siRNA and 48-hours post-transfection cells were plated on BSA, LAP or fibronectin (FN) as ligands of  $\alpha v \beta 6$  and  $\alpha 5 \beta 1$ , respectively. BSA was used as a negative control. SCC25 cells adhere to LAP through  $\alpha v \beta 6$  which can be inhibited by an  $\alpha v \beta 6$ -specific inhibitory antibody, 63G9. We confirmed that SCC25 cells adhere to LAP solely through  $\alpha v \beta 6$ ; incubating the cells with 63G9 completely inhibited adhesion (Figure 6.1A-B). Similarly, in H357 cells adhesion on fibronectin was solely mediated by integrin  $\beta 1$  as treating the cells with the  $\beta 1$ -blocking antibody (P5D2) completely blocked adhesion of these cells (Figure 6.1C-D).

Graphs produced by xCELLigence RTCA software show adhesion as a measurement of cell index (CI) (Figure 6.1A and C). CI measurements were taken every 2 minutes for up to 20 hours during each adhesion assay which produced coloured line graphs where each line represents a different condition. From these graphs we were able to see how long it took for cells to begin to adhere in a non-specific manner to BSA. Adhesion to BSA suggests that cells are beginning to secrete their own extracellular matrix (ECM) proteins in order to adhere. As we were only interested on the effect of CtBP1 or Eps15 knockdown effect on integrin-specific adhesion we examined time-points prior to cell adhesion on BSA. By showing adhesion of each condition to BSA or ligand over time, graphs taken from the xCELLigence software could be used to pick appropriate time-points for analysis of ligand-specific adhesion.

Our xCELLigence graph shows adhesion measurements of SCC25 cells taken up to 2 hours after the cells were plated (Figure 6.1A). At this point we begin to see an increase in CI of cells plated on BSA (control cells = red line, CtBP1 knockdown cells = lilac line). Non-specific adhesion to BSA began to occur at approximately 1 hour 30 minutes after the cells were plated. To measure integrin-specific adhesion we took the CI measurements of SCC25 cells at 1 hour 1 minute after we plated the cells (Figure 6.1B). At this time-point, CtBP1 siRNA treated cells show significantly more adhesion to LAP than control (non-targeting siRNA treated) cells. When expressed as percentage of control adhesion, we see CtBP1 knockdown is able to increase cell adhesion by 150% compared to control cells. The adhesion which we are measuring in both cell conditions is  $\alpha v\beta 6$ -specific as treatment with 63G9 antibody significantly decreased cell adhesion onto LAP of both conditions. Thus, we show that CtBP1 knockdown is increasing integrin-dependent cell adhesion.

Similar results were achieved with the H357 cell line. Figure 6.1C is a graph taken from the xCELLigence RTCA showing visually the amount of cell adhesion of each cell condition up to 2 hours after the cells were plated onto BSA or FN. Cells began to adhere to BSA after 1 hour 30 minutes so the time-point which we took for analysis was at 1 hour 15 minutes. CtBP1 knockdown cells showed a non-significant trend for increased cell adhesion compared with control cells, (Figure 6.1D). Treatment with P5D2 significantly reduced cell adhesion confirming that H357 adhesion to FN is solely mediated by  $\beta 1$  integrins. These results show that CtBP1 knockdown increases cell adhesion in two cell lines utilising two different integrins.

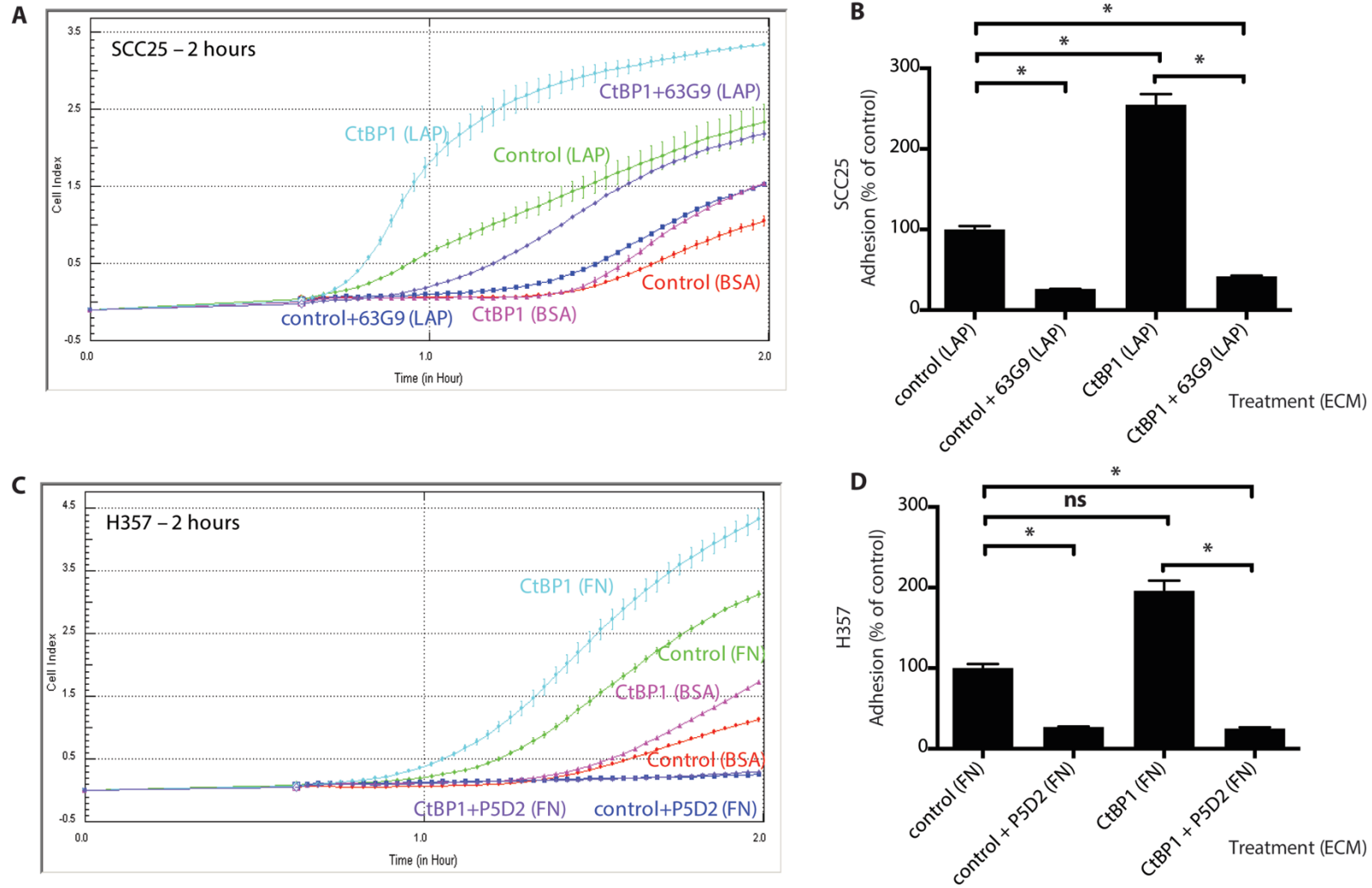


Figure 6.1 CtBP1 knockdown increases cell adhesion via an integrin dependent mechanism

Data shows one experiment confirming CtBP1 knockdown effect on cell adhesion already established (Chrzan 2014). (A) xCELLigence RTCA data of SCC25 cells treated with control (non-targeting) siRNA or CtBP1 siRNA and treated with or without the  $\beta 6$  blocking antibody 63G9. Graphs shows CI measured every 2 minutes up to 2 hours after the cells were plated. Each coloured line represents a different condition and differs between conditions plated onto BSA or LAP extracellular matrix (ECM). Non-specific adhesion onto BSA begins to occur after the cells have been plated for 1 hour 30 minutes so we chose time-points prior to this to analyse integrin-specific adhesion (B) CI measurements taken at 1 hour 1 minute after cells were plated. CtBP1 knockdown significantly increased cell adhesion compared with control (non-targeting) siRNA treated cells ( $p < 0.05$ ). Specific inhibition of  $\beta 6$  integrin with 63G9 antibody significantly reduced cell adhesion of both control and CtBP1 siRNA treated cells plated on LAP ( $p < 0.05$ ) (C) xCELLigence RTCA data of H357 cells treated with control (non-targeting) siRNA or CtBP1 siRNA and treated with or without the  $\beta 1$  blocking antibody P5D2. Graphs show CI measured every 2 minutes up to 2 hours after the cells were plated. Each coloured line represents a different condition and differs between conditions plated onto BSA or FN ECM. Non-specific adhesion onto BSA begins to occur after the cells have been plated for 1 hour 30 minutes so we chose time-points prior to this to analyse integrin-specific adhesion (D) CI measurements taken at 1 hour 15 minutes after cells were plated. CtBP1 knockdown increased cell adhesion compared with control (non-targeting) siRNA treated cells but not significantly. Specific inhibition of  $\beta 1$  integrin with P5D2 antibody significantly reduced cell adhesion of both control and CtBP1 siRNA treated cells plated on FN ( $p < 0.05$ ) (B) + (D) Graphs show adhesion measured as percentage of control adhesion. Graphs show mean and standard error of the mean (SEM) of 2 technical repeats and an unpaired t-test analysed significance.



After confirming that knockdown of CtBP1 increased cell adhesion we investigated the effect of Eps15 knockdown on cell adhesion also using xCELLigence RTCA technique (Figure 6.2A-C). Two Eps15 siRNA were used to exclude the possibility of off-target effects. In SCC25, cells which endogenously express high levels of  $\alpha v\beta 6$  integrins, and VB6, cells genetically engineered to express high levels of  $\alpha v\beta 6$  integrins, both Eps15 siRNA led to a significant decrease in cell adhesion to LAP compared to control siRNA treated cells (Figure 6.2A-B). A similar result was observed in H357 cells, which adhere to fibronectin in an  $\alpha 5\beta 1$  integrin-dependent manner, again a decrease in cell adhesion was observed (Figure 6.2C), however, this decrease was only seen with Eps15\_5 siRNA. As this result only occurred in H357 cells, we assume this result to be cell line specific with Eps15\_7 siRNA. Overall these results show that Eps15 appears to have a positive effect on cell adhesion across various cell lines (Figure 6.2A-C).

The time-point taken for analysis of SCC25 cell adhesion was 2 hours 1 minute after cells were plated onto BSA or LAP (Figure 6.2A). Control cells showed increased adhesion compared to cells treated with Eps15 siRNA from around 40 minutes post-plating but this difference was greatest after 2 hours. Control cells showed consistently more adhesion to LAP than cells treated with Eps15 siRNA from early in the experiment.

VB6 cells adhered even more quickly to LAP than SCC25 cells, probably due to having over-expression of  $\beta 6$  integrin able to initiate adherence to LAP. Control cells show double the CI of Eps15 knockdown cells after just half an hour so an early time-point of 40 minutes was taken for VB6 cell analysis (Figure 6.2B).

H357 control cells adhered to FN at similar speeds as were observed previously. CI measurements were taken at 1 hour 1 min after the cells were plated (Figure 6.2C) and cells treated with Eps15\_5 siRNA have significantly lower CI measurements than control siRNA treated cells.

CI measurements of the amount of adhesion that control cells adhered to LAP/FN varied between experiments on a day-to-day basis. While, cells treated with Eps15 knockdown were consistently less adherent to LAP/FN than control cells, variations in the amount of adhesion meant that experiments could not be pooled and still maintain significance. Thus the graphs shown are representative of all experiments performed.

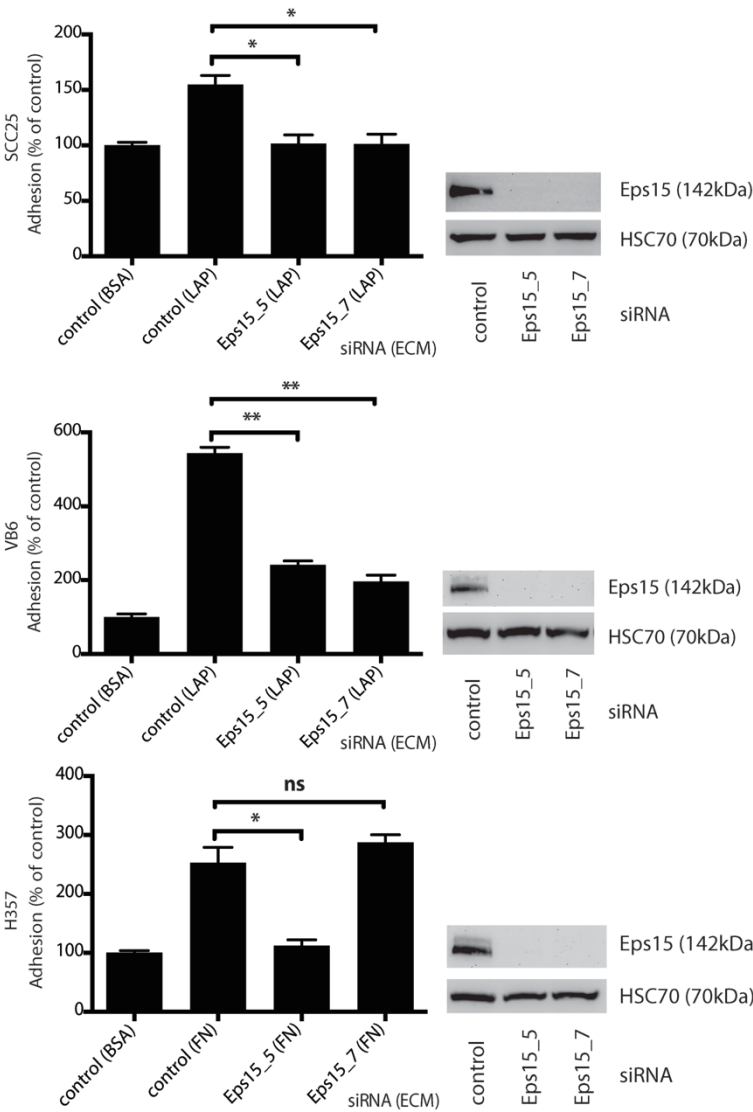
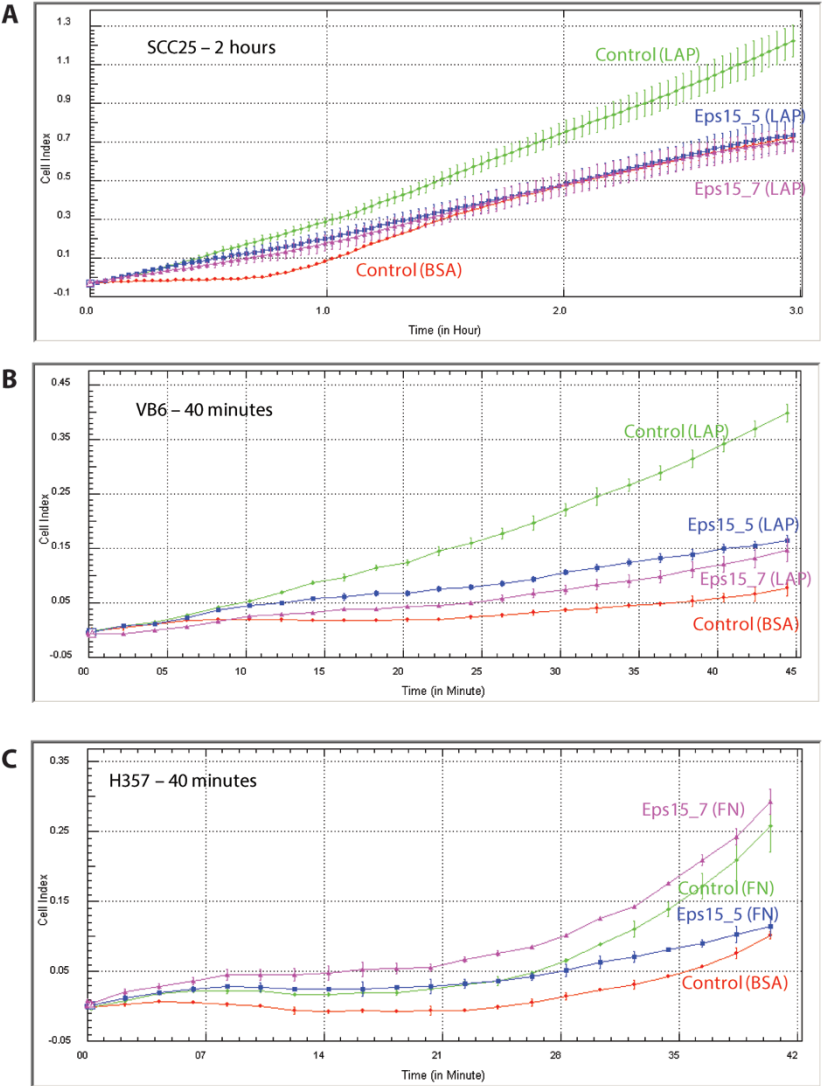
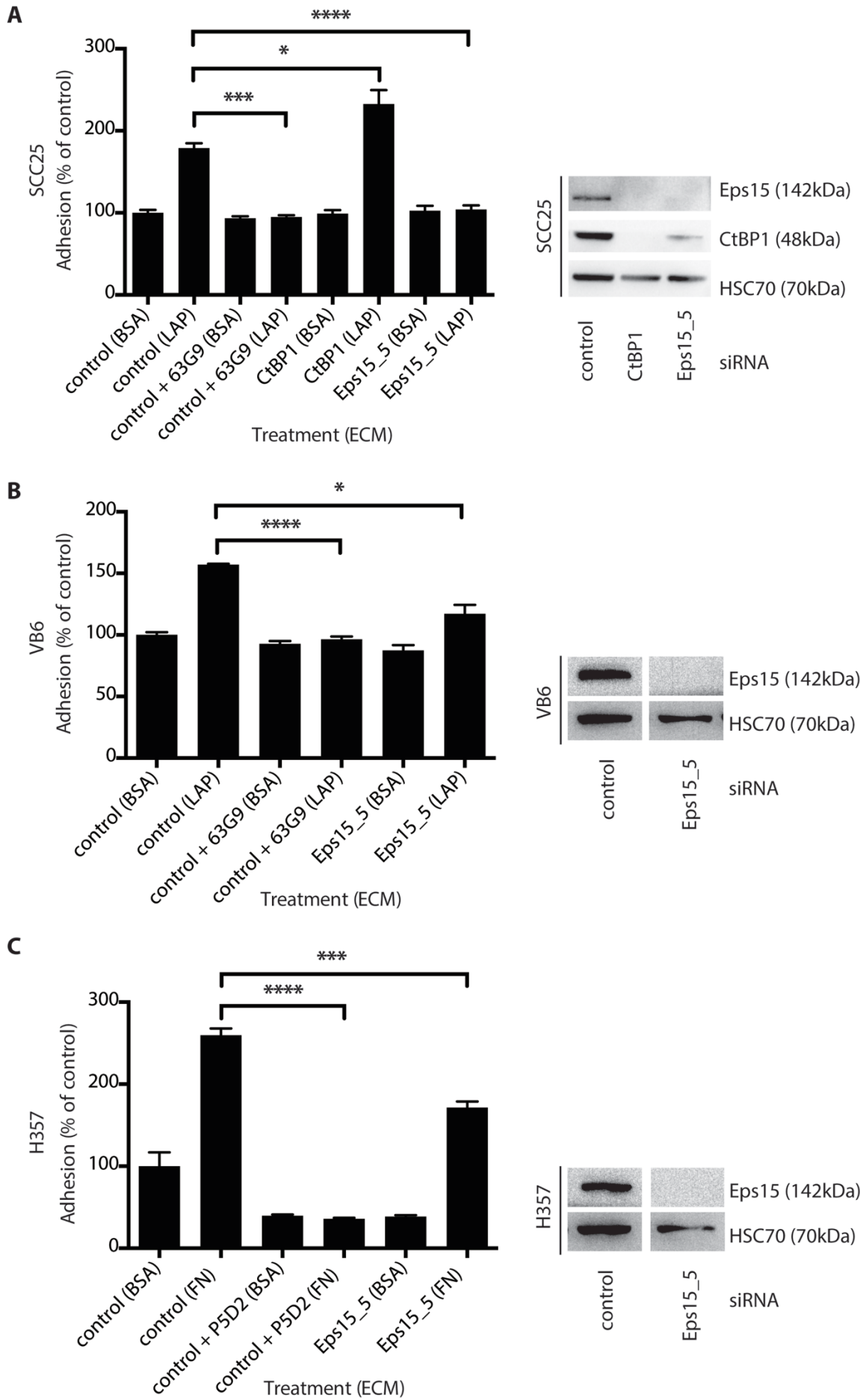


Figure 6.2 Eps15 knockdown inhibits tumour cell adhesion in xCELLigence RTCA analysis

xCELLigence Real Time Cell Analyser (RTCA) was used to study the effect of Eps15 knockdown on speed of cell adhesion. Cells were transfected with control or Eps15 siRNA and 72-hours post-transfection cells were plated on BSA, LAP or FN as ligands of  $\alpha v \beta 6$  and  $\alpha 5 \beta 1$ , respectively. Graphs on the left-hand side were taken from xCELLigence RTCA software and show adhesion of each condition to BSA or ligand over time. These graphs were used to pick appropriate time-points for analysis of ligand-specific adhesion. Bar graphs show adhesion measured as percentage of control adhesion on BSA. Each graph is representative of all independent adhesion assays performed (SCC25; n=4, VB6, n=8, H357; n=6). Graphs show mean and standard error of the mean (SEM) and an unpaired t-test was used to analyse significance. Eps15 knockdown using Eps15 siRNA led to a significant decrease in cell adhesion on LAP compared to control siRNA treated cells in all 3 cell lines (A) SCC25 cells (Eps15\_5,  $p < 0.05$ ; Eps15\_7,  $p < 0.05$ ) (B) VB6 cells (Eps15\_5,  $p < 0.01$ ; Eps15\_7,  $p < 0.01$ ) and (C) H357 cells (Eps15\_5,  $p < 0.05$ ; Eps15\_7,  $p = \text{ns}$ ). Eps15 knockdown efficiency was confirmed for each cell line via Western blotting techniques from cell lysates collected 72 hours post-transfection. 40  $\mu\text{g}$  protein was loaded for each condition.

This result by Eps15 knockdown on cell adhesion was surprising, as we had expected an increase in cell adhesion similar to that of CtBP1 knockdown, therefore, we also examined the effect of Eps15 knockdown on cell adhesion using a different type of adhesion assay performed in a 96-well plate. While the xCELLigence RTCA system is particularly suitable to detecting a difference in the adhesion kinetics, adhesion assays performed on a 96-well plate can also suggest a difference in the strength of adhesion between the cell and the ECM due to the mechanical interventions (washing steps) during the experiment. Using this technique we once again confirmed that SCC25 and VB6 cells adhere on LAP using solely their  $\alpha\beta6$  integrins, as incubating the cells with the  $\alpha\beta6$ -blocking antibody (63G9) completely blocked adhesion (Figure 6.3A-B). Similarly, in H357 cells adhesion on fibronectin was solely mediated by integrin  $\beta1$  as treating the cells with the  $\beta1$ -blocking antibody (P5D2) completely blocked adhesion of these cells (Figure 6.3C). We also confirmed that down-regulation of Eps15 by RNA interference significantly inhibits adhesion across all three cell lines (Figure 6.3A-C) and confirmed previous results showing the positive effect of CtBP1 knockdown on cell adhesion in SCC25 cells (Figure 6.3A). We saw that while CtBP1 knockdown significantly increases tumour cell adhesion Eps15 knockdown has the opposite effect (Figure 6.2-Figure 6.3) suggesting that CtBP1 and Eps15 are affecting cell adhesion and potentially cell motility via different mechanisms.



## Figure 6.3 Eps15 knockdown inhibits tumour cell adhesion in a 96-well adhesion assay

A different type of adhesion assay was used to confirm the effect of Eps15 knockdown on tumour cell adhesion to the ECM using 96-well plates. Cells were transfected with control or Eps15 siRNA and 72-hours post-transfection cells were plated on BSA, LAP or FN as ligands of  $\alpha v\beta 6$  and  $\alpha 5\beta 1$ , respectively. Cells were allowed to adhere only up until ligand non-specific adhesion onto BSA began and then all non-adherent cells were washed away, thus this method potentially measures differences in the strength of adhesion between the cell and the ECM (A-B) The  $\beta 6$ -blocking antibody (63G9) significantly blocked adhesion of SCC25 and VB6 cells to LAP (SCC25;  $p < 0.001$ , VB6;  $p < 0.0001$ ) and down-regulation of Eps15 by RNA interference significantly inhibited adhesion of SCC25 cells ( $p < 0.0001$ ) and VB6 cells ( $p < 0.05$ ) to LAP (C) The  $\beta 1$ -blocking antibody (P5D2) significantly blocked adhesion of H357 to FN ( $p < 0.0001$ ) and down-regulation of Eps15 by RNAi significantly inhibited adhesion of H357 ( $p < 0.001$ ). Graphs show a representative experiment of all adhesion assays performed (SCC25,  $n=4$ ; VB6,  $n=3$ ; H357,  $n=3$ ) and adhesion is calculated as percentage of control cell adhesion on BSA. Graphs show mean and standard error of the mean (SEM) and an unpaired t-test was used to analyse significance. CtBP1 and Eps15 knockdown efficiency was confirmed via Western blotting techniques from cells collected 72 hours post-transfection. 40 $\mu$ g protein was loaded for each condition.

## 6.1.1 Clathrin and HAX-1 knockdown increase tumour cell adhesion

Our endocytosis results with Eps15 knockdown were inconsistent and we wanted to confirm that change in endocytosis is not the mechanism by which Eps15 affects cell adhesion. Therefore, we investigated the effect of known  $\alpha v\beta 6$  integrin endocytosis regulators; clathrin and HAX-1, on cell adhesion using xCELLigence RTCA. Our previous observations showed that down-regulation of the endocytosis adaptor protein clathrin, with clathrin heavy-chain (CHC) siRNA, caused a consistent decrease in integrin  $\alpha v\beta 6$  endocytosis (Figure 5.1) and an increase of cell surface levels of integrin  $\alpha v\beta 6$  (Figure 5.2). HAX-1, a protein which binds the integrin subunit  $\beta 6$ , has been shown to regulate its endocytosis (A. G. Ramsay et al. 2007). CHC- and HAX-1-knockdown both decrease tumour cell motility (A. G. Ramsay et al. 2007) similar to Eps15 knockdown (Figure 4.1 and Figure 4.2), yet knockdown of clathrin also causes a consistent decrease in  $\beta 6$  integrin internalisation not seen following Eps15 knockdown (Figure 5.5 and Figure 5.6). Thus we wanted to investigate the role of HAX-1 and clathrin in tumour cell adhesion and compare their role in cell adhesion to that of Eps15 knockdown. HAX-1 knockdown was only investigated in the two  $\beta 6$  expressing cell lines; SCC25 and VB6, as it has no known association with  $\beta 1$  integrins, used by H357 to bind to FN. We hypothesised that because CHC knockdown inhibits integrin internalisation this inhibition would cause significant increase of tumour cell adhesion. Our investigations support our hypothesis and show that knockdown of both HAX-1 and CHC significantly increase tumour cell adhesion in SCC25 (Figure 6.4) and VB6 cells (Figure 6.5A) as well as just CHC knockdown in H357 cells (Figure 6.5B). These observations reiterate that Eps15 is potentially affecting cell motility via an endocytosis-independent mechanism, as knockdown of two known regulators of integrin endocytosis cause the opposite effect on cell adhesion as that of Eps15 knockdown.

In addition to treating cells with CHC or HAX-1 siRNA we also treated these knockdown cells with the  $\beta 6$  integrin blocking antibody 63G9 (Figure 6.4B). Our investigations show that blocking of the integrin  $\beta 6$  significantly reduces increased cell adhesion as a result of CHC and HAX-1 knockdown to low levels of adhesion such as those seen on the control surface BSA. Similar to CtBP1 knockdown, these results indicate that both CHC and HAX-1 knockdown affect cell adhesion via an integrin-dependent mechanism. By producing the opposite effect on cell adhesion, knockdown of these proteins further suggests that Eps15 knockdown is inhibiting cell adhesion via an endocytosis-independent mechanism.

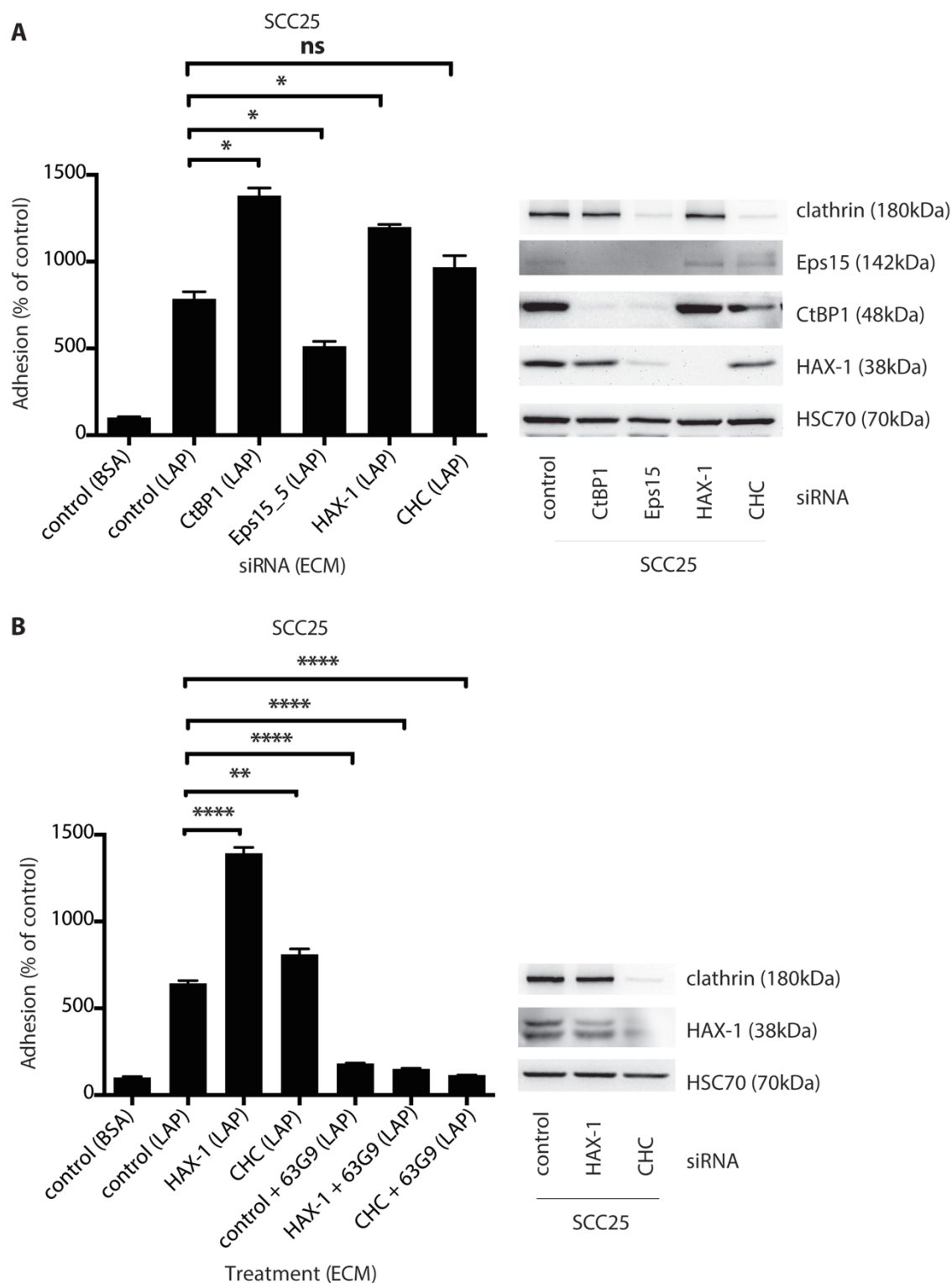


Figure 6.4 Clathrin and HAX-1 knockdown significantly increase tumour cell adhesion of SCC25 cells

xCELLigence Real Time Cell Analyser (RTCA) was used to study the effect of clathrin-heavy chain (CHC) and HAX-1 knockdown on SCC25 cell adhesion. Graphs show adhesion measured as percentage of control adhesion on BSA. Each graph is a representative experiment of all independent adhesion assays performed (SCC25; n=3). Graphs show mean and standard error of the mean (SEM) of 2 technical repeats and an unpaired t-test was used to analyse significance of treated cells against control cells plated on LAP. (A) SCC25 cells were transfected with control, CtBP1, Eps15, HAX-1 or CHC siRNA and plated on BSA or LAP (ligand of  $\alpha v \beta 6$ ). CtBP1 and HAX-1 siRNA treated cells adhered to LAP significantly more than control siRNA treated cells, CHC



knockdown cells showed non-significant increased cell adhesion and Eps15 knockdown cells significantly decreased cell adhesion (CtBP1;  $p < 0.05$ , Eps15\_5;  $p < 0.05$ , HAX-1;  $p < 0.05$ , clathrin;  $p = ns$ ). (B) SCC25 cells were treated with HAX-1 or CHC siRNA then 48 hours post-transfection cells were also treated with or without integrin blocking antibody 63G9 for 30 minutes. HAX-1 and CHC siRNA treated cells had significantly increased cell adhesion compared with control cells (HAX-1;  $p < 0.0001$ , CHC;  $p < 0.01$ ). Treatment of all cells with  $\beta 6$  integrin blocking antibody; 63G9, significantly reduced cell adhesion (63G9 alone;  $p < 0.0001$ , HAX-1 + 63G9;  $p < 0.0001$ , clathrin + 63G9;  $p < 0.0001$ ). CtBP1, Eps15, HAX-1 and clathrin knockdown efficiency was confirmed from cell lysates collected 72 hours post-transfection, via Western blotting techniques. 40  $\mu$ g protein was loaded for each condition.

We also examined the knockdown of both HAX-1 and CHC in VB6 cells; genetically engineered to express high levels of  $\alpha v \beta 6$ . Once again HAX-1 knockdown significantly increased cell adhesion compared with control cells (Figure 6.5A). CHC knockdown failed to show a significant effect on cell adhesion in individual experiments but consistently showed a trend of increased cell adhesion compared with control cells plated on LAP (Figure 6.5A). Lack of significance here could be due to the speed at which VB6 cells adhered to LAP. In this experiment control cells plated onto LAP show 5X more adhesion than control cells plated on BSA while control cell adhesion onto FN by H357 cells only increases 1.5X than of control cells plated onto BSA (Figure 6.5B). Perhaps such large increases in cell adhesion make increases as a consequence of CHC knockdown more difficult to detect as control cell adhesion is already a large CI measurement. This experiment is representative of all adhesion experiments involving VB6 cells treated with CHC siRNA. H357 cells also showed an increase in tumour cell adhesion when treated with CHC siRNA, an increase which was significantly more than control (non-targeting) siRNA treated cells plated onto FN (Figure 6.5B). These experiments confirm that CHC knockdown causes an increase in tumour cell adhesion in two further cell lines. These results reiterate that Eps15 is affecting tumour cell adhesion via an endocytosis-independent mechanism as the effect of Eps15 knockdown on cell adhesion opposes that of the endocytic proteins clathrin and HAX-1 in three different cell lines and in the context of two different integrins.

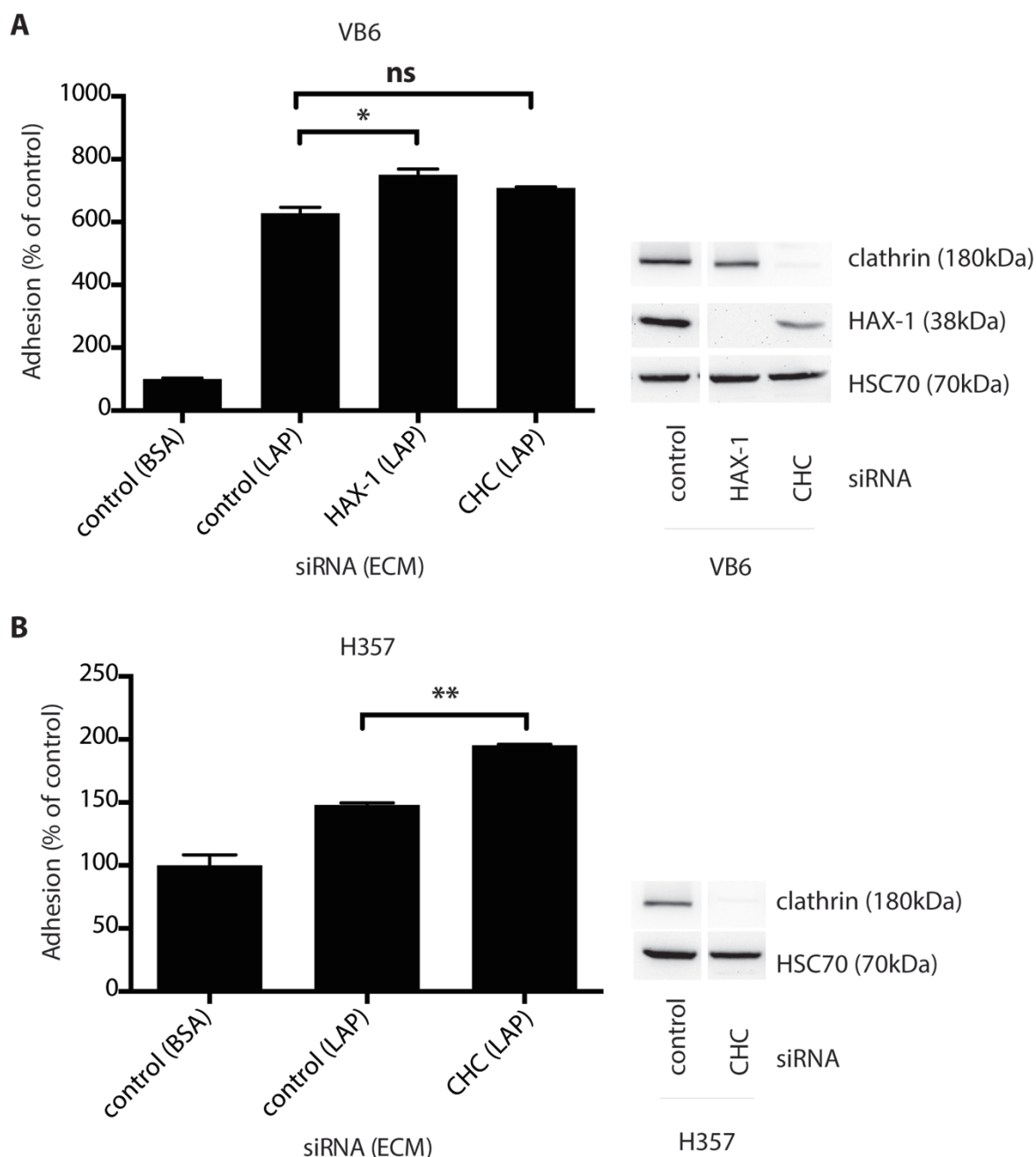


Figure 6.5 Clathrin and HAX-1 knockdown significantly increase tumour cell adhesion of VB6 and H357 cell lines

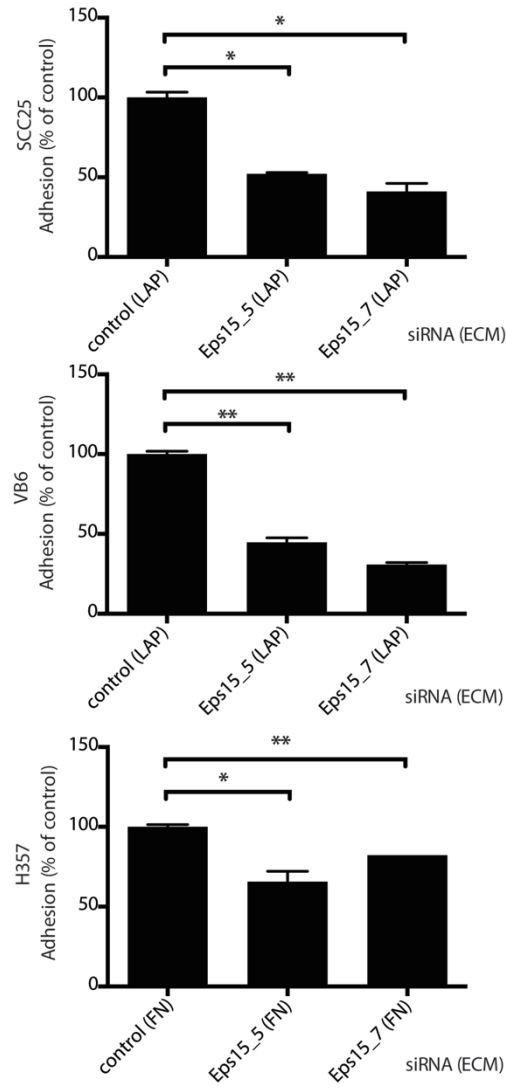
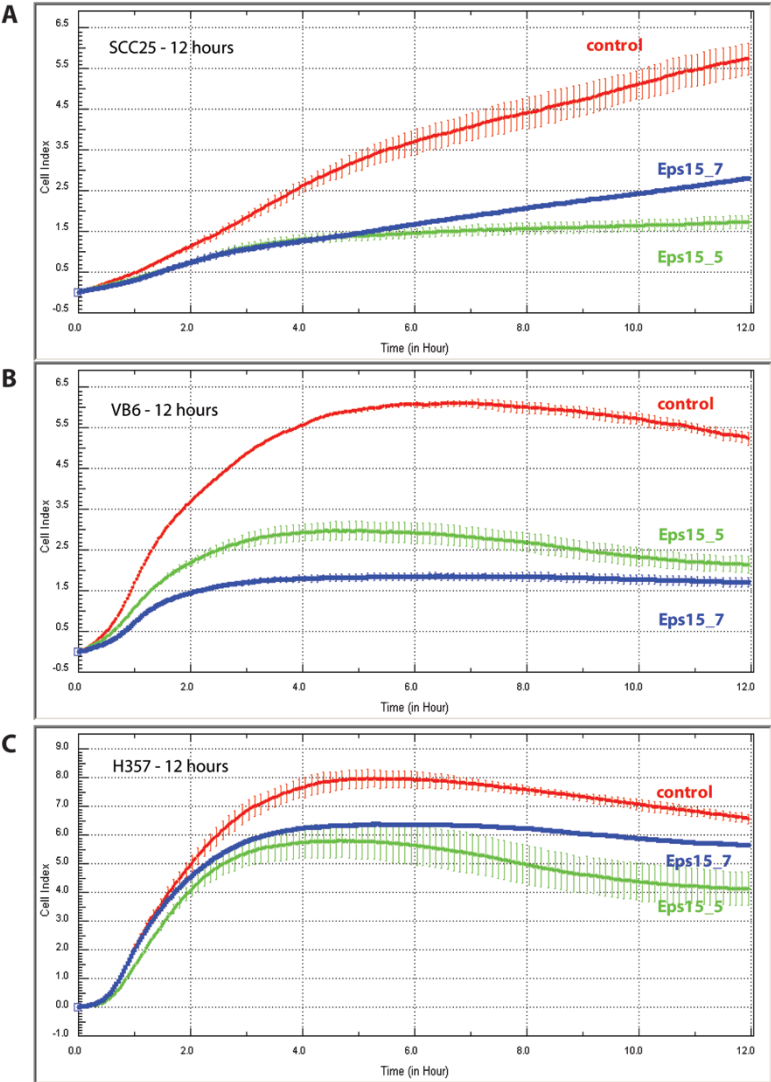
xCELLigence Real Time Cell Analyser (RTCA) was used to study the effect of clathrin-heavy chain (CHC) and HAX-1 knockdown on cell adhesion. Cells were transfected with control, HAX-1 or CHC siRNA and 72-hours post-transfection cells were plated on BSA, LAP or FN as ligands of  $\alpha v \beta 6$  and  $\alpha 5 \beta 1$ , respectively. Graphs show a representative experiment of all adhesion assays performed (VB6; n=4, H357; n=3). Adhesion shown is calculated as percentage of control adhesion on BSA. Graphs show mean and standard error of the mean (SEM) and an unpaired t-test was used to analyse significance. (A) In VB6 cells, HAX-1 knockdown significantly increased tumour cell adhesion and CHC knockdown also increased tumour cell adhesion but the effect was not significant (HAX-1;  $p < 0.05$ , CHC;  $p = ns$ ). (B) In H357 cells, HAX-1 knockdown was not tested but CHC knockdown significantly increased tumour cell adhesion compared with control cells (CHC;  $p < 0.01$ ). HAX-1 and clathrin knockdown efficiency was confirmed via Western blotting techniques on cells collected 72-hours post-transfection. 40 $\mu$ g protein was loaded for each condition.

## 6.2 Eps15 knockdown inhibits cell spreading

### 6.2.1 Eps15 significantly inhibits cell spreading over time

As discussed previously, cell index (CI) measured by xCELLigence RTCA after short time points is indicative of cell adhesion. However, as CI is just a measurement of the amount of electrical impedance, which can be affected by numerous things such as number of cells adhering (short time-points), cell spreading (intermediate time-points) or proliferation (long time-points). After longer time points the xCELLigence RTCA is no longer measuring cell adhesion but also the spreading of already adherent cells. While previously we were only interested in early time-points which showed integrin-specific adhesion to a ligand, we noticed that differences between our conditions were still noticeable at longer time-points suggesting that perhaps not just adhesion but also cell spreading is affected by Eps15 knockdown.

All previous xCELLigence RTCA data was taken at early time-points between 40 minutes and 2 hours as these time-points were representative of integrin-dependent cell adhesion, however, most of our adhesion experiments were left to run for up to 24 hours. Because of this we were able to analyse the longer time-point of 8 hours after plating the cells, which we assumed was no longer indicative of cell adhesion but of cell spreading. We can see the change in cell adhesion kinetics in graphs from xCELLigence software. The curve produced by cell adhesion begins to plateau at 6 hours post-plating and earlier, at 4 hours, in VB6 and H357 cells. These CI measurements suggest that cells are fully spread as the CI is no longer increasing exponentially. While, the xCELLigence RTCA does not conclusively measure cell spreading or adhesion, the time-course of the effects and consequent CI measurements suggest that adhesion or spreading is affected. The effect of Eps15 knockdown on CI was quantified in all three cell lines at 8 hours following cell plating when we assumed that cells would no longer be adhering but would be fully spread. We found that Eps15 knockdown cells still had a CI that was significantly lower than control siRNA treated cells at these later time-points suggesting that Eps15 is involved in the regulation of cell spreading as well as cell adhesion. Eps15 knockdown significantly reduced cell spreading at 8 hours in all three cell lines tested (Figure 6.6).



#### Figure 6.6 Eps15 knockdown inhibits cell spreading

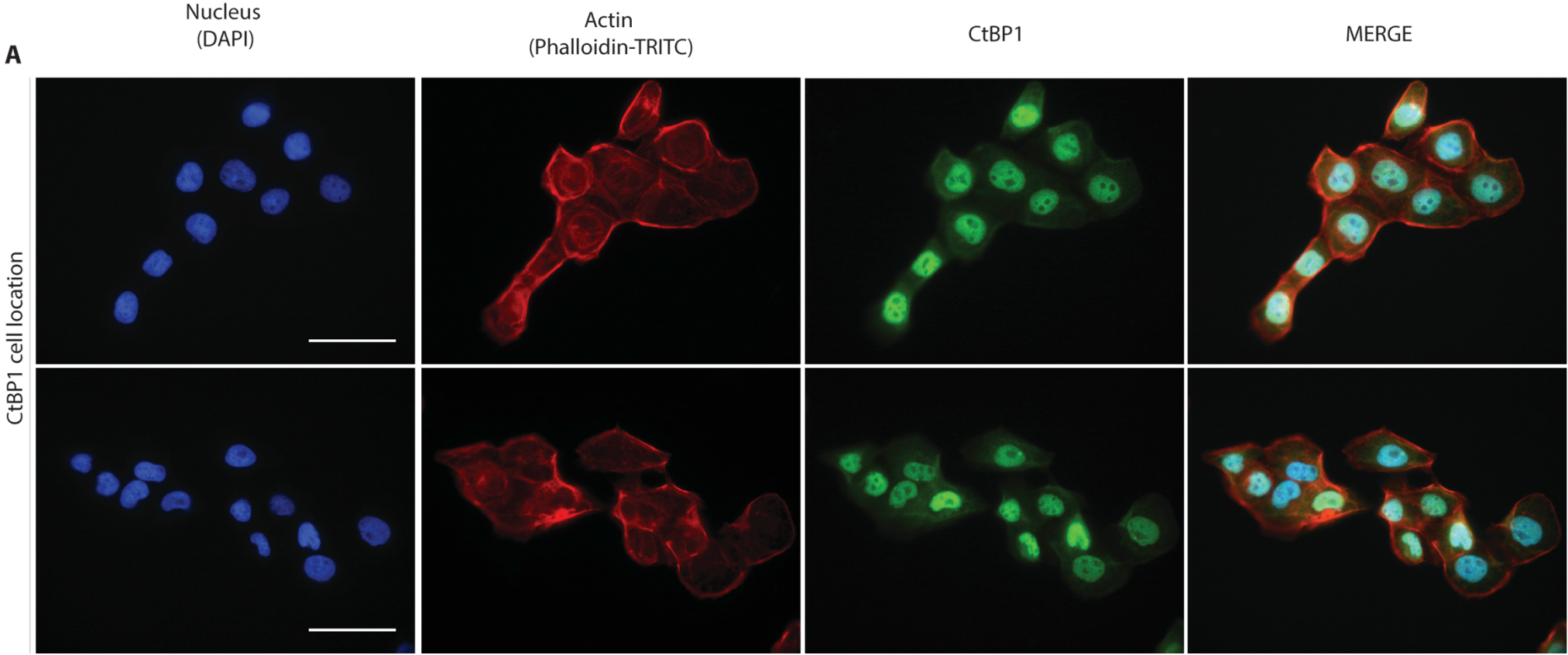
Figures show data from xCELLigence machine at 12 hour time-points following cell plating. Coloured lines represent the amount of cell impedance (cell index; CI) of cells of each condition. Red = control siRNA treated cells, green = Eps15\_5 siRNA treated cells and blue = Eps15\_7 siRNA treated cells. Cell index at this later time-point of 8 hours indicates cell spreading and not cell adhesion. Bar graphs are representative of all adhesion assays which were performed and left to adhere for 12 hours, all quantitative analysis was performed on data at 8 hours adhesion (SCC25; n=3, VB6; n=7 H357; n=6). Graphs show mean with standard error of the mean (SEM) and adhesion is measured as percentage of control cell adhesion on LAP. Significance was measured by unpaired t-test (A) SCC25: Eps15\_5;  $p < 0.05$ , Eps15\_7;  $p < 0.05$  (B) VB6: Eps15\_5;  $p < 0.01$ , Eps15\_7;  $p < 0.01$  (C) H357: Eps15\_5;  $p < 0.05$ , Eps15\_7;  $p < 0.01$ .

### 6.2.2 Eps15 localisation is peri-nuclear

Our investigations indicate that Eps15 is involved in the regulation of cell adhesion and cell spreading. We found that knockdown with Eps15 by siRNA consistently reduced cell adhesion and possibly cell spreading of three tumour cell lines. To investigate potential mechanisms by which Eps15 knockdown could be affecting cell functions we began by studying the localisation of Eps15 within the cell using immunofluorescence (Figure 6.7B). We also investigated the localisation of CtBP1 in the cell to see if the two proteins were co-localised in adherent cells (Figure 6.7A).

As proteins, which are heavily involved in the transcription of other proteins, activated CtBPs are mostly found located within the nucleus, dependent on the nuclear localisation signal (NLS) available on CtBP2 (Verger et al. 2006). Eps15 on the other hand has been found to be localised both in the cell cytoplasm as well as having some expression within the cell nucleus (Offenhauser et al. 2000). Our investigation confirmed the localisation of CtBPs within the cell nucleus (Figure 6.7A) and we found Eps15 to be mostly peri-nuclear localised, with some dotted staining spread throughout the cytoplasm (Figure 6.7B).

We next investigated whether the localisation of Eps15 changes during cell spreading. To this end cells were plated on LAP and fixed at various states of cell spreading and localisation of Eps15 was detected using immunofluorescence. While Eps15 showed peri-nuclear staining shortly after cell adhesion (Figure 6.8A), staining appeared more dispersed as cells spread on coverslips coated with LAP (Figure 6.8B). Eps15 stain optimisation and secondary only images are given in the appendix (Appendix 2 – Figure S2.1).





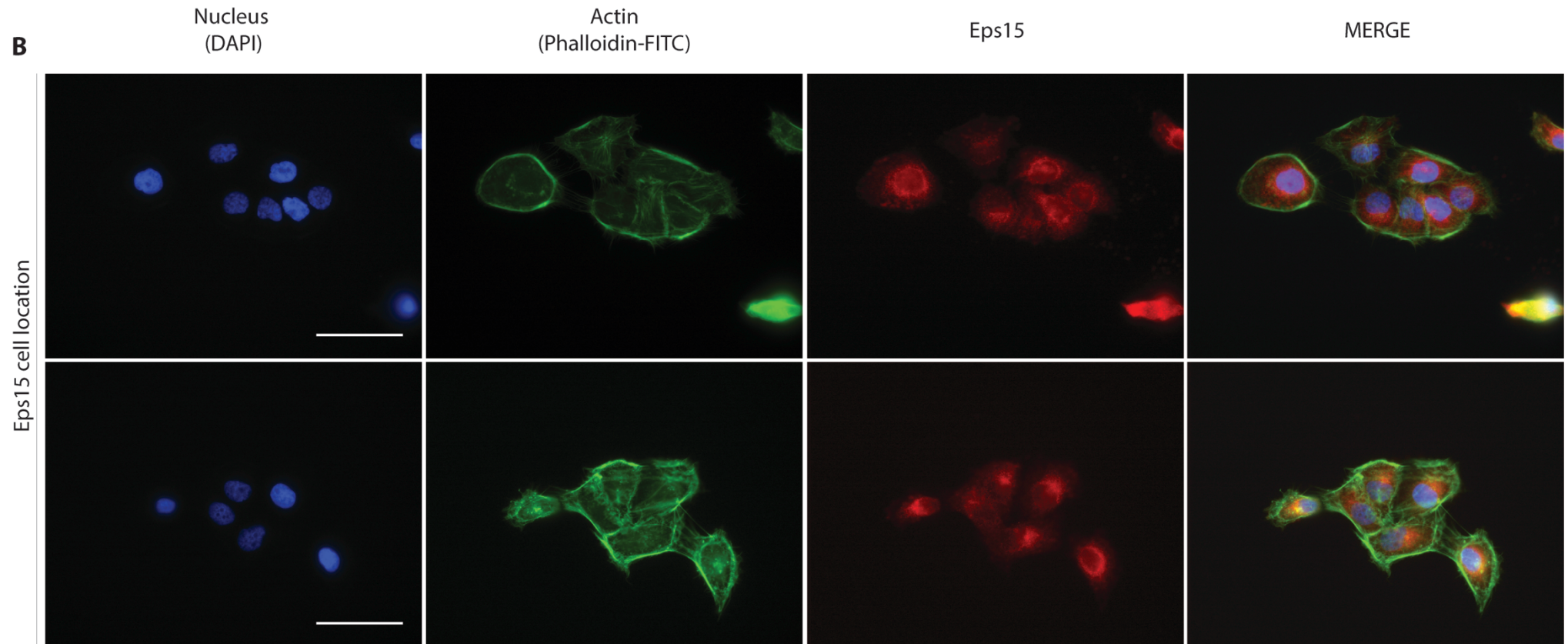


Figure 6.7 CtBP1 is nuclear localised and Eps15 is peri-nuclear localised

SCC25 cells were sparsely plated onto glass coverslips coated with ECM protein collagen I. Cells were then stained for the nucleus (DAPI = blue), actin filaments (Phalloidin-FITC/TRITC = green/red) and either CtBP1 (E12; green) or Eps15 (#8855; red) following overnight incubation in serum-containing media. Scale bar represents 50µm (A) CtBP1 localisation was analysed by binding with the primary antibody E12 (Qiagen; 1:50) and then with anti-mouse Alexa 488 (1:250). CtBP1 was shown to be localised in the cell nucleus (CtBP1 = green; actin = red) (B) Eps15 is mostly localised outside the nucleus, perhaps co-localised with the Golgi. Eps15 was imaged in red using anti-mouse Alexa 546 secondary bound to primary antibody for Eps15 (#8855; Cell Signalling; 1:250).



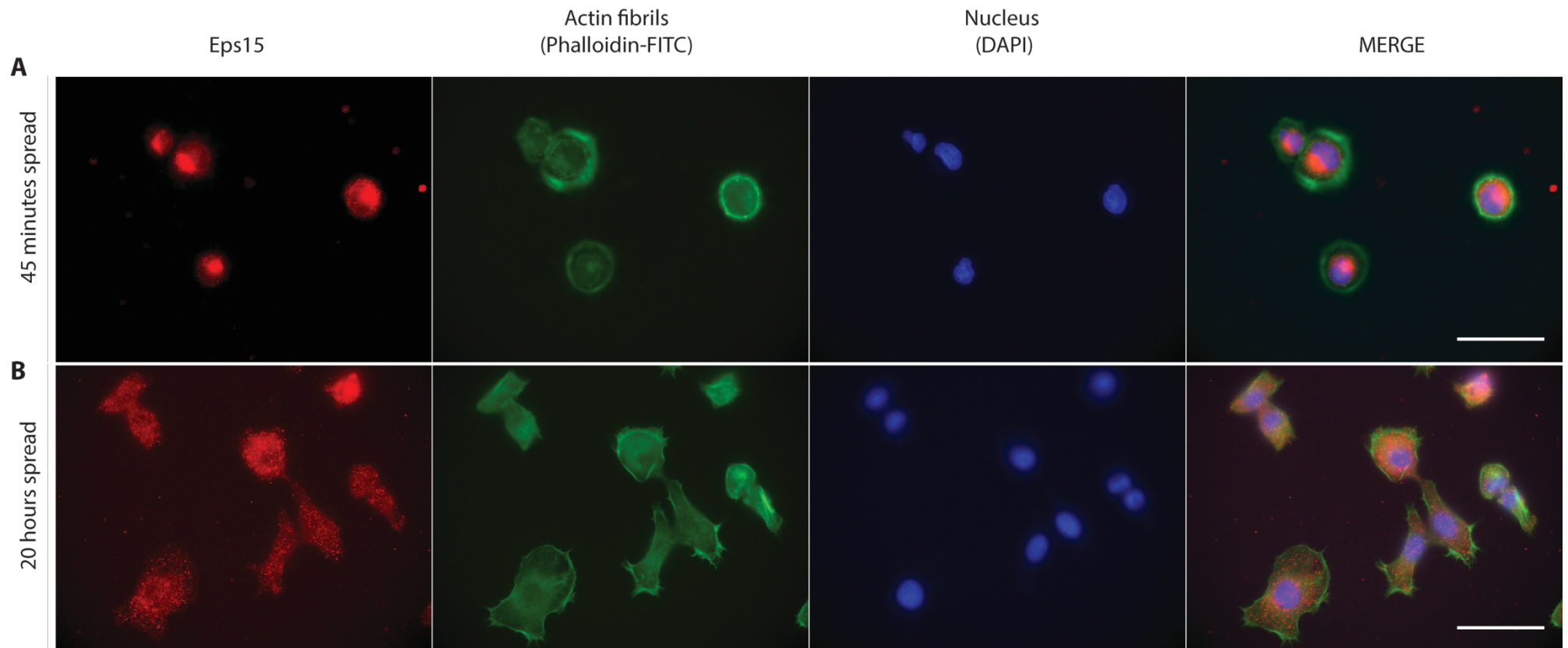


Figure 6.8 As cells spread Eps15 disperses away from the nucleus

SCC25 cells were sparsely plated onto glass coverslips coated with ECM protein LAP. Cells were then stained for the nucleus (DAPI = blue), actin filaments (Phalloidin-FITC = green) and Eps15 (#8855; red) at (A) 45 minutes post-plating and (B) 20 hours post-plating. Scale bar represents 50 $\mu$ m. In adherent cells we see that Eps15 disperses away from the nucleus into the cells' cytoplasm.

### 6.2.3 Eps15 inhibits cell spreading

Analysis of xCELLigence RTCA time curves suggested that Eps15 does not only affect cell adhesion but could also potentially affect cell spreading. To confirm this SCC25 cells were plated on LAP and cell spreading was measured by measuring cell areas after various times using immunofluorescence. Our adhesion assays showed that clathrin heavy-chain (CHC) knockdown has the opposite effect on cell adhesion compared to Eps15 knockdown, therefore we wanted to investigate whether they also affect cell spreading differently. Initially we chose short time-points up to 20 hours, based on the times we got from our xCELLigence RTCA experiments. However, we found that the cells adhered and spread much slower than expected on the glass coverslips and we only observed fully spread SCC25 control cells at 24 hours post-plating (Figure 6.9).

We fixed cells up to 24 hours and then stained for actin with phalloidin. Eps15 knockdown cells appeared smaller than control cells, indicating that even though the cells were adherent, that they were unable to spread on LAP coated cover slips as well as control cells did. In order to quantify this size difference, we measured the area of cells using Fiji software, and then calculated the average area of the cells in each condition. To ensure that we were measuring the mean of individual cell area, the amount of phalloidin was divided by the amount of DAPI also pictured in each field of view. We found that Eps15 knockdown significantly reduced the area of adherent cells compared to control siRNA treated cells suggesting that Eps15 knockdown not only causes an inhibitory effect on cell adhesion but also on cell spreading (Figure 6.9B). CHC knockdown on the other hand, caused no significant effect on cell area compared with the area of control cells (Figure 6.9B). These results suggest that Eps15 is involved in the regulation of cell spreading, while clathrin is not. These observations reiterate once more that Eps15 and clathrin operate to affect tumour cell motility in different ways. Eps15 knockdown inhibits cell adhesion causing a consequent reduction in cell spreading while clathrin knockdown causes an increase in cell adhesion and no significant change in cell spreading once cells are adherent.

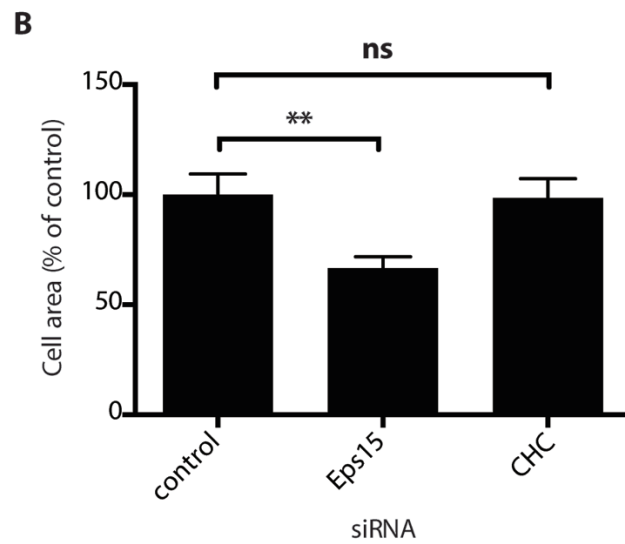
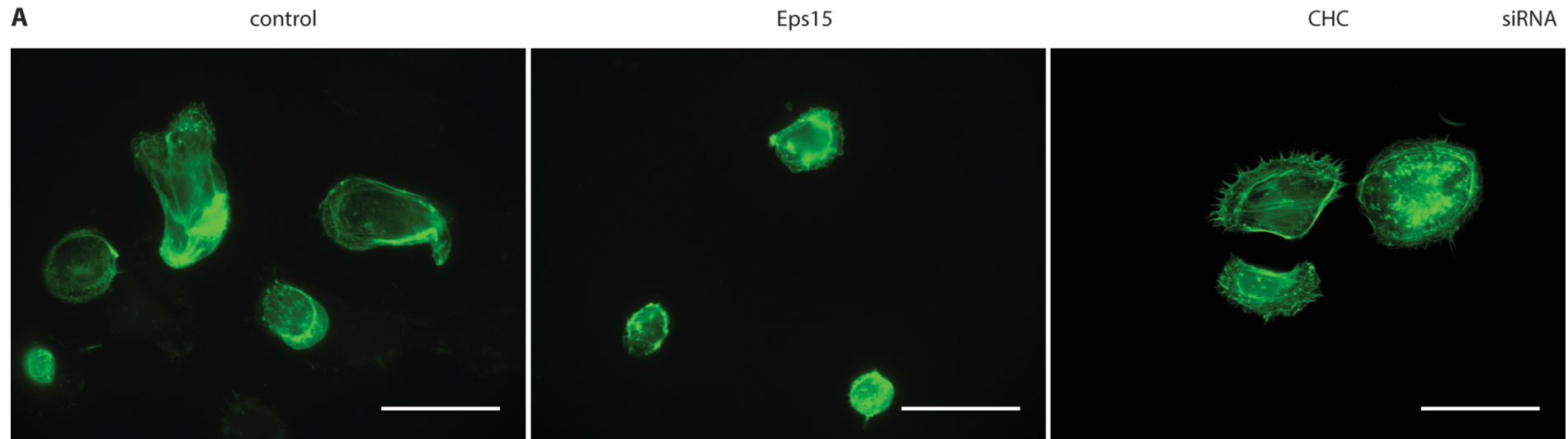


Figure 6.9 Eps15 knockdown significantly reduces cell spreading while clathrin knockdown has no effect

SCC25 cells treated with control, Eps15 or clathrin heavy-chain (CHC) siRNA were sparsely plated onto glass coverslips coated with ECM protein LAP 24 hours post-transfection in migration media. Cells were then stained for actin filaments (Phalloidin-FITC). (A) 24 hours post-plating we observed the cells and noticed that adherent cells treated with Eps15 siRNA appeared smaller than control siRNA treated cells, while CHC siRNA treated cells were of a similar size to control treated cells. (B) Quantification of phalloidin cell area by Fiji showed that cells treated with Eps15 siRNA were significantly smaller than control treated cells, while CHC siRNA treated cells showed no significant difference to control siRNA treated cells (Eps15;  $p < 0.01$ , CHC;  $p = \text{ns}$ ). Quantification was carried out on 10 fields of view for each condition and observations are representative of two independent experiments ( $n=2$ ). Scale bar represents  $50\mu\text{m}$ .

#### 6.2.4 Eps15 knockdown could inhibit cell spreading by causing actin filament disorganisation

Our observations suggest that Eps15 has a role in the regulation of cell spreading. As discussed previously, too little or too much adhesion and cells cannot migrate effectively. We have shown that Eps15 knockdown consistently inhibits cell adhesion which is most likely the cause of reduced cell migration following Eps15 knockdown. To better observe the effect of Eps15 knockdown on cell spreading we fixed and observed SCC25 cells treated with siRNA at much longer time-points. Our initial observations showed that SCC25 control cells needed up to 20 hours to be fully spread on glass coverslips therefore we observed time-points up to 70 hours following plating of cells onto coverslips. In order for cells to survive and remain healthy for up to 70 hours we plated them in 1% serum.

Eps15 knockdown caused a significant decrease in cell area compared to control cells at 20 hours (similar to that seen at 24 hours previously) as well as at the much later time-point of 50 hours post plating (Figure 6.10A). We also observed that actin organisation was markedly different between Eps15 siRNA treated cells and control cells across all time-points (Figure 6.10B). Eps15 knockdown cells were much smaller and often actin stress fibres were absent in these cells, when they were visible throughout the cell cytoplasm of control cells. Additionally, Eps15 knockdown cells exhibited fewer protrusions from the cell membrane in comparison to control cells. These observations suggest that Eps15 could be involved in actin cytoskeleton organisation, including the organisation of fibres to produce protrusions such as filopodia (Figure 6.10B). These observations are very preliminary and while we can only speculate that Eps15 is affecting actin organisation and filopodia production, as we did not quantitatively measure these observations, we can conclude that Eps15 knockdown does inhibit the size of adherent cells, suggesting Eps15 to have a role in the regulation of cell spreading.

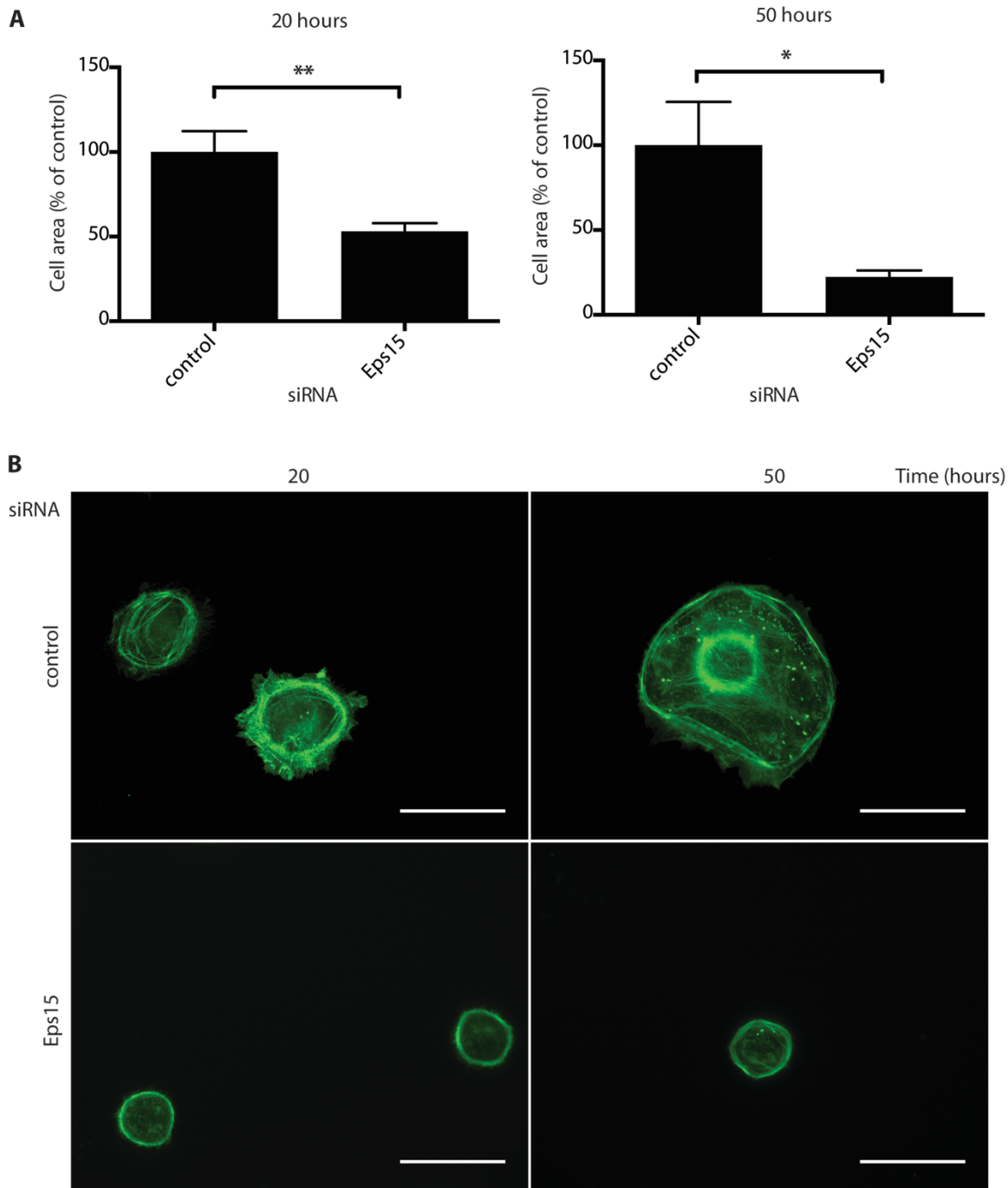


Figure 6.10 Eps15 knockdown inhibits spreading of cells over time

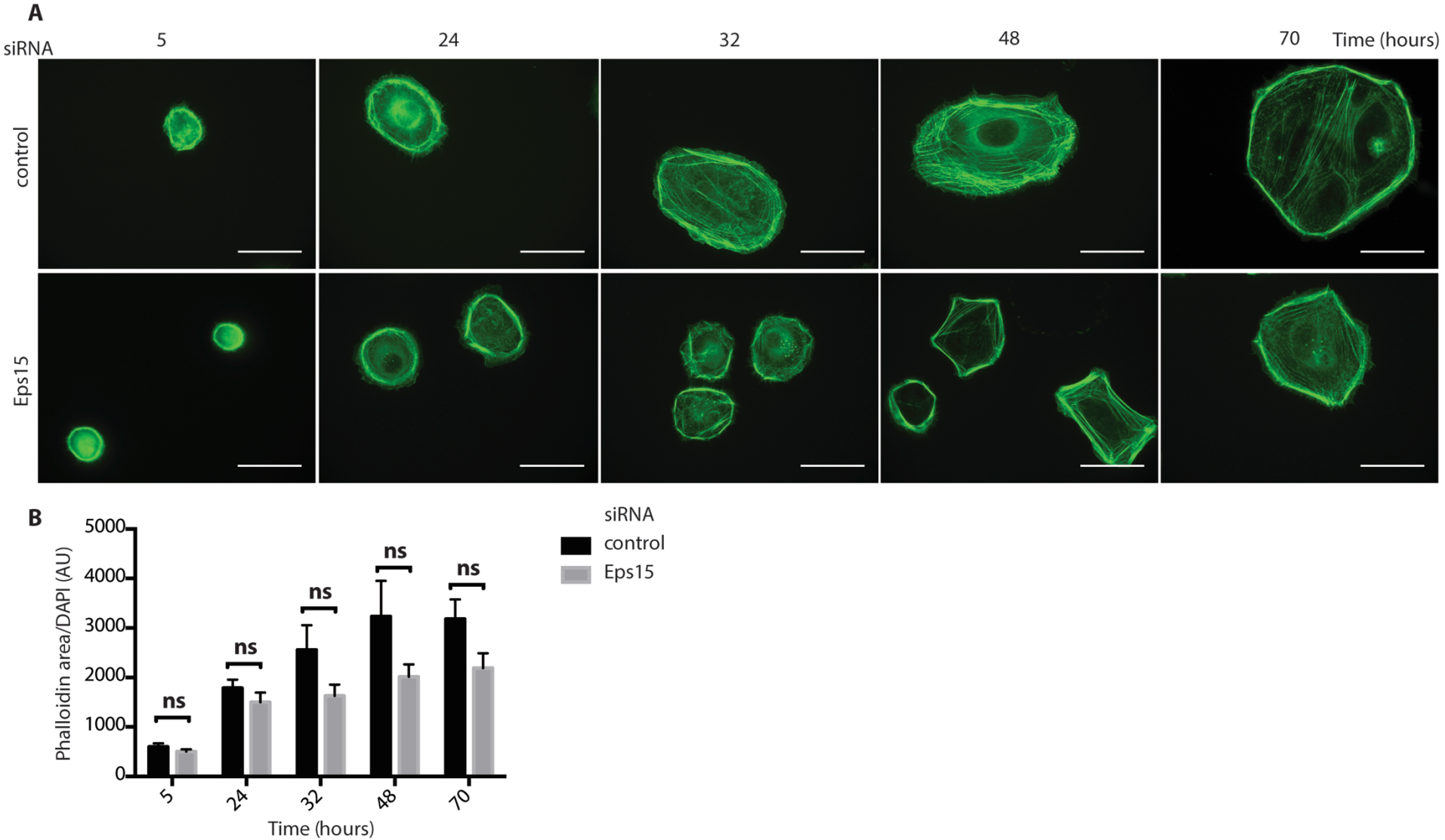
SCC25 cells treated with control or Eps15 siRNA were sparsely plated onto glass coverslips coated with extracellular matrix (ECM) protein LAP in 1% serum 24 hours post-transfection. Cells were then stained for actin filaments (Phalloidin-FITC). (A) Eps15 knockdown significantly reduces the area of spread cells at 20 hours and still at 50 hours post-plating (20 hours;  $p < 0.01$ , 50 hours;  $p < 0.05$ ). Quantification of phalloidin cell area was performed by Fiji software and carried out on 10 fields of view for each condition. Observations are representative of two independent experiments. (B) Cells treated with Eps15 siRNA were visibly smaller than control siRNA treated cells and appeared to produce fewer cell protrusions, such as filopodia. Actin organisation was also disrupted in Eps15 siRNA treated cells; cells show thick cortical actin and less actin fibres throughout the cell. Scale bar represents  $50\mu\text{m}$ .

We have observed that Eps15 knockdown significantly reduces the cell area of spreading cells at 20 hours and 50 hours after plating onto coverslips. A second repeat showed that Eps15

knockdown was able to consistently reduce cell size up to 70 hours post-plating onto LAP coated glass coverslips (Figure 6.11). SCC25 cells treated with Eps15 siRNA were consistently smaller than control siRNA treated cells at all time-points fixed; 5-70 hours following cell plating onto coverslips. Control cells consistently increase in area until 70 hours when a slight decline in cell size does occur, however, but this is possibly due to cells becoming less healthy after such a long time in just 1% serum. Eps15 knockdown cells also continue to increase in cell area over time but their cell area is consistently less than their control counterparts (Figure 6.11A). This difference in size is consistent but unfortunately not significant. This could be because too few images were pictured for each condition. Despite cells in each condition displaying a similar phenotype, there was a large variation in the size of individual cells. While control cells were phenotypically larger than Eps15 knockdown cells, cells within each condition displayed large variation so perhaps 10 pictures was not enough needed to allow significance between condition (Figure 6.11B).

By observing the cells at each time-point we saw that not only were Eps15 knockdown cells smaller at each time-point but that they were also phenotypically different from their control counterparts. Control cells at each time-point (except the earliest time-point of 5 hours) displayed stress fibres across their cytoplasm and often displayed fanned membrane at the edge of spreading cells suggesting the production of filopodia protrusions from their membranes, while Eps15 knockdown cells showed mostly cortical actin. Many Eps15 knockdown cells were devoid of stress fibres across their cytoplasm, and instead showed clumping green staining and thick cortical actin (Figure 6.11A) While we did not quantify these changes in actin organisation we observed that Eps15 knockdown cells consistently showed actin cytoskeleton organisation that was phenotypically different to control cells at all time-points, suggesting that Eps15 knockdown can affect actin cytoskeleton organisation.







#### Figure 6.11 Eps15 knockdown inhibits cell spreading

SCC25 cells treated with control or Eps15 siRNA were sparsely plated onto glass coverslips coated with ECM protein LAP in 1% serum 24 hours post-transfection. Cells were then fixed at 5, 24, 32, 48 and 70 hours and stained for actin filaments (Phalloidin-FITC). (A) Cells treated with Eps15 siRNA are phenotypically different to control treated cells. They are visibly smaller and appear to have disorganised actin filaments. Scale bar represents 50 $\mu$ m. (B) Quantification of phalloidin cell area by Fiji software shows a trend of consistently smaller cells with Eps15 knockdown compared with control treated cells but that this difference is not significant. Quantification was carried out on 10 fields of view for each condition and observations are representative of two independent experiments (n=2) (AU = arbitrary units).

### 6.3 Eps15 over-expression is able to partially rescue the effect of Eps15 knockdown on cell spreading

SCC25 cells treated with Eps15 siRNA are unable to spread effectively to the size of control SCC25 cells. Eps15 knockdown cells also display a phenotype which differs to that of control siRNA treated cells. The inability of Eps15 knockdown cells to spread to the size of control cells could be due to disruption of the actin cytoskeleton. Eps15 knockdown cells were consistently smaller in size, and phenotypically showed reduced actin fibre formation and reduced filopodia formation, although these observations were not quantified. Following these observations, we investigated cells transfected with an Eps15 over-expression vector following Eps15 knockdown to see if control cell phenotype could be rescued by re-introducing Eps15 into cells which had previously had Eps15 expression inhibited with siRNA. In order to rescue Eps15 expression we first transfected the cells with Eps15 siRNA and after 24 hours we transfected them again with an Eps15 over-expression vector. In order to ensure that we could over-express Eps15 once it had been knocked out, we needed to transfect cells with an siRNA that only targets the untranslated region of Eps15 mRNA, which is not present in the over-expression vector, as the vector only contains the coding sequence for Eps15. We were unable to use Eps15\_5 as its sequence targeted Eps15 coding region, but Eps15\_7 did not. Therefore, treatment with Eps15\_7 siRNA was able to inhibit endogenous Eps15 expression, then by introducing our Eps15 over-expression vector we could re-introduce Eps15 back into the cells and observe its effect on cell morphology by immunofluorescent techniques.

Re-introduction of Eps15 is able to partially rescue the abnormal morphology of Eps15 knockdown cells to a morphology more similar to that of control cells. We observed that cells with Eps15 re-introduced were more phenotypically similar to control cells than to Eps15 knockdown cells. These cells showed similar actin stress fibres across their cytoskeleton as well as fan-like protrusions from their membrane, indicating cell spreading (Figure 6.12A). Eps15 knockdown cells once again had reduced actin stress fibre formation and more cortical actin as well as fewer cell protrusions. Eps15 over-expression rescued this phenotype of Eps15 knockdown cells to a phenotype more similar to control treated cells. Cells treated with Eps15 over-expression vector following Eps15 knockdown were larger and appeared to have better actin fibre organisation across the cell compared to Eps15 knockdown cells (Figure 6.12A). Eps15 over-expression cells were not fully rescued from Eps15 knockdown as the majority of cells still appeared smaller than

control cells and appeared to have thicker cortical actin layer than control cells (Figure 6.12A), but these features were less pronounced.

Another observation was the way in which the cells appeared to interact with one another in each of the different conditions. It was more difficult to find lone control cells to image than Eps15 knockdown cells. Control cells tended to adhere together in small cell 'islands' and so finding individual cells was more difficult. Eps15 knockdown cells on the other hand were often adherent as single cells. It appeared that cell-cell contacts were less abundant in Eps15 knockdown cells compared to control cells. While we did not quantify cell-cell contacts between the cells, this observation is interesting and could suggest that Eps15 inhibition could also be affecting cell-cell adhesion as well as possibly having an effect on cell-ECM interaction, but this would need further investigation (Figure 6.12A).

Once again we measured the cell area of individual cells in each condition in order to quantitatively analyse the effect of Eps15 knockdown on cell size. SCC25 cells were fixed at 48 hours post-plating. 10 fields of view for each condition were pictured and cell area was analysed using Fiji software by quantifying phalloidin (Figure 6.12B). Eps15 knockdown cells were significantly smaller than control treated cells, as seen previously. Cells treated with just Eps15 over-expression vector and cells with re-introduced Eps15 expression showed no significant difference in size compared with control cells. Perhaps endogenous Eps15 is sufficient for optimal cell spreading, and introduction of more causes no obvious increases in cell size. Cells treated with Eps15 siRNA and then Eps15 over-expression also showed no significant difference in cell area compared to control cells (Figure 6.12B). This result suggests that Eps15 does have a role in regulating cell spreading, as re-introducing Eps15 into cells which had previously had their Eps15 expression inhibited was able to rescue the amount of cell spreading to similar levels as control cells (Figure 6.12B). Although cells treated with Eps15 over-expression vector did not produce cells which were significantly smaller, they did appear to still show a trend of smaller cells. This could indicate that Eps15 re-introduction is not able to completely rescue the effect of Eps15 inhibition. Additionally, cells with Eps15 over-expression did not produce cells which were significantly larger than just Eps15 siRNA treated cells. The lack of a significant difference between these two samples could suggest that Eps15 over-expression is unable to rescue Eps15 knockdown however, lack of significance could be as a result of too few photos taken. This could also be as a result of the cell's being less healthy than control cells. Perhaps both a decrease and an increase from endogenous Eps15 can affect the balance of normal cell processes and affect cell morphology, but more research would be needed to conclude on the effects of Eps15 over-expression on cell function. This investigation was only performed once and so a repeat would be needed to confirm the effects seen.

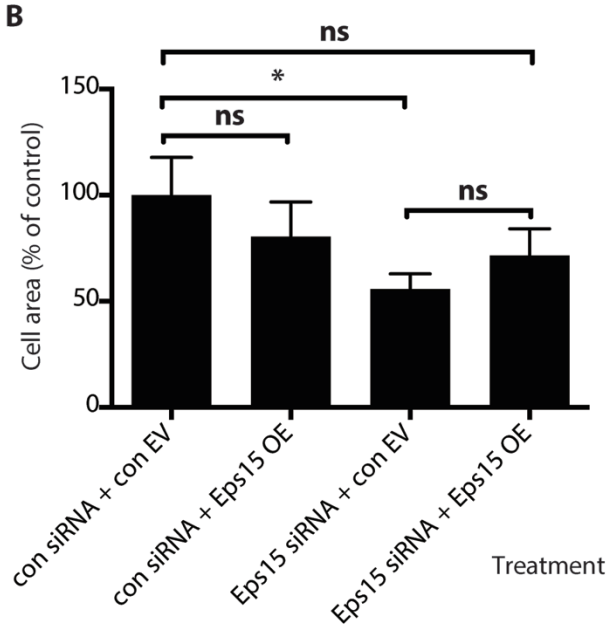
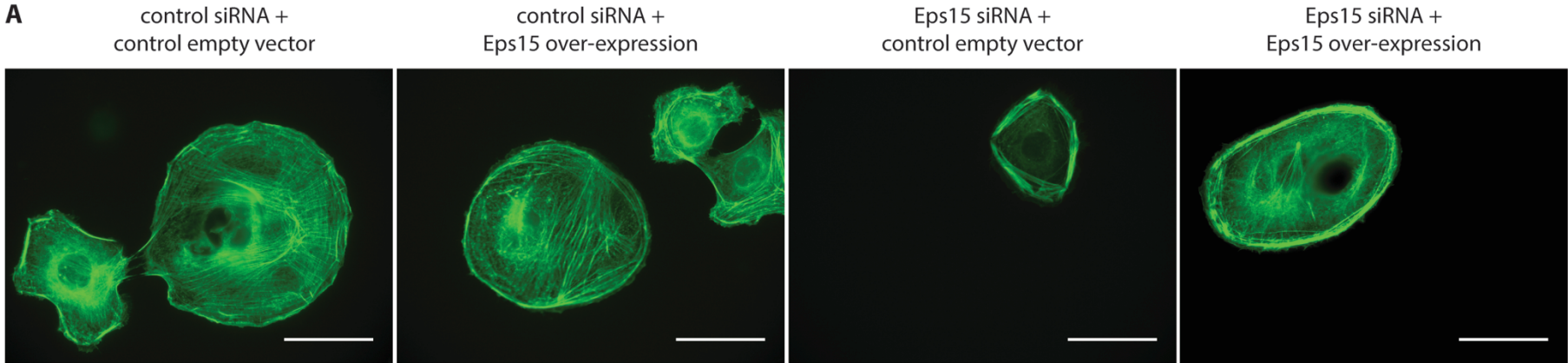


Figure 6.12 Eps15 over-expression is able to partially rescue the effect of Eps15 knockdown on cell spreading

SCC25 cells were sparsely plated onto glass coverslips coated with extracellular matrix (ECM) protein LAP in 1% serum. SCC25 cells were treated with control or Eps15 siRNA or control empty vector (con EV) or Eps15 over-expression vector (Eps15 OE) in combination to produce four conditions; control siRNA + con EV, control siRNA + Eps15 OE, Eps15 siRNA + con EV and Eps15 siRNA + Eps15 OE. Cells were transfected with siRNA on day 0, then with expression vector on day 1, plated on day 2 and fixed on day 4; 48 hours post-plating and stained for actin filaments (Phalloidin-FITC). (A) Phenotypically, Eps15 knockdown cells appeared smaller, with disorganised actin and reduced filopodia production compared with control cells while cells treated with Eps15 over-expression had a phenotype more like control cells. They were larger with more stress fibres visible but still thicker cortical actin than seen in control cells. Scale bar represents 50µm. (B) Quantification of phalloidin cell area by Fiji showed that cells treated with Eps15 knockdown had a significantly smaller cell area compared with control treated cells ( $p < 0.05$ ) but that this condition was slightly rescued by Eps15 over-expression so that the difference in cell area was no longer significant. There was also no significant difference between Eps15 knockdown and Eps15 over-expression conditions. Quantification was carried out on 10 fields of view for each condition ( $n=1$ ).

## 6.4 Discussion

Cell migration is an important process during cancer progression and as discussed previously optimal cell motility requires optimal cell adhesion; too strong or too weak adhesion to the ECM and cells cannot migrate effectively (Cox & Huttenlocher 1998). The ability for tumour cells to migrate drives tumour progression and leads to worse prognosis for patients as approximately 90% of cancer patients die as a result of tumour metastasis (Spano et al. 2012), therefore, understanding how and why tumour cells migrate and invade is important in order to try and reduce tumour cell metastasis.

Using two different adhesion assays we observed that Eps15 knockdown inhibits tumour cell adhesion. We confirmed again that endocytosis is not the mechanism of how Eps15 affects cell function as knockdown of CHC and HAX-1, both proteins known to regulate integrin endocytosis (A. Ramsay et al. 2007) had the opposite effect and caused a consistent increase in cell adhesion. We also confirmed previous data that inhibition of the glycolytic sensor CtBP1 increased cell adhesion (Chrzan 2014) and have shown in Chapter 3 that it also results in a significant reduction in Eps15 expression. While we originally hypothesised that CtBP1 affects cell adhesion through regulating Eps15 expression, the fact that knockdown of these two proteins has the opposite effect suggests that they are regulating cell adhesion through different mechanisms. We also showed that Eps15 knockdown caused significant decrease in cell spreading compared with control cells and the morphological difference between these cells suggests that Eps15 knockdown could disrupt actin cytoskeleton organisation, but confirmation of this would require further investigation. These results are novel as there are no papers showing these effects of Eps15 on cell spreading. However, there are several ways which we could explain these effects by previous results in the literature.

The Eps15-like protein; Pan1p, has been shown to bind actin in yeast and one paper even suggests that it is required for normal organisation of the actin cytoskeleton in *S. cerevisiae* (Tang & Cai 1996), therefore we cannot exclude that Eps15 could do the same in cancer cells indicating a potential role for Eps15 in directly affecting the actin cytoskeleton. Tang and Cai found that *pan1* mutants failed to maintain a proper distribution of the actin cytoskeleton and were unable to reorganise actin during the cell cycle. Using immunofluorescent techniques they imaged *pan1* mutant budding yeast cells and found that cortical actin patches in the *pan1-4* cells were not confined to the bud, and a large amount of them was retained in the mother. Many of the cortical actin patches seen in the mutant cells were also larger in size. These data mirror the thick cortical actin distribution seen in our Eps15 knockdown cells. In addition, the authors found that the actin

cytoskeleton in some *pan1-4* cells aggregated into thick cables or bars not seen in wild-type cells. These actin cables in the mother, when visible, were not arrayed orderly as in wild-type cells and did not display any regular pattern in respect of bud size, suggesting that the mutant had lost its ability to reorganise the actin structure in accordance with cell cycle progression (Tang & Cai 1996). These data mirror what we saw in regards to loss of actin fibre organisation of our Eps15 knockdown cells. The Pan1 protein contains an EF-hand calcium-binding domain, a putative Src homology 3 (SH3)-binding domain, and two EH domain motifs. Staining for Pan1 was partly localised to the membrane with some cytosol staining and was also found to be co-localised with cortical actin patches but not the actin cables (Tang & Cai 1996).

Pan1p is described as having a role in both yeast endocytosis and organisation of the cortical actin cytoskeleton (Tang et al. 1997). Toshima *et al* discuss Pan1p as a regulator of the interaction between endocytic vesicles, endosomes and the actin cytoskeleton (Toshima et al. 2016). The actin cytoskeleton plays important roles in the formation and internalisation of endocytic vesicles (Mooren et al. 2012) as in both yeast and mammals, endocytic internalisation is accompanied by a transient burst of actin polymerisation (Toshima et al. 2005). Toshima *et al* show that the phospho-regulator Prk1p is a key regulator in this burst of actin polymerisation through phosphorylation of Pan1p. As discussed, Pan1p is essential for endocytic internalisation and for proper actin organisation and is also a Prk1p substrate (Toshima et al. 2005). In yeast, endocytic vesicles move towards early endosomes along actin cables but Toshima *et al* showed that the dephosphorylated form of Pan1p caused stable associations between endocytic vesicles and actin cables, and between endocytic vesicles and endosomes, thus inhibiting endocytic vesicle movement along actin cables, therefore phosphorylation states of Pan1p is important in its regulatory role (Toshima et al. 2016). Pan1p is able to regulate actin cytoskeleton polymerisation as it is an Arp2/3 activator. Pan1p is able to regulate Arp2/3 complex by directly binding F-actin (Toshima et al. 2005). Phosphorylation by Prk1p inhibits the ability of Pan1p to bind to F-actin and to activate the Arp2/3 complex. This regulation is used by cells to allow endocytic vesicles to fuse with endosomes (Toshima et al. 2005). Other papers have also investigated the role of protein Pan1p in the regulation of actin cytoskeleton organisation by activating the Arp2/3 complex (Duncan et al. 2001). Duncan *et al* found that Pan1p forms the core of an endocytic complex and physically couples actin polymerisation nucleated by the Arp2/3 complex to the endocytic machinery, thus providing the forces necessary for endocytosis (Duncan et al. 2001). We found that Eps15 knockdown did not consistently increase or decrease integrin-endocytosis. While papers have shown that knockdown of Eps15 disrupts clathrin-mediated endocytosis (Benmerah et al. 1999) perhaps its mechanism is through regulation of actin cytoskeleton organisation.

While there are no papers that show a direct association of human Eps15 with the actin cytoskeleton, it is possible that human Eps15 could link to the actin cytoskeleton indirectly through interaction with an Eps15 binding protein, for example, Epsin1 (Lin et al. 2011). Eps15 is involved during actin accumulation and pedestal formation required for cell infection by *Escherichia coli* (EPEC) (Lin et al. 2011). Induction of EPEC pathogenesis is accomplished by the delivery of pathogenic effector proteins of which the translocated intimin receptor (Tir), is crucial for pedestal formation. Once secreted into the host cells, Tir becomes inserted into the host cell plasma membrane, where it functions as a receptor for the bacterial outer membrane ligand intimin. This Tir-intimin interaction firmly anchors the pathogen to the epithelial cell surface and recruits host cell kinases to induce Tir phosphorylation on a variety of tyrosine residues (Lin et al. 2011). This phosphorylation event recruits host adaptor molecules to Tir, ultimately triggering actin rearrangement beneath the attached bacteria. Clathrin is recruited to EPEC pedestals as are Eps15 and Epsin1 (Lin et al. 2011). Lin *et al* were interested in understanding how endocytic proteins are involved in infection by extracellular pathogens and found that HeLa cells treated with Eps15 siRNA showed no pedestal formation or actin accumulation when subjected to EPEC infection (Lin et al. 2011). The authors infected both HeLa and Caco-2 cells with wild-type (WT) EPEC and found that Eps15 was restricted specifically to the tips of the EPEC actin-rich pedestals in over 80% of all pedestals (Lin et al. 2011). Following Eps15 knockdown with siRNA treatment, actin accumulation was abolished beneath the attached bacteria and almost no actin pedestals were formed at sites of infection (Lin et al. 2011). Actin-rich pedestals were also severely depleted in cells treated with Epsin1 siRNA and thus the authors suggest that the presence of Eps15 or Epsin1 at sites of bacterial attachment is a prerequisite for subsequent actin accumulation to create EPEC pedestals (Lin et al. 2011). Eps15 and Epsin1 are known binding partners (van Bergen En Henegouwen 2009) and Lin *et al* found that knockdown of either Epsin1 or Eps15 caused failure of the other protein to localise to the site of EPEC attachment but did not influence the protein expression of the other protein (Lin et al. 2011). The authors suggest that although the expression of Eps15 and Epsin1 are independent of each other, these proteins are dependent on one another for proper targeting to EPEC pedestals (Lin et al. 2011). Additionally, the authors found that only the ubiquitin interacting motif (UIM) region of Eps15 and Epsin1 were sufficient for proper targeting to EPEC pedestals (Lin et al. 2011). While Eps15 has not been shown to have a role in actin reorganisation previously, Epsin1 deficiency has been shown to impair endocytosis by stalling the actin-dependent invagination of endocytic clathrin-coated pits (Messa et al. 2014). Messa *et al* generated embryonic fibroblasts from conditional Epsin1 triple knockout (KO) mice which displayed a dramatic cell division defect and a robust impairment in clathrin-mediated endocytosis. Furthermore, the authors found at least two actin-binding sites in Epsin (Messa et al. 2014). The authors incubated recombinant Epsin1 fragments with purified F-actin followed by co-



sedimentation which revealed that both the DPW and NPF motif containing regions of the protein but not the ENTH domain, bind actin (Messa et al. 2014). The authors suggest that the site in the NPF motif containing region likely corresponds to the actin cytoskeleton-binding (ACB) site, previously identified in yeast Epsin1; Ent1 (Skruzny et al. 2012). Given these observations it is possible that knockdown of Eps15 is able to exert an effect on actin cytoskeleton organisation in certain situations through its binding to Epsin1. Lin *et al* showed that Eps15 and Epsin1 were required for actin accumulation during EPEC infection (Lin et al. 2011) and Epsin1 has a direct link to actin (Messa et al. 2014). So while there is no evidence yet to suggest that human Eps15 can directly bind actin, Eps15 may be able to affect actin indirectly through association with other proteins.

The majority of the literature focuses on binding partners of Eps15 as explanation for the role of Eps15 in endocytosis (Benmerah et al. 1999; Cai et al. 2013; Carbone et al. 1997) however, recent publications have highlighted other roles of these proteins in the regulation of actin reorganisation. Therefore, it is possible that Eps15 could have multiple functions within the cell through interaction with these binding partners (Table 1.1) (van Bergen En Henegouwen 2009; Salcini et al. 1999).

There are three human STAM proteins; STAM 1, STAM 2A (53% identity to STAM 1) and STAM 2B (64% identity to STAM 1) (Lohi & Lehto 2001). STAM 1 and STAM 2 are regulators of receptor trafficking as STAM proteins interact with the same coiled-coil domain that is involved in targeting Hrs (hepatocyte growth factor-regulated tyrosine substrate kinase) to endosomes (Bache et al. 2003). Bache *et al* used glutathione-S-transferase (GST) gene fusion system to investigate how STAM and Hrs bind together and found that STAM and Eps15 bind Hrs at distinct locations to form a ternary complex. GST-STAM 1 and GST-STAM 2 were incubated with recombinant Eps15 and in the presence or absence of recombinant Hrs and they found that Eps15 was only associated with GST-STAM 1 and GST-STAM 2 in the presence of Hrs (Bache et al. 2003). While binding of Eps15 to STAM proteins supports a link between Eps15 and the endocytic machinery, EAST, a chick homolog of STAM 2A (78% identity) also associates directly with actin filaments (Lohi & Lehto 1998). Both STAM 2A and EAST are expressed in a wide variety of tissues and cell lines (Lohi & Lehto 2001). Lohi and Lehto discovered that EAST, was able to interact with Eps15 and focal adhesions and actin filaments (Lohi & Lehto 1998). The authors found extensive co-distribution of EAST with Vinculin, Paxillin and actin filaments and discovered that over-expression of the NH<sub>2</sub> terminus of EAST caused the formation of actin-rich micro-spikes and membrane protrusions (Lohi & Lehto 1998). They conclude that EAST is involved in the EGFR-regulated reorganisation of the actin cytoskeleton and may be part of a link between cytoskeleton and endocytic machinery (Lohi & Lehto 1998). Understanding the functions of Eps15 binding proteins such as EAST and

their role in actin organisation is the first step to establishing a possible connection between Eps15 and the actin cytoskeleton.

Intersectin (ITSN) is a multi-domain scaffold protein and a high throughput yeast two-hybrid screen by Wong *et al* found over 100 binding proteins across two mammalian ITSN genes; ITSN1 and ITSN2, including Eps15 (Wong et al. 2012; Yamabhai et al. 1998). ITSN1 has at least 24 splice variants and ITSN2 has at least 4 splice variants, which have altered interactions with specific targets. ITSN1 and ITSN2 share 59% identity and each encode a short and long isoform (Wong et al. 2012). Both ITSN short (ITSN-S) isoforms possess two amino-terminal Eps15 homology (EH) domains followed by a coiled-coil (CC) domain and five Src homology 3 (SH3 A-E) domains (Yamabhai et al. 1998). The ITSN long isoform (ITSN-L) contains all these domains in addition to an extended carboxy-terminus encoding a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, and a C2 domain. The DH and PH domains function together as a guanine nucleotide exchange factor (GEF) that regulates the activation of the Rho family GTPase Cdc42 (Wong et al. 2012), involved in actin reorganisation through its interaction with N-WASP (Hussain et al. 2001). By acting as a GEF of Cdc42, Intersectin is able to cause actin rearrangements specific for Cdc42. Hussain *et al* investigated a role for ITSN1 in a novel mechanism of N-WASP activation and in regulation of the actin cytoskeleton (Hussain et al. 2001). They found that N-WASP binds directly to ITSN1, up-regulating its GEF activity, generating GTP-bound Cdc42 (GTP-Cdc42), a critical activator of N-WASP, in a positive feedback loop (Hussain et al. 2001). Whiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) family proteins are scaffold proteins linking upstream signals, such as those from GTP-Cdc42, to the activation of the Arp2/3 complex leading to a burst of actin polymerisation (Takenawa & Suetsugu 2007). Two forms of WASP exist; WASP (expression restricted to haematopoietic cells) and N-WASP (so called due to its abundance in neural tissue, but is found in other tissue types) (Takenawa & Suetsugu 2007). Both possess an important VCA domain required to activate the Arp2/3 complex. N-WASP activates Arp2/3 by binding to it at its VCA region along with an actin monomer. Actin polymerisation is initiated by the assembly of three actin monomers, the Arp2/3 complex has two-actin related molecules, so binding of a third initiated by N-WASP binding causes a burst of actin polymerisation (Takenawa & Suetsugu 2007). Actin polymerisation induced by WASP and WAVE proteins occurs during several biological functions such as the formation of filopodia and lamellipodia in cell migration, membrane trafficking and cell adhesion, among others. Rapid actin polymerisation at the leading edge is required for cells to migrate and these processes are highly governed by the Rho family of GTPases; specifically, filopodia formation is mediated by the Rho GTPase Cdc42, which we know to be activated by the GEF ITSN1. Lamellipodia formation is mediated by Rac and retraction of the rear of the cell is mediated by RhoA (Takenawa & Suetsugu

2007). Actin reorganisation requires both the activation and inactivation of Rac1 and Cdc42 in the regulation of filopodia and lamellipodia production. Not only is ITSN1 involved in Cdc42 activation but it has also been found bound to the Cdc42 GTPase-activating protein (CdGAP) at its SH3D domain (Primeau et al. 2011), regulating activity towards both Rac1 and Cdc42 (Lamarche-Vane & Hall 1998). ITSN1 and ITSN2 are also involved in the regulation of the production of invadopodias; actin-rich protrusions formed by invasive cancer cells. Immunofluorescent analysis revealed co-localisation of ITSN1 and WIP (WASP-interacting protein) at sites of invadopodia formation and in clathrin-coated pits and the two were found to interact via the SH3 domains of ITSNs and the middle part of the WIP proline-rich motifs (Gryaznova et al. 2015). Furthermore, a study by Friesland *et al* showed that a small molecule inhibitor of the interaction between Cdc42 and Intersectin was able to disrupt Golgi organisation and suppress cell motility (Friesland et al. 2013), both characteristics that we observed in our cells treated with Eps15 siRNA. Perhaps Intersectin-Eps15 complexes are required for further protein-Intersectin complexes such as binding Cdc42 or even CdGAP and a loss of Eps15 makes these complexes impossible. Friesland *et al* used the small molecule inhibitor of Cdc42; ZCL278, to directly bind to Cdc42 and inhibit its functions. They found that in 3T3 fibroblast cultures, ZCL278 abolished micro-spike formation and disrupted GM130-docked Golgi structures. GM130 is a peripheral cytoplasmic protein that is tightly bound to Golgi membranes, they found that following ZCL278 treatment cells showed a clear reduction of perinuclear GM130 immunoreactivity (Friesland et al. 2013). These studies show the importance of the Cdc42-ITSN complex once more highlighting the importance of protein-protein complexes in cell function.

The relationship of Eps15 binding to Intersectin was defined in a paper by Sengar *et al*. They discuss that Intersectin is an orthologue of the endocytic adaptor proteins Ese1 and Ese2 as *Xenopus* Intersectin is 81% identical to mouse Ese1 and 54% identical to mouse Ese2 (Sengar et al. 1999). The authors discuss that the binding capabilities of Ese1 and Eps15 allows a minimum of 14 protein-protein interaction surfaces of the Ese-Eps15 complex due to six protein-protein interaction surfaces on Eps15 (three EH domains, a central coiled-coil domain, an  $\alpha$ -adaptin-binding domain and a proline-rich motif) and a minimum of eight across the Ese1/2 proteins' protein-protein interaction surfaces (two EH domains, a central coiled-coil domain and five SH3 domains). The ability of Eps15 to produce dimers and tetramers allows even more binding capabilities (Cupers et al. 1997). Wong *et al* expand on this relationship and discuss that since ITSN1 has at least 24 splice variants and ITSN2 has at least 4 splice variants, then the number of potential unique tetrameric complexes that could be formed in the cell is  $30^4$  if it's assumed that each of these isoforms has the potential to form homo- or hetero-tetramers with each other and Eps15/Eps15R (Wong et al. 2012). Sengar *et al* began to show the importance of these complexes

by investigating the endogenous Ese1-Eps15 complex. They discuss that Ese1-Eps15 allows the formation of higher order protein complexes and found that over-expression of Ese1 was able to disrupt the production of these complexes to significantly inhibit clathrin-mediated endocytosis (Sengar et al. 1999). The authors transfected cells with myc-tagged Ese1 and, 48 hours post transfection, added fluorescein isothiocyanate (FITC)-labelled transferrin to cultures for 30 minutes. They then fixed and analysed the cells for expression of mycEse1 and for internalisation of transferrin (a marker for constitutive endocytosis). 96% of Ese1 over-expressing cells did not internalise labelled transferrin compared with 100% of un-transfected cells that were capable of clathrin-mediated endocytosis (Sengar et al. 1999).

Crk is another binding protein of Eps15 which is also involved in the activation of WASP (Sasahara et al. 2002). Crk proteins are thought to transduce signals from tyrosine kinases to downstream effectors and an expression library screened for Crk binding partners revealed that both Eps15 and Eps15R could bind to the amino-terminal of the SH3 domain of Crk (Schumacher et al. 1995). Furthermore, both c-Crk and v-Crk co-precipitated equivalently with Eps15 and Eps15R (Schumacher et al. 1995). Crk is involved in the activation of WASP in activated T-cells by forming a complex with WIP following release of WASP inhibition from a WIP-WASP complex (Schumacher et al. 1995). During T-cell activation the T-cell receptor (TCR) becomes engaged with its ligand causing the phosphorylation of WIP. This breaks the WIP-WASP complex, releasing WASP from inhibition. Crk then binds WIP in a ZAP-70-Crk-WIP complex to allow continued activation of WASP in activated TCRs. Here the importance of interaction between complexes is shown as the ZAP-70-Crk-WIP complex maintains WASP activation by inhibiting the formation of further WIP-WASP complexes in activated T-cells (Schumacher et al. 1995). The ZAP-70-Crk-WIP complex first requires binding of ZAP-70 to Crk as WASP activation was markedly reduced in ZAP-70 deficient cells. Once again the importance of appropriate protein complexes is highlighted. A loss of ZAP-70 reduced WASP activation as the ZAP-70-Crk-WIP complex could not form. If Crk proteins are involved in the downstream signalling of tyrosine kinases, then perhaps downstream signalling of Eps15 activation is occurring in activated T-cells. Crk may be able to form larger complexes after forming an initial complex with Eps15 and loss of Eps15 as a result of Eps15 knockdown could inhibit further Crk complexes such as Crk-WIP from forming. It's possible that inhibition of Crk-WIP could lead to constitutively inactive WASP due to stable WIP-WASP complexes, disrupting actin organisation. This hypothesis assumes that Eps15 is constitutively bound to Crk, which has not been investigated, but an interesting discussion can be made on how loss of Eps15 could be disrupting protein-Eps15 interactions consequently affecting actin organisation.

These data highlight the importance of protein-protein complexes in cell function and, in the context of our own data, while we found that Eps15 knockdown did not affect integrin

endocytosis, it is possible that knockdown of Eps15 could still be disrupting cell function by disrupting other protein-Eps15 complexes and consequently higher order protein complexes. I have discussed here some interesting hypotheses on how Eps15 knockdown could be affecting cell motility by discussing the known binding partners of Eps15. However, to elucidate an exact mechanism of how Eps15 is affecting cell motility would require further investigation. In order to better understand how Eps15 knockdown can affect cell processes we performed RNA sequencing on SCC25 cells transfected with Eps15 siRNA compared with cells transfected with control siRNA to see what genes are affected by Eps15 knockdown and if any of these could be responsible for the effect on actin organisation seen.

## 6.5 Summary

1. Eps15 knockdown significantly reduces tumour cell adhesion, via an endocytosis-independent mechanism
2. Eps15 knockdown is affecting cell function via a mechanism different to that of CtBP1 as CtBP1 knockdown significantly increases adhesion
3. Eps15 knockdown significantly affects cell spreading possibly by disrupting actin organisation
4. Eps15 could have a direct or indirect effect on actin cytoskeleton organisation, but confirming this would require further investigation



## Chapter 7: Other roles of Eps15

The metabolic sensor CtBP1 is involved in the regulation of tumour cell motility. Loss of CtBP1 reduces tumour cell motility by increasing tumour cell adhesion (Chrzan 2014). Our initial hypothesis suggested that CtBP1 could be regulating cell motility through a mechanism involving the endocytosis adaptor protein Eps15. We hypothesised that loss of Eps15, as a consequence of CtBP1 knockdown, would disrupt integrin endocytosis, causing an increase of integrin on the cell surface and a consequent increase in cell adhesion. However, not only did we find that loss of Eps15 did not consistently inhibit integrin endocytosis or cell surface levels of  $\beta 1$  or  $\beta 6$  integrin (Figure 5.1 – Figure 5.4), but loss of Eps15 also resulted in decreased tumour cell adhesion (Figure 6.2 and Figure 6.3). While investigating the effect of Eps15 on tumour cell adhesion we observed that loss of Eps15 also resulted in inhibition of tumour cell spreading and that this inhibition was perhaps the result of disorganisation of the actin cytoskeleton. Whilst Eps15 is a known endocytosis adapter protein, there is sparse evidence discussing other functions of Eps15. Due to the availability of a number of binding sites throughout its structure Eps15 is capable of interacting with a variety of proteins to produce many higher order structures (van Bergen En Henegouwen 2009), therefore it is possible that loss of Eps15 could disrupt a number of downstream processes to affect cell function, some of which could be involved in the regulation of tumour cell adhesion or actin cytoskeleton organisation.

### 7.1 RNA sequencing of Eps15 knockdown samples

In order to identify a possible mechanism by which Eps15 knockdown could be inhibiting cell adhesion and causing disruption to the actin cytoskeleton we performed RNA sequencing on SCC25 cells treated with Eps15 siRNA. Three independent paired sample transfections of control and Eps15 siRNA were carried out on SCC25 cells of similar passages. Cells were collected 48 hours post-transfection with media changed 24 hours post-transfection. RNA was then extracted using RNeasy extraction kit from Qiagen according to manufacturer's instructions. The RNA underwent quality control testing using Bioanalyzer analysis (Agilent Technologies Inc.) to obtain RNA integrity numbers prior to downstream processing. Eps15 knockdown was confirmed by RT-PCR in all three paired samples as previously described. 250-300 ng of total RNA at a minimum concentration of 25 ng/ $\mu$ l was sent to Expression Analysis Genomic Services (Durham, USA). RNA sequencing was performed using Illumina Truseq Stranded protocol, with paired end sequencing and 25 million reads per sample. Reads were mapped to the human genome (hg19) with Tophat

2.0.13 (Trapnell et al. 2012), indexed and sorted with Samtools-1.2 (Li et al. 2009) and counted using HTSeq (Anders et al. 2013) to allow normalisation and differential gene expression analysis.

Once the raw count matrix was achieved 'R' software was used with bespoke macros, and a Venn diagram representing the 11,345 expressed genes differentially expressed between Eps15 knockdown cells and control SCC25 cells was produced to determine which of these gene changes was significantly different with a p value less than 0.001 (Figure 7.1).

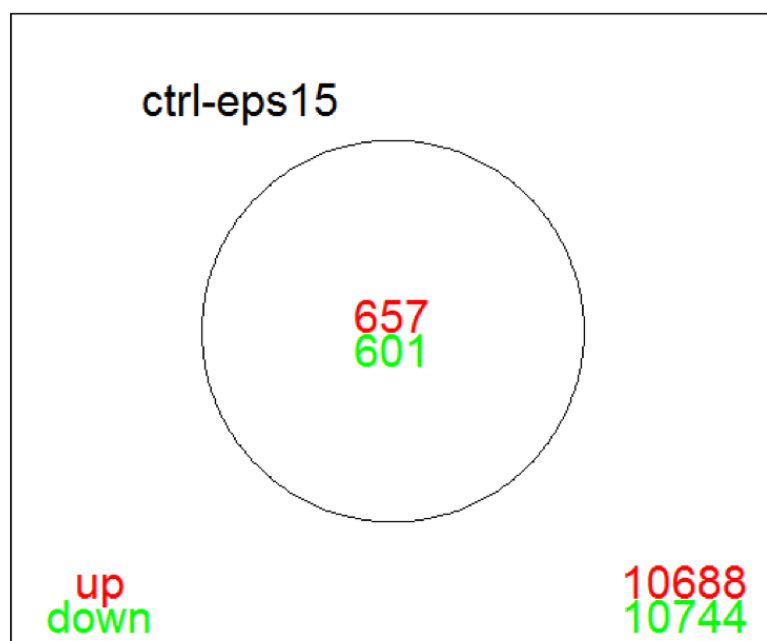


Figure 7.1 Gene expression following Eps15 knockdown  
RNA sequencing revealed 11,345 differentially expressed genes in SCC25 cells treated with Eps15 siRNA compared with cells treated with control (non-targeting) siRNA. When a significance cap of  $p < 0.001$  was applied 657 genes were significantly down-regulated, while 601 genes were significantly up-regulated in Eps15 siRNA treated cells compared with control cells.

'R' software with bespoke macros was also used to produce a heat map of the top 60 genes differentially expressed following Eps15 knockdown across the three paired samples. The genes were well defined between control samples and Eps15 samples with 34 genes down-regulated (fold change  $< -1$ ,  $p < 0.001$ ) and 26 genes up-regulated (fold change  $< 1$ ,  $p < 0.001$ ). This heat map includes several genes involved in actin morphology regulation (*e.g.* FERMT2) or GTPase activity (*e.g.* RAB8A, ASAP1) among others (Figure 7.2), and provides the first insight into genes affected by Eps15 knockdown and are discussed in greater detail in the discussion.



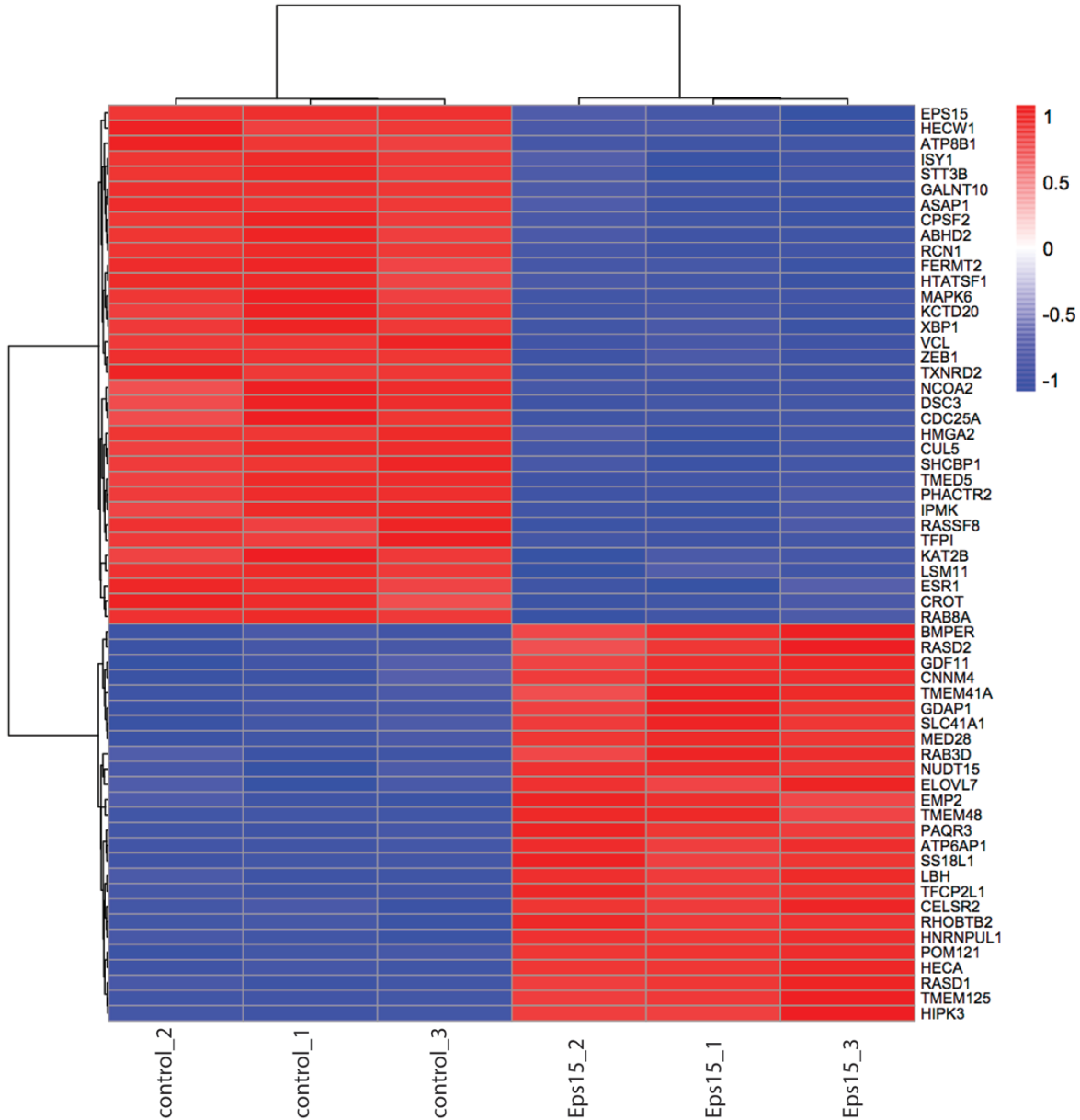
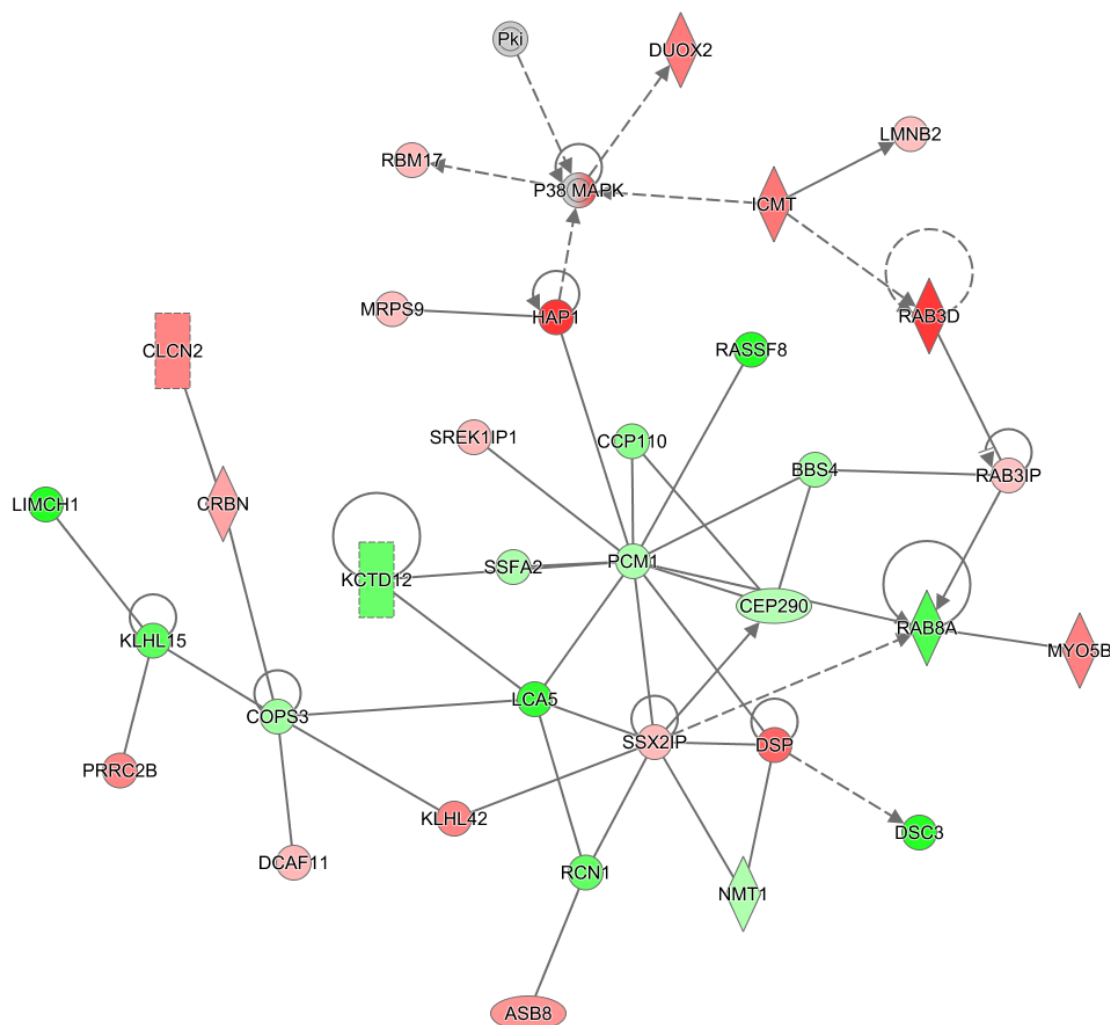


Figure 7.2 RNA sequencing top 60 genes  
Heat map depiction of the top 60 genes differentially expressed in SCC25 cells treated with Eps15 siRNA compared with control (non-targeting) siRNA produced with 'R' software. Key indicates the fold change of all genes. Red = up-regulated genes. Blue = down-regulated genes. Heat map shows 34 genes down-regulated (fold change <-1, p<0.001) and 24 genes up-regulated (fold change <1, p<0.001).

In order to investigate gene networks, which are affected in cells treated with Eps15 siRNA Ingenuity Pathway analysis (IPA) was used. Network analysis is produced by IPA based on what is already known in the literature about the affected genes and their interaction with one another. Only genes, which were significantly (p<0.001) differentially expressed in Eps15 knockdown cells were analysed by IPA network analysis, thereby reducing the number of genes from 11,345 to 1024. IPA used this gene list and provided 25 possible networks affected by Eps15 knockdown. Of the 25 networks given, 14 were involved in regulating cell or tissue morphology, cell regulation and organisation, the first such network is presented in Figure 7.3. It includes at least 3 of the top

60 differentially expressed genes (RAB8A, RAB3D, DSC3) and is a network involved in regulating cellular morphology, cellular assembly and organisation and cellular function and maintenance. This is actually the second of the top 25 possible networks, the first is given in the appendix (Appendix 3 – Figure S3.1).

Network 2 : RNA seq - adj  $p < 0.001$  : RNA seq - adj  $p < 0.001$



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Figure 7.3 Network 2 of genes changed following Eps15 knockdown  
Ingenuity Pathway Analysis (IPA) revealed network analysis of gene changes associated with Eps15 knockdown. The network ranked second using IPA is important in regulating cellular morphology, cellular assembly and organisation. Genes involved in this network are differentially expressed in Eps15 knockdown cells compared to control cells with a significance value of  $p < 0.001$ .

The goal with RNA sequencing was to identify potential genes, which could explain the effect of Eps15 on actin reorganisation, cell adhesion and motility. Therefore, all genes within the top 60 were briefly researched and genes involved in actin remodelling (*e.g.* FERMT2, VCL) or in regulating signalling, which could affect actin reorganisation (*e.g.* GTPases) were selected to be validated by quantitative RT-PCR analysis. Genes were briefly investigated by searching the

Human Gene Database; GeneCard.org, which integrates data from more than 125 web sources and includes genomic, transcriptomic, proteomic, genetic, clinical and functional information on all genes. The 34 down-regulated genes are listed in Table 7.1, and the top 26 up-regulated genes are listed in Table 7.2.

Table 7.1 Top 34 down-regulated genes as a result of Eps15 knockdown

All 34 most down-regulated genes were briefly researched and genes which fit our selection criteria were chosen to be validated by quantitative RT-PCR analysis. Selection criteria included genes involved in actin remodelling or in regulating signalling, which could affect actin reorganisation.

Gene name (down-regulated)	Function	Selection criteria
HECW1 (HECT, C2 and WW Domain Containing E3 Ubiquitin Protein Ligase 1)	Protein Coding gene involved in ligase activity and ubiquitin protein ligase activity.	
ATP8B1	This gene encodes a member of the P-type cation transport ATPase family, which belongs to the subfamily of aminophospholipid-transporting ATPases. The aminophospholipid translocases transport phosphatidylserine and phosphatidylethanolamine from one side of a bilayer to another.	
ISY1 (ISY1 Splicing Factor Homolog)	Protein Coding gene involved in DNA double-strand break repair and mRNA splicing	
STT3B	The protein encoded by this gene is a catalytic subunit of a protein complex that transfers oligosaccharides onto asparagine residues.	
GALNT10 (GalNAc polypeptide N-acetylgalactosaminyltransferase 10)	These enzymes catalyze the first step in the synthesis of mucin-type oligosaccharides. The protein encoded by this locus may have increased catalytic activity toward glycosylated peptides compared to activity toward non-glycosylated peptides.	
ASAP1	This gene encodes an ADP-ribosylation factor (ARF) GTPase-activating protein. The GTPase-activating activity is stimulated by phosphatidylinositol 4,5-bisphosphate (PIP2). Gene involved in regulation of membrane trafficking and cytoskeleton remodelling.	Involved in cytoskeleton remodelling (Randazzo et al. 2000)

Gene name (down-regulated)	Function	Selection criteria
CPSF2 (Cleavage And Polyadenylation Specific Factor 2)	Protein coding gene involved in mRNA splicing and RNA binding.	
ABHD2	This gene encodes a protein containing an alpha/beta hydrolase fold, which is a catalytic domain found in a very wide range of enzymes. The function of this protein has not been determined.	
RCN1 (Reticulocalbin 1)	Calcium-binding protein located in the lumen of the endoplasmic reticulum (ER). In human endothelial and prostate cancer cell lines this protein localises to the plasma membrane.	
FERMT2 (Fermitin Family Member 2)	FERMT2 encodes Kindlin-2 protein involved in integrin activation, Erk signalling and adhesion.	Binds actin and certain integrins (Montanez et al. 2008)
HTATSF1	The protein encoded by this gene functions as a cofactor for the stimulation of transcriptional elongation by HIV-1 Tat, which binds to the HIV-1 promoter through Tat-TAR interaction. This protein may also serve as a dual-function factor to couple transcription and splicing and to facilitate their reciprocal activation.	
MAPK6	The protein encoded by this gene is a member of the Ser/Thr protein kinase family, and is most closely related to mitogen-activated protein kinases (MAP kinases). MAP kinases also known as extracellular signal-regulated kinases (ERKs), are activated through protein phosphorylation cascades and act as integration points for multiple biochemical signals.	Extracellular signalling related kinase 3, known to regulate cancer cell morphology and motility (Al-Mahdi et al. 2015)

Gene name (down-regulated)	Function	Selection criteria
KCTD20 (Potassium Channel Tetramerization Domain Containing 20)	Protein Coding gene that promotes the phosphorylation of AKT family members.	
XBP1	This gene encodes a transcription factor that regulates MHC class II genes. It may increase expression of viral proteins by acting as the DNA binding partner of a viral trans-activator.	
VCL (Vinculin)	Vinculin is a cytoskeletal protein associated with cell-cell and cell-matrix junctions, where it is thought to function as one of several interacting proteins involved in anchoring F-actin to the membrane.	Cytoskeletal protein involved in cell-cell junctions (Mierke 2009)
ZEB1	This gene encodes a zinc finger transcription factor. The encoded protein plays a role in transcriptional repression.	Known links with CtBPs and actin cytoskeleton remodelling (Ahn et al. 2012)
TXNRD2	This gene encodes a member of the class I pyridine nucleotide-disulfide oxidoreductase family. Which plays a key role in regulating the cellular redox environment.	
NCOA2	Encodes nuclear receptor coactivator 2, which aids in the function of nuclear hormone receptors.	
DSC3 (Desmocollin-3)	The protein encoded by this gene is a calcium-dependent glycoprotein that is a member of the Desmocollin subfamily of the cadherin superfamily. These Desmosomal family members, along with the Desmogleins, are found primarily in epithelial cells where they constitute the adhesive proteins of the Desmosome cell-cell junction and are required for cell adhesion and Desmosome formation.	Known protein involved in cell adhesion and cell junctions (Schmidt & Koch 2007)

Gene name (down-regulated)	Function	Selection criteria
CDC25A (Cell division cycle 25A)	CDC25A is required for progression from G1 to the S phase of the cell cycle. It activates the cyclin-dependent kinase CDC2. CDC25A is specifically degraded in response to DNA damage, which prevents cells with chromosomal abnormalities from progressing through cell division.	
HMG2 (High Mobility Group AT-Hook 2)	HMG proteins function as architectural factors and are essential components of the enhancosome. This protein contains structural DNA-binding domains and may act as a transcriptional regulating factor.	
CUL5 (Cullin 5)	CUL5 (Cullin 5) is a Protein Coding gene involved in protein hetero-dimerisation activity and ubiquitin protein ligase binding.	
SHCBP1	Protein Coding gene linked SH2 domain binding.	
TMED5 (Transmembrane P24 Trafficking Protein 5)	Protein Coding gene involved in signalling by GPCR and signalling by Wnt.	
PHACTR2 (Phosphatase And Actin Regulator 2)	Protein Coding gene relating to actin binding and protein phosphatase inhibitor activity.	Regulates cell migration through integrin and cofilin binding (Zhang et al. 2012)
IPMK (Inositol Polyphosphate Multikinase)	This gene encodes a member of the inositol phosphokinase family. This gene may play a role in nuclear mRNA export.	

Gene name (down-regulated)	Function	Selection criteria
RASSF8 (Ras Association (RalGDS/AF-6) Domain Family (N-Terminal) Member 8)	This gene encodes a member of the Ras-association domain family (RASSF) of tumour suppressor proteins. This gene is essential for maintaining adherens junction function in epithelial cells and has a role in epithelial cell migration.	Role in adherens junction maintenance and cell migration (Lock et al. 2010)
TFPI (Tissue Factor Pathway Inhibitor)	This gene encodes a protease inhibitor that regulates the tissue factor (TF)-dependent pathway of blood coagulation.	
KAT2B	The protein encoded by this gene associates with p300/CBP and competes with E1A for binding sites in p300/CBP. It has histone acetyl transferase activity with core histones and nucleosome core particles, indicating that this protein plays a direct role in transcriptional regulation.	
LSM11 (U7 Small Nuclear RNA Associated)	Component of the U7 snRNP complex that is involved in the histone 3-end pre-mRNA processing. Required for cell cycle progression from G1 to S phases.	
ESR1 (Estrogen Receptor 1)	This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription.	
CROT	This gene encodes a member of the carnitine/choline acetyltransferase family which plays a role in lipid metabolism and fatty acid beta-oxidation.	
RAB8A (Member RAS Oncogene Family)	The protein encoded by this gene is a member of the RAS superfamily which are small GTP/GDP-binding proteins.	GTP/GDP binding and a regulator of cell shape (Peränen 2011)



Table 7.2 Top 26 up-regulated genes as a result of Eps15 knockdown

All 26 most up-regulated genes were briefly researched and genes which fit our selection criteria were chosen to be validated by quantitative RT-PCR analysis. Selection criteria included genes involved in actin remodelling or in regulating signalling, which could affect actin reorganisation.

Gene name (up-regulated)	Function	Criteria
BMPER (Bone Morphogenetic Protein-Binding Endothelial Cell )	This gene encodes a secreted protein that interacts with, and inhibits bone morphogenetic protein (BMP) function.	
RASD2	The product of this gene binds to GTP and possesses intrinsic GTPase activity.	GTPase activity (Vargiu et al. 2004)
GDF11 (Growth Differentiation Factor 11)	The protein encoded by this gene is a member of the bone morphogenetic protein (BMP) family and the TGF- $\beta$ superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues.	
CNNM4 (Cyclin And CBS Domain Divalent Metal Cation Transport Mediator 4)	This gene encodes a member of the ancient conserved domain containing protein family. Members of this protein family contain a cyclin box motif and have structural similarity to the cyclins.	
TMEM41A	Transmembrane protein.	
GDAP1	This gene encodes a member of the ganglioside-induced differentiation-associated protein family, which may play a role in a signal transduction pathway during neuronal development.	
SLC41A1	Solute Carrier Family 41 (Magnesium Transporter), Member 1 involved in cation transmembrane transportation.	

Gene name (up-regulated)	Function	Criteria
MED28	Merlin and Grb2-Interacting Cytoskeletal Protein involved in actin binding. Part of a complex containing NF2/merlin that participates in cellular signalling to the actin cytoskeleton downstream of tyrosine kinase signalling pathways.	Actin cytoskeleton signalling (Wiederhold et al. 2004)
RAB3D	Member of the RAS Oncogene Family involved in GTP binding and GTPase binding. Probably involved in regulated exocytosis.	GTPase activity and regulator of intracellular vesicle transport (Millar et al. 2002)
Nudix (Nucleoside Diphosphate Linked Moiety X)-Type Motif 15)	Can degrade 8-oxo-dGTP in vitro, suggesting that it may remove an oxidatively damaged form of guanine from DNA and the nucleotide pool, thereby preventing mis-incorporation into DNA, preventing A:T to C:G transversions.	
ELOVL7 (ELOVL Fatty Acid Elongase 7)	Protein involved in transferase activity. May participate to the production of saturated and polyunsaturated VLCFAs of different chain lengths that are involved in multiple biological processes as precursors of membrane lipids and lipid mediators.	
EMP2 (Epithelial Membrane Protein 2)	The encoded protein regulates cell membrane composition. It has been associated with various functions including endocytosis, cell signalling, cell proliferation, cell migration, cell adhesion, cell death, cholesterol homeostasis, urinary albumin excretion, and embryo implantation. It is known to negatively regulate caveolin-1, a scaffolding protein which is the main component of the caveolae plasma membrane invaginations found in most cell types.	Involved in the regulation of cell membrane composition (Wadehra et al. 2004)

Gene name (up-regulated)	Function	Criteria
TMEM48 (Transmembrane Nucleoporin)	Protein Coding gene for a component of the nuclear pore complex (NPC), which plays a key role in <i>de novo</i> assembly and insertion of NPC in the nuclear envelope. Required for NPC and nuclear envelope assembly, possibly by forming a link between the nuclear envelope membrane and soluble nucleoporins, thereby anchoring the NPC in the membrane.	
PAQR3	Progestin And AdipoQ Receptor Family Member III functions as a spatial regulator of RAF1 kinase by sequestering it to the Golgi.	
ATP6AP1 (ATPase, H <sup>+</sup> Transporting, Lysosomal Accessory Protein 1)	This gene encodes a component of a multi-subunit enzyme that mediates acidification of eukaryotic intracellular organelles necessary for intracellular processes such as protein sorting and receptor-mediated endocytosis.	Involved in regulating receptor degradation in lysosomes (Hsin et al. 2012)
SS18L1 (Synovial Sarcoma Translocation Gene On Chromosome 18-Like 1)	Transcriptional activator which is required for calcium-dependent dendritic growth and branching in cortical neurons.	
LBH (Limb Bud And Heart Development)	Transcriptional activator which may act in mitogen-activated protein kinase signalling pathway.	
TFCP2L1	Transcription Factor CP2-Like 1 protein involved in regulating transcription factor activity, sequence-specific DNA binding and transcription co-repressor activity.	
CELSR2 (Cadherin, EGF LAG Seven-Pass G-Type Receptor 2)	The protein encoded by this gene is a member of the flamingo subfamily, part of the cadherin superfamily, located at the plasma membrane.	Cadherin protein involved in cell adhesion (Halbleib & Nelson 2006)

Gene name (up-regulated)	Function	Criteria
RHOBTB2	Rho-Related BTB Domain Containing 2 encodes a small Rho GTPase and a candidate tumour suppressor.	
HNRNPUL1	Represses basic transcription driven by several virus and cellular promoters.	
POM121	This gene encodes a transmembrane protein that localises to the inner nuclear membrane and forms a core component of the nuclear pore complex, which mediates transport to and from the nucleus.	
HECA (Hdc Homolog, Cell Cycle Regulator)	This gene encodes the homolog of the <i>Drosophila</i> headcase protein, a highly basic, cytoplasmic protein that regulates the re-entry of imaginal cells into the mitotic cycle during adult morphogenesis.	
RASD1	This gene encodes a member of the Ras superfamily of small GTPases.	GTPase that may suppress cell growth (Vaidyanathan et al. 2004)
TMEM125	TMEM125 (Transmembrane Protein 125) is a protein coding gene.	
HIPK3	Serine/threonine-protein kinase involved in transcription regulation, apoptosis and steroidogenic gene expression. Phosphorylates JUN and RUNX2. Seems to negatively regulate apoptosis by promoting FADD phosphorylation.	

16 genes (9 down-regulated and 7 up-regulated) were selected for validation by RT-PCR including actin cytoskeleton regulators; FERMT2, ASAP1, cell-cell junction protein; Desmocollin-3 (DSC3) and the cytoskeletal protein; Vinculin (VCL) (Figure 7.4). Validation by RT-PCR was performed on samples from the same isolated RNA used for RNA sequencing (Figure 7.4A-B). In order to compare how CtBP1 regulates these genes paired samples of RNA isolated from SCC25 cells treated with control siRNA or CtBP1 siRNA were also prepared and analysed by RT-PCR (Figure 7.4C-D). Primer sequences from Roche's Universal Probe Library Assay design centre were ordered from Eurofins and used for validation. Each primer correlated to a specific fluorophore within the Roche Universal Probe library. It is important to point out that while these *unvalidated* primers allow us to quantify gene changes between samples, further investigation using *validated* Taqman<sup>®</sup> primers would be a more reliable technique, especially for results following CtBP1 knockdown for which this technique is our only source of information.

Differential regulation of all 9 of the chosen down-regulated genes and 7 up-regulated genes were confirmed by RT-PCR (Figure 7.4A and B). All 7 genes, which were significantly up-regulated in Eps15 knockdown cells were also significantly up-regulated in cells treated with CtBP1 siRNA (Figure 7.4B and Figure 7.4D). However, of the 9 genes down-regulated following Eps15 RNAi, only one (MAPK6) was significantly down-regulated in cells treated with CtBP1 siRNA (Figure 7.4A and Figure 7.4C). None of the other 8 genes showed significant difference in expression in CtBP1 knockdown compared with control treated cells except Desmocollin-3 (DSC3). However, while Eps15 significantly down-regulated DSC3 (Figure 7.4A), CtBP1 knockdown resulted in a significant up-regulation of the same gene (Figure 7.4C). Although these results are preliminary they suggest that the differences we see in terms of cell adhesion between CtBP1 siRNA and Eps15 siRNA treated cells could be a result of differential gene expression patterns between Eps15 and CtBP1 knockdown cells.

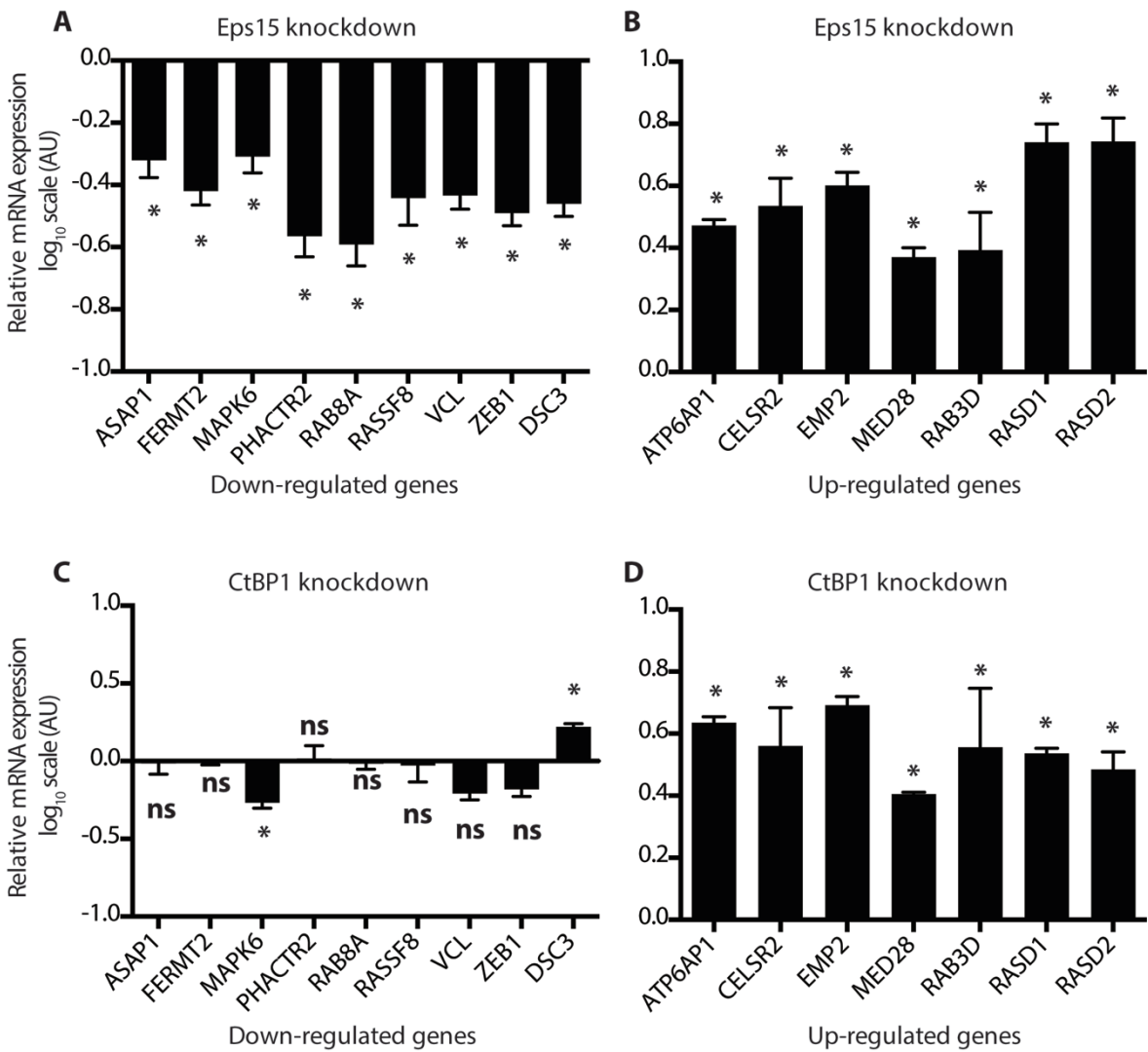
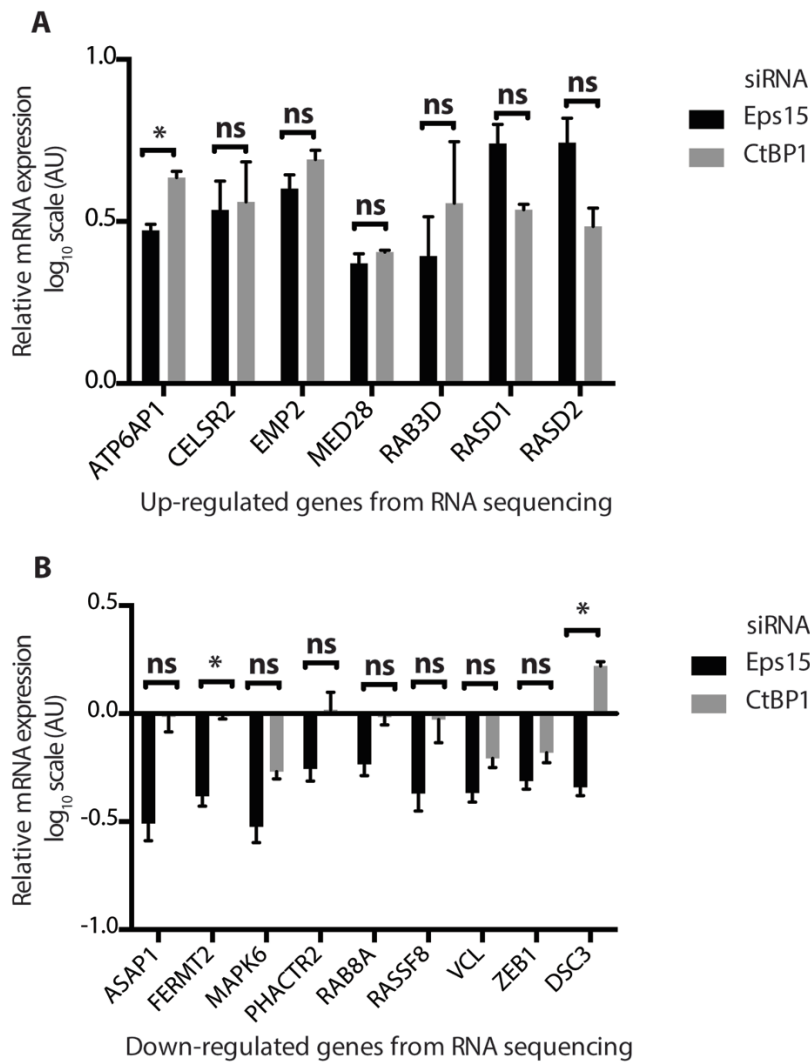


Figure 7.4 RT-PCR validation of genes differentially expressed in Eps15 and CtBP1 knockdown cells compared with control cells

A Roche Universal Probe library was used to validate 16 genes differentially expressed in SCC25 cells treated with Eps15 siRNA found by RNAseq techniques. Validation by RT-PCR was performed on samples from the same isolated RNA that were sent for RNA sequencing. Bars represent mean and error bars indicate standard error of the mean (SEM) of 3 independent transfections (n=3). Graphs show relative mRNA expression of each of the genes of interest. Data is normalised to ubiquitin and to 1 for mRNA expression of control siRNA treated cells. Data is shown on log<sub>10</sub> scale to make control value 0 to better represent mRNA changes as a consequence of Eps15 or CtBP1 siRNA treatment (AU = arbitrary unit). Significance was calculated by multiple unpaired t-tests (A) RT-PCR validation of 9 down-regulated genes following Eps15 knockdown identified by RNAseq. All genes are significantly down-regulated in Eps15 knockdown cells compared to control cells (p<0.05, n=3) (B) RT-PCR validation of 7 up-regulated genes following Eps15 knockdown identified by RNAseq. All 7 genes are significantly up-regulated in Eps15 knockdown cells compared to control cells (p<0.05, n=3) (C) RT-PCR validation of 9 RNAseq down-regulated genes following CtBP1 knockdown. With the exception of MAPK6 and DSC3, no changes in mRNA expression were significant compared to control cells. MAPK6 is significantly down-regulated (p<0.05, n=3), while DSC3 is significantly up-regulated in CtBP1 knockdown cells compared with control siRNA treated cells (p<0.05, n=3) (D) RT-PCR validation of 7 RNAseq up-regulated genes following CtBP1 knockdown. All 7 genes are significantly up-regulated in CtBP1 knockdown cells compared to control cells (p<0.05, n=3).



**Figure 7.5 Combined effect of Eps15 and CtBP1 knockdown on chosen target genes**  
 Graphs compare the effect of Eps15 and CtBP1 knockdown on each of the chosen genes, shown individually in Figure 7.4. Bars represent mean and error bars indicate standard error of the mean (SEM) of 3 independent transfections (n=3). Graphs show relative mRNA expression of each of the genes of interest. Data is normalised to ubiquitin and to 1 for mRNA expression of control siRNA treated cells. Data is shown on log<sub>10</sub> scale to make control value 0 to better represent mRNA changes as a consequence of Eps15 or CtBP1 siRNA treatment (AU = arbitrary unit). Statistical significance is indicative of gene changes as a result of CtBP1 knockdown compared to Eps15 knockdown and were calculated by un-paired t-tests (A) 7 genes, which were up-regulated in RNAseq as a consequence of Eps15 knockdown. Both CtBP1 knockdown and Eps15 knockdown increase expression of the chosen genes significantly and to similar levels. Only expression of the gene ATP6AP1 is significantly different between Eps15 knockdown cells and CtBP1 knockdown cells but both show increased gene expression (p<0.05, n=3) (B) 9 genes, which were down-regulated in RNAseq as a consequence of Eps15 knockdown. Eps15 knockdown significantly decreased expression of the chosen genes while CtBP1 shows minimal change in many of the genes from control cell expression. mRNA expression is not significantly different between CtBP1 and Eps15 knockdown cells except with FERMT2 and DSC3. The change in FERMT2 expression as a consequence of CtBP1 and Eps15 knockdown is significantly different from each other (p<0.05, n=3), showing that Eps15 knockdown decreases expression of FERMT2 from its expression in control cells significantly more than CtBP1 decreases expression of the same gene. DSC3 expression is down-regulated in Eps15 knockdown cells and up-regulated in CtBP1 knockdown cells compared with expression in control cells and this difference is significant between the two treatments (p<0.05, n=3).

To confirm that similar changes also occur on the protein level, expression of key targets (Kindlin-2 (FERMT2) and Vinculin (VCL)) were also examined by Western blotting (Figure 7.6-Figure 7.7). Both Kindlin-2 and Vinculin are focal adhesion proteins (Bandyopadhyay et al. 2012; Carisey & Ballestrem 2011) able to interact with Talin-1, which we have previously shown to be down-regulated by CtBP1 (Chrzan, 2014). Furthermore, although their expression was not significantly changed at the mRNA level, we also examined expression of Talin-1 and Kindlin-1. Talin-1 was examined because we have previously shown that it is a downstream target of CtBP1, while Kindlin-1 was investigated due to its close homology with Kindlin-2 and because it is known to regulate integrin activation in cooperation with Talin-1 (Bandyopadhyay et al. 2012). Figure 7.6A-B shows the effect of Eps15 siRNA on protein expression of Kindlin-1, -2, Talin-1, Vinculin and CtBP1. Densitometric analysis confirmed that, compared with protein expression in control cells, Eps15 knockdown resulted in a non-significant increase in the expression of adaptor protein Talin-1 and a significant decrease in both Vinculin and Kindlin-2 protein expression. No significant change was observed in the expression of Kindlin-1 and we once again confirmed that Eps15 siRNA has no significant effect on CtBP1 protein expression. Decrease in the expression of Kindlin-2 and Vinculin correspond to the decrease seen using RNAseq and RT-PCR, and suggest that expression of these proteins is transcriptionally regulated by Eps15.

Figure 7.7A-B shows changes in the expression of the same proteins in cells treated with CtBP1 siRNA. Compared with protein expression in control cells, CtBP1 knockdown resulted in a significant increase in the protein expression of adaptor protein Talin-1, which confirms our previous results (Chrzan, 2014). Furthermore, CtBP1 knockdown significantly increased expression of Kindlin-2, while no change was detected in Kindlin-1 and Vinculin protein expression. Despite no significant change in FERMT2 at the RNA level, a prominent increase is seen at the protein level suggesting that it is the result of a post-transcriptional regulation. The increase in Kindlin-2 protein expression is the opposite of the inhibitory effect seen following Eps15 knockdown (Figure 7.6B) and suggests that Eps15 and CtBP1 regulate this focal adhesion protein differently and such differences could help us understand how Eps15 and CtBP1 knockdown both decrease cell motility but have opposing effects on cell adhesion.



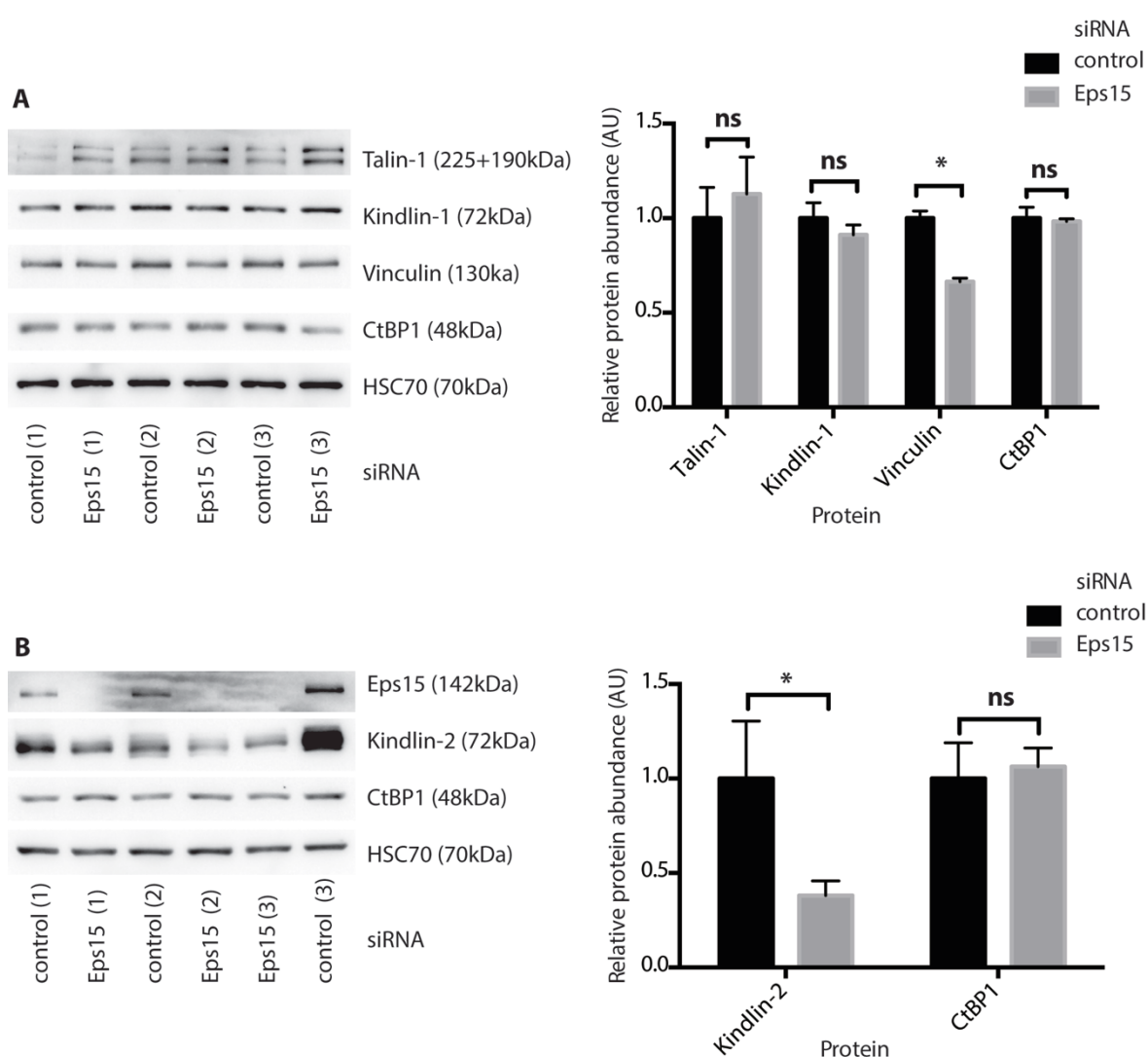


Figure 7.6 Eps15 knockdown effect on protein expression

Western blotting was used to investigate the effect of Eps15 knockdown on protein expression of certain focal adhesion genes in three matched pair samples of SCC25 cells treated with control siRNA or Eps15 siRNA. HSC70 was used as a loading control. Densitometric analysis was carried out to quantify each protein independently. Data were adjusted for HSC70 and then normalised to control lysate protein abundance (AU = arbitrary unit). Bar graphs represent the mean protein abundance relative to controls from the three independent experiments with error bars representing standard error of the mean (SEM) ( $n=3$ ). Statistical analysis was carried out using multiple t-tests (A) 20 $\mu$ g protein was loaded from cell lysates to assess Talin-1, Kindlin-1, Vinculin and CtBP1 protein expression. Densitometric analysis shows a significant decrease in Vinculin protein abundance in cell lysates treated with Eps15 siRNA compared to control cell lysates, all other protein changes are not significant (Talin-1;  $p=ns$ , Kindlin-1,  $p=ns$ , Vinculin;  $p<0.05$ , CtBP1;  $p=ns$ ) (B) 40 $\mu$ g protein from the same lysates as in (A) was loaded from cell lysates to assess Eps15 and Kindlin-2 expression levels. CtBP1 protein expression was also examined in both gels as a control for a protein whose expression is not changed with Eps15 knockdown. Eps15 siRNA effectively reduced Eps15 protein expression in all three matched pairs but densitometry could not be carried out due to the high exposure of the blot. Densitometric analysis confirmed that cells treated with Eps15 siRNA had significantly less Kindlin-2 protein expression compared with control cells ( $p<0.05$ ) and that Eps15 siRNA treatment has no significant effect on CtBP1 protein expression ( $p=ns$ ).

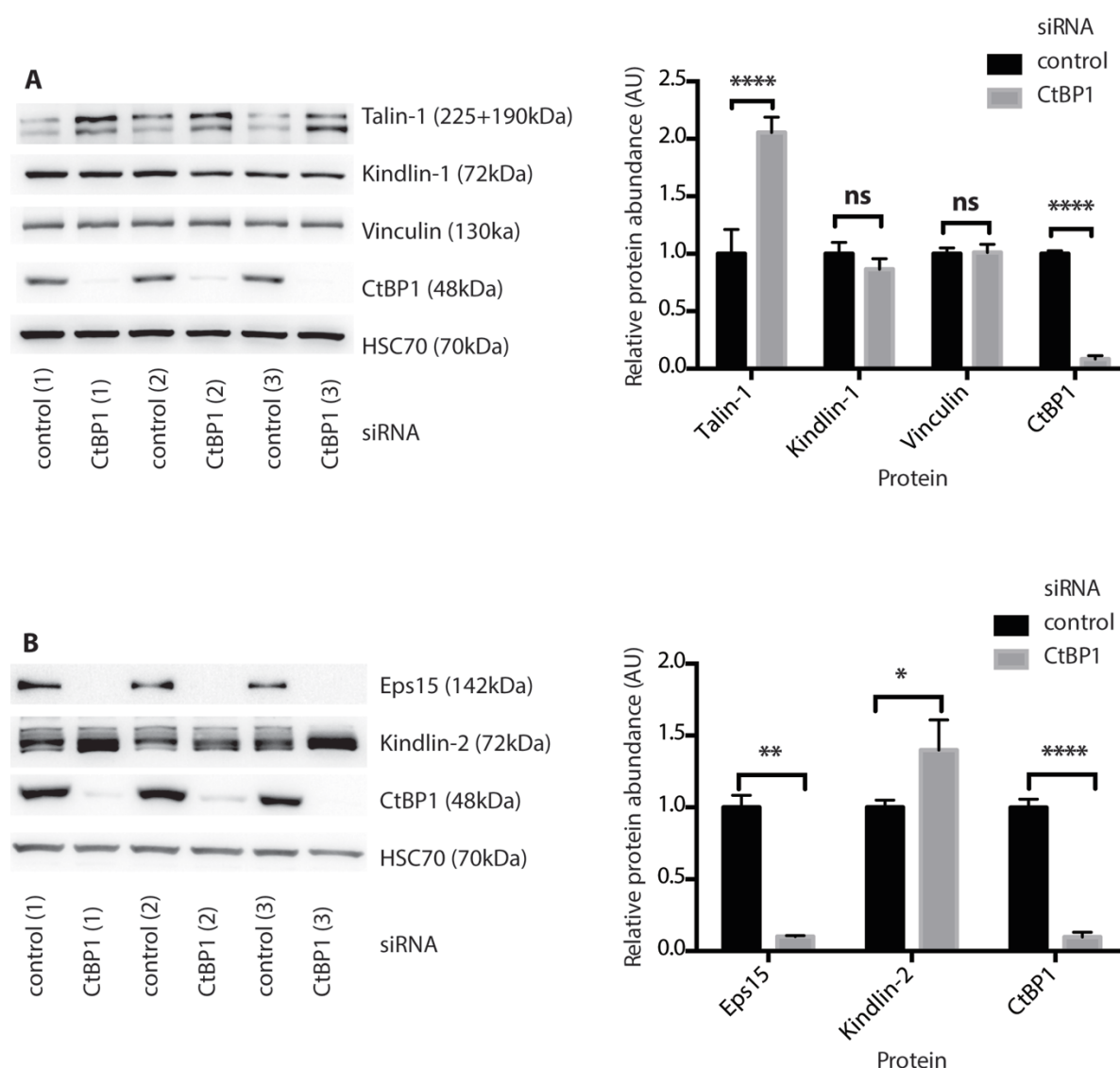
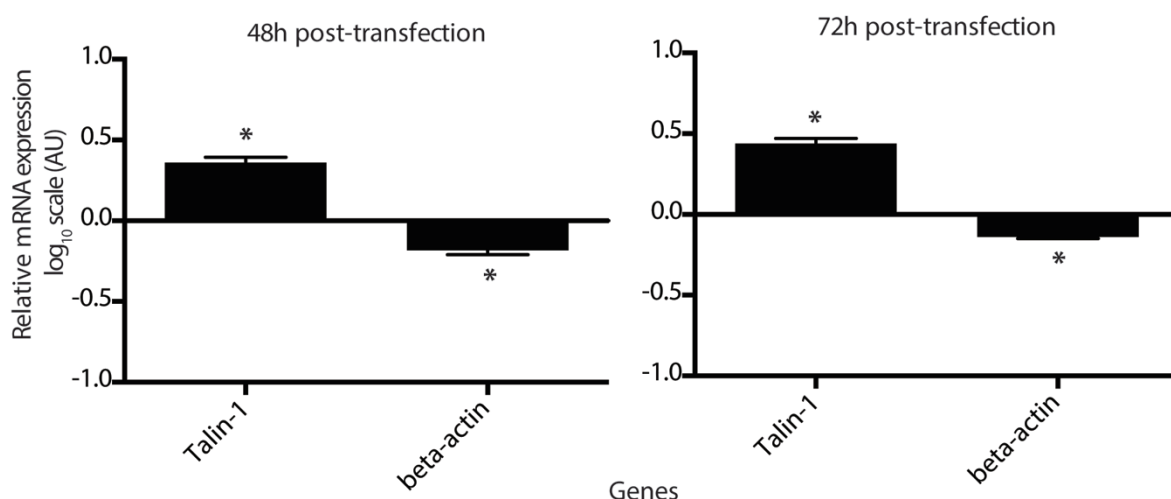


Figure 7.7 CtBP1 knockdown effect on protein expression

Western blotting techniques were used to investigate the effect of CtBP1 knockdown on protein expression of three matched pair samples of SCC25 cells treated with control siRNA or CtBP1 siRNA. HSC70 was used as a loading control. Densitometric analysis was carried out to quantify each protein independently. Data were adjusted for HSC70 and then normalised to control lysate protein abundance (AU = arbitrary unit). Bar graphs represent the mean protein abundance relative to controls from the three independent experiments with error bars representing standard error of the mean (SEM) ( $n=3$ ). Statistical analysis was carried out using multiple t-tests (A) 20 $\mu$ g protein was loaded from cell lysates to assess Talin-1, Kindlin-1 and Vinculin protein expression. Densitometric analysis confirmed that cells treated with CtBP1 siRNA have significantly increased Talin-1 protein expression ( $p<0.0001$ ), significantly decreased CtBP1 protein expression, as expected ( $p<0.0001$ ) but no significant difference in Kindlin-1 or Vinculin protein expression compared with control cell protein expression (B) 40 $\mu$ g protein was loaded from the same cell lysates to assess Eps15 and Kindlin-2 protein expression. CtBP1 protein expression was also examined in both gels as a control for a protein whose expression is changed with CtBP1 knockdown. Eps15 expression was examined to check that CtBP1 knockdown is reducing Eps15 expression as previously seen. CtBP1 knockdown was effective in all three matched pairs. Compared to control cells, CtBP1 knockdown cells had significantly less Eps15 and CtBP1 protein expression (Eps15;  $p<0.01$ , CtBP1;  $p<0.0001$ ) and significantly more Kindlin-2 protein expression ( $p<0.05$ ).

## 7.2 Eps15 knockdown affects gene expression of adhesion proteins

Previous data in our group showed that CtBP1 knockdown increased Talin-1 levels significantly. We have just shown that Eps15 knockdown also causes an increase in Talin-1 protein expression (Figure 7.6A). Previously we have shown that Eps15 knockdown affected beta-actin expression consequently causing us to use ubiquitin as our housekeeping gene in RT-PCR analysis (Figure 3.10). While beta-actin did not come up on our RNAseq analysis the result was consistent using Taqman<sup>®</sup> PCR and could be of interest in light of the effects we have seen of Eps15 knockdown on actin regulation. We also investigated the effect of Eps15 knockdown on Talin-1 at the RNA level (Figure 7.8) These results, in addition to the decrease in Kindlin-2 protein expression seen with Eps15 knockdown, show that Eps15 knockdown can affect the expression of adhesion proteins which could consequently be causing the inhibitory effect on adhesion.



**Figure 7.8 Eps15 knockdown affects gene expression of adhesion proteins**  
VB6 cells were transfected with control siRNA or Eps15 siRNA and collected 48 hours and 72-hours post-transfection. Following RNA extraction and cDNA synthesis using a High Capacity cDNA Reverse Transcriptase Kit, TaqMan<sup>®</sup> RT-PCR was carried out to quantify Talin-1 and beta-actin expression in Eps15 siRNA treated cells in comparison to control siRNA treated cells. Data is normalised to ubiquitin and to 1 for control mRNA expression. Data is shown as log<sub>10</sub> scale to make control values 0 to better represent changes in Talin-1 and beta-actin mRNA expression as a consequence of Eps15 knockdown. Graphs show mean and standard error of the mean (SEM) and significance was calculated by unpaired t-test. Eps15 siRNA treatment significantly increased Talin-1 mRNA expression ( $p < 0.05$ ) and significantly decreased beta-actin mRNA expression ( $p < 0.05$ ) at 48-hours post-transfection and still at 72-hours post-transfection.

## 7.3 Discussion

We have observed that Eps15 plays a crucial role in cell motility and knockdown of Eps15 by RNAi causes a significant decrease in cell adhesion as well as cell spreading. These observations suggest that Eps15 could be involved in actin reorganisation however, very little is known about the role

of Eps15 in cell signalling, apart from its role in endocytosis (van Bergen En Henegouwen 2009; Carbone et al. 1997; Salcini et al. 1999). Therefore, in order to establish a possible mechanism between Eps15 knockdown and actin cytoskeleton regulation we performed an RNAseq analysis to test for genes whose expression is altered by Eps15 knockdown (Figure 7.1-Figure 7.5).

Validation of 16 of the top 60 differentially expressed genes was carried out using Roche Universal probe library and RT-PCR techniques. 9 down-regulated and 7 up-regulated genes were chosen for validation. The genes were chosen if they fit our initial criteria; involvement in actin cytoskeleton remodelling, involvement in cell-cell junctions, ATPase or GTPase activity. Currently, there is no published information available on the interaction between any of these genes and Eps15, however these genes have been shown to regulate actin reorganisation, cell spreading, adhesion and/or motility and could therefore either individually or most probably in combination they could contribute to the phenotype observed in cancer cells following down-regulation of Eps15. The repertoire of possible potential explanations and interactions between Eps15 and these proteins is endless, therefore in the following paragraphs I am going to give examples of how some of these genes could contribute to the effect of Eps15 knockdown on cell adhesion, spreading and motility.

### **Kindlin-2 and Talin-1**

Kindlin-2 (FERMT2) was one of the most significantly down-regulated genes following Eps15 knockdown with a fold change of -1.5 compared with control samples. Pathway analysis using IPA showed FERMT2 is involved in a network of genes regulating cellular function and maintenance and is discussed in the literature as a regulator of integrin signalling; both inside-out and outside-in (Montanez et al. 2008; Bledzka et al. 2016). Kindlins are a family of proteins recruited to integrin-containing adhesion sites (focal adhesions; FAs) and consists of three members; Kindlin-1 (expressed in epithelial cells), Kindlin-2 (ubiquitously expressed) and Kindlin-3 (expressed in haematopoietic cells) (Montanez et al. 2008). Kindlins all contain a FERM domain, which the integrin adaptor protein Talin-1 also contains. The FERM domain of Talin-1 has the highest homology to that of the Kindlin FERM domain of all other FERM containing proteins (Bledzka et al. 2016). It has been found that both Kindlin and Talin-1 must bind to the  $\beta$ -cytoplasmic tail for optimal integrin activation *in vivo* (Plow et al. 2014) but one study has shown that it is Kindlin-2, which is required for Talin-1-induced integrin activation (Montanez et al. 2008). Montanez *et al* used mice embryonic stem cells (ESCs) and embryoid bodies (EBs) lacking Kindlin-2 expression and found that Kindlin-2-integrin interaction enhanced Talin-1-mediated integrin activation via a mechanism which did not alter Talin-1 levels (Montanez et al. 2008). The authors performed pull-down assays and found that Kindlin-2 and Talin bound distinct sites on  $\beta 1$  and  $\beta 3$  integrin tails but

Talin levels were not decreased in Kindlin-2<sup>-/-</sup> ESCs (Montanez et al. 2008). Montanez *et al* therefore show that, the loss of Kindlin-2 severely impairs activation of  $\beta 1$  and  $\beta 3$  integrins (Montanez et al. 2008), and in doing so perhaps the loss of Kindlin-2 abrogates Talin-mediated integrin activation, regardless of Talin-1 expression. In our own data, we observed that Talin-1 levels increase following Eps15 knockdown, but adhesion still decreases. Perhaps the additional loss of Kindlin-2 following Eps15 knockdown impairs Talin-mediated integrin activation so that even though an increase in Talin-1 expression is seen it cannot activate integrins in order to increase cell adhesion. Our increase in Talin-1 levels could be a result of Eps15 knockdown on expression of other genes and not affected by just Kindlin-2 inhibition, as Montanez *et al* saw no effect on Talin protein expression in their Kindlin-2<sup>-/-</sup> ESCs. Furthermore, Kindlin-2<sup>-/-</sup> EBs experienced abrogated adhesion and spreading to fibronectin (FN) even when cells were also treated with manganese chloride (MnCl<sub>2</sub>) to activate integrins. Kindlin-2<sup>-/-</sup> cells were only able to develop a few FA-like structures and were still incapable of spreading. These data as well as those which show that Kindlin-2 enhances Talin-1-mediated integrin activation suggest that Kindlin-2 is required for both integrin inside-out and outside-in signalling (Montanez et al. 2008). Our data mimics that seen in this study by Montanez *et al*. We found that adherent cells treated with Eps15 siRNA showed reduced spreading compared to control cells, despite increased Talin-1 expression and no change in cell surface integrin expression.

A recent study by Bledzka showed similar decreased spreading as well as disorganised actin cytoskeleton in murine aortic endothelial (MAE) cells from Kindlin-2<sup>+/-</sup> mice as well as C2C12 cells treated with Kindlin-2 siRNA, which corresponds to our results. The authors noted marked disorganisation of actin filaments and a significant decrease in cell area of Kindlin-2<sup>+/-</sup> MAE cells on vitronectin compared with control cells (Bledzka et al. 2016). The authors also found that this decrease in adhesion on vitronectin was not caused by a decrease in expression of the primary vitronectin receptor  $\alpha v\beta 3$  as reported levels of  $\beta 3$  by flow cytometry were found to be similar between MAE Kindlin-2<sup>+/-</sup> cells and control MAE cells. Similarly, we also found unchanged levels of  $\beta 1$  and  $\beta 6$  cell surface integrin levels following Eps15 knockdown compared with control cells (Figure 5.1 – Figure 5.4). These results demonstrate the importance of Kindlin-2 in mediating integrin responses regardless of the level of integrins present on the cell surface and could explain how Eps15 knockdown results in a reduction of cell spreading of our cells.

Bledzka *et al* also discuss similar actin disorganisation in cells following Kindlin-2 siRNA treatment to what we have observed in cells following Eps15 siRNA treatment. C2C12 cells treated with Kindlin-2 siRNA had distinct actin organisation; actin was located only at the cell periphery and actin stress fibres were absent. Cell area, measured after 1 hour on fibronectin was also significantly reduced in Kindlin-2 knockdown cells compared with control cells. The similar

phenotype of Kindlin-2 knockdown cells to Eps15 knockdown cells could suggest that Eps15 knockdown is affecting cell spreading through a loss of Kindlin-2. However, the effect of Kindlin-2 knockdown on cell spreading is much quicker than that seen with Eps15 knockdown. This could either be a result of the different cell types or techniques used (different cell types display different adhesion and spreading kinetics, which partly depends on the ECM used); or could be because Eps15 knockdown not only affects Kindlin-2 knockdown but other adhesion proteins such as Talin-1, leading to a slower effect on cell spreading than that of cells which experience direct Kindlin-2 knockdown.

Although these data correlate with our data in SCC25 cell spreading utilising the integrin  $\alpha v\beta 6$ , Bandyopadhyay *et al* showed that Kindlin-2 does not bind the cytoplasmic domain of  $\beta 6$  in keratinocytes (Bandyopadhyay *et al.* 2012). The authors found that integrin- $\beta 1$ -null keratinocytes could adhere to fibronectin through the integrin  $\beta 6$  but that the cells experienced defective cell spreading (Bandyopadhyay *et al.* 2012), which could not explain our results showing reduced cell spreading on the  $\alpha v\beta 6$  ligand, LAP. However, Kindlin-2 has also been shown to affect integrin outside-in signalling (not just inside-out) through its ability to bind actin directly (Bledzka *et al.* 2016). Bledzka *et al* discovered an actin binding site within the F0 domain of Kindlin-2 at LK<sup>47</sup> residue and showed that mutations of this site reduced actin-binding activity of F0 domain of Kindlin-2 using co-immunoprecipitation. The authors also conclude that the spreading defect is not secondary to an activation defect seen in Kindlin-2 knockdown C1C12 cells and it was not overcome when cells were treated with  $MnCl_2$  suggesting that the actin binding-site of Kindlin-2 is capable of influencing integrin outside-in signalling independent of its role in integrin activation (Bledzka *et al.* 2016). Therefore, the ability of Kindlin-2 to regulate integrin outside-in signalling could potentially explain how the  $\alpha v\beta 6$ -mediated spreading of our SCC25 cells could be affected by Eps15 knockdown.

Furthermore, a paper by Theodosiou has discussed that although Talin-1 and Kindlin cooperatively activate integrins leading to cell ECM binding and adhesion, Kindlin is required for the assembly of a subsequent signalling node in a Talin-1-independent manner which is required for cell spreading. The authors report that fibroblasts lacking either Talin or Kindlin-2 failed to activate  $\beta 1$  integrins, adhere to fibronectin (FN) or maintain their integrins in a high affinity conformation induced by manganese treatment ( $Mn^{2+}$ ). The authors discovered that despite compromised integrin activation and adhesion,  $Mn^{2+}$  enabled Talin-1 deficient cells were able to initiate cell spreading following adhesion but that spreading could not be rescued in Kindlin-2 deficient cells. They went on to investigate how Kindlin-2 was able to induce spreading and discovered that cell spreading was induced by the ability of Kindlin-2 to directly bind Paxillin, which in turn bound focal adhesion kinase (FAK) resulting in FAK activation and the formation of

lamellipodia (Theodosiou et al. 2016). Pull down experiments concluded that binding of Kindlin-2 to Paxillin was direct. They found that the PH domain of Kindlin-2 directly binds the LIM3 domain of Paxillin and recruits Paxillin into small adhesions present on the protruding membrane (nascent adhesions, NAs) but not to mature FAs. The Kindlin-2-Paxillin complex was then shown to bind, cluster and activate FAK in NAs, which lead to the recruitment of p130Cas, Crk (another known binding partner of Eps15 (Table 1.1)) and Dock followed by the activation of Rac1 and the induction of cell spreading. The authors then investigated FAK and found that by over-expressing FAK they could increase lamellipodia formation and increase cell spreading in both Talin-1 and Kindlin-2 knockdown cells. They conclude that the Kindlin-2-Paxillin complex in NAs is required to recruit and activate FAK which in turn is necessary to induce cell spreading (Theodosiou et al. 2016). Therefore, it may be possible that when Kindlin-2 levels reduce upon Eps15 knockdown this could have an effect on cell spreading and adhesion.

The differences that we see comparing cell adhesion with CtBP1 knockdown (increased adhesion) and Eps15 knockdown (decreased adhesion) may also be a consequence of loss of Kindlin-2. Our Western blots (Figure 7.6-Figure 7.7) show that while Kindlin-2 levels *decrease* as a result of Eps15 knockdown, they *increase* as a result of CtBP1 knockdown. Although previously we showed increased expression of Talin-1 was responsible for increased integrin activation, adhesion and reduced motility following CtBP1 down-regulation, based on previously described effects of Kindlins in integrin activation and adhesion (Theodosiou et al. 2016) one could hypothesise that this increased expression of Kindlin-2 could also contribute to the phenotype of CtBP1 knockdown cells. Further investigations using Kindlin-2 siRNA would be needed to confirm such a relationship. Additionally, we found that Eps15 knockdown results in an increase of Talin-1 expression at the RNA and protein level but that this was not sufficient to rescue the reduced adhesion seen as a consequence of Eps15 knockdown. Perhaps Kindlin-2 is required for effective cell adhesion even with high Talin-1 expression and that without Kindlin-2 cells are unable to adhere effectively.

### **Rab8a**

GTPases are essential components of cell signalling to orchestrate the interplay between the plasma membrane and the actin cytoskeleton and the expression of a few were shown to be significantly changed in our RNA sequencing data following Eps15 knockdown. The expression of GTPase Rab8a, a known regulator of cell shape, was significantly down-regulated in Eps15 knockdown cells (Peränen 2011). Endogenous Rab8 is found in dynamic cell structures such as filopodia and lamellipodia among others (Peränen 2011). It regulates a membrane recycling pathway that is linked to Eps15-homology domain containing protein 1 (EHD1) and inhibition of its expression causes similar cell disruption to that seen following Eps15 inhibition (Peränen

2011). Rab8 depletion from cells reduces the production of cell protrusions (extended cell surface domains with a leading edge containing filopodia and lamellipodia) and decreases cell invasiveness; both characteristics of Eps15 knockdown cells (Peränen 2011). These characteristics suggest that Eps15 could be interacting with Rab8 within the cell to produce effective cell protrusions however, their relationship, if any, is a complex one as inhibition of Rab8 also promotes cell-cell adhesion and increases the appearance of actin stress fibres (Peränen 2011), both opposite characteristics to that seen in Eps15 knockdown cells.

### **ASAP1**

The expression of the GTPase activating protein (GAP) ASAP1, a known regulator of the actin cytoskeleton (Randazzo et al. 2000), was also significantly decreased in our Eps15 knockdown cells. ASAP1 localises to focal adhesions and cycles with focal adhesion proteins including Arf1 and the tyrosine kinase Src, when cells are stimulated to move. ASAP1 also contains a C-terminal SH3-domain that binds to the focal adhesion tyrosine kinase FAK, which has already been discussed in relation with Kindlin-2 as important in the regulation of cell spreading (Theodosiou et al. 2016). Regulation of actin cytoskeleton by ASAP1 is complex; over-expression of ASAP1 alters the morphology of focal adhesions and blocks both cell spreading and formation of dorsal ruffles induced by platelet-derived growth factor (PDGF) while a mutated form of ASAP1 with disrupted GAP activity, also reduces cell spreading but increases the number of cells forming dorsal ruffles in response to PDGF. Additionally, the PH domain of ASAP1 binds phosphatidylinositol 4,5-bisphosphate (PtdInsP2) which is known to regulate the cytoskeletal remodelling that occurs during cell spreading (Randazzo et al. 2000). Although the relationship of ASAP1 with the cell cytoskeleton is a complex one, it is clear that changes in its expression, such as those initiated by a loss of Eps15, can have drastic impacts on cell morphology. Additionally, ASAP1 is the only protein for which we have found a possible indirect connection with Eps15. POB1; a binding partner of Eps15 (van Bergen En Henegouwen 2009; Nakashima et al. 1999) is also able to interact with ASAP1 (Oshiro et al. 2002). POB1, is a binding protein of RalBP1 (a small GTPase) and although it is not known how these three proteins interact; POB1 could interact with ASAP1 and Eps15 simultaneously or perhaps Eps15 could affect the formation of the complex between POB1 and ASAP1, it could be an interesting link.

### **Vinculin**

Eps15 knockdown has also been shown to regulate other adaptor proteins such as Vinculin, which is part of a complex interaction network within cells. Vinculin is an adaptor protein with binding sites for over 15 proteins. Vinculin is localised to both integrin-mediated cell–matrix adhesions as well as cadherin-mediated cell–cell junctions (Carisey & Ballestrem 2011). Loss of Vinculin causes



phenotypic changes in the cell such as reduced cell adhesion and an increase in cell motility, which is thought to drive the formation of tumour metastasis. Re-expression of Vinculin in these Vinculin diminished cells suppresses their tumorigenic ability and increases cell adhesion strength (Carisey & Ballestrem 2011). It has been found that the interaction of Vinculin with Talin has a key role in regulating FA assembly and that Vinculin is able to hold adhesion receptors in a high affinity state thus promoting focal adhesion growth (Carisey & Ballestrem 2011). It is possible that loss of Eps15 leads to a consequent loss of Vinculin resulting in an initial decrease in cell adhesion but that the increase in cell motility, which can follow Vinculin loss is suppressed by another mechanism, such as a loss of Kindlin-2, as discussed previously. Vinculin is also important for the organisation of cell-cell junctions, a role analysed by Peng *et al* using a short hairpin-RNA (shRNA) based knockdown system, which was able to perturb Vinculin preferentially at sites of cell-cell adhesion (Peng et al. 2010). They found that following Vinculin knockdown, cadherin-dependent adhesion was reduced resulting from impaired E-cadherin cell-surface expression (Peng et al. 2010). They found that impaired E-cadherin expression could be rescued by re-introducing Vinculin but not with a Vinculin mutant defective for binding  $\beta$ -catenin. Further analysis revealed that  $\beta$ -catenin and Vinculin interaction is crucial for stabilising E-cadherin at the cell surface (Peng et al. 2010). Our initial, preliminary observations in wound-healing assays suggested that cell-cell interactions appeared inhibited following Eps15 knockdown, therefore the loss of Vinculin could potentially contribute to such a phenotype.

### **RASSF8**

RASSF8 is another protein known to be involved in stabilising cell-cell adhesion (Lock et al. 2010) which was significantly decreased in Eps15 knockdown cells. RASSF8 was found to co-localise with the adherens junction (AJ) of cell-cell contacts and RASSF8 siRNA resulted in AJ destabilisation. Furthermore, RASSF8 could be required for actin-cytoskeletal organisation as RASSF8 depletion also causes actin-cytoskeleton disorganisation (Lock et al. 2010).

### **ZEB1 and Desmocollin-3**

The down-regulation of ZEB1 as a result of Eps15 knockdown also provides an interesting discussion on the complex nature of cell motility. ZEB1 is a transcriptional repressor involved in driving epithelial to mesenchymal transition (EMT), which is thought to drive tumour cell metastasis in some cases and increased ZEB1 levels correlate with poor prognosis in a variety of epithelial tumour types (Browne et al. 2010). EMT leads to a loss of polarised features, detachment from neighbouring cells, increased motility and invasion into the surrounding matrix (Ahn et al. 2012) and has also been shown to promote tumour cell dedifferentiation by repressing master regulators of epithelial polarity such as the cell polarity genes Crumbs and HUGL2 (Aigner

et al. 2007). ZEB1 is able to induce EMT by down-regulating the expression of epithelial genes including E-cadherin, which is also known to be regulated by CtBPs (Shi et al. 2003; Grooteclaes & Frisch 2000). ZEB1 regulates E-cadherin in both a CtBP1-dependent (Grooteclaes & Frisch 2000) and independent (Sánchez-Tilló et al. 2010) manner and is thought to repress E-cadherin expression by recruitment of histone deacetylases (HDACs) (Aghdassi et al. 2012). Loss of E-cadherin-mediated adhesion characterises the transition from benign lesions to invasive, metastatic cancer (Pećina-Slaus 2003).

ZEB1 can also drive metastasis at the post-transcriptional level by regulating microRNAs (miRs) (Ahn et al. 2012). The tumour-suppressive miR-34a, is negatively regulated by ZEB1 driving tumour cell metastasis through actin cytoskeletal remodelling (Ahn et al. 2012). Ahn *et al* performed a microarray on a pancreatic cell line; 393P, which undergoes EMT and gains invasive properties following ectopic ZEB1 expression labelled 393P\_ZEB1 cells and found 46 miRs that were differentially expressed in 393P\_ZEB1 cells; 27 down-regulated and 19 up-regulated (Ahn et al. 2012). miR-200a-c along with miR-34a were among the down-regulated miRs. miR-34a is known for its tumour suppressing activity and Ahn *et al* suggest that this is due to its role in inhibiting pro-migratory cytoskeletal processes and attenuating Rho GTPase activity (Ahn et al. 2012). Ahn *et al* found that cells which exogenously expressed miR-34a abrogated TGF- $\beta$ -induced formation of invasive cellular protrusions. They formed no filopodia, generated more focal adhesions per surface area and exhibited increased cell cross-sectional area (Ahn et al. 2012). Such phenotypic changes involve regulation of the actin cytoskeleton and Ahn *et al* found that miR-34a attenuated cytokine-induced Rho GTPase activation, known as master regulators of the cytoskeleton (de Curtis & Meldolesi 2012) Once again these data highlight the complex regulation involved in processes regulating cell motility. In relation to our own data, Eps15 knockdown causes a decrease in ZEB1 gene expression. Given the above research we would hypothesise that a decrease in ZEB1 should release its inhibition on miR-34a. Expression of miR-34a is anti-invasive and results in cells which cannot produce filopodia; both characteristics of Eps15 knockdown cells. However, cells expressing miR-34a also generated more focal adhesions per surface area and increased cell size; both characteristics which oppose those of Eps15 knockdown cells. The loss of Kindlin-2 in Eps15 knockdown cells could once again explain why we don't see increased cell spreading in Eps15 knockdown cells. Eps15 knockdown cells may show increased miR-34a expression as a result of reduced ZEB1 expression leading to reduced filopodia production, but increased cell spreading may not be possible due to loss of Kindlin-2. miR-200c expression was also down-regulated as a consequence of ZEB1 expression. Loss of this inhibition following Eps15 knockdown could result in activation of miR-200c leading to the post-transcriptional regulation of all genes associated with miR-200c, including ASAP1. Antagomir treatment of mir-300c resulted in

significant increase in the GAP ASAP1 (Rebustini et al. 2012) which we know to be involved in actin cytoskeleton remodelling. This observation is interesting as it raises the possibility that down-regulation of ZEB1 following Eps15 knockdown could potentially explain the increased expression of other genes, such as ASAP1.

Another epithelial-specific gene known to be repressed by ZEB1 is Desmocollin-2 (DSC2) (Aigner et al. 2007). Our RNAseq data showed a significant loss of another Desmocollin, Desmocollin-3 (DSC3) (Figure 7.2). Interestingly, Desmocollin-3 was the only validated gene whose expression following CtBP1 knockdown showed the opposite to that following Eps15 knockdown. DSC3 expression was significantly increased following CtBP1 knockdown (Figure 7.5). Desmocollins (Dscs) are transmembrane glycoproteins of a subfamily of cadherins which are thought to establish cell-cell adhesion through heterophilic interactions with each other and other glycoproteins known as Desmogleins (Dsg) at sites known as Desmosomes (Schmidt & Koch 2007). Desmosomes are multi-protein complexes assembled at the plasma membrane, connecting adjacent cells and providing anchoring points for the intermediate filament cytoskeleton. Dsc and Dsg are connected to intermediate filaments through a protein complex consisting of Plakoglobin (Pg), Desmoplakin (Dp) and Plakophilin(s) (Pkp) (Schmidt & Koch 2007). Desmosomes are required to maintain tissue cohesion, especially in organs exposed to considerable mechanical stress, such as the skin (Schmidt & Koch 2007). Studies have shown that alterations in tissue cohesion can have a marked effect on cell rearrangement (David et al. 2014) and this could potentially explain the differences that we have seen on collective cell motility following CtBP1 and Eps15 knockdown. The ability of cells to form cell-cell adhesions allows collective cell motility (Friedl et al. 2004). It is interesting that CtBP1 and Eps15 knockdown cause the opposite effect on collective cell motility as well as Dsc3 expression. Perhaps by causing an increase in Dsc3 CtBP1 knockdown cells become too adhesive to one another. While cell-cell adhesion is required for collective cell motility, movement as a whole still requires cells at the leading edge to orient themselves away from the group. Perhaps if cells are too adherent to one another cells at the leading edge are unable to initiate movement away from the group. Perhaps an increase in Dsc3 would cause cell-cell adhesions, which do not encourage collective cell motility, resulting in the inhibition of collective cell motility seen as a result of CtBP1 knockdown. Inversely, Eps15 knockdown results in a decrease in Dsc3 expression, which might reduce the strength of cell-cell adhesions just enough to allow cells at the leading edge to begin moving away from the group. If Dsc3 expression is just reduced and not abolished then cells will still adhere to one another, albeit not as effectively as control cells, but still allow collective cell motility, just as we have seen with our Eps15 knockdown cells. Cell-cell adhesions appeared broken in patches of the confluent monolayer produced by

Eps15 knockdown cells, compared with the monolayer produced by control cells, yet cells were still able to move as a collective layer and close the wound.

### **EMP2**

Not all genes of interest were down-regulated as a consequence of Eps15 knockdown. Epithelial membrane protein-2 (EMP2) gene expression was significantly up-regulated by Eps15 down-regulation. EMP2 has shown a role in regulation of caveolin expression (Wadehra et al. 2004). The authors investigated recombinant over-expression of EMP2 in NIH-3T3 cells and found that it decreased caveolin-1 and caveolin-2 protein levels while increasing the surface expression of glycosylphosphatidyl inositol-anchored proteins GPI-APs (lacking in caveolae lipid rafts). The authors specifically cleaved the EMP2 transcript and found reduced surface GPI-APs and increased caveolin protein expression (Wadehra et al. 2004). These findings suggest that EMP2 facilitates the formation and surface trafficking of lipid rafts bearing GPI-APs, and reduces caveolin expression, resulting in impaired formation of caveolae. While the authors did not investigate the importance of reduced caveolin expression on caveolin-dependent endocytosis or discuss how this could implicate receptor translocation, it is interesting that Eps15 knockdown regulates a protein involved in another form of endocytosis and raises the possibility that Eps15 is not exclusively involved in clathrin-mediated endocytosis.

It is important to point out that while RNA sequencing provides information on the genes that are affected by Eps15 knockdown it gives no information on other processes, for example, protein-protein interactions, which could also explain the effect of Eps15 knockdown on cell functions. While the majority of the literature focuses on these binding partners as explanation for the role of Eps15 in endocytosis (Benmerah et al. 1999; Cai et al. 2013; Carbone et al. 1997) recent publications highlighted other roles of these proteins in the regulation of actin reorganisation and a number of these binding proteins are discussed in Chapter 6 including Epsin1, STAM proteins, Intersectin (ITSN1) and Crk. Interestingly, both ITSN1 and Crk gene expression were also shown to be marginally decreased in our RNAseq data (ITSN1; fold change <-1, Crk; fold change <-0.5). The fact that the expression levels of these proteins was only marginally affected by Eps15 does not exclude the possibility that they play a role in regulating the Eps15 knockdown phenotype. We cannot exclude for example that Intersectins and Crk need to be in complex with Eps15 to achieve their function; therefore down-regulation of Eps15 could reduce the formation of such complexes and inhibit the function of these proteins.

To elucidate an exact mechanism of how Eps15 is affecting cell motility would require further investigation, but we uncovered a novel effect of Eps15 on a number of proteins already known to regulate cell motility. We would hypothesise that Eps15 is able to regulate cell motility by

regulating the actin cytoskeleton and many of the proteins discussed show complex but often direct interactions with the actin cytoskeleton and its regulation. Further work would involve the production of Eps15 mutants to narrow down specific sites of Eps15, which cause the characteristics previously observed. This would allow us to narrow down the specific binding sites and potential protein complexes, which could be important for regulation of cell functions. Investigations attempting to rescue the phenotype of Eps15 knockdown cells would also be key to determine which genes, when re-expressed, rescue cell phenotype to that of control cells most effectively. Due to the capabilities of Eps15 as a scaffold protein elucidating an exact mechanism may not be simple. It is possible that while Eps15 is most certainly involved in actin cytoskeleton organisation that this effect is not reliant on just one mechanism but the accumulation of a few proteins regulating many downstream processes.

## 7.4 Summary

1. Eps15 knockdown causes significant differential expression of over 1200 genes ( $p < 0.001$ )
2. Eps15 knockdown causes the down-regulation and up-regulation of genes involved in actin cytoskeleton remodelling
3. Certain previously known Eps15 binding partners, which have previously only been known as endocytosis regulators now have been described in the regulation of actin remodelling
4. Eps15 has a novel role in the regulation of actin remodelling



## Chapter 8: Final discussion and future work

Tumour cell metabolism differs from normal cells. Regardless of available oxygen levels, tumour cells often metabolise glucose by glycolysis in a process known as the Warburg effect (Upadhyay et al. 2013). While the exact benefits this method of metabolism confers to tumour cells is not understood, it is thought to provide an advantage to highly proliferating cells, such as tumour cells. Proliferating cells require a large amount of nucleotides, amino acids and lipids in their biomass ready for mitosis; production of which requires the consumption of more carbon equivalents and nicotinamide adenine dinucleotide (NADH) than of ATP; both of which are produced more rapidly by aerobic glycolysis (Vander Heiden et al. 2009). Changes in the metabolic state of cells is not without consequence and the high levels of NADH produced can cause the activation of metabolic sensors; such as CtBPs, which function as transcriptional regulators (Chinnadurai 2002).

Cancer metastases, the movement of cancer cells from the primary site of development to secondary sites across the body, are responsible for over 90% of cancer patient deaths (Spano et al. 2012). The understanding of how and why tumour cells metastasise is therefore extremely important. Regulation of tumour cell motility is complex and relies on regulation of many downstream processes to organise the actin cytoskeleton appropriately for motility. Cell motility relies on a balance between optimal cell adhesion and cell motility (Cox & Huttenlocher 1998). The main receptors of cell adhesion to the extracellular matrix (ECM) are the transmembrane family of proteins; integrins, which consist of an extracellular domain, to bind to the ECM and an intracellular domain, to regulate downstream signalling following cell adhesion (Bridgewater et al. 2012). Binding to the ECM causes integrin activation and vice versa, which can then allow downstream signalling to occur and regulate many cellular processes including cell motility. Cell motility is dependent on expression and localisation of integrins on the cell surface, which is regulated by receptor endocytosis driving the constant internalisation, recycling and re-expression of integrins from the rear of the cell to the leading edge of the cell (Caswell & Norman 2006).

Previous results generated in our group showed that the metabolic sensors, CtBPs, regulate integrin-dependent cell motility in various cancer types and a gene array, found that the endocytic adaptor protein, Eps15, is down-regulated upon knockdown of CtBP1 (Figure 1.10) (Chrzan 2014).

Based on these previous findings, we hypothesised that CtBPs regulate cell motility by up-regulation of Eps15 in cancer cells to allow increased integrin endocytosis and therefore increased cell motility. Our initial aims were as follows:

- To investigate the role of CtBP1 and CtBP2 in regulation of Eps15 expression using CtBP siRNA and Western blotting techniques in a variety of cancer cell lines
- To investigate the role of Eps15 in the regulation of  $\beta 6$  and  $\beta 1$  integrin endocytosis using Eps15 RNA interference and a functional endocytosis assay
- To investigate the role of CtBP1 in the regulation of  $\beta 6$  and  $\beta 1$  integrin endocytosis using RNA interference and a functional endocytosis assay
- To determine the role of Eps15 in integrin-dependent tumour cell motility through functional assays including adhesion, cell spreading, Transwell® migration and Matrigel® invasion along with organotypic models

### 8.1 CtBP1 regulates Eps15 expression

Eps15 is ubiquitously expressed. We have shown that Eps15 expression can be regulated by CtBP1 expression, but independent of CtBP2. Knockdown of CtBP1 was able to reduce Eps15 protein expression substantially as well as Eps15 mRNA. This result is interesting as in the context of Eps15, CtBP is able to act as a transcriptional activator while it is best known as a transcriptional repressor (Chinnadurai 2009). This effect was seen across six cell lines; four oral squamous cell carcinoma (H357, VB6, BICR6 and SCC25), a metastatic colorectal adenocarcinoma (SW620) and a breast cancer cell line (MCF7) suggesting that this is a generic effect in cancer cells (Figure 3.3 – Figure 3.5). These results are novel as little is known about the regulation of Eps15 expression. A limited number of papers discuss the possibility that Eps15 is regulated by certain microRNAs (miRs) and indeed target sites for both miR-23b (Nicholls et al. 2011) and miR-186 (Babenko et al. 2012) have been found in the 3'UTR of Eps15, but little else is known.

We have also found that the decrease in Eps15 expression as a consequence of CtBP1 knockdown occurs relatively quickly as the interaction between CtBP1 and Eps15 is direct, shown by a chromatin immunoprecipitation (ChIP) assay (Figure 3.7). This interaction does not form part of a positive feedback loop as changes of Eps15 expression do not affect levels of CtBP1, neither CtBP1 protein nor CtBP1 mRNA were affected (Figure 3.9 - Figure 3.11).

As CtBP1 is a metabolic sensor we investigated whether Eps15 expression was sensitive to changes in cell metabolism. We subjected cells to varying concentrations of glucose (above 10 mM and below 2 mM) and compared the effect to environmental glucose concentrations (5 mM) but saw no effect either on Eps15 protein (Figure 3.12) or mRNA expression (Figure 3.13) in three cell lines and at several time-points up to 72 hours post-treatment. Changing the glucose concentration is perhaps not the most appropriate method to measure the effects of metabolism due to the number of downstream effects that could occur as a consequence so we also tested



the effect of hypoxia on Eps15 expression in order to determine if the result is protocol dependent. We once again saw no significant effect of hypoxic conditions on Eps15 mRNA expression (Figure 3.14). We concluded that changes in metabolism probably do not affect Eps15 expression, however further investigations would be required to confirm this using other techniques such as using NADH inhibition/NAD<sup>+</sup> activation (Wilkinsons & Williams 1981), regulating the levels of pyruvate in the medium (Huckabee 1958) or by changing the levels of other enzymes in the metabolic pathway such as lactate dehydrogenase (Brooks et al. 1999). Additionally, the effect of other cell stresses, such as radiation or chemotherapy, could provide interesting insight into the regulation of Eps15 expression.

High expression of both CtBP1 (Deng et al. 2013) and Ehd1 (Meng et al. 2015) are associated with poor prognosis in cancer patients and we also found a significant positive correlation between Eps15 and CtBP1 expression in human tissue samples (Figure 3.15). This association is a novel finding, which not only confirms our previous *in vitro* results but could also suggest that both could potentially be good prognostic markers of tumour progression. As the sample number in our cohort was relatively low, investigation into the expression of CtBP1 and Eps15 using a much larger cohort of human tissue samples would be required. Furthermore, to confirm previously published results in other cancer types the correlation between Eps15/CtBP1 expression and various clinicopathological parameters could be investigated. As there is relatively little evidence on the role of Eps15 and CtBP1 in cancer and based on our *in vitro* results correlation between the two protein exists in other tumour cell lines, the investigation could be extended to other tumour types.

## 8.2 Eps15 knockdown decreases tumour cell motility

Previous data in our group had already shown that CtBP1 inhibition reduced tumour cell migration and invasion (Chrzan 2014). We were able to confirm this effect and show that Eps15 knockdown was able to have the same negative effect on tumour cell migration and invasion. Eps15 knockdown reduced cell motility in both in single cell 2D migration and invasion assays (Figure 4.1 and Figure 4.2) as well invasion in more physiologically relevant 3D organotypic assays (Figure 4.4), suggesting that CtBP1 and Eps15 could be affecting cell motility via the same mechanism.

Interestingly, we found that although single cell motility was inhibited by Eps15 knockdown, sheet cell motility, a form of collective cell motility, was not. Cells treated with Eps15 siRNA did not significantly inhibit wound closure compared with control siRNA treated cells (Figure 4.6). Despite the fact that sheet cell motility appeared un-hindered, closer inspection by immunofluorescence

revealed that Golgi polarisation was disrupted in Eps15 knockdown cells (Figure 4.7). A paper by Desai *et al* found similar results in cells transfected with a dominant-negative mutant of E-cadherin (Desai et al. 2009). Desai *et al* found that cells lacking E-cadherin were unable to orient the Golgi network to face the wound but that these cells were still able to migrate directionally to close the wound with a speed comparable to control cells (Desai et al. 2009). These results highlight the complexity of cell migration and suggest that while Eps15 may be required for single cell motility, reliant on cell-ECM interactions, it might be redundant during collective cell motility, which is more reliant on cell-cell adhesions. Future work investigating the role of Eps15 in cell-cell contacts would allow us to confidently conclude the role of Eps15 in different situations involving different cell interactions.

*In vivo*, stable Eps15 knockdown cells produced tumours, which were significantly smaller in size than control cells (Figure 4.11 – Figure 4.13). We had initially begun *in vivo* work with the aim of assessing tumour cell metastasis and the effect of Eps15 knockdown. However, we did not see any metastasis in mice injected with control tumour cells nor mice injected with tumour cells transfected with Eps15 knockdown, so effects on metastasis could not be concluded. The significantly smaller size of tumours produced by cells treated with Eps15 knockdown suggest a role for Eps15 in tumour cell proliferation *in vivo*. We assessed cell proliferation as a consequence of Eps15 knockdown and found that Eps15 did not significantly affect tumour cell proliferation *in vitro*. However, we cannot rule out that Eps15 could have different effects *in vivo* and to test this Ki67 (a proliferation marker) staining of the tumour sections is going to be performed during follow-up investigations. Optimisation of a more appropriate metastatic model, most likely one that involves orthotopic injection would also be better for assessing the role of Eps15 on tumour metastasis and could be optimised in the future.

### 8.3 Eps15 knockdown does not regulate $\beta 1$ or $\beta 6$ integrin endocytosis

The transmembrane integrin family of proteins have been shown to be internalised by clathrin-mediated endocytosis (CME) (Caswell et al. 2009). Eps15 is an endocytic adaptor protein known to function in CME of epidermal growth factor receptors (EGFR) and transferrin (Benmerah et al. 1998) but to our knowledge no-one has previously investigated the requirement of Eps15 in CME of integrins in detail. Eps15 has been revealed as a regulator of integrin activity; as loss of Eps15 by RNAi resulted in decreased  $\beta 1$  activation (Pellinen et al. 2012) and Eps15 has even been shown to interact directly with  $\beta 1$  integrin tail (Humphries et al., 2009; Supplementary Material).

We have shown that Eps15 knockdown does not have a consistent effect on neither  $\beta 1$  nor  $\beta 6$  integrin internalisation or subsequent cell surface levels of these integrins (Figure 5.1 – Figure

5.4). It's possible that  $\beta 1$  integrins are able to alternate between endocytic pathways such as; CME and clathrin-independent endocytosis (CIE) if one pathway becomes perturbed.  $\alpha 5\beta 1$  has been shown to localise to both clathrin-coated structures as well as alternative cholesterol-sensitive caveolar routes (Shi & Sottile 2008).  $\alpha 5\beta 1$  internalisation normally depends on NXXY motifs and clathrin but can be internalised independent of clathrin and NXXY motifs by over-expressing Rab21 (Pellinen et al. 2008). Perhaps knockdown of Eps15 is able to disrupt CME of  $\beta 1$  integrins but they are still able to internalise via a different mechanism if possible, ultimately revealing no effect of Eps15 knockdown on  $\beta 1$  internalisation. To confidently conclude that  $\beta 1$  integrins are switching endocytic routes would require further investigation. We could possibly investigate this by blocking other forms of endocytosis i.e. caveolin siRNA treatment, to see if Eps15 knockdown is able to have an effect on integrin internalisation when other forms of internalisation are inhibited. The only study investigating the endocytosis of  $\beta 6$  integrins showed that they internalise by CME (A. G. Ramsay et al. 2007), however the authors have not studied any other forms of endocytosis, therefore we cannot exclude that other forms of endocytosis could also be involved. Our investigations supported the previous conclusion by showing that clathrin heavy-chain (CHC) knockdown by siRNA consistently reduced  $\beta 6$  integrin endocytosis suggesting it is reliant on CME. It is possible that CME is not always dependent on Eps15, which has been found for other cargo (Pu & Zhang 2008). Infection by mouse hepatitis virus type 2 (MHV-2) was significantly inhibited in cells where the clathrin-mediated pathway was blocked and viral gene expression was significantly inhibited when cells were transfected with CHC siRNA. Over-expression of a dominant-negative mutant of caveolin-1 did not have any effect on MHV-2 infection suggesting internalisation to be reliant on clathrin-mediated endocytosis. Additionally, over-expression of a dominant-negative form of Eps15 also had no effect on viral gene expression or infectivity suggesting MHV-2 entry is mediated through clathrin-dependent but Eps15-independent endocytosis (Pu & Zhang 2008).

CtBP1 knockdown also had no consistent effect on integrin internalisation of neither  $\beta 1$  nor  $\beta 6$  integrin internalisation or consequent cell surface levels (Figure 5.1 – Figure 5.3). CtBP1 has been shown to regulate macropinocytosis (Liberali et al. 2008) and CtBPs have also been implicated in clathrin- and dynamin-independent endocytotic pathways (Bonazzi et al. 2005; Hansen & Nichols 2009), however there is no evidence that they are involved in CME, which is consistent with our findings, if we exclude the possibility that in our cells  $\beta 1$  and  $\beta 6$  integrins internalise excluding this pathway.

Another possibility is that, while Eps15 knockdown might not affect integrin endocytosis, it could affect integrin activation and thereby motility. Pellinen *et al* used a cell spot microarray (CSMA) and found that silencing of *EPS15* resulted in the most frequent  $\beta 1$  integrin inactivation among

the different cell lines that they tested (Pellinen et al. 2012). While this does not give us information on how Eps15 could be affecting integrin internalisation it does show that Eps15 can affect integrin activation which could consequently affect integrin internalisation (Margadant et al. 2011). Little work has been carried out discussing the role of active versus inactive integrins and their subsequent endocytosis but new evidence is emerging, which suggests that integrins in active and inactive conformation have distinct recycling routes (Arjonen et al. 2012). Arjonen *et al* found that both active and inactive conformations of  $\beta 1$  are endocytosed in a clathrin and dynamin-dependent manner but the net endocytosis rate of the active  $\beta 1$  is higher. The authors concluded that inactive  $\beta 1$  integrin undergoes rapid recycling back to the plasma membrane, causing localisation mainly at the plasma membrane when cells are in a steady state, while the active conformation is predominantly intracellular (Arjonen et al. 2012). Future work would investigate the effect of Eps15 knockdown on integrin activation using activation-specific antibodies.

We concluded that perhaps Eps15 is affecting cell motility by a mechanism that is independent of integrin-internalisation. The role of integrins in the regulation of cell motility is entwined with their regulation of cell adhesion, so we investigated the effect of Eps15 knockdown on cell adhesion to begin to better understand the cell processes affected by Eps15 and how they are regulated.

### 8.4 Eps15 knockdown decreases tumour cell adhesion and cell spreading

Previous work in our group showed that CtBP1 knockdown increases ligand-specific cell adhesion thus, if CtBP1 is affecting cell adhesion through a mechanism involving Eps15 then Eps15 knockdown should have the same effect. We investigated the effect of Eps15 knockdown on tumour cell adhesion, however, two different types of adhesion assays conclusively showed that Eps15 knockdown decreased tumour cell adhesion (Figure 6.2 and Figure 6.3). We confirmed again that Eps15 is most likely not affecting cell adhesion by regulating integrin endocytosis because RNAi of both CHC and HAX-1 had the opposite effect and increased cell adhesion. We concluded that CtBP1 and Eps15 probably affect adhesion via different mechanisms because while CtBP1 increased adhesion, Eps15 significantly reduced it.

We observed, using immunofluorescence staining, that Eps15 knockdown also inhibited cell spreading (Figure 6.10 and Figure 6.11), and that this effect is most likely due to disruption of the actin cytoskeleton. These results are novel, as there are no papers showing these effects of Eps15 knockdown. There are, however, several explanations for these effects. While human Eps15 has never been shown to link directly with the actin cytoskeleton, the Eps15-like protein; Pan1p, has

been shown to bind actin in yeast (Tang & Cai 1996), therefore we cannot exclude that it does the same in human cells. Furthermore, there are several binding partners of Eps15, which could explain these results. Eps15 contains multiple binding sites (van Bergen En Henegouwen 2009) and while most are only related to endocytosis, some have wider functions, such as Epsin, Intersectin and Crk. Epsin1 is able to bind directly to F-actin (Messa et al. 2014) and the interaction of Eps15 and Epsin1 has been shown to be crucial in the accumulation of actin for the production and maintenance of actin-rich pedestals during *Escherichia coli* (EPEC) infection (Lin et al. 2011). Intersectin too is an important regulator of the actin cytoskeleton by its ability to function as a guanine nucleotide exchange factor (GEF) that regulates the activation of the Rho family GTPase Cdc42 (Wong et al. 2012). Hussain *et al* found that N-WASP binds directly to Intersectin, up-regulating its GEF activity, generating GTP-bound Cdc42 (GTP-Cdc42), a critical activator of N-WASP, in a positive feedback loop (Hussain et al. 2001). Crk is another binding protein of Eps15 which is also involved in the activation of WASP (Sasahara et al. 2002). Crk is involved in the activation of WASP in activated T-cells by forming a complex with WIP following release of WASP inhibition from a WIP-WASP complex (Schumacher et al. 1995).

In light of the information gathered about these known binding partners of Eps15, it is possible that knockdown of Eps15 could be disrupting cell function by disrupting other protein-Eps15 complexes and consequently higher order protein complexes involved in actin organisation. However, further investigation into the effect of Eps15 knockdown on the regulation of these cell complexes would be required to confirm a mechanism. Future work would involve additional siRNA knockdown of potential binding partners to investigate their effects on cell function, such as cell motility and adhesion and compare the effect of their loss with that of Eps15 on cells.

In order to better understand how Eps15 knockdown can affect cell processes we performed RNA sequencing on SCC25 cells transfected with Eps15 siRNA compared with cells transfected with control siRNA to see what genes are affected by Eps15 knockdown and if any of these could be responsible for the effect on actin organisation seen.

First we isolated the top 60 genes regulated by Eps15; 26 up-regulated genes and 34 down-regulated. We then picked 9 down-regulated (ASAP1, FERMT2, MAPK6, PHACTR2, RAB8A, RASSF8, VCL, ZEB1, DSC3) and 7 up-regulated genes (ATP6AP1, CELSR2, EMP2, MED28, RAB3D, RASD1, RASD2) to validate using RT-PCR with *unvalidated* primers and fluorophores from the Roche Universal Probe library. In addition to validating the effects of Eps15 knockdown we also assessed the effect of CtBP1 knockdown on our chosen genes. It is important to point out that while these *unvalidated* primers allow us to quantify gene changes between samples, further investigation using *validated* Taqman<sup>®</sup> primers would be a more reliable technique, especially for results

following CtBP1 knockdown for which this technique is our only source of information. The effect of Eps15 knockdown on all genes chosen, whether positive or negative, was validated by RT-PCR (Figure 7.4 and Figure 7.5). Interestingly, while all 7 up-regulated genes were also found to be up-regulated following CtBP1 knockdown, the 9 genes down-regulated as a result of Eps15 knockdown were either not significantly affected (ASAP1, FERMT2, MAPK6, PHACTR2, RAB8A, RASSF8, VCL, ZEB1) or significantly up-regulated (DSC3) as a result of CtBP1 knockdown (Figure 7.4 and Figure 7.5). It is therefore possible that the opposing effect of Eps15 or CtBP1 knockdown on cell adhesion lies in the regulation of one or all of these genes, but further investigation using RNAi techniques would be required to confirm which of these genes, if any, is most responsible for the effects on cell motility that occur as a result of Eps15 knockdown.

Perhaps the most likely gene to be responsible for the differences observed on cell adhesion between CtBP1 and Eps15 knockdown is FERMT2; which is the gene name of the protein Kindlin-2. FERMT2 was one of the most significantly down-regulated genes following Eps15 knockdown with a fold change of -1.5 compared with control samples and is discussed in the literature as a regulator of integrin signalling; both inside-out and outside-in (Montanez et al. 2008; Bledzka et al. 2016). Both Kindlin and Talin-1 must bind to the  $\beta$ -cytoplasmic tail for optimal integrin activation *in vivo* (Plow et al. 2014) and it has been suggested that that it is Kindlin-2, which is required for Talin-1-induced integrin activation (Montanez et al. 2008). The loss of Kindlin-2 severely impairs activation of  $\beta$ 1 and  $\beta$ 3 integrins (Montanez et al. 2008), and in doing so perhaps the loss of Kindlin-2 abrogates Talin-1-mediated integrin activation, regardless of Talin-1 expression. In our own data, we observed that Talin-1 levels increased following Eps15 knockdown, but adhesion still decreased. Perhaps the additional loss of Kindlin-2 following Eps15 knockdown impairs Talin-1-mediated integrin activation so that even though an increase in Talin-1 expression is seen it cannot activate integrins in order to increase cell adhesion. Additionally, marked disorganisation of actin filaments and a significant decrease in the cell area of *Kindlin-2*<sup>+/-</sup> cells occurs when plated on vitronectin compared with control cells (Bledzka et al. 2016), similar to the effect on cell spreading seen with our cells treated with Eps15 siRNA. Perhaps most convincing are our observations of CtBP1 and Eps15 knockdown on Kindlin-2 protein expression. Our Western blots show that while Kindlin-2 levels decrease as a result of Eps15 knockdown, they increase as a result of CtBP1 knockdown (Figure 7.6 and Figure 7.7). Given the association between loss of Kindlin-2 and integrin activation and cell adhesion, the opposing effect of Eps15 and CtBP1 on FERMT2 protein expression could explain their opposing effects on cell adhesion. However, further investigations using Kindlin-2 siRNA would be needed to confirm such a relationship.

## 8.5 Summary

In summary, this report discusses some novel findings involving the endocytic adaptor protein; Eps15, including the following;

- CtBP1 regulates the expression of the endocytic adaptor protein Eps15; a generic function across tumour cell lines
- CtBP1 and Eps15 show a significant positive correlation in human tissue sections
- Eps15 knockdown significantly decreases cell migration and invasion, in a number of cell lines, in both 2D and 3D assays *in vitro*
- Eps15 knockdown significantly reduces tumour size *in vivo*
- Eps15 does not regulate  $\beta 1$  or  $\beta 6$  integrin endocytosis
- Eps15 knockdown significantly decreases tumour cell adhesion and spreading
- Eps15 knockdown has a novel role in the regulation of actin remodelling

Future work involving mutant forms of Eps15 would be able to determine which sections of Eps15 are most important in regulating these functions. By systematically removing sections of Eps15 we could elucidate which domains of Eps15's structure is most important for appropriate actin organisation, and whether the effect of Eps15 is direct or indirect and if indirect which binding partners are required most. By fully understanding how tumour cells regulate their actin cytoskeleton we can begin to better understand how tumour cells move and form metastasis, therefore new therapies can be developed. Additionally, the observation that CtBP1 and Eps15 show a significant positive correlation in human tissue sections and that both proteins are associated with poor prognosis suggest that with further investigation they could potentially be developed into new prognostic markers of tumour progression.





## Appendix 1: Chapter 4 Supplementary Figures

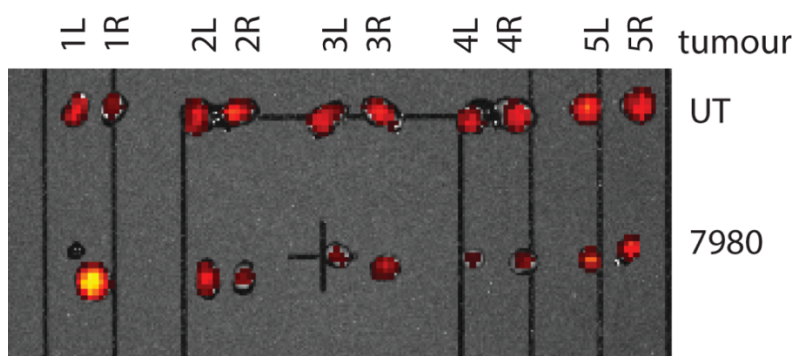


Figure S1.1 A tumour from one of the 7980 mice was excluded from analysis  
 Mouse 1 of the 7980 condition only developed one tumour on the left flank (1L) and this was much larger than any other tumour, even UT tumours produced. This tumour was therefore excluded from analysis.

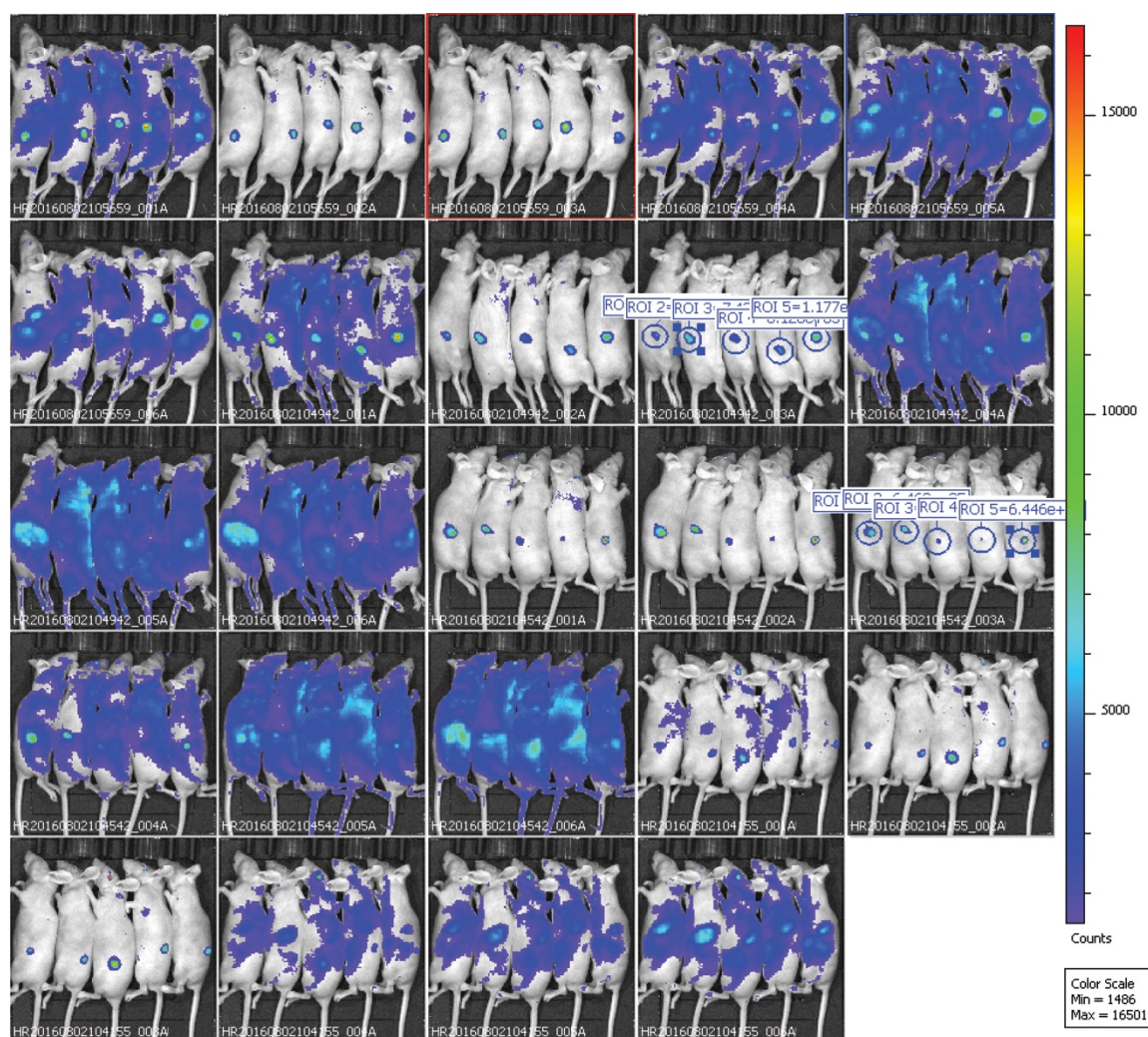


Figure S1.2 Sequential spectrum and mixing  
 Living Image<sup>®</sup> software takes 6 fluorescent pictures across the red spectrum. These images can then be used to remove background fluorescence so that just the tumours can be analysed.

## Appendix 2: Chapter 6 Supplementary Figures

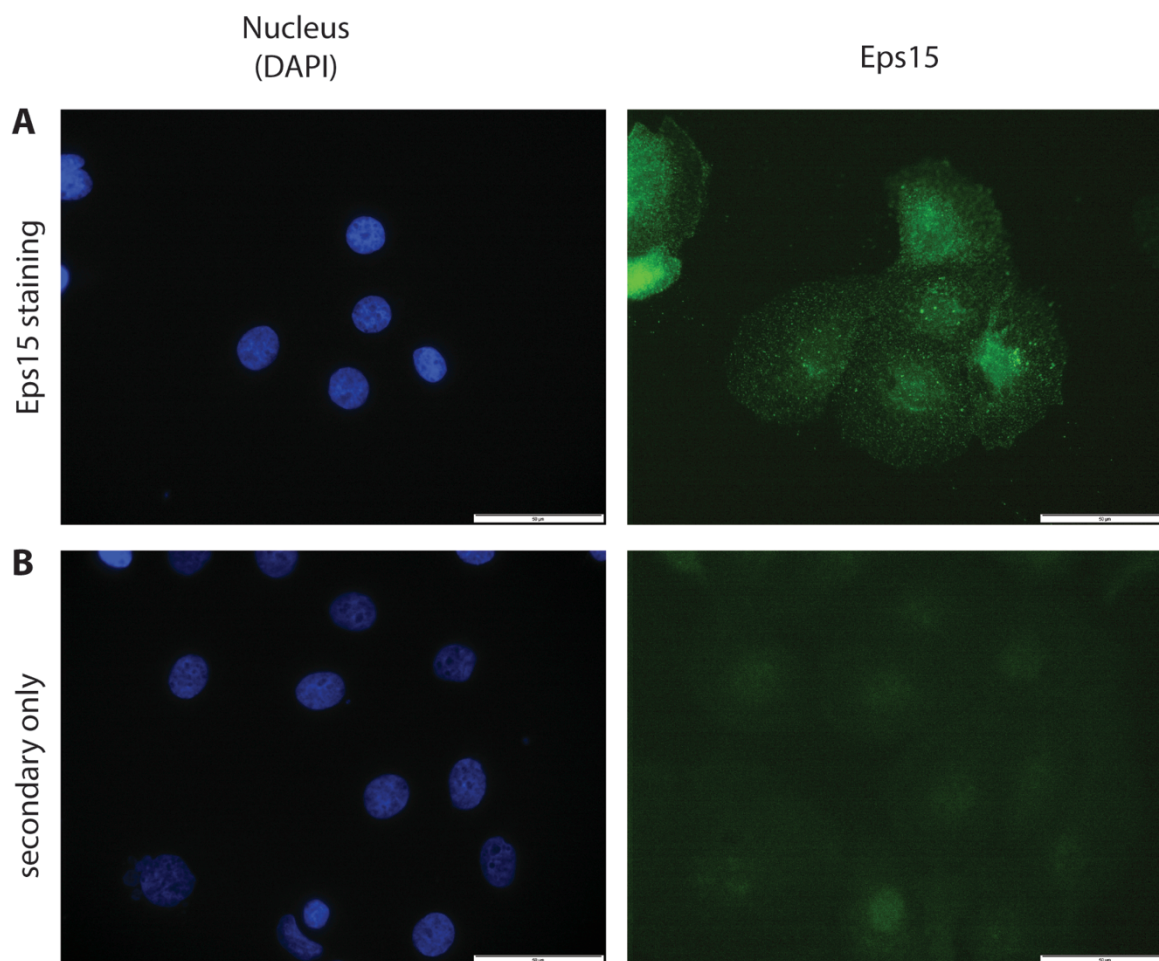
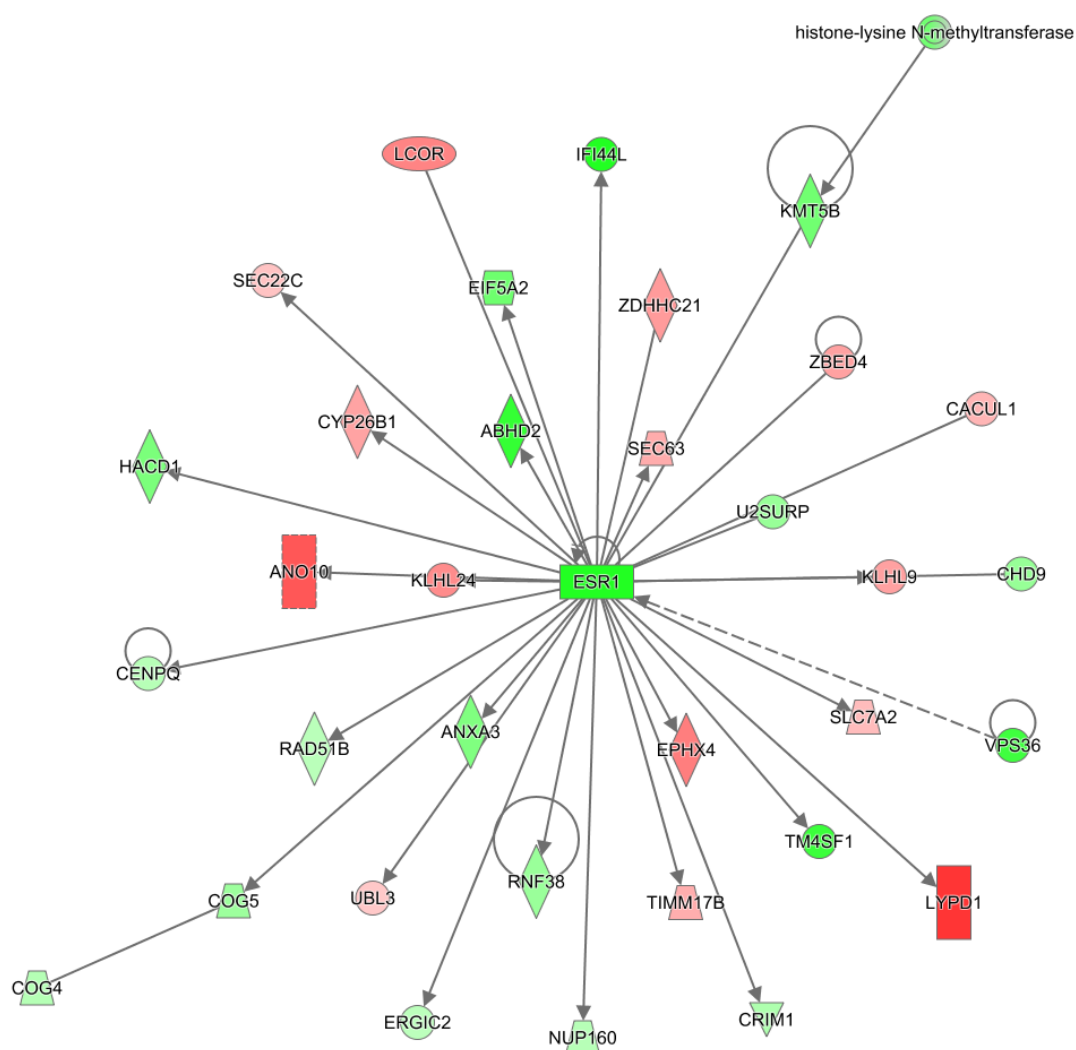


Figure S2.1 Immunofluorescence of Eps15 within VB6 cells

VB6 cells were used to optimise Eps15 immunofluorescence. Cells were stained with Eps15 antibody (#8855) at 1:250 concentration (A) Eps15 staining appears as a dotted staining and is localised throughout the cytoplasm but mostly at the peri-nuclear site (B) secondary only staining shows minor background fluorescence. Scale bar represents 50 $\mu$ m.

## Appendix 3: Chapter 7 Supplementary Figures

Network 1 : RNA seq - adj p<0.001 : RNA seq - adj p<0.001



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Figure S3.1 Network 1 from IPA analysis

Ingenuity Pathway Analysis (IPA) revealed network analysis of gene changes associated with Eps15 knockdown. The network ranked first using IPA is important in regulating developmental disorder, hereditary disorder and metabolic disease. Genes involved in this network are differentially expressed in Eps15 knockdown cells compared to control cells with a significance value of  $p < 0.001$ .



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