UNIVERSITY OF SOUTHAMPTON

MECHANISMS OF TRANSCRIPTIONAL REPRESSION BY THE PROTO-ONCOGENE c-Myc

ABIGAIL LAPHAM

SUBMITTED FOR THE AWARD OF DOCTOR OF PHILOSOPHY

DEVELOPMENTAL AND CELL BIOLOGY

AUGUST 2005

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

DEVELOPMENTAL AND CELL BIOLOGY

Doctor of Philosophy

Mechanisms of transcriptional repression by the proto-oncogene c-Myc

By Abigail Lapham

The Myc family of proto-oncogenes fulfils a pivotal role in controlling cell growth and differentiation. Myc responses are mediated by both stimulatory and inhibitory effects on the transcriptional responses of particular gene sets. Several mechanisms of c-Myc-mediated transcriptional repression have been reported, but repression is best described when c-Myc interacts with the initiator element(Inr)-binding protein Miz-1. c-Myc displaces co-activator proteins from binding Miz-1 and thereby represses transcription. An alternative mechanism involves c-Myc binding Sp1 in a large inactive complex. Mechanisms of c-Myc-mediated transcriptional repression have been examined and compared using the *Nramp1* and NCAM genes. Both genes have TATA box-deficient promoters, with NCAM, encoding a repertoire of adhesion molecules, and incorporates two Inr and one consensus Sp1 binding site. *Nramp1*, encoding a macrophage-restricted divalent cation transporter, has a unique arrangement of a tandem duplication of Inrs and one consensus Sp1 binding site.

Nramp1 is uniquely responsive to Miz-1-mediated transactivation and inhibition by RNAi directed against Miz-1, whereas, both promoters show a dependence on Sp1 for basal transcription. A c-MycV394D mutant that prevents c-Myc-Miz-1 interaction, attenuates c-Myc's ability to repress *Nramp1* transcription. The same mutation is silent with regard to attenuating NCAM repression. These data indicate that *Nramp1* is regulated specifically via Miz-1 and NCAM is subjected to Miz-1-independent regulation. Data using a chromatin immuoprecipitation assay for Miz-1 are supportive of the functional data, as Miz-1 only is bound to the *Nramp1* Inr. Data are supportive of a mechanism by which c-Myc is recruited to the NCAM promoter through a conserved Sp1 site and repression is sensitive to TSA treatment suggesting active repression. *Nramp1* repression is TSA insensitive and repression is likely through a passive mechanism of co-activator displacement. Both mechanisms proposed require intact Myc-Max heterodimers. This thesis identifies model genes for the study of c-Myc transcriptional repression by Miz-1-dependent and -independent pathways and the data highlight the complexity of the biology of Myc in that similar processes i.e. transcriptional repression, are mediated via distinct and gene-specific mechanisms.

ACKNOWLEDGEMENTS

I wish to acknowledge the following people for their help during my PhD.

HOWARD- Thank you for giving me this opportunity, I have loved (almost) every minute of it! I couldn't have done it without all your help and support. KAREN- Queen of the ChIP assay, I am really grateful for all your help and advice throughout my PhD. EMMA- Thank you for putting up with my moaning when things were going wrong and for all you technical help and expertise along the way. ED- Thanks for giving me the confidence and encouragement to do this in the first place and for putting up with me while I was doing it.

PUBLICATIONS

Some of the work presented in this study has been published as listed below.

Lapham AS, Phillips ES, Barton CH. Transcriptional control of *Nramp1*: a paradigm for the repressive action of c-Myc. Biochem Soc Trans. 2004;32(Pt 6):1084-6.

Bowen H, Lapham A, Phillips E, Yeung I, Alter-Koltunoff M, Levi BZ, Perry VH, Mann DA, Barton CH. Characterization of the murine Nramp1 promoter: requirements for transactivation by Miz-1. J Biol Chem. 2003;278(38):36017-26.

CONTENTS

FIGURE CONTENTS

CHAPTER 4

CHAPTER 6

APPENDIX

ABBREVIATIONS

AdML- Adenovirus Major late
ATP- Adenosing Tri-Phosphate
BCG- Bacille Calmette Guerin
bHLHLZ- basic Helix Loop Helix Leucine Zipper
cDNA- Copy Deoxyribose nucleic acid
c-Myc- Cellular Myelomatosis
CTM- Consensus Transport Motif
DCT1- Divalent Cation Transporter 1
DPE- Downstream Promoter Element
dsRNA- Double Stranded RNA
eCFP-Enhanced Cyan Fluorescent Protein
eGFP-Enhanced Green Fluorescent protein
EM- Electron microscopy
FCCP- Carbonyl Cyanide 4-(trifluoromethoxy)phenylhydrazone
GAS- Growth Arrest Supression
GADD- Growth Arrest and DNA Damage
HAT- Histone Acetyl Transferase
HDAC- Histone De-Acetylase
HFE- Hereditary hemochromatosis protein
HLH- Helix loop Helix
IFN- Interferon
Inr- Initiator
IgG- Immunoglobulin G
IL3- Interleukin 3
iNos- Inducible Nitric Oxide Synthase
Inr- Initiator
IRE- Iron Response Element
IREG-1- Iron Regulatory Protien 1
IRP1/2- Iron Response Protein ½
ISG- Interferon Stimulated Genes
Ity- Immunity to Typhimurium
Jak- Janus Kinases
Kb- Kilobase
KDa- Kilodalton
LA- Lipofectamine
LPS- Lipopolysaccaride
Max- Myc Associated factor x
MB1/2- Myc Box 1/2
MH1/2/3-Mad Homology Domain 1/2/3
Miz-1-Myc Interacting Zinc finger protein 1
MxiI-Max interactor 1
NCAM- Neural Cell Adhesion Molecule

NF-IL6- Neutrophil- interleukin6
NLS- Nuclear Localisation Signal
Nramp1-Natural resistance associated macrophage protein 1
Nramp-rs –Natural resistance associated protein –related species
ORF- Open Reading Frame
PCR- Polymerase Chain Reaction
PKC- Protein Kinase C
POZ- Poxvirus and Zinc finger
PTGS- Post- Transcriptional Gene Silencing
RdRp- RNA dependent RNA polymerase
RISC- RNA Induced Silencing Complex
RNAi- RNA interference
SH3- Src Homology 3
shRNA- Short Hairpin RNA
SID-Sin3 Interacting domain
siRNA- Small Interfering RNA
Slc11a1- Solute carrier family 11 member a1
Sp1- Stimulatory Protein 1
Stat- Signal Transducers and Activators of Transcription
TAD- Transactivation domain
TfR- Transferrin Receptor
TGF-β- Transforming Growth Factor Beta
TMD- Transmembrane domain
TPEN- N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine
TSA- Trichostatin A
TRRAP- transformation/transcription domain-associated protein
Y2H- Yeast two Hybrid
YAC- Yeast Artificial Chromosome
YY1-Yin Yang1

CHAPTER 1

Introduction

1 Introduction

Myc was originally defined as an oncogene (*v-Myc)* transduced by the Avian Myelocytosis Virus that was capable of inducing neoplastic disease (Hayward *et al,* 1982). Following this discovery, the cellular homologue of *v-Myc* was identified, termed c-*Myc*, and found to be a member of a family of proto-oncogenes consisting of *c-Myc*, *N-Myc* and *L-Myc*. Alterations in the structure and expression of these genes has been linked to a wide variety of human and animal cancers, with changes in the expression levels of c-Myc accounting for one in seven cancer deaths (Dang & Lee, 1995). c-Myc is amplified in lung carcinoma (Little *et al*, 1983), Breast carcinoma (Mariani-costnantini *et al*, 1998) and colon carcinoma (Augenlicht *et al*, 1997) to give just a few examples

1.1 Structure of the c-Myc transcription factor

The Myc transcription factor has two major domains. The first of these is the C-terminal bHLHLZ domain and consists of approximately 90 amino acids. This domain is required for dimerization with Max (as described later in this section) and sequence specific DNA binding (Blackwood & Eisenman, 1991). DNA binding is essential for Myc function as deletion or disruption of this domain destroys all biological activity (Stone *et al,* 1997).

The second domain consists of the remaining three quarters of Myc, the N-terminal region and the central region. The N-terminal region is needed for transactivation of transcription (Kato *et al,* 1990), and contains two Myc boxes termed MBI and MBII. DNA sequences of these boxes are highly conserved and unique to Myc family members (Grandori *et al*, 2000). The central region also contains an NLS as well as the recently re-discovered MBIII (Herbst *et al*, 2005), a highly conserved region involved in Myc mediated repression, originally identified by Atchley and Fitch (1995) 10 years prior to this and termed box C.

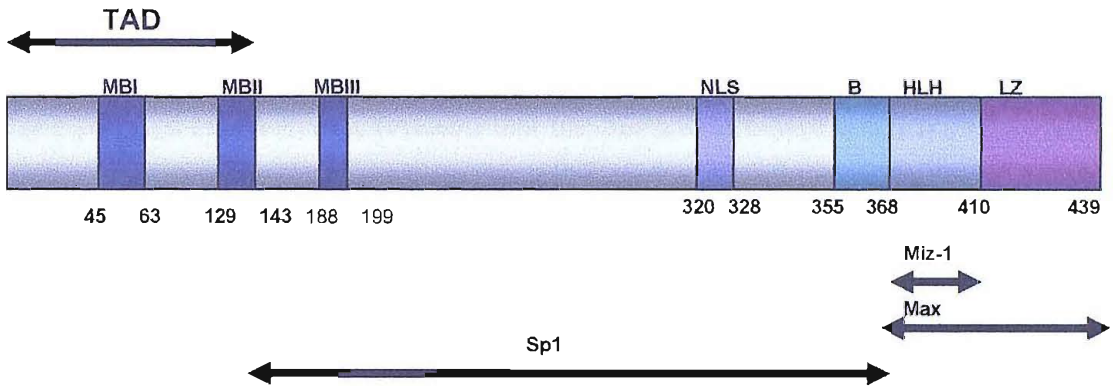


Figure 1.1 Schematic diagram of c-Myc

N-terminal region: containing a transactivation domain that stimulates transcription. Also present are two Myc boxes termed MBI and MBII. These regions are highly conserved and unique to Myc family members (Grandori *et al*, 2000) Also shown is MBIII (Herbst *et al*, 2005).

C-terminal bHLHLZ region: This region is necessary for interaction with Max and Miz-1 as detailed later in this chapter.

Central region: This region contains an NLS as well as the recently identified MBIII (Herbst *et al*, 2005), a highly conserved region involved in Myc mediated repression.
The numbers correspond to amino acid residues and the regions highlighted underneath indicate regions that interact with the transcription factors shown.

Myc family proteins are predominantly localized in the nucleus. These proteins were first shown to function as transcription factors in the late 80's / early 90's, when it was shown that the N-terminal region of c-Myc displayed transcriptional activation activity (Kato *et al*, 1990). These experiments used an N-terminal fragment of c-Myc fused to a heterologous DNA binding domain and demonstrated that this region was sufficient to stimulate transcription. Around this time the C-terminal region of Myc was found to possess significant sequence identity to bHLHLZ proteins (Murre *et al*, 1989). Despite these findings initial studies revealed that the full-length c-Myc could not function as a transcription factor due to its inability to self-dimerise or bind DNA directly (reviewed in Grandori *et al*, 2000). These results led to a search for novel interacting proteins. In 1991 Blackwood identified Max, Myc associating factor X, a HLHLZ protein capable of forming a sequence specific DNA binding complex with c-Myc. These studies revealed that Max was capable of interaction with all Myc family proteins.

The resulting Myc /Max heterodimers were shown to recognise the DNA sequence CAT/CGTG belonging to the class of sequences called E-boxes –CANNTG (Blackwood and Eisenman, 1991). It has since been shown that Myc/Max heterodimers activate transcription from genes containing E-box sites. It was not until 1994, when transcriptional activation by Myc/Max heterodimers had been widely confirmed that reports of Myc repression of a number of genes was reported. Work by Li *et al*, 1994 showed that Myc was also able to repress transcription of a number of genes. Repression activity occurs independently of E-boxes and many repressed genes contained Initiator elements rather than TATA boxes at their transcriptional initiation sites.

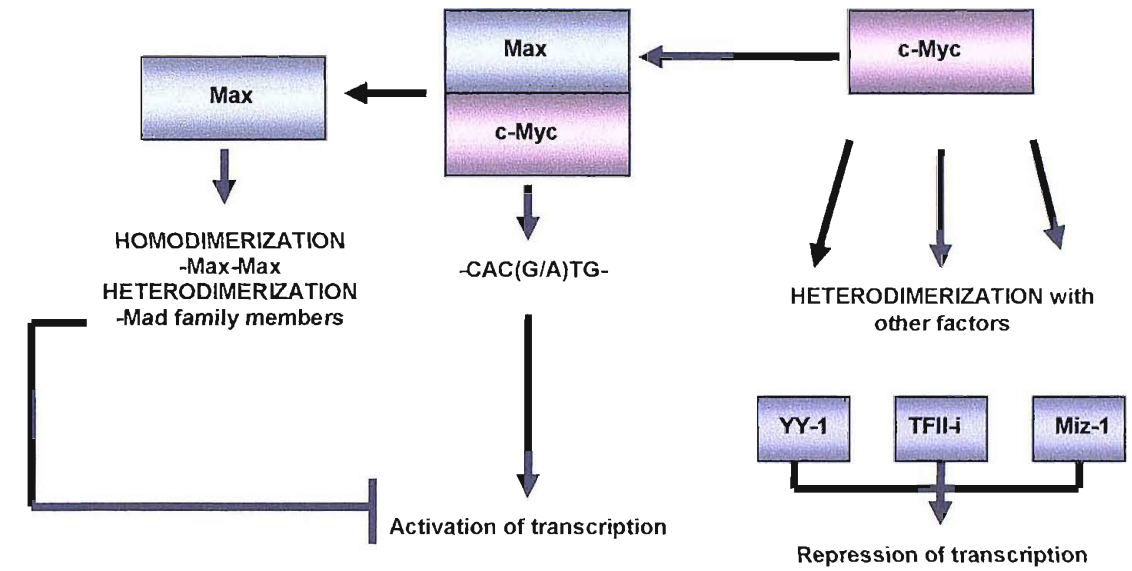
Later work revealed that Max was expressed in the absence of Myc raising the possibility that there were likely additional binding partners for Max. A two Hybrid screen led to the discovery of Mad1 (Ayer *et al*, 1993) and MxiI (Zervos *et al*, 1993). These proteins were termed the Mad (Max associated protein) family. Since their initial discovery other members of the family including Mad3 and Mad4 (Hurlin *et al*, 1995) have been identified. These proteins are very similar to Myc in that they form weak homodimers capable of only very weak DNA binding. Mad proteins bind E-box consensus sequences

as partners with Max and work suggests that they can bind identical sequences as Myc-Max heterodimers.

Mad/Max heterodimers antagonise Myc/Max function by acting as transcriptional repressors. This repression is via their interaction with the mSin3 transcriptional repressor complex. mSin3 proteins function by modifying chromatin structure via association with HDAC's 1&2, as demonstrated by a yeast two hybrid screen with Mad1 and MxiI as bait (Ayer *et al*, 1995). Mad repression is therefore a result of deacetylation of histone tails within the nucleosomes leading to the formation of a closed chromatin, transcription factor-inaccessible structure.

Expression of Mad family proteins is associated with post-mitotic cells and terminal differentiation (Queva et al, 1998), whereas Myc is expressed in proliferating cells. Consistent with these observations and the idea that Mad proteins antagonizes Myc function, the overexpression of Mad protein has been shown to inhibit Myc activated reporter genes and prevent apoptosis (Henriksson & Luscher, 1996). Studies looking at the effects of ectopically over-expressed Mad proteins in both cultured cells and mice, have also shown that Mad1 over expression interferes with the proliferation of the non-transformed cell and blocks transformation by Myc (McArthur & Eisenmann 1997).

As well as members of the Max and Mad families, other proteins have been shown to interact with Myc (Shrivastava *et al*, 1993). These include YY1, TFIIi and Miz-1, which have all been shown to activate transcription from initiator elements. Binding of Myc to YY1 precludes the interaction between YY1, the TATA binding protein and TFIIB leading to transcriptional repression (Seto *et al*, 1991). TFIIi has been found to bind to and activate transcription from initiator elements and has also been shown to form a complex with Myc (Roy *et al*, 1991, 1993). Myc also binds Miz-1, a zinc finger protein which binds to and activates transcription from initiator elements. The binding of Myc prevents Miz-1 binding to its co-factor P300 (Peukert *et al*, 1997). This interaction is discussed in more detail in section 4.1



Adapted from Bouchard *et al* 1998

Figure 1.2: The Myc Network

Myc is only capable of binding DNA by Heterodimerization with other factors. Heterodimer formation with transcriptional activators, such as YY1, TFIIi or Miz-1 results in repression of transcription. Myc/Max heterodimers bind to E-boxes within the promoters of target genes and activate transcription. This process is antagonised by the formation of Mad/Max heterodimers which are also capable of binding to E-boxes and repress transcription via HDAC recruitment.

c-Myc has been the subject of intense study since its discovery in the early 1980's. This interest is largely due to its involvement in a large range of fundamental cellular processes such as cell growth and proliferation, differentiation, apoptosis and tumourogenesis. c-Myc is evolutionary conserved and has been identified in all vertebrates including zebrafish (Langenau *et al*, 2003) and Drosophila (Gallant *et al*, 1996). These provide useful model organisms in which to study c-Myc function. c-Myc is widely expressed during embryogenesis and is an essential gene, as embryos with deletions in cMyc die before birth at day E10.5 of gestation (Davis *et al*, 1993).

1.2 c-Myc activation of target genes

Myc/Max transcriptional activity, as demonstrated using synthetic reporters or putative cellular target genes is very low, typically in the region of 2-5 fold from RNApolII transcribed genes (Kretzner et al, 1992) and much lower than other transcriptional activators. Recent data have indicated Myc is a more powerful transcriptional activator from polI and polIII genes (Gomez-Roman *et al*, 2003; Grandori *et al*, 2005). Myc/Max heterodimers recognise the DNA sequence CAT/CGTG which belongs to the class of sequences called E-boxes –CANNTG (Blackwood and Eisenman, 1991). Studies in yeast (Amati *et al*, 1992) revealed that Myc/Max heterodimers activated transcription of genes containing E-boxes. In 2003 (Nair & Burley) the crystal structures of both the Myc/Max and Mad/Max heterodimers were solved. The bonds linking the heterodimers were found to be much stronger than those linking the Max/Max homodimers due to the differences in the amino acid composition of the leucine zipper region. This tighter binding causes the leucine zipper region of the heterodimers to flare, allowing four contacts between each basic region and the DNA/E-box sites. These heterodimers have been shown to be capable of recognising both canonical and non-canonical (containing a TG or CG di-nucleotide core). Mad/Max and Myc/Max heterodimers make almost identical protein DNA contacts with E-boxes. Max/Max heterodimers bind DNA weakly as the flaring of the leucine zipper does not occur.

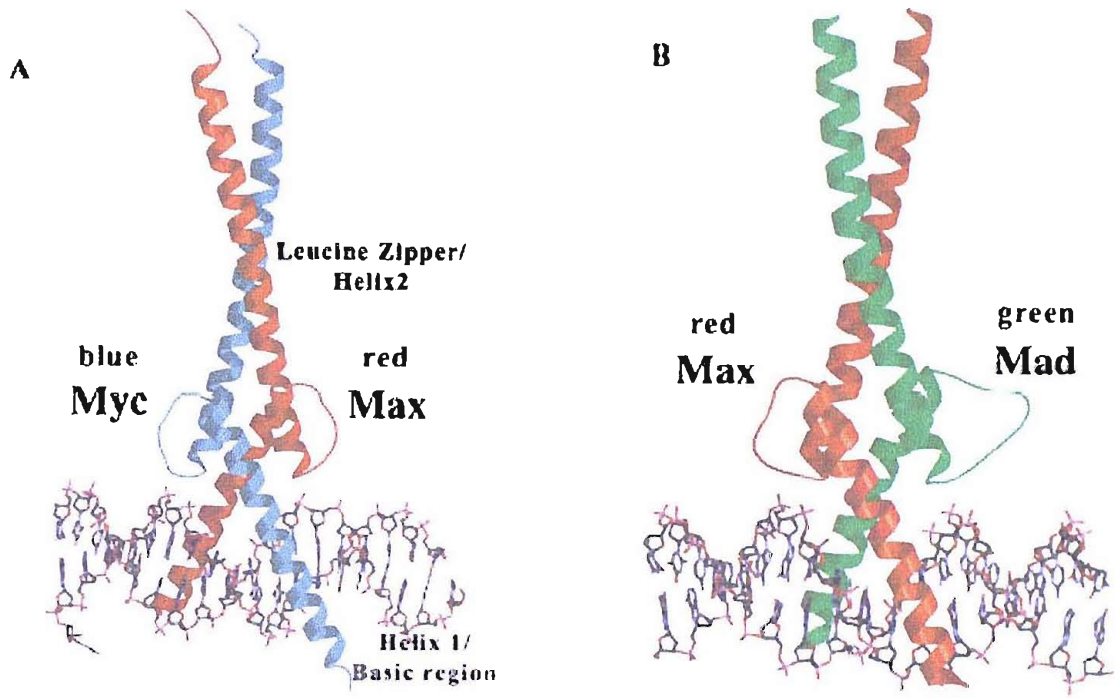


Figure 1.3 Myc/Max & Mad/Max ribbon diagrams (Nair & Burley, 2003)

The crystal structures of Myc/Max and Mad/Max heterodimers reveal that both heterodimers make almost identical contacts with the DNA. Flaring of the leucine Zipper region is as a result of the differing amino acid composition of the two leucine zippers. This flaring allows the heterodimers to make four contacts between the basic region and the DNA/E-box sites.

As detailed earlier in this section, Myc contains a trans-activation domain at its N-terminal region. This region was defined (Kato et al, 1990) by fusing fragments of c-Myc to the DNA binding domain of yeast Gal4 protein. The precise region required for transcriptional activation has been mapped to amino acids 1-143, and includes the two highly conserved regions MBI (amino acids 45-63) and MBII (amino acids 128-143). Using the Gal4 system, deletion of either MBI or MBII reduced transcriptional activation by 10-fold (MBI) or 50-fold (MBII) that of the wild type Myc chimera. In functional assays deletion of MBI reduced the transforming ability of c-Myc whilst deletion of MBII totally abolished it (Stone *et al*, 1987).

The precise mechanism of c-Myc activation was not fully understood until the discovery of the Myc binding protein TRRAP. TRRAP was identified in studies using the transactivation domain of c-Myc as bait to isolate interacting proteins and encodes a 400kDa protein (McMahon *et al*, 1998). Co-immunoprecipitation studies carried out both *in-vivo* and *in-vitro* have shown that TRRAP is contained in a complex which associates with the N-terminal region of c-Myc (McMahon *et al*, 2000).

TRRAP displays sequence identity to the P-I3 Kinase/ATM family. However, it lacks the kinase catalytic residues in the active site making it unlikely to have kinase activity (McMahon *et al*, 1998). The discovery of TRRAP as a co-factor of Myc was followed by the finding that TRRAP was a component of the SAGA complex (Saleh *et al*, 1998). SAGA (SPT/ADA/GCN5/Acetyltransferase) is an 18MDa complex consisting of around 20 proteins. SAGA regulates transcription through chromatin remodelling. Studies highlighted a role for this complex in TBP positioning. SAGA is highly conserved from yeast to humans (Martinez *et al*, 1998). GCN5 is one component of the SAGA complex and GCN5p, the yeast homologue, mediates the acetylation of histone tails. This results in an open chromatin structure and increased transcription (Grant *et al*, 1997). Co-immunoprecipitation studies show that GCN5 associates with the N-terminus of c-Myc both *in-vivo* and *in-vitro* (McMahon *et al*, 2000).

Histone acetylation by transcriptional co-factors recruited by Myc is complemented by Mad/Max heterodimers that function by recruitment of an mSin3-HDAC complex acetylating nearby histones resulting in closed chromatin structure at Myc target genes. Therefore a model can be proposed in which the antagonistic effects of Myc and Mad are due to the recruitment of HAT hGCN5 by Myc whose activity is directly opposed by the recruitment of HDAC by Mad. (see figure 1.4).

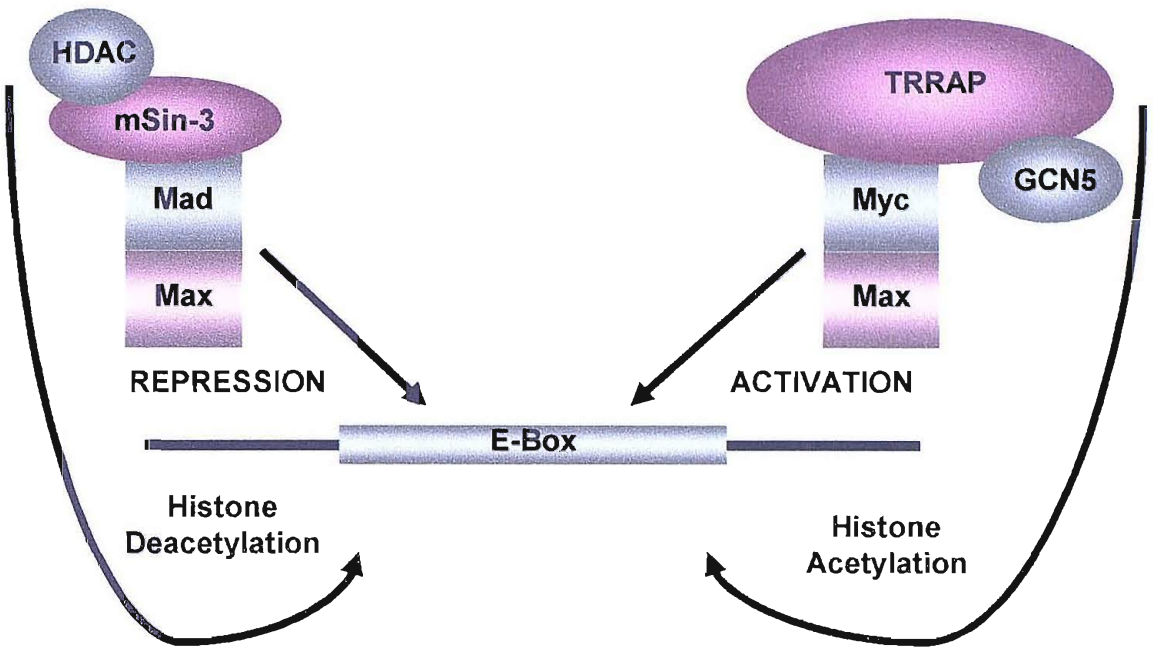


Figure 1.4 **A model of the opposing biochemical functions of Myc and Mad.**
Mad/Max heterodimers repress the activation of Myc/Max target genes blocking Myc function. Mad represses via recruitment of a Sin3-HDAC complex which de-acetylates histones leading to closed chromatin structure. Myc recruits HAT hGCN5 via TRRAP and acetylates histones leading to an open chromatin structure. These opposing biochemical activities are likely to be responsible for the antagonistic effects of Myc and Mad on target genes.
Adapted from McMahon *et al*, 2000

1.3 c-Myc in cell growth and proliferation.

One of the key biological functions of c-Myc is to promote cell cycle progression. De-regulated expression of c-Myc prevents cell cycle withdrawal in response to anti proliferative stimuli such as TGF-β or p53 activation (reviewed in Amati *et al*, 1998).

c-Myc synthesis is rapidly induced, upon mitogenic stimulation, as cells enter the G1 or gap phase of the cell cycle. Following rapid induction of c-Myc, protein levels within the cell drop, but are still detectable for the duration of proliferation (Pelengaris & Khan, 2003). The creation of a c-Myc deficient fibroblast with both alleles of Myc ablated has revealed interesting effects of c-Myc on the cell cycle. In these cells proliferation rates were greatly reduced and an overall marked reduction in global mRNA and protein synthesis was observed (Mateyak *et al*, 1997).

c-Myc also has a role in the growth of invertebrates using *D.melanogaster* as a model (Johnson *et al*, 1999). In these studies Myc plays an important role in regulating growth rates during development. This has also been shown in the growth of mammalian cells using B-lymphocytes where it was demonstrated that c-Myc overexpression enhanced both cell size and protein synthesis (Iritani & Eisenman, 1999).

The key role of Myc in the cell cycle is largely due to its ability to activate or repress a number of target genes involved in cell cycle progression (Amanti *et al*, 1988). Activation of Myc switches on rapid induction of cyclinE-CDK2 kinase activity (Steiner, *et al*, 1995), an essential event in G1-S phase progression. CCND2 which encodes cyclin 2 and CDK4 are also targets of Myc (Bouchard *et al*, 1999; Hermeking *et al*, 2000). Expression of both genes promotes the sequestering of the CDK inhibitor p27 in cyclin D2-CDK4 complexes. This in turn prevents p27 being able to bind cyclinE-CDK2 complexes leaving inhibitor free cyclinE-CDK2 complexes to become phosphorlylated by CAK (Cyclin Activating Kinase). This increased CDK2-CDK4 activity results in the hyper-phosphorylation of Rb and subsequent release of E2F allowing progression into S-phase.

As well as activation, Myc has also been shown to repress some genes involved in cell cycle regulation. These include the CDK inhibitors p15 and p21, involved in cell cycle arrest. Their repression is mediated through interaction of the Myc/Max heterodimer with positively acting transcription factors such as Miz-1 and Sp1 (Reviewed in Wanzel *et al*, 2003; Gartel & Shchors, 2003). The precise mechanisms will be discussed in more detail later in this chapter.

1.4 c-Myc- Differentiation

A large number of studies (reviewed Grandori *et al*, 2000) have highlighted the important role that the Myc/Max/Mad network fulfils in the regulation of proliferation and differentiation. Myc levels fall rapidly during terminal differentiation, as demonstrated in a large number of cell types including myoblasts, erythroleukaemia cells, adipocytes, B-lymphocytes and myeloid cells (reviewed Henriksson & Luscher, 1996). This is consistent with the finding that Mad levels are elevated at this time. Recruitment of Mad/Max heterodimers to the same E-box elements on target genes as Myc/Max heterodimers prevents Myc/Max binding. Mad/Max heterodimers then repress transcription by recruitment of a chromatin modifying co-repressor complex as described in 1.1.

1.5 c-Myc and apoptosis

De-regulated or elevated levels of c-Myc are observed in a large percentage of human cancers. In order for a cell to become cancerous it must avoid apoptosis. c-Myc induced apoptosis was first observed in the early 1990's (Askew *et al*, 1991) in cells of the 32D.3 myeloid progenitor cell line. Expression of c-Myc in these cells is under the control of IL-3. Over expression of c-Myc in 32D.3 cells deficient of IL-3 drove cells into S-phase and accelerated cell death.

Apoptosis due to c-Myc over-expression was also observed in Rat-1 fibroblasts (Evan *et al*, 1992). Rat-1 fibroblasts over-expressing Myc or expressing Myc-ER (c-Myc Oestrogen Receptor chimera) were unable to arrest growth under conditions of low

serum. However, cell numbers were found to decrease due to substantial cell death, dependent on the expression of c-Myc protein and occurring by apoptosis.

The precise mechanisms implicated in c-Myc induced apoptosis are not well understood. However, expression of c-Myc sensitises the cell to a range of pro-apoptotic stimuli including hypoxia, DNA damage and depleted survival factors (Reviewed, Pelengaris & Khan, 2003). c-Myc induces the release of cytochrome c from the mitochondria. Once released into the cytoplasm cytochrome c associates with the apoptotic protease factor 1 (APAF1) and activates pro-caspase 9 (Acehan *et al*, 2002). This in turn leads to the activation of the caspase effector cascade and cell death.

p53 and CD95 (Fas) signaling pathways have also been implicated in c-Myc induced cell death (Huber *et al*, 1997). Work by Seoane *et al* in 2002 on the protein dependent kinase inhibitor $p21^{cip1}$ uncovered a novel mechanism by which c-Myc was able to switch the p53 response from growth arrest to apoptosis. These studies identified a mechanism independent of p53, which blocked the expression of a p53 induced inhibitor of cell death, $p21^{cip1}$. Expression of the $p21^{cip1}$has been shown to be regulated by the zinc finger transcription factor Miz-1(Herold *et al*, 2002; Seoane *et al*, 2002 Van de Wetering *et a*l, 2002). As described in section 1.5 Miz-1 activates transcription from Inr containing promoters via its interaction with its co-activator P300. This interaction is inhibited by c-Myc binding to the Inr via interaction with Miz-1, displacing P300 and causing transcriptional repression (Seoane *et al*, 2001; Staller *et al*, 2001).

Work by Seoane *et al* (2002) showed that Miz-1, like p53, was able to activate the $p21^{cip1}$ promoter. Activated p53 could not overcome the repression by c-Myc. This means that the cell can be protected from apoptosis without altering p53 activity, thus allowing activation of the expression of other p53 target genes such as those encoding pro-apoptotic proteins including PIG3 and PUMA (Seoane et al, 2002).

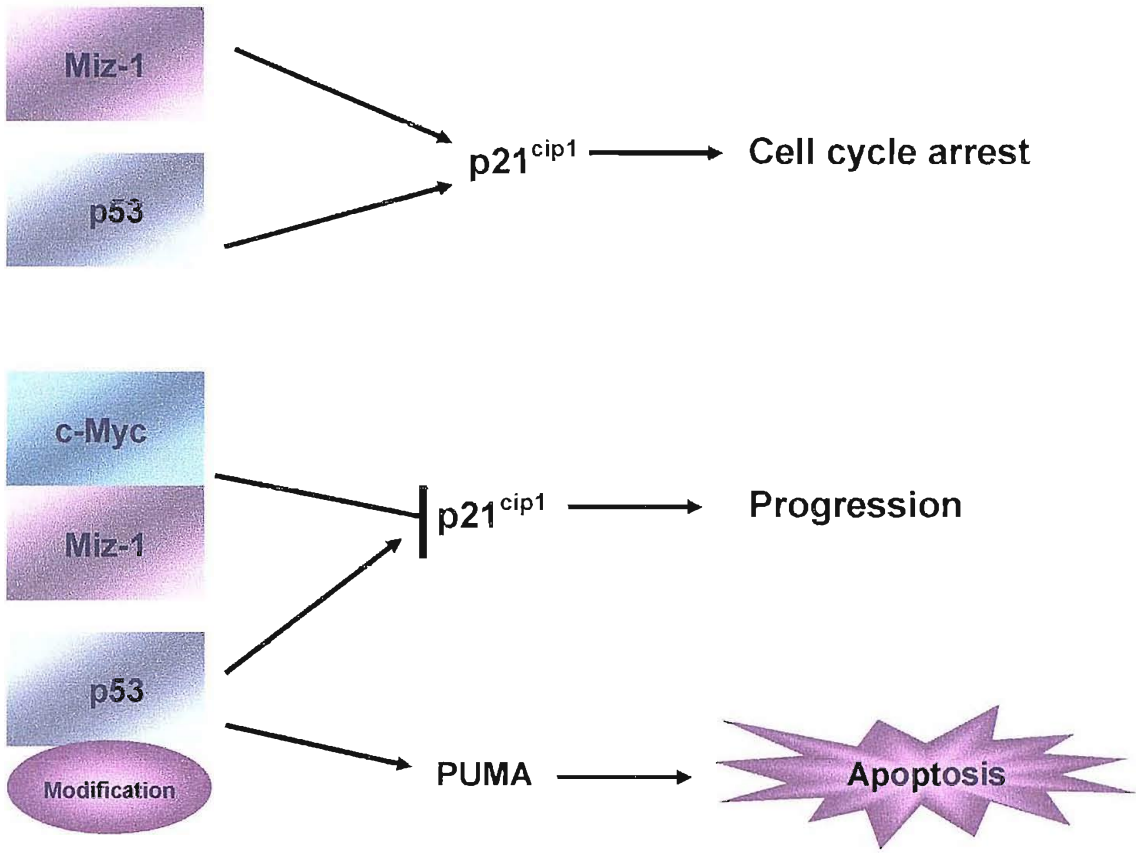


Figure 1.5 **c-Myc switches the response to p53 from cell cycle arrest to apoptosis.**

In normal cells $p21^{cip1}$ a mediator of cell cycle arrest is activated in response to p53 or Miz-1

$P21^{cip1}$ activation by Miz-1 can be inhibited by interaction with c-Myc preventing the cell from apoptosis. This allows p53 to activate other proapoptotic genes such as PUMA. (Adapted from Vousden, 2002)

1.6 Myc mediated repression of target genes

The transcription factor C/EBPα inhibits proliferation of pre-adipocytes to adipocytes in culture. Myc was shown to repress C/EBPα through a core promoter region (Freitag & Geddes, 1992). Later work on the regulation of the adenovirus major late (AdML) promoter (Roy *et al*, 1993) showed repression by Myc in a similar manner. Mutagenesis was carried out in order to identify the structural elements of Myc required for core promoter repression. The HLH domain was shown to be essential (Philips *et al*, 1994), as was the integrity of the LZ and basic region of the dimerization domain and MBII (Li *et al*, 1994). Studies on the promoters themselves found that the integrity of the Inr, a pyrimidine rich sequence element at the start of transcription (described in more detail in section 1.7), was essential for repression of both the AdML and C/EBPα promoters used in this study.

The finding that the Inr element was essential for Myc mediated repression was supported by the observation that other genes which had been reportedly repressed by Myc also contained Inr elements. These include; NCAM, a cell-cell adhesion molecule (Akeson & Bernards, 1990), MHC class1 (Bernards *et al*, 1986) and Cyclin D1 (Philip *et al*, 1994).

A yeast two-hybrid screen was carried out using a DNA fragment encoding the bHLHLZ region of c-Myc (amino acids 355-439) (Schneider *et al*, 1997; Peukert *et a*l, 1997). This region was selected as previous studies on the Cyclin D1 promoter (Philip *et al*, 1994) had shown that the HLH of Myc was essential for repression in stable cell lines. The protein encoded by an identified clone was seen to interact with Myc if the leucine zipper was deleted (amino acids 412-434), but not if the HLH (amino acids 355-412) was deleted. Interaction was demonstrated with the HLH of both c and N-Myc but not with the HLH of other proteins closely related to Myc. 5' RACE was then used to isolate full length cDNA. The identified gene, termed Miz-1 (Myc interacting zinc finger protein 1) encoded an 803 amino acid polypeptide with predicted molecular weight of 87,970KDa. Sequence analysis revealed a zinc finger protein consisting of thirteen C_2H_2 zinc fingers, twelve centrally located and one nearer to the C-terminus. This protein also contained a

BTB/POZ domain within the N-terminal region. BTB/POZ domains are evolutionarily conserved consisting of approximately 120 amino acids found at the extreme N-terminus of some C_2H_2 zinc finger transcription factors and are known to mediate protein-protein interactions (Bardwell & Treisman, 1994), and homo- or hetero-dimer formation (Zipper *et al*, 2002)

Figure 1.6 A schematic diagram of Miz-1

Miz-1 contains 13 zinc fingers, 12 located centrally and one at the c-terminus. The N-terminus contains a BTB/POZ domain known to mediate dimer formation and protein-protein interactions.

Using structure prediction programmes (Peukert *et al*, 1997) it was revealed that amino acids 641-715 of Miz-1, which lay between zinc fingers twelve and thirteen, had a high tendency to form an amphipathic α-helix, similar to the HLH of c-Myc. This led to the hypothesis that these two domains may interact. Deletion and mutation of this region indeed inhibited the interaction with c-Myc (Peukert *et al*, 1997). Miz-1 itself transactivates a number of reporter constructs repressed by Myc; AdML & Cyclin D1 (Peukert *et al*, 1997), $p15^{Ink4B}$ (Staller *et al*, 2001; Seoane *et al*, 2001), $p21^{Cip1}$ (Herold *et al*, 2002; Seoane *et al*, 2002; Wu *et al*, 2003), LDLR (Zeigelbauer *et al*, 2001) the Myc agonist Mad4 (Kime & Wright, 2002), *Nramp1*(Bowen *et al*, 2002,2003) and the DNA damage gene GADD153 (Barsyte-Lovejoy *et al*, 2004). Deletion of the Zinc finger domains inhibits transactivation by Miz-1 (Peukert *et al*, 1997).

EMSA and ChiP analysis identified a Miz-1, Myc and Max complex bound at the Inr element of the $p15^{Ink4B}$ promoter (Seoane *et al*, 2001; Staller *et al*, 2001). Treatment with the cytokine TGF-β results in loss of this complex from the $p15^{Ink4B}$ promoter. c-Myc also forms a stable complex with Smad and Sp1 proteins that blocks transcription (Feng *et al*, 2002,2002).

In the case of at least four Miz-1 activated / c-Myc repressed genes namely $p15^{Ink4B}$ (Staller *et al*, 2001, Seoane *et al* 2001), $p21^{cip1}$ (Herold *et al*, 2002; Seoane *et al*, 2002; Wu *et al*, 2003), Mad4 (Kime & Wright, 2002) and *Nramp*1 (Bowen *et al*, 2003, 2002) the mechanism of Myc repression is thought to be due to competition for binding to Miz-1 with the its co-factor P300.

This mechanism was first proposed by Staller *et al* (2001) in studies on the $p15^{Ink4B}$ promoter. P300 was investigated as a co-factor for Miz-1 as it had previously been implicated in the transcriptional regulation of $p15^{Ink4B}$ (Datto *et al*, 1997). In order to look at a possible role for transcriptional activation for Miz-1 *in-vitro* binding assays were carried out using recombinant fragments of P300 and *in-vitro* translated Miz-1 (Staller *et al*, 2001). These studies showed that P300 indeed interacted with Miz-1 and these findings were confirmed by immunoprecipitation and two-Hybrid assays.

Staller et al, (2001) also demonstrated that the binding of Myc to Miz-1 interferes with binding of P300. Mammalian two-hybrid assays showed that amino acids 190-248 and 683-715 of Miz-1 were essential for interaction with P300 and that deletion of either of these regions abolished transactivation by Miz-1. This was consistent with previous findings (Peukert et al, 1997) that interaction of Miz-1 with Myc was via a putative alpha helix located between zinc fingers twelve and thirteen, amino acids 683-715. Therefore the binding domains of P300 and Myc overlap and Myc competes with P300 for binding to Miz-1.

P300 was identified as a protein targeted by the adenovirus E1A oncoprotein (Eckner *et al*, 1994), and shown to interact with the CREB binding protein CBP. P300/CBP transcriptional co-activator proteins play roles in the activities of a number of diverse transcription factors, serving to regulate a number of physiological processes such as proliferation, differentiation and apoptosis. This mode of action is not fully understood and the precise mechanism is likely to differ dependent on the pattern of transcription factor expression and the context of the target promoter (reviewed, Man Chan & Thange, 2001).

Translocation to the nucleus is essential for any transcription factor to modulate gene expression. Analysis of Miz-1 (Peukert *et al*, 1997) revealed that it lacked a classical NLS. Findings of immuno-fluorescence studies were consistent with this, showing that Miz-1 was predominantly located in the cytosol (Peukert *et al*, 1997). Microtubule association was suggested and was later confirmed using co-immunoprecipitation (Zeigelbauer et al, 2001). It is not clear exactly how Miz-1 enters the nucleus. The generation of an NLSMiz-1 chimera revealed that in order for Myc to effectively inhibit transactivation and subsequent cell cycle arrest by Miz-1, the amount of Miz-1 in the nucleus must be limited. NLS Miz-1 was found to be more resistant to Myc repression and inhibited cell cycle arrest more effectively than the wild type Miz-1.

It has been proposed that the Miz-1 must somehow enter the nucleus via association with another protein, and P300 or Myc are candidates. Other transcription factors lacking a functional NLS use a related process. Similarities have been drawn between Miz-1 and E2F-4 whose nuclear import has is stimulated via an association with the pocket proteins p107 and p130 which are themselves negative regulators of E2F-4 (Linderman *et al*, 1997).

Studies on the stability of Myc have provided support for the idea that Miz-1 is dependent on Myc for transport into the nucleus. Myc is dependent on Miz-1 for stability and to prevent ubiquitin-mediated proteolysis. Miz-1 over-expression also stabilises Myc (Salghetti *et al*, 1999). Myc has a very short half-life of around thirty minutes (Hann &

Eisenmann, 1984). This is fairly typical of transcription factors that are involved in the regulation of cell growth. The rapid destruction of these proteins is essential if the cellular levels are to remain responsive to extra-cellular stimuli (Salghetti *et al*, 1999) for example alterations in Myc stability can enhance cell proliferation and lead to tumourigenesis.

As is the case most commonly observed for short-lived transcription factors Myc is destroyed by ubiquitin-mediated proteolysis (Ciechanova *et al*, 1991). Salghetti *et al* (1999) sought to identify the regions of Myc necessary for this process. A panel of Myc deletion mutants were transiently expressed in HeLa cells and the stability and ubiquitylation status of each were measured. The N-terminal TAD of Myc was shown to be essential for destruction by ubiquitin mediated proteolysis. Deletion of the C-terminus had no effect on ubiquitin mediated proteolysis, but did have considerable effects on stability in combination with deletion of amino acids 370-412, reducing the half-life of the protein to around fifteen minutes. Taken together it can be seen that these two regions act in opposition to regulate the stability of Myc.

1.7 Biological relevance of Myc repression

Myc exerts transforming function by activation of a critical set of target genes by Myc/Max heterodimers. In transformed cells a number of genes are repressed by Myc including those involved in proliferation, differentiation and cell adhesion. Therefore it can be said that repression of genes by Myc significantly affects the phenotype of the transformed cell.

De-regulation of c-Myc inhibits differentiation of a number of cell types (Reviewed Faccini & Penn, 1998). In support of this Myc represses several genes involved in differentiation i.e. C/EBP-α (Li *et al*, 1994), forcing cells to remain in a proliferative state therefore pre-disposing cells to transformation. Repression of cell cycle inhibitory genes promotes cell growth, for example GADD's. These are induced by DNA damage or treatment with agents that induce cell cycle arrest. Myc represses GADD's (Barsyte-Lovejoy, 2004). Additionally Gas1 (Growth Arrest Suppression) has also been shown to

be repressed by c-Myc (Lee *et al*, 1997). Gas1 is normally down regulated following growth induction (Schneider et al, 1998).

Myc also represses a number of cyclin dependent kinase inhibitors including $p15^{Ink4B}$ (Staller *et al*, 2001; Seoane *et al*, 2001), and $p21^{Cip1}$ (Herold *et al*, 2002; Seoane *et al*, 2002; Wu et al, 2003) . Cyclin dependent kinase inhibitors block cell cycle progression by binding cyclin/cdk complexes and inhibiting their activity (reviewed Sherr & Roberts, 1995).

The link between Myc mediated repression and transformation has been most clearly demonstrated in studies using Burkitts Lymphoma derived Myc. A mutant protein called MB2 containing a point mutation on the region of Myc shown to be important for transcriptional repression was found to repress the AdML promoter more strongly and to have more potent transforming ability than the wild type Myc (Lee *et al*, 1996). A second mutant, MycW136E, had decreased repression and transformation ability (Lee & Dang 1997).

The link between Myc-mediated repression and cellular transformation was also supported by the discovery of a shorter form of c-Myc found in Human and Mouse cells, c-MycS (Spotts *et al*, 1997). This Myc variant lacks the first 100 amino acids and arises from translational initiation at a conserved and internal AUG codon. Although most of the N-terminal transactivation domain is absent the C-terminal dimerization and DNA binding domains are still present. c-MycS is similar to full length Myc in that it is localised in the nucleus and is relatively unstable. The levels of this shorter form of Myc are similar to levels of the full-length protein, although it cannot activate transcription. This shorter form of Myc retains the ability to repress transcription of promoter constructs tested including Gadd 45, Gas1 and the AdML promoter. This shorter form of Myc can stimulate proliferation, induce apoptosis under low serum conditions and transform Rat 1a fibroblasts (Xiao *et al*, 1998). These studies on c-MycS, taken together, reveal that the ability to repress target genes is sufficient for many aspects of Myc function.

1.8 Transcription and Inr containing promoters

In the early 1950's Francis Crick defined what is now known as the central dogma of molecular biology- Uni-directional flow of genetic information from DNA through to RNA to protein. DNA is transcribed into RNA, which is then in turn translated into protein (Turner *et al*, 2001).

Transcription in Eukaryotic cells is divided into three categories with each being transcribed by a different RNA polymerase. Ribosomal RNA is transcribed by RNA PolI, messenger RNA by RNA PolII and tRNA and other small RNA's by RNA PolIII (Lewin *et al*, 1994). The focus of the work presented in this thesis will be RNA PolII transcription as this catalyses the synthesis of mRNA, which is the precursor for all protein-coding genes.

In order for transcription to occur in a eukaryotic cell a number of events need to occur-

- Decondensation of the loci in a cell-specific fashion.
- Nucleosome remodelling
- Histone modifications
- Binding of transcriptional activators and enhancers to promoters
- Recruitment of the basal transcription machinery to the core promoter.

(Reviewed in Smale & Kadonaga 2003).

The core promoter element can be defined as the region of DNA which can extend up to ~35bp either upstream or downstream of the transcription initiation site. Most promoters are capable of interaction with the basal transcription machinery (Smale & kadonaga, 2004).

The basal transcription machinery includes RNA Pol II and the other factors required for the mechanics of initiating RNA synthesis at all promoters. A series of these nuclear transcription factors have been identified, purified and cloned. These factors have been largely studied with respect to TATA containing promoters and have been shown to assemble in specific order ;TBP/TFIID/TFIIA, TFIIB, RNA PolII/TFIIF, TFIIE & finally

TFIIH (Reviewed in Turner et al, 2001). Recruitment of these factors to Inr containing promoters is believed to be via a mechanism similar to that which occurs in TATA containing promoters.

The TATA box

The TATA box, also known as the Goldberg-Hogness box, after those who discovered it, was identified in 1979 and is defined as an AT rich sequence located ~25/30 nucleotides upstream of the transcription start site (Breathnach and Chambon, 1981). It was the first core promoter element to be identified in eukaryotic protein-coding genes. Almost all of the genes identified in the late 1970's and early 1980's were found to contain a TATA box.

The optimal sequence for TBP recognition and binding has been identified as- **5'-TATATAAG-3'** (Wong & Bateman, 1994) although, since this sequence was identified, a large number of A/T rich sequences have been shown to be capable of functioning as TATA boxes (Patikoglou et al, 1999).

Initiatior (Inr) elements

The initiator element was first identified in 1989 during studies on the promoter of the lymphocyte specific terminal transferase gene (TdT) (Smale & Baltimore, 1989). This gene lacked a TATA box, but instead contained what was termed an initiator element (Inr), 'a discrete promoter element functionally similar to the TATA box'.

The consensus sequence for the Inr element was later defined as-

- **Py, Py A+1, N, (T/A), Py Py** (Py is a pyrimidine base) (Javahery et al, 1994).

This sequence was discovered by analysis of a number of randomly generated Inr's and Inr mutants analysed by *in-vitro* transcription. Transcription from the TdT core promoter was initiated from a single start site. Using a series of promoter mutants, the region between -3 and +5 was necessary and sufficient to direct transcription *in-vivo*.

The Inr element was capable of directing transcription by itself. However, it was strengthened when activators such as Sp1 were bound upstream of the core promoter

(Smale & Baltimore, 1989). Inr elements are capable of unidirectional transcription (O'Shea-Greenfield & Smale, 1992). However, the direction of transcription is dependent on the orientation of activators and core promoter elements.

Transcription initiation from Inr promoters

DNase1 footprinting on the AdML promoter, as well as others containing both TATA boxes and Inr elements, provide evidence that the Inr is capable of direct interaction with TFIID (Sawadogo & Roeder, 1985). This interaction was reduced by mutation of the Inr element. Analysis of a series of mutants revealed the TFIID-Inr interaction corresponds with the nucleotides required for Inr element function (Emami *et al*, 1997). The specific subunit of the TFIID complex that binds to the Inr is unknown and the involvement of other proteins in recruitment and Inr element recognition has not been ruled out. The TFIID-Inr interaction is weaker than the TFIID-TATA interaction (Emami *et al*, 1997), and as yet no studies have shown TFIID binding to an Inr promoter lacking either a TATA box upstream or a DPE downstream.

Studies have suggested (Weis & Reinberg, 1997) that RNA PolII may itself be responsible for initial recognition of the Inr element. This is supported by previous observations (Carmico *et al*, 1991), showing that purified RNA PolII is capable of initiating transfection, albeit inefficient, from Inr elements in the absence of other basal transcription factors. Interaction between RNA PolII and Inr elements has also been shown using gel shift experiments with an Inr containing probe. Although it is known that RNA PolII can bind DNA non-specifically, these studies indicate that it is possible RNA PolII forms stable complexes on Inr promoters.

Other factors including TFIIi, YY1 and Miz-1 have been shown to be capable of binding to Inr elements. TFIIi was discovered in 1991 (Roy *et al*) as a factor able to bind to the Inr of the AdML promoter. TFIIi has an unusual binding domain and six HLH motifs and this factor is capable of forming homomeric and heteromeric interactions (Roy *et al*, 1997). TFIIi can stimulate transcription from Inr elements and can form complexes with Myc (Roy *et al*, 1991, 1993).

YY1, a C_2H_2 zinc finger protein and was identified in 1991(Shi *et al*). YY1 activates transcription from the AAV (Adenovirus associated) P5 core promoter by binding to its Inr element (Seto *et al*, 1991). In addition another Zinc finger protein, Miz-1 (Peukert *et al*, 1997) was identified that activates transcription from Inr containing promoters. Like TFIIi, both YY1 and Miz-1 have been shown to form complexes with Myc, although the precise role of factors such as TFIIi, YY1 and Miz-1 in Inr function is not fully understood.

Downstream promoter elements

A DPE is a distinct 7bp element located at ~+30 relative to the start site of transcription. DPE's have been identified in a number of TATA less promoters (Reviewed Burke *et al*, 1998), and they specifically bind TFIID. (Burke & Kadonaga, 1996). Studies carried out using both *D.melanogaster* and human promoter constructs have shown that the presence of an Inr in addition to the DPE is essential for the efficiency of DPE-containing promoters (Burke & Kadonaga, 1997). These studies also revealed that the spacing between the Inr and DPE is critical for binding of TFIID, with native spacing between these two elements found to be essential.

1.9 Aims

Previous studies have shown that both the NCAM and *Nramp1* genes were repressed by the Myc family of oncogenes. In addition, a role for Miz-1 was identified in *Nramp1* repression. Since both *Nramp1* and NCAM genes have a core promoter architecture with common features, NCAM was examined as another potential gene for regulation by Miz-1. It soon became clear that NCAM, whilst being repressed by Myc, was not a Miz-1 target gene. The aims of this project were:

- to develop systems to compare the repression of Nramp1 and NCAM genes by Myc
- To compare the mechanism of Myc recruitment to the core promoters
- To established how Myc recruitment brings about transcriptional repression.

Regulation of gene expression is critical for determining cell phenotype and for the maintenance of cellular homeostasis. Transcriptional regulation is important as it precedes other mechanisms of regulation in the hierarchy of information flow. Gene specific transcription factors such as Myc are capable of either activation or repression of basal transcription. Mutation or de-regulation of these transcription factors plays a large role in a huge variety of human diseases such as cancer, and have pleiotropic effects as these factors control the expression of so many genes.

The Myc family of proto-oncogenes fulfils a pivotal role in controlling cell fate, specifically in determining choices between proliferation, cell growth arrest and apoptosis. Responses are mediated by modulating transcriptional rates, both gene repression and activation. Recent evidence has accumulated to indicate that Myc's role in repression is of greater relevance for cell transformation. c-Myc repressed genes can be broadly defined as-(i) those essential for cell cycle arrest, such as the CDK inhibitors, (ii) genes associated with the terminally differentiated state and not expressed within cycling cells or (iii) genes that antagonise Myc function.

Several mechanisms for c-Myc mediated repression of gene transcription have been reported including those which involve the Inr binding protein Miz-1 and those which suggest a role for Sp1. The paradigm for control of higher eukaryotic gene transcription is that they contain a TATA box a number of bases upstream of the transcriptional start site. However, several genome studies are now indicating that these may represent a minority of eukaryotic promoters and that there is significant variation in terms of promoter configuration.

Work presented in this thesis uses two model Inr promoters in order to study mechanisms of Myc repression. The first of these, *Nramp1*, encodes the Natural resistance associated macrophage protein 1, a divalent cation transporter expressed in the murine macrophage. *Nramp1* plays an important role in controlling the response of the macrophage to infection with a number of antigenically unrelated pathogens. The second promoter is from the NCAM gene that encodes a neural cell adhesion molecule expressed by a

variety of cell types including most nerve cells. Down regulation of NCAM has been shown to induce tumour metastasis by up-regulating lymphangeogenesis (Crnic *et al*, 2004).

Initial sequence inspection of the two Inr promoters reveals a number of similarities. However, detailed promoter analysis carried out and presented as part of this thesis provides evidence that Myc is able to repress Inr promoters by a variety of gene specific mechanisms, demonstrating that sequence inspection alone is insufficient to determine regulation of a promoter by a particular transcription factor.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

All chemicals used in experiments (unless otherwise stated) were purchased from Sigma-Aldrich , Poole, Dorset, UK.

Reagents used in cell culture including DMEM and antibiotics were purchased from Lifesciences Gibco BRL.

Radioactive isotopes used in CAT assays were purchased from ICN biochemicals, Thane, UK.

2.1.2 Media for bacterial culture

Luria-Bertani Medium (LB medium) was used for bacterial culture.

10g Sodium Chloride

10g Bactotryptone

5g Yeast Extract

1L dH_20

(The media was sterilized by autoclaving)

In order to produce a solid medium for bacterial growth 1.5% w/v was added to the above solution before autoclaving.

Ampicillin was added to the culture media to enable the selection of bacteria that contained plasmids encoding Ampicillin resistance. A stock solution of Ampicillin was made at a concentration of 50mg/ml in dH_2O. Ampicillin was then filter sterilised and stored in 1ml aliquots at -20°c. Ampicillin was added to media after autoclaving, at a final concentration of 100µg/ml

Kanamycin was added to the culture media to enable the selection of bacteria that contained plasmids encoding Kanamycin resistance. A stock solution of Kanamycin was made at a concentration of 15mg/ml in dH_2O. Kanamycin was filter sterilized and stored

in 1ml aliquots at -20°c. Kanamycin was added to media after autoclaving, at a final concentration of 30µg/ml.

X-Gal and IPTG were added to media to allow blue white selection of colonies cloned using the T-easy vector (Promega). A stock solution of IPTG was made at a concentration of 100mM in dH_2O , filter sterilized and stored in 1ml aliquots at -20°c. A 2% solution of X-Gal was made in di-methylformamide. This stock was stored in 1ml aliquots and stored at -20°c. The X-Gal and IPTG were used to supplement LB agar plates containing ampicillin. 100µl of each solution was applied to the surface of each plate using a sterile glass spreader and allowed to dry before the bacteria were plated out.

2.1.3 DMEM media for cell culture

Cos-1, ND7 and RAW 264.7 cell lines used in transfections were cultured in low endotoxin Dulbecco Modified Eagles Medium (DMEM). This media was supplemented with-

Myoclone foetal calf serum (10% v/v)

L-Glutamine 2mM

Penecillin 10 units/ml

Streptomycin 100mg/ml.

2.1.4 Sterilization

All materials with the exception of those which were heat labile were autoclaved at 15psi for 15 minutes. Heat labile solutions were filter sterilized using 0.2mm filter from Millipore

2.1.5 Water

Deionised water (dH_20) was used in all experiments and was purchased from BDH laboratory suppliers, Poole, Dorset, UK.

2.1.6 Preparation of siRNA insert for pSilencer™

Annealing buffer

100mM K-acetate

30mM HEPES KOH pH 7.4

2mM Mg acetate

2.1.7 Preparation of Plasmids

Small scale- Mini-preps

Nucleospin Mini plasmid prep kit.

Machery Nagel

Medium scale- Midi preps

Midi prep kits

Sigma Aldrich

Large scale- Maxi preps

Nucleospin Mini plasmid prep kit.

Machery Nagel

DNA Gel extraction / PCR clean up - Quiagen Kit

10X Tris Boric EDTA (TBE)

108g Tris-Base

55g Boric Acid

40 ml 0.5M EDTA pH8

Agrose Gel

1% Electrophoresis grade agrose (unless otherwise stated)

1X TBE

2.1.8 Chloramphenicol Acetyl Transferase assay (CAT assay)

Tris.Cl pH 7.8 (1M)

121.1g Tris Base

800ml dH2O

pH adjusted using concentrated HCl

Running solvent for Thin Layer Chromatography

95% (v/v) Chloroform

5%(v/v) Methanol

2.1.9 Luciferase Assay

All reagents for the Luciferase assay were Purchased from Promega UK and stored in accordance with the manufacturers instructions.

2.1.10 Polyacrylamide Gel Electrophoresis

Resolving gel buffer

29.04g Tris-base

8ml 10% SDS

Make up to 200ml with dH_2O

PH to 8.8 using concentrated HCl

Stacking gel buffer

60.4g Tris-base

8ml 10% SDS

Make up to 200ml with dH_2O

PH to 6.8 using concentrated HCl

Resolving Gel Mix (10mls)

2.5ml 4X resolving gel buffer

3.3ml Protogel Polyacrylamide

4.1ml dH_20

100ul	Ammonium Persulphate (APS)
10ul	N,N,N,N-Tetra-methylenediamide

Stacking Gel Mix (5mls)

2.5ml	Stacking gel buffer
0.835ml	Protogel Polyacrylamide
1.67ml	dH_20
50ul	Ammonium Persulphate (APS)
5ul	N,N,N,N-Tetra-methylenediamide

Running Buffer

25mM	Tris Base
190mM	Glycine
0.1% (w/v)	SDS

Coomasie Blue Stain

0.25g	Coomassie Brillian Blue
50g	TCA

Make up to 100ml volume using dH_2O

Destain

450ml	Acetic acid
100ml	Methanol
450ml	dH_2O

2.1.11 Western Blotting

Western blotting transfer buffer

1:4 Methanol:SDS PAGE running buffer

2.1.12 Antibody detection

PBS (Phosphate Buffered Saline)

Prepared from tablets as per manufacturers instructions

PBS/Tween

0.1% (v/v) Tween 20 in PBS

Blocking Agent

10% (w/v) Marvel powdered milk in PBS/Tween

Antibody diluent

5% (w/v) Marvel powdered milk in PBS/Tween

2.1.13 Purification of Glutathione-s-transferase fusion proteins

PBS (Phosphate Buffered Saline)

Prepared from tablets as per manufacturers instructions

PBS Triton (0.1%)

400ml	PBS
400µl	10X Triton

Elution buffer

50mM	Tris.Cl pH8.0
5mM	Reduced glutathione

2.1.14 Biotinylated oligonucleotide pulldown assays

Biotinylated oligonucleotides

Puchased from Sigma-Genosys

HKMG

10mM Hepes pH7.9

100mM Kcl

5mM $MgCL_2$

10% Glycerol

1mM DTT

0.5% NP40

2.1.15 Flow Cytometry

Sheath Fluid, FACS safe and FACS rinse

The above reagents used for all flow cytometry experiments was purchased from Beckton Dickinson UK

2.1.16 Chromatin immunoprecipitation assay (ChIP assay)

Salmon sperm DNA/Protein A agarose

Purchased from Upstate, cell signaling solutions.

Chip dilution buffer

0.01% SDS

1.1% Triton X-100

1.2mM EDTA

20mM Tris-HCl pH8.1

167mMNacl

Low salt immune complex wash buffer

0.1% SDS

1% TritonX-100

2mM EDTA

20mM Tris-HCl pH8.1

150mM NaCl

High salt immune complex wash buffer

0.1% SDS

1% Triton X-100

2mM EDTA

20mM Tris-HCl pH8.1

500mM NaCl

LiCl Immune complex wash buffer

0.25M LiCl

1% NP40

1% Deoxycholate

1mM EDTA

10mM Tris HCl pH8.1

TE Buffer

10mM Tris-HCl

1mM EDTA pH 8.0

SDS Lysis Buffer

1% SDS

10mM EDTA

50mM Tris-HCl pH 8.1

ChIP elution buffer

1% SDS

1.1M $NaHCO_3$

2.2 METHODS

2.2.1.1 Transformation into Competent *E.coli*

50µl of competent *E.coli* (Promega, UK) was aliquoted into pre-chilled 1.5ml microfuge tubes. 2µl of the ligation reactions was added to each 50µl of cells. Cells were immediately returned to the ice for 30 minutes. Following this period of incubation the cells were then heat shocked at 42° c for 45 seconds in a waterbath, before being returned to the ice for further two minute incubation. 500µl of ice cold LB media was then added to the cells before they were incubated at 37° c for 45 minutes (shaking).

Finally the tubes were centrifuged for 1 minute at 12,000rpm and 400µl of the media was removed. The bacteria were then resuspended in the remaining media and plated onto LB agar plates. The plates were incubated at 37° c overnight.

2.2.1.1 Transformation into DH5α

A single colony of *E.coli* DH5a was used to inoculate 10ml of LB media and allowed to grow to stationary phase overnight at 37°c with agitation. A 1:100 dilution of this culture was then made into 10mls of fresh LB and grown at 37°c with agitation for approximately three hours until the cells reached an OD_{600} of 0.6. Following centrifugation at 3000rpm for 15 minutes at 4°c, media was removed and the cells were re-suspended in 5ml of sterile filtered 100mM $CaCl_2$ at 4°c. Cells were then centrifuged at 3000rpm for 5 minutes at 4°c, $CaCl_2$ was removed and cells were then re-suspended in 500µl of fresh $CaCl_2$ and divided into 100µl aliquots. 2µl of DNA was then added to each aliqot and cells were placed on ice for 30 minutes Following this period of incubation the cells were then heat shocked at 42° c for 2 minutes in a waterbath, before being returned to the ice for further two minute incubation. 500µl of ice cold LB media was then added to the cells before they were incubated at 37° c for 45 minutes (shaking).

Finally the tubes were centrifuged for 1 minute at 12,000rpm and 400µl of the media was removed. The bacteria were then resuspended in the remaining media and plated onto LB agar plates. The plates were incubated at 37° c overnight.

2.2.2 Plasmid preparation

All of the plasmids used were maintained in JM109 *E.coli*. Frozen stocks were kept of all clones and these were stored in 25% glycerol in LB broth, at -80° c. When required these were streaked onto LB agar plates containing the appropriate antibiotic. Following overnight incubation of these plates at 37° c single colonies were picked and used to inoculate 10ml of LB broth, again containing the appropriate antibiotic. For small scale preps the cells were harvested by centrifugation at 4000rpm for 10 minutes. Plasmid DNA was then isolated Nucleospin plasmid kit (Malchery Nagel), as per manufacturers instructions. When larger amounts of DNA were required such as for transfection, the 10ml culture was incubated until the OD_{600} reached ~0.6, and was then added to 500ml of pre-warmed LB, before being incubated overnight at 37° c with agitation. The cells were then harvested by centrifugation at 4000rpm for 20 minutes. Plasmid DNA was then isolated using the Nucleospin Plasmid large-scale prep kit (Machery Nagel) as per manufacturers instructions

2.2.3 Purification of DNA for tissue culture.

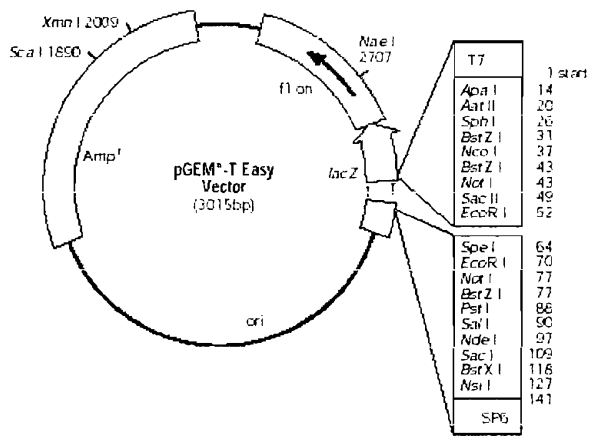
The OD260 of the DNA was taken and used to calculate the concentration of the DNA. DNA was then alcohol precipitated by adding 1/10 volume of sodium acetate and 3 volumes of 100% ethanol and centrifuging for 15 minutes at 4000rpm. Following centrifugation the supernatant was removed and discarded to leave a white pellet of DNA. This pellet was then washed in 100ul of 80% alcohol and following 5 minutes of centrifugation at 4000rpm the alcohol was removed and the pellet was resuspended in sterile water at a concentration of 1μg/μl.

2.2.4 TA cloning using the T-easy vector system (Promega).

Inserts synthesised using the polymersase chain reaction were cloned into their appropriate vectors using TA cloning into the pGEM-T Easy vector. The terminal transferase activity of Taq DNA polymerase was utilized to add template independent 'A' residues to the 3' end of PCR products. This tailing reaction was carried out at 74°c for 30 minutes. Following the tailing reaction, the insert was then purified using the Quiagen PCR purification kit. The tailed PCR product was then ligated with the linearized T-

Easy vector, which has single 3'T overhangs on both ends. The DNA was then transformed into competent JM109 cells and plated onto X-Gal/IPTG/Ampicillin plates and incubated overnight at 37°c. The resulting colonies were then analyzed to determine if they were recombinant. Successful cloning of an insert into pGEM-T Easy interrupts the coding sequence of β-galactosidase, enabling the recombinant clones to be identified by colour screening, with recombinant clones white in colour and non-recombinant clones blue. The white colonies were picked and used to inoculate 10ml of fresh LB (Amp) broth. Following overnight incubation the plasmid DNA was isolated and digested using the restriction enzyme EcoR1. The pGEM-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoR I, which enables single-enzyme digestions for release of the insert. Once the presence of the insert had been confirmed the DNA was digested using the appropriate restriction enzymes and run out of an agarose gel. The insert was then excised from the gel and purified using the Quiagen gel extraction kit before ligating with the prepared vector and transforming into competent JM109 *E coli*.

Figure 2.2.4. T-easy (Promega)



2.2.5 Cell Culture

Cos-1 Cells

The Cos-1, African green monkey kidney cells were cultured in DMEM containing the antibiotics and serum as detailed previously. Cells were maintained in 75mm^2 flasks, at 37° c and at 5% CO_2. When cells became confluent the media was removed from them and the surface of the cells was washed in sterile PBS. Following washing 2.5ml of sterile Trypsin-EDTA is added to the cells, which were then incubated at 37° c for 5 minutes, in order to detach the cells from the surface of the flask. In order to neutralize the Trypsin-EDTA and 2.5ml of DMEM (with antibiotics and serum) was added, and then 0.5ml of this was then added to a fresh flask containing approximately 12mls of DMEM (with antibiotics and serum).

If the cells were to be harvested for experiments the above procedure was carried out but instead of returning some cells to the flask and discarding the rest the cells were counted. This was done by staining a small portion of the cells with Trypan blue and using a haemocytometer to count the number of viable cells. Calculation of the number of cells in solution allowed us to distribute appropriate numbers of cells when plating out the cells. For transfections using Cos-1 cells 1.5x 10^5 cells were distributed into each well of six well plates (35mm diameter wells).

Transfection into Cos-1 cells

As stated previously, 1.5x 10^5 cells were distributed into each well of a six well tissue culture plate in 2ml of DMEM with antibiotics and serum. The plates were then incubated under the conditions described previously for 24 hours until the cells were between 50-80% confluent. For each transfection two sterile microfuge tubes were prepared. In one the DNA was added to 100µl of DMEM with no antibiotics or serum. In the other tube 5µl of LipofectAMINE reagent (Life Sciences, GibcoBRL,UK) was diluted with 100µl of DMEM with no antibiotics or serum. The contents of the two tubes were then combined and mixed gently before incubating at room temperature for 20 minutes. This incubation was in order to allow the formation of a DNA liposome complex which could be taken up by the Cos-1 cells. During the 20-minute incubation

the plates of cells were removed from the incubator and the media containing serum and antibiotics was removed. The cells were then washed using DMEM to ensure no serum remained and then 1ml of DMEM without antibiotics or serum was added to each well of the plate. After the DNA/Liposome complex had formed 100µl of the complex was added to the appropriate well of the plate.

The cells were incubated for 5hrs (conditions as previously) before 1ml of Media containing 20% serum, but no antibiotics was added back to the cells. The DNA/Liposome complex was not removed. If a Chloramphenicol Acetyl Transferase assay was to be carried out, the cells were then incubated under the conditions previously stated for a further 48 hours. If the cells were to be used for flow cytometry the media was removed after 24 hours and replaced with 2mls of colourless DMEM containing 10% serum before being incubated for a further 24 hours (conditions as previously).

Raw 264.7 cells

RAW 264.7 murine macrophage like cells were cultured in DMEM containing the antibiotics and serum as detailed previously. Cells were maintained in 75mm^2 flasks, at 37° c and at 5% CO_2. When cells became confluent the cells were scraped off of the surface of the flask and removed with the media. If the cells were to be used for transfection they were counted. This was done by staining a small portion of the cells with Trypan blue and using a haemocytometer to count the number of viable cells. Calculation of the number of cells in solution allowed us to distribute appropriate numbers of cells when plating out the cells. For transfections using RAW 264.7 cells 5x 10^5 cells were distributed into each well of a twelve well plate in 1ml of complete media.

Transfection of RAW 264.7 cells

As stated previously, 5x 10^5 cells were distributed into each well of a twelve well tissue culture plate in 1ml of DMEM with antibiotics and serum. The plates were then incubated under the conditions described previously for 24 hours until the cells were between 50-80% confluent. For each transfection two sterile microfuge tubes were prepared. In one the DNA was added to 100µl of DMEM with no antibiotics or serum.

In the other tube 5µl of LipofectAMINE reagent (Life Sciences, GibcoBRL,UK) was diluted with 100µl of DMEM with no antibiotics or serum. The contents of the two tubes were then combined and mixed gently before incubating at room temperature for 20 minutes to allow the formation of a DNA liposome complex. During the 20-minute incubation the plates of cells were removed from the incubator and the media containing serum and antibiotics was removed. The cells were then washed using DMEM to ensure no serum remained and then 0.5ml of DMEM without antibiotics or serum was added to each well of the plate. After the DNA/Liposome complex had formed 60µl of the complex was added to the appropriate well of the plate.

The cells were incubated for 5hrs (conditions as previously) before 0.5ml of Media containing 20% serum, but no antibiotics was added back to the cells. The DNA/Liposome complex was not removed.

ND7 Cells

ND7 mouse-rat chimeric neuroblastoma cells were cultured in DMEM containing the antibiotics and serum as detailed previously. Cells were maintained in 75mm^2 flasks, at 37° c and at 5% CO_2. When cells became confluent the cells were scraped off of the surface of the flask and removed with the media. If the cells were to be used for transfection they were counted. This was done by staining a small portion of the cells with Trypan blue and using a haemocytometer to count the number of viable cells. Calculation of the number of cells in solution allowed us to distribute appropriate numbers of cells when plating out the cells.

Transfection of ND7 cells

$0.5x10^5$ cells were distributed into each well of a twelve well tissue culture plate in 1ml of DMEM with antibiotics and serum. The plates were then incubated under the conditions described previously for 24 hours until the cells were between 50-80% confluent. For each transfection two sterile microfuge tubes were prepared. In one the DNA was added to 50µl of DMEM with no antibiotics or serum. In the other tube 2.5µl of LipofectAMINE reagent (Life Sciences, GibcoBRL,UK) was diluted with 50µl of

DMEM with no antibiotics or serum. The contents of the two tubes were then combined and mixed gently before incubating at room temperature for 20 minutes to allow the formation of a DNA liposome complex. During the 20-minute incubation the plates of cells were removed from the incubator and the media containing serum and antibiotics was removed. The cells were then washed using DMEM to ensure no serum remained and then 0.5ml of DMEM without antibiotics or serum was added to each well of the plate. After the DNA/Liposome complex had formed 60ul of the complex was added to the appropriate well of the plate.

The cells were incubated for 5hrs (conditions as previously) before 0.5ml of Media containing 20% serum, but no antibiotics was added back to the cells. The DNA/Liposome complex was not removed.

2.2.6 Protein Assay

Protein assay- for CAT assay

Transfected cells were harvested from the six well plates as follows; Media was removed from the cells and before washing in PBS. Once the cells had been washed they were then scraped into 1ml of fresh PBS and transferred to clean microfuge tubes. These tubes were then spun briefly to pellet the cells so that the PBS could be removed. The cell pellets were then re-suspended in 100µl of 0.25MTris.Cl. The cells were then subjected to three cycles of freezing and thawing (1minute liquid Nitrogen, 3 minutes 37° c) before being centrifuged again at 12,000rpm for 10 minutes to allow recovery of the cell extracts. The soluble material was then taken into fresh microfuge tubes and stored at -20° c

A Micro BCA TM (Pierce, Rockford, USA) was used in accordance with manufacturers instructions to determine the protein concentration of samples. 100µl of PBS +0.1%SDS and 100µl of protein assay reagent (made as per manufacturers instructions) was added to 5µl of cell extract. In addition to the samples a standard curve was also produced using BSA at 2mg/ml. Samples were incubated at 37° c on a 96 well microtitre plate for 45

minutes before being read at 570nm, and the protein concentration of each sample calculated.

Protein assay-for luciferase assay

The growth medium was removed from the cultured cells and the cells were then washed in PBS, care was taken not to dislodge any of the cells and as much of this wash as possible was removed. A minimal amount of the 1x Lysis reagent (100µl for a six well plate or 50µl for a 12 well plate) was dispensed into each well of the culture plate. The attached cells were then scraped from the dishes and the cells and solution was transferred to a clean microfuge tube. Cell debris was pelleted by centrifugation at 12,000 rpm for 5 minutes and the supernatant was removed and transferred to fresh microfuge tubes. Biorad protein assay reagent was diluted and used in accordance with manufacturers instructions. In addition to the samples a standard curve was also produced using BSA at 0.22mg/ml. Samples were incubated at room temperature on a 96 well microtitre plate for 5 minutes before being read at 570nm, and the protein concentration of each sample calculated

2.2.7 Chloramphenicol Acetyl Transferase Assay (CAT assay)

Before carrying out the CAT assay the concentration of protein in each sample was calculated as described previously. The amounts of protein used and the incubation times are not the same for each assay and are detailed in each of the individual experiments.

For each transfection a set amount of protein was placed in a microfuge tube and the volume was made up to 90µl with 0.25M Tris.Cl pH7.8. In addition to this 20µl of 4mM Acetyl Coenzyme A, 35ul dH_20 and 0.5ul of [^{14}C] Chloramphenicol. Samples were then incubated for between 1-5 hours at 37° c, depending on the amount of protein used and the activity of the promoter.

After incubation 1ml of Ethyl acetate was added to each sample and vortexed for 30 seconds in order to extract the chloramphenicol and thus terminate the reaction. Samples were centrifuged at 12,000rpm for five minutes separated the mixture into two phases.

The top phase containing the [^{14}C] Chloramphenicol in it's mono, di and un-acetylated forms was removed and transferred to a fresh microfuge tube, the lower phase was discarded.

In order to evaporate off the ethyl acetate and leave only the [^{14}C] Chloramphenicol and its acetylated derivatives, samples were dried down under vacuum in a univap dryer for approximately 1 hour. Once the samples had dried down, they were re-suspended in 10µl of ethyl acetate and spotted onto a Thin Layer Chromatography plate. The plates were then subjected to ascending chromatography in solvent. Once the solvent front had reached the top of the plate, the plate was removed and allowed to air dry before being wrapped in cling film and placed against a phosphorus screen overnight. The screen was then examined using a 'Storm scanner' as per manufacturer's instruction and the percentage conversion of Chloramphenicol and hence the activity of the promoter was calculated.

2.2.8 Luciferase Assay

The growth medium was removed from the cultured cells and the cells were then washed in PBS, care was taken not to dislodge any of the cells and as much of this wash as possible was removed. A minimal amount of the 1x Lysis reagent (100µl for a six well plate or 50µl for a 12 well plate) was dispensed into each well of the culture plate. The attached cells were then scraped from the dishes and the cells and solution was transferred to a clean microfuge tube. Cell debris was pelleted by centrifugation at 12,000 rpm for 5 minutes and the supernatant was removed and transferred to fresh microfuge tubes. 20µl of cell lysate was mixed with 100µl of Luciferase Assay Reagent and the light produced was measured using a TD-20/20 Luminometer (Turner designs, UK), as per manufacturers instructions.

2.2.9 Analysis of transfected cells using Flow-Cytometry

As mentioned previously cells to be analysed by flow cytometry spent 24 hours in colourless media. As it was important that all cells remained in tact these cells were not harvested by scraping but were instead removed from the plates using Trypsin-EDTA.

Media was removed from the cells, which were then washed using PBS. 400ul of Trypsin EDTA was then added to each well and incubated for 5 minutes at 37°c. Following this incubation 1ml of colourless media was then added back to the wells to neutralize the Trypsin-EDTA. The cells and media were then transferred to FACS tubes (Beckton Dickinson, UK) and centrifuged at 1000rpm for 5 minutes to pellet the cells. The cells were then washed three times in PBS to ensure no media remained. After washing the cells were then re-suspended in 0.5ml of fresh PBS before being subjected to analysis using FACScalibur flow cytometer (Becton Dickinson, UK) in accordance with manufacturers instructions. The software used to analyze the data was the Cellquest package (Beckton Dickinson, UK) , and the parameters used for measuring fluorescence were as follows-

P2-372 (Linear)
P3-386 (Linear)
P4-550 (Logarithmic)
P5-650 (Logarithmic)

For each experiment an untransfected sample was analysed first to allow gates to be set which would exclude the background fluorescence. This enabled measurements to be made of fluorescence that was due to the Green Fluorescence that had been transfected into the cells.

2.2.10 Purification of proteins expressed in *E.Coli* as fusions with Glutathione-S-transferase

Screening transformants for expression of protein

Small-scale overnight cultures were set up, by inoculating 10mls of LB (with ampicillin) with single colonies. Following overnight incubation at 37°c, 1ml of this culture was then diluted with 9mls of fresh media. These cultures were then incubated at 37°c with agitation until they reached and OD_{600} of 0.7 (approximately 1 hour). The cultures were then induced with IPTG to a final concentration of 0.1mM, and incubated under the same conditions as previously for a further three hours, or overnight.

Following incubation the bacteria were harvested by centrifugation at 4000rpm for 10 minutes. The resulting pellet of cells was then washed in STE before being re-suspended in 1ml of PBS-Triton. In order to minimize degradation of any protein present in the sample the following protease inhibitors were added at this stage-Leupeptide 5mg/ml (1/100), PMSF 100mM (1/100) and Aprotinin (1/100). The cells were then lysed on ice by mild sonication. The samples were then centrifuged at 12,000rpm for 5 minutes and the supernatent was then transferred to fresh micrifuge tubes. 100ul of preswollen Glutathione-s-transferase beads (50% v/v PBS triton) were added to each sample and incubated with agitation at 4°c. After two washes in PBS Triton and one wash in PBS 100µl of 1x SDS PAGE buffer was added to the samples. The samples were then boiled for 5 minutes to release the protein bound to the beads. Samples were then run on a SDS PAGE gel, alongside prestained protein markers (Sigma, Poole, UK). After destaining the gel was then photographed using the 'Genesnap' (Syngene, UK in accordance with manufacturers instructions.

Large-scale protein purification

50ml overnight cultures were used to inoculate 500ml of pre-warmed LB media (with ampicillin). The cultures were then incubated with agitation at 37°c until they reached OD_{600} of 0.7, when they were then induced with IPTG to a final concentration of 0.1mM. After further three hours incubation the bacteria were harvested by centrifugation at 4000rpm for 20 minutes. The pellet was then washed in STE before being resuspended in 50ml of PBS Triton. Protease inhibitors (concentrations as previously) were then added and the cells were then lysed on ice by mild sonication. Samples w0ere then centrifuged at 12,000rpm for 5 minutes. The supernatant was then mixed with and equal volume of GST beads (50%v/v PBS triton) and incubated for 10 minutes at 4°c with agitation. The beads were then placed in a glass chromatography column where they were washed at 4°c with PBS Triton for 1 hour. Following this wash a further wash was also done using PBS for an additional hour, also at 4°c. The fusion protein was then eluted by competition with free glutathione using elution buffer.

24x 1ml samples were collected and then analyzed by measuring the OD_{260} to identify which fractions contained the protein. The protein containing fractions were then pooled and 20ul of this was run out on a SDS PAGE gel alongside pre-stained protein markers to ensure they were of the correct Molecular weight.

2.2.11 Production of Nuclear and Cytoplasmic extracts

Cells were harvested and scraped into eppendorf tubes. Cells were then resuspended in 800µl of buffer A and incubated on ice for 10 minutes. Following incubation 50µl of NP40 (10%) was added to cells and mixed by vortexing. Cells were centrifuged at 12,000rpm for 30 seconds (4°C) and the supernatant (cytoplasmic extract) was removed to a fresh eppendorf tube). The remaining pellet was then resuspended in 30-50µl of buffer C and incubated on ice for 15-30 minutes before centrifugation at12,000rpm for 30 seconds (4°C). The supernatant (nuclear extract) was removed to a fresh eppendorf tube and the remaining pellet was discarded.

2.2.12 SDS-PAGE

Polypeptide-SDS complexes were fractionated by electrophoretic molecular sieving in polyacrylamide gels according to their size. The proteins were denatured by boiling in SDS loading dye.

Glass plates were cleaned and degreased using 100% Ethanol. The apparatus was assembled in accordance with manufacturer's instructions (Biorad). SDS-PAGE was performed using 10% resolving gels and 4% stacking gels unless otherwise stated. Resolving and stacking gel mixtures were prepared as described in 2.1.10. The resolving gel was poured and allowed to polymerise at room temperature before the stacking gel was poured on top and the comb to allow formation of the wells was inserted before the gel had polymerized..

Once the stacking gel had set the comb was carefully removed and the wells were washed using SDS-PAGE running buffer, as described in 2.1.10. The gel tank was then filled with SDS-PAGE running buffer before the samples were loaded into the wells. Electrophoreisis was carried out at 30mA constant current per gel.

2.2.13 Western Blotting

Following SDS-PAGE the glass plates were separated and the stacking gel removed. The gel was then soaked in transfer buffer, as described in 2.1.10 until it was needed. The PVDF membrane (Millipore) was soaked in methanol for five minutes prior to being soaked for 15 minutes in transfer buffer. 3 pieces of 3mm Whatman paper which had been pre-soaked for fifteen minutes in transfer buffer where then stacked onto the dry graphite electrode of the semi-dry blotter (Biorad) with the membrane placed on top. The gel was placed on top of the membrane underneath a further 3 pieces of pre-soaked 3mm Whatman paper. The stack was then rolled using a pipette tip to ensure air bubbles were removed. The blot was then run at 100mA constant current for 1 hour.

2.2.14 Antibody detection

Detection of proteins bound to the membrane following transfer was by immune detection with specific antibodies. Following transfer the membrane was incubated for one hour with agitation at room temperature in blocking agent. Primary antibody was then diluted to the required concentration in antibody diluent. The membrane was then incubated with the antibody for one hour with agitation at room temperature. Following 3x 5 minute washes in PBS/Tween, the blot was then incubated with the secondary antibody (Horseradish peroxidase conjugated) under the same conditions as for the primary antibody. The blot was then given 2x 10 minute washes in PBS/Tween and 2x 5 minute washes in DI water before detection was carried out using the Enhanced Chemiluminesence (ECL) reagent (Santa-Cruz) in accordance with manufacturer's instructions.

2.2.15 Biotinylated oligonucleotide pull down assay

Oligonucleotides were incubated with approximately 100ng or recombinant protein, 500ng of annealed oligonucleotides and 5µg of salmon sperm DNA in HKMG buffer overnight at 4°c. 20µl of pre-washed (in HKMG) Streptavidin Dynabeads (Pierce) was then added and samples were then incubated at room temperature for 15 minutes to allow the biotinylated oligonucleotide to bind to the beads. Following binding the beads were

then washed 3x in HKMG and then boiled for 5 minutes in SDS-PAGE loading dye. Samples were then analyzed by western blotting.

2.2.16 Chromatin immunoprecipitation assay

$1x10^6$ cells were plated onto 90mm dishes and treated under conditions for which transcriptional activation/repression of the gene of interests had previously been demonstrated. Following treatment and appropriate incubation time histones were cross-linked to DNA by adding formaldehyde directly to culture media at a final concentration of 1% and incubating at 37°c for 10 minutes. Following incubation cells were aspirated to remove culture media and washed twice with PBS containing protease inhibitors. Cells were then scraped into a sterile eppendorf tube and pelleted for 4 minutes at 2000rpm at 4°C.

Following centrifugation excess PBS was removed and cells were re-suspended in 200µl of SDS lysis buffer and incubated on ice for 10 minutes. The lysate was then sheared using sonication to shear DNA into lengths between 200 and 1000 base pairs. Samples were then centrifuged for 10 minutes at 13,000rpm at 4°C and the sonicated supernatant was then transferred to a fresh eppendorf tube, the pellet was discarded. The sonicated supernatant was then diluted 10 fold in ChiP dilution buffer. In order to reduce nonspecific background the samples were pre-cleared using 80µl of DNA/proteinA agarose slurry overnight at 4°C with agitation.

The agarose was then pelleted by brief centrifugation and the supernatant fraction was collected. The immuno-precipitating antibody was then added at the appropriate dilution to the supernatant fraction and incubated at 4°C overnight with rotation. 60ml of salmon sperm DNA/protein A agarose slurry was then added to the samples for 1 hour at 4°C with rotation to collect the antibody/histone complex. The agarose was then pelleted by gentle centrifugation and the supernatant containing the non-bound DNA was carefully removed. The protein A agarose/antibody/histone complex was then washed on a rotating platform for 3-5 minutes with each of the buffers listed in the order given below.
Low salt immune complex wash buffer-two washes

High salt immune complex wash buffer-two washes

LiCl immune complex wash buffer-two washes

TE buffer-two washes

Following washing the histone complex was then eluted from the antibody by adding 250ml of elution buffer to the pelleted protein A agarose/antibody/histone complex, vortexing briefly and then incubating at room temperature with rotation. The agarose was then spun down and the supernatant fraction was then transferred to another tube. The elution was then repeated and the eluates combined. 20µl of NaCl was then added to the eluate and histone-DNA cross links were then reversed by heating at 65°C for 4 hours. DNA was then recovered by phenol/chloroform extraction and ethanol precipitation. Pellets were then washed in 70% ethanol and air dried. Pellets were then resuspended in DNA grade H_2O and samples were then used in PCR reactions with primers for the promoter region of interest.

2.2.17 Construct production

Production of RNAi constructs

As described (3.1.4) the pSilencer ™ 1.0-U6 expression plasmid was used to generatie siRNA within cells. This method, despite the disadvantage of being more labour intensive than some of its alternatives, was selected as it enabled the screening of a number of sequences at a lower cost. Following the recommendations for insert design and using the Ambion siRNA target finder and design tool-
Http://www.ambion.com/techlib/misc/siRNA_Finder.html
siRNA constructs were designed against the following targets using their cDNA sequences (with the exception of the murine Miz-1 sequence which was provided by Steffi Herold (Herold *et al,* 2002)). Once selected sequences were subjected to a BLAST search to ensure there was no significant sequence identity with other genes.

- Human Miz-1- AAG GCC GAG ATC AGC AAA G
- Murine Miz-1- AAC TGG GCC TCC TCT GTG A
- Murine c-Myc- AAG GAC TAT CCA GCT GCC A

- Murine Sp1- AAT AAT GGG GGT AGC GGC A
- Human IREG1- AAT TGA ATC TGA AAG AGG C

Oligonucleotides were produced as described for the target sequences shown above. These were then cloned into the ApaI and EcoR1 restriction sites present in both the oligonucleotide insert and the pSilencer ^{TM}1.0-u6 vector. Following ligation and subsequent transformation into competent *E.coli*, positive clones containing the siRNA insert were selected via a *Hind* III digest. (See Figure 3.3.1).

Production of eGFP-chimeric constructs for validation of RNAi efficacy.

c-Myc-GFP

The effectiveness of the RNAi process on the endogenous gene can only be assessed if cells can be transfected with high efficiency. Raw264.7 used for some of these studies are notoriously difficult to transfect (<1%) and therefore an alternative strategy was developed to monitor the RNAi process. c-Myc ORF was amplified using the PCR to create a c-Myc cDNA fragment with synthetic *EcoR1* and *BamHI* site at the 5' and 3' ends respectively. This was then inserted into the pEGFP-N3 (Clontech) vector via the *EcoRI* and *BamHI* sites at positions 631 (*EcoRI*) and 661(*Bam HI*) in the multiple cloning site of the pEGFP vector (figure 3.3.2).

Miz-1-GFP and IREG-1- GFP

The Miz-1-GFP (Bowen *et al*, 2003) and IREG1-GFP (McKie *et al*, 2000) were both produced in the pEGFP-N3 vector using the same method as for the c-Myc GFP constructs.

***Nramp1* Promoter constructs- pL4/ pL4M6M /pL4Sp1M**

The *Nramp1* luciferase construct pL4 was prepared, by Alter Koltunoff (Bowen *et al*, 2003). A 1.654 Kbp region from the XbaI site at -1555Kbp, to a synthetic BamHI site introduced immediately downstream of exon 1 at position +99bp was ligated into the pGL3 and is a derivative of the Nramp1 construct pHB4, figure 4.3.1. (Bowen *et al*, 2002). The constructs pL4M6M and pL4Sp1M were generated by Emma Phillips

(Bowen *et al*, 2003) from pL4 using the CAT constructs pHB4M6M and pHB4Sp1M (Bowen et al, 2002) respectively in a similar manner to pL4.

***Nramp1* Inr mutant promoter constructs**

The *Nramp1* Inr mutant promoter constructs were produced using the following pairs of synthetic oligonucleotides-

Pair 1: *Nhe* Linker

Sense 5'- CTAGAGGTCTGGAGGG-3'

Antisense 5'-TCCCCTCCAGACCT-3'

Pair 2

NRP#5: 5'- AGCACCCACAGAAGGGGACAGATTGAG-3'

NRP#6: 5'- GATCTCCAATCTGTCCCCTTCTGTGGGTG-3'

Pair 3

NRP#3:5'- GATGGGAAGGGCGTGGGT**TC**CCACTCTTACTCACTCGGACC-3'

NRP#4:5'- CTGGTCCGAGTGAGTAAGAGTGGGAACCCACGCCCTTCCCA-3'

Pair 4

NRP#15: 5'-GATGGGAAGGGCGTGGGTTCCCACTCTTGCTCACGGGGACC-3'

NRP#16: 5'-CTGGTCCCCGTGAGCAAGAGTGGGAACCCACGCCCTTCCCA-3'

Pair 5

NRP#17: 5'-GATGGGAAGGGCGTGGGTGCCCACGGGTACTCACTCGGACC-3'

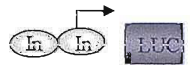
NRP#18: 5'- CTGGTCCGAGTGAGTACCCGTGGGCACCCACGCCCTTCCCA-3'

Pair 6

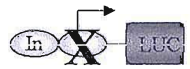
NRP#19-5'-GATGGGAAGGGCGTGGGTGCCCACGGGTGCTCACGGGGACC-3'

NRP#20-5'-CTGGTCCCCGTGAGCACCCGTGGGCACCCACGCCCTTCCCA-3'

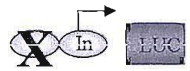
Pairs of oligonucleotides were annealed by combining equimolar amounts of constituent oligonuleotides, placing in a water bath at 95°C and cooling to room temperature.

Wild Type 

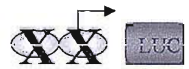
Pairs 1, 2 and 3 were inserted into a *Nhe/BglII* digested pGL3 basic vector. This produced an 84bp fragment corresponding to -34bp to +34bp (Plus the *Nhe* linker fragment) of the *Nramp1* promoter.

Mutant 1 

Pairs 1, 2 and 4 were inserted into a *Nhe/BglII* digested pGL3 basic vector. This produced an 84bp fragment corresponding to -34bp to +34bp (Plus the *Nhe* linker fragment) of the *Nramp1* promoter, with the second Inr/Inr like element mutated.

Mutant 2 

Pairs 1, 2 and 5 were inserted into a *Nhe/BglII* digested pGL3 basic vector. This produced an 84bp fragment corresponding to -34bp to +34bp (Plus the *Nhe* linker fragment) of the *Nramp1* promoter, with the first Inr element mutated.

Double mutant 

Pairs 1, 2 and 5 were inserted into a *Nhe/BglII* digested pGL3 basic vector. This produced an 84bp fragment corresponding to -34bp to +34bp (Plus the *Nhe* linker fragment) of the *Nramp1* promoter, with both the first Inr and the second Inr/Inr-like element mutated.

Myc Mutant V394D

The Myc mutant V394D (Valine-Aspartic Acid substitution) was created via a two-step PCR amplification from the c-Myc expression vector pEF-c-Myc. The first fragment was produced using a 5' vector arm primer and the anti-sense primer carrying the mutation-
(5'GAA AAG GCC CCC AAG GTA GAT ATC CTT AAA AAA GCC ACA GCA 3')
The second reaction was carried out using sense primer carrying the mutation-
(5'TGC TGT GGC TTT TTT AAG GAT ATC CTT GGG GGC CTT TTC 3')
and the 3' vector arm primer. The primers carrying the mutation were designed to substitute a non-polar Valine residue for a negatively charged Aspartic acid residue in the

HLH domain of c-Myc preventing c-Myc-Miz-1 interaction (Herold *et al*, 2002). After PCR the two products were purified, annealed and 10 PCR cycles were carried out in absence of primers to extend anneal mutant product. Then the 1.4Kbp sequence with 5'BamHI site and 3' EcoRI site was then amplified by adding the 5'vector arm and 3'vector arm primers. The resultant fragment was then cloned via ligating between BamHI and EcoRI sites in pcDNA3.1.

Miz-1 NLS

The Miz-1 NLS construct was created by the insertion of the following pair of synthetic oligonucleotides (Sigma Genosys, UK) between the *EcoR1* and *BglII* restriction endonuclease sites (positions 631 and 611 respectively in the pEGFP-N3 vector) in the Miz-1 GFP construct.

Sense- 5' GATC TCC GCC ACC ATG GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTG 3'

Antisense- 5' AAT TCA CCT TTC TCT TCT TTT TTG GAT CTA CCT TTC TCT TCT TTT TTG GAT CTA CCT TTC TCT TCT TTT TTG GAT CCA TGG TGG CGG A 3'

This sequence contains the nuclear localization sequence (NLS) from the construct pECFP-Nuc (a variant of the pEGFP protein), and a consensus Kozak sequence to increase the translational efficiency of the Miz-1 NLS mRNA.

The oligonucleotide pair was annealed by combining equimolar amounts of each which are then placed in a water bath at 94°C and allowed to cool slowly to room temperature

pGex-2TK constructs

pGex-2TK/Miz-1

Miz-1 was amplified via the polymerase chain reaction (PCR) to create a Miz-1 cDNA with synthetic *EcoRI* and *BglII* sites at the 5' and 3' ends respectively. This was then cloned into the pGex-2TK expression vector via the *EcoRI* and *BamHI* sites present in the MCS of this vector (Figure 4.3.4)

pGex-2TK/c-Myc

The pGex-2TK/c-Myc construct was produced using the same method as for the Miz-1 construct with the only difference being the introduction of a synthetic *BamHI* site instead of *BglII* at the 3' end of the c-Myc insert produced by the PCR reaction. This was possible, as unlike Miz-1, c-Myc does not have an internal *BamHI* site.

pGex-2TK/Miz-1 S46A

The S46A mutant Miz-1 was produced using the Quikchange technique with the pGex-2TK/c-Miz-1 as a template. The serine to alanine substitution was made using the following primers-

Sense: 5'-GGCCCGCTGTGCGGAGTACTTCAAGATGCTCTTCG-3'
Anti-sense:5'-GAAGTACTCCGCACAGGCCGCCAGCACTGCTTTATG-3'

The PCR conditions for the reaction were as follows- 17X (94°C 1min, 60°C 1min, 72°C 16 mins).
Following digestion with *Dpn1* (37°C-2 hours) DNA was transformed into XL-1 blue ultra-competent cells (as per manufacturer's instructions). Resulting colonies were picked and DNA was purified as described in 2.2.2. The presence of the S46A mutation was confirmed by sequencing (MWG, UK).

pCDNA3.1/S46A/Δ33-60 Miz-1

The Miz-1 ORF was amplified using PCR with the pGex-2TK/Miz-1 S46A construct as a template to create a mutant Miz-1 fragment with synthetic *BglII* and *EcoR1* sites at the 5'

and 3' ends respectively. This was then inserted into the pCDNA3.1 vector via *BamHI* and *EcoR1* sites in the MCS of the pCDNA3.1 vector. The presence of the S46A mutation was confirmed by sequencing (GRI, UK). During production of the point mutant a 27-codon deletion mutant spontaneously arose (Δ33-60 Miz-1). These mutants were analysed in parallel.

Production of a Miz-1 Antibody

A rabbit polyclonal Miz-1 antibody was produced by injection of a synthetic polypeptide corresponding to a sequence located at the C-terminus of the murine Miz-1 protein, as shown below-

AESPPTAPDCLPPAE

The peptide was injected into two rabbits #24 and #38 (Borek Vojtesek) and immune sera from the second bleed was tested for its reactivity (by means of a dot blot) against the synthetic peptide, the Miz-1 GST fusion protein and as a negative control, a solution of 2mg/ml BSA. Appendix 4 shows the results of the dot blot carried out using the immune sera from rabbit #38 at a dilution of 1:500. This dilution was deemed to be appropriate for use as compared to others tested (not shown) and to give better results than the immune sera from rabbit #24, as a strong signal was detected without significant background.

NCAM promoter constructs

WT NCAM promoter

A 2.095Kb promoter fragment was removed from the CAT NCAM construct NCAM#1 (Barton *et al*, 1990) via a BamHI/SacI digest. This insert was then cloned into the pGL3 basic vector via the SacI and BglII sites present at in the MCS of the pGL3 basic vector.

NCAM Sp1M/ Sp1 GAL4

A unique EcoR1 restriction site was introduced in place of the Sp1 site by 2-step PCR with PFU to create the NCAM-Sp1M construct. A synthetic double stranded oligonucleotide pair incorporating a DNA-GAL4 binding site was cloned into a partial fill in EcoR1 site;

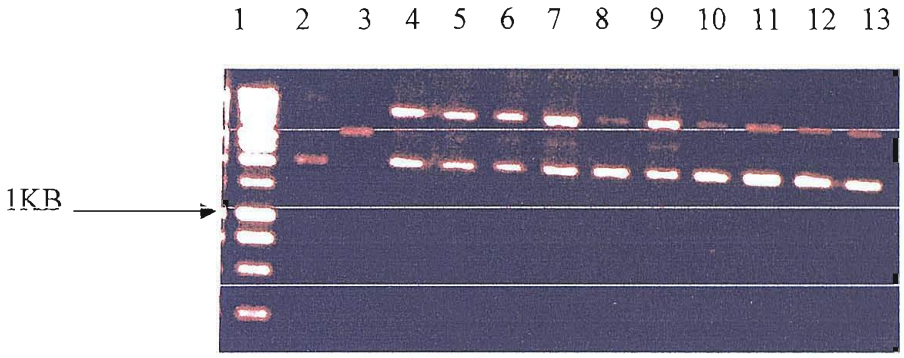
Sense 5'- tttCGGGTGACAGCCCTCCGA-3'

Antisense 3'-aGCCCACTGTCGGGAGGCTtt-5'

(Lower case letters represent bases introduced to enable cloning).

Recombinant clones were sequenced to confirm the presence of the insert in the correct orientation (GRI, UK)

A)



B)

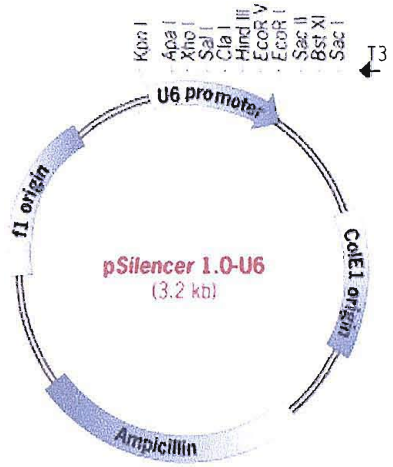
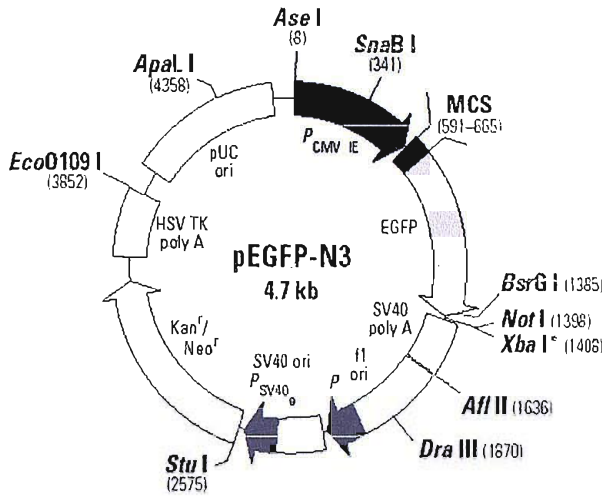


Figure 2.3.1 Restriction digests confirming presence of RNAi insert in pSilencer™ 1.0 U6 Vector, and plasmid map . A) 2µl of DNA mini-preparations were incubated with 1µl of *HindIII* restriction endonuclease at 37°c for 45 minutes. If the RNAi insert is present the *HindIII* site present in the vector will be lost and the plasmid will fail to linearise. Digests were loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes. (1) 1Kb ladder (Promega, UK), (2) pSilencer empty vector-Uncut,
(3) pSilencer empty vector-cut, (5) Human Miz-1 RNAi-uncut, (6) Human Miz-1 RNAi-*HindIII*, (7) Murine Miz-1 RNAi-uncut, (8) Murine Miz-1 RNAi-*HindIII*,
(9) c-Myc RNAi-uncut, (10) c-Myc RNAi-*HindIII*, (11) Sp1 RNAi-uncut, (12) Sp1 RNAi-*Hind III*. B) pSilencer™ 1.0U6 plasmid ma

A)



B)

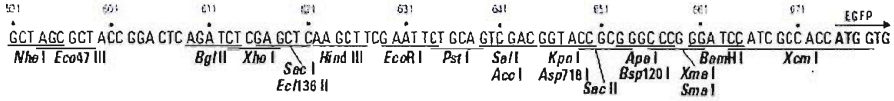


Figure 2.3.2 Plasmid map and multiple cloning site of the pEGFP N3 vector.

A) pEGFP N3 vector

B) Multiple cloning site (MCS) of pEGFP N3 vector.

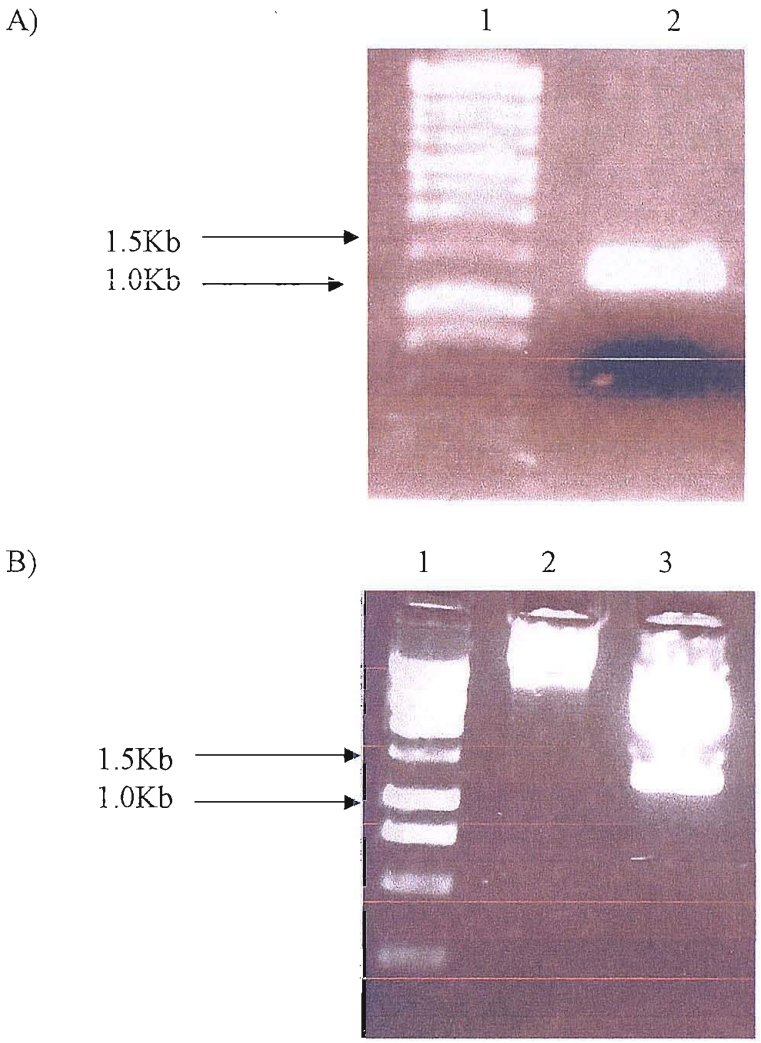
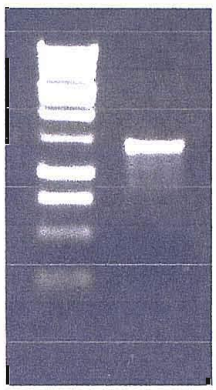


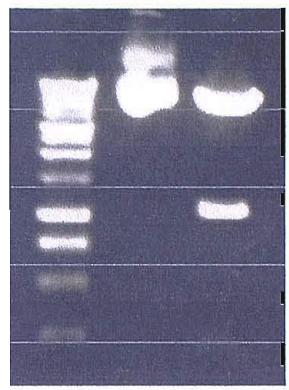
Figure 2.3.3 Production of an N3 Myc GFP construct in the N3 eGFP vector cloned in via *Bam/Eco* (insert) and *BglII/Eco* (Vector). **A)** PCR product following Gel extraction and purification , (1) 1Kbp ladder (Promega, UK), (2) PCR product of c-Myc ORF.

B) Recombinant N3/Myc clone. 2µl of DNA mini preparation was incubated with 0.5µl of *NheI* and 0.5µl of *EcoRI* restriction endonucleases at 37°c for 45 minutes. If correct the digest should drop out the 1.4kbp c-Myc insert. Digests were loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes. (1) 1Kbp ladder (Promega, UK), (2) c-Myc GFP clone 1- uncut, (3) c-Myc GFP clone 1- *NheI/EcoRI*

A)



B)



C)

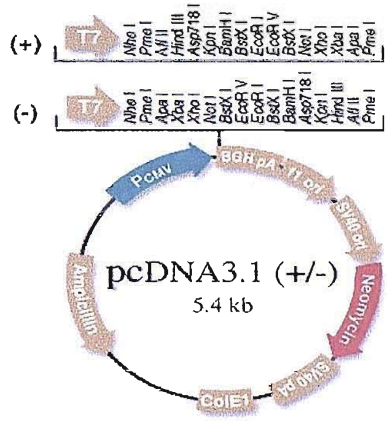


Figure 2.3.4 Myc Mutant V394D PCR product, restriction digest confirming presence of Myc V394D mutant in vector pcDNA 3.1 and pcDNA3.1 plasmid map.
A) Product of PCR reaction to produce V394D Myc mutant. (1) 1Kbp ladder (Promega, UK), (2) 5µl of PCR product.

B) EcoRV restriction digest confirming presence of V394D mutation. 2µl of DNA mini-preparation were incubated for 45 minutes at 37° with 1µl of *EcoRV* restriction endonuclease (Promega,UK). If correct this digest should result in two fragments dropping out due to the introduction of an *EcoRV* site at +1182 in c-Myc as a result of the mutation, the EcoRV site already present in c-Myc at +144 and the *EcoRV* site in the vector. (1) 1Kbp Ladder (Promega, UK), (2) V394D Mutant, clone 1-Uncut, (3) V394D mutant, clone 1- *EcoRV*. For A and B all DNA was loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes .

C) pcDNA3.1 plasmid map and Multiple Cloning sequence (MCS)

1 2 3 4 5

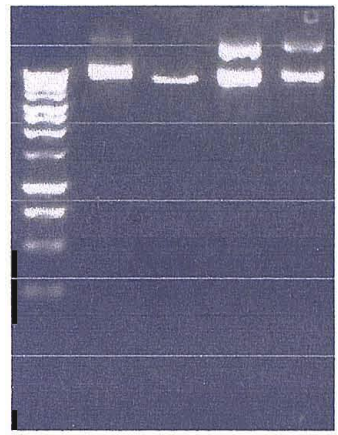


Figure2.3.5 Miz-1 NLS GFP construct.

2µl of DNA Mini-preparations were incubated for 45 minutes at 37° with 1µl of *HindIII* restriction endonuclease (Promega,UK). If correct this digest should show the loss of the *HindIII* restriction endonuclease site at position 621 in the pEGFP-N3 vector and therefore DNA should not cut. Digests were loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes (1)1Kbp DNA ladder (Promega) (2) pEGFP-N3 empty vector -uncut (3) pEGFP-N3 empty vector-*HindIII* (4) Miz-1 NLS clone 1- uncut (5) Miz-NLS clone 1- *Hind III*.

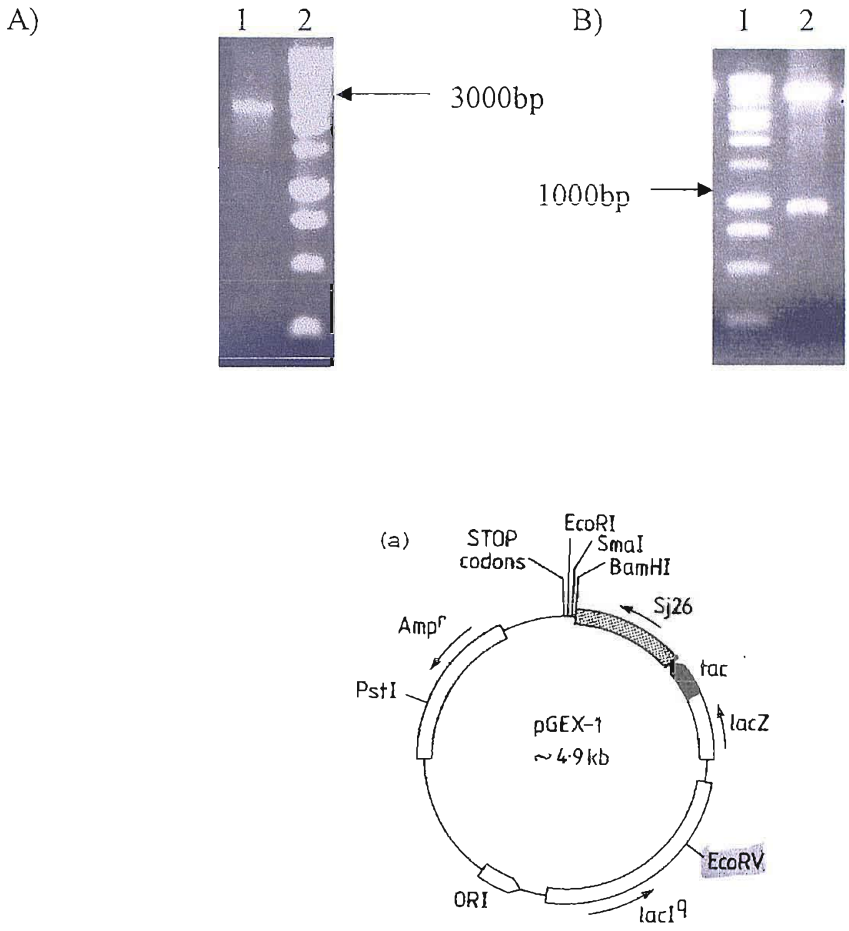


Figure 2.3.6 Production of pGex/Miz-1 constructs to enable expression of Miz-1/GST fusion protein. A) 2.4Kb Miz-1 PCR product, (1) PCR product, (2) 1Kbp ladder (Promega, UK) B) Recombinant pGex/Miz-1 Clone 1. 2µl of DNA mini preparation was incubated with 0.5µl of *BamHI* and 0.5µl of *EcoR1* restriction endoculeases at 37°c for 45 minutes. If correct the digest should drop out a 1Kb fragment. The digest was loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 45 minutes. (1) 1Kbp ladder (Promega, UK), (2) pGex/Miz-1 Clone 1-*BamH1/EcoR1*.

A) 1 2 3

B)

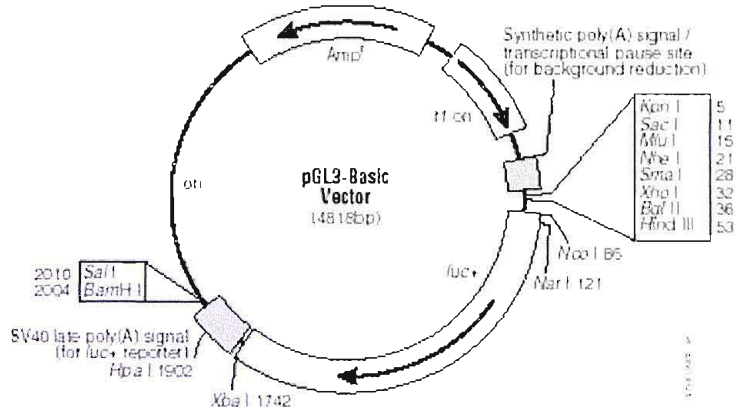


Figure 2.3.7 Cloning the *NCAM* promoter region into pGL3 basic. The NCAM promoter region was dropped out of an NCAM CAT promoter construct (Barton *et al*, 1990) using a *SacI/BamHI* digest and was cloned via *SacI/BglII* into the pGL3 vector (as shown in figure 5.3.1). To confirm the presence of the insert the empty vector DNA should run more slowly than the plasmid containing the insert. A) 2µl of mini-prep DNA was loaded onto a 1% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes (1) 1Kbp ladder, (Promega, UK), (2)pGL3 basic empty vector, (3) pGL3 basic/NCAM clone 1 .

CHAPTER 3

RNA interference

3.1 Introduction

3.1.1 What is RNA interference?

RNAi is a phenomenon leading to Post-Transcriptional Genes Silencing (PTGS) following either artificial introduction or endogenous production of siRNA that has sequences complementary to that of a particular target gene. This prevents translation of the mRNA and expression of the respective protein due to degradation of its mRNA. RNA interference was initially discovered in the nematode worm *Caenorhabditis elegans* (Fire *et al*, 1998), when it was noted that the injection of double stranded RNA into cells had much more of an effect on gene silencing than antisense RNA which had previously been shown to inhibit translation of proteins by hybridizing with target mRNA and blocking translation or causing mRNA degradation.

It is thought that the natural function of RNA interference is to serve as a primitive immune response against parasitisation by foreign nucleic acids such as RNA from viral pathogens (Ramaswamy *et al*, 2002). In addition to this RNAi is thought to suppress the expression of transposons which may otherwise destabilize the genome by acting as insertional mutagens (Hannon *et al*, 2002).

3.1.2 Molecular mechanisms of RNA interference

The mechanism of RNA interference is not fully understood. However, there is a general consensus of initial steps. On entering a cell double stranded RNA is first processed into 21-23 nucleotide double stranded fragments with two nucleotide overhangs on the 3' end, called siRNA's (Bernstein *et al*, 2001). The RNASE III enzyme Dicer carries out this process. Dicer contains helicase, dsRNA binding and PAZ domains. The helicase and dsRNA binding domains are involved in unwinding the double stranded RNA and mediate protein-RNA interaction. The role of the PAZ domain (so called after Piwi, Argonaute and Zwille proteins) is not known (Bernstein *et al*, 2001). Following the production of siRNA's by Dicer there are two proposed mechanisms for RNAi.

The first of these is the 'Random degradative PCR model'. In this model it is proposed that the siRNA's act as primers and bind to the target mRNA. This mRNA is then extended using nucleotide addition using the enzyme RdRp. This leads to the production of double stranded mRNA. The enzyme Dicer then degrades the double stranded mRNA resulting in the destruction of the target sequence and the production of more siRNA's (Lipardi *et al,* 2001) to sustain the RNAi process.

The second model, that is favored by many is the 'Endonucleolytic cleavage model'. Here it is proposed that the siRNA's are bound to a complex of proteins called RISC. Activation of this complex by ATP causes the siRNA to unwind and guide the activated complex to the target mRNA by Watson-Crick complementary base pairing followed by cleavage at a site located approximately in the middle of the double stranded region (Nykanen *et al,* 2001. Bernstein *et al,* 2001). This specifically interrupts gene expression at a post-transcriptional level.

Each RISC complex is believed to contain only one of the two strands from the siRNA (Martinez *et al,* 2002). Recent studies have shown that each of the two strands is not equally eligible for entry into the RISC. Work by Schwarz *et al,* has suggested that assembly of the RISC is controlled by an ATP-dependent Helicase which selects which strand of the siRNA is taken into the RISC. The strand which has a less tightly paired 5' end is always chosen. It has been suggested (Schwarz *et al,* 2003) that siRNA should be designed based on the ease of entry of the anti-sense strand into the RISC and that siRNA duplex structure can be used to explain ineffective siRNA.

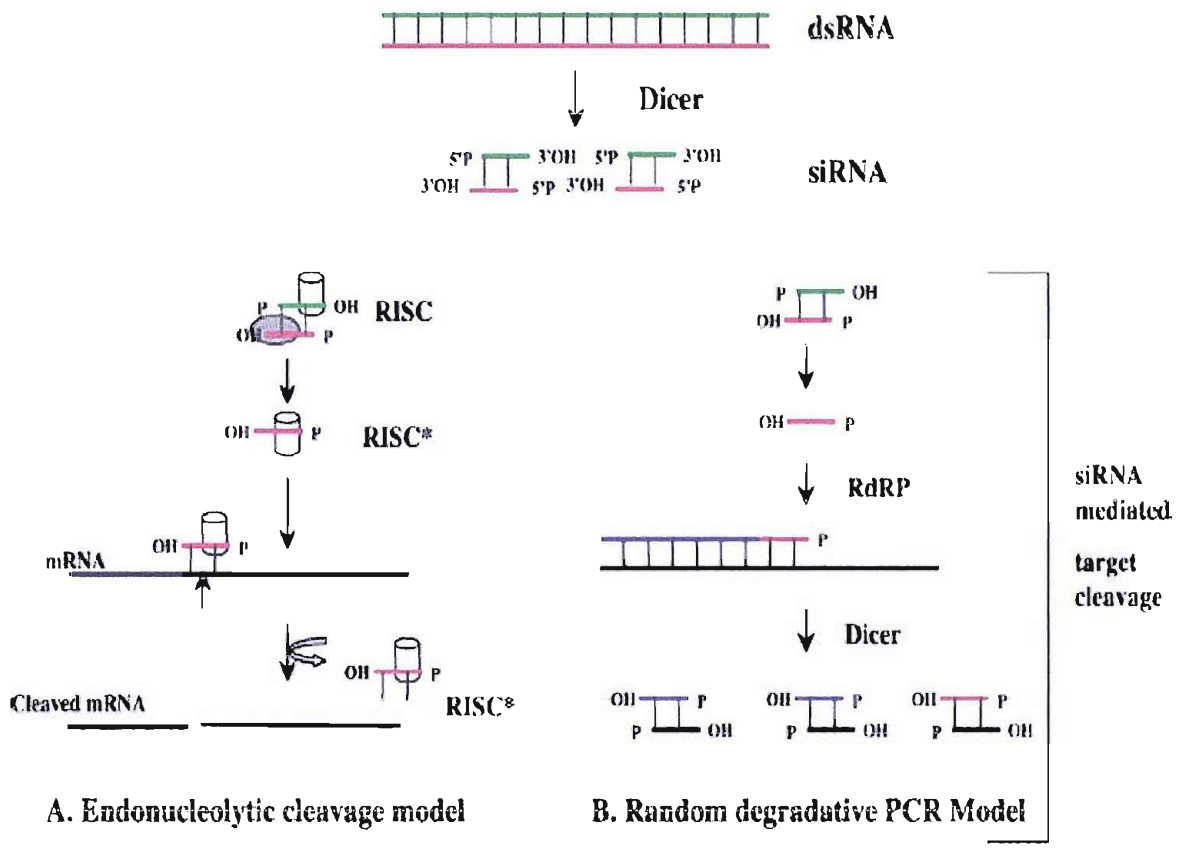


Figure 3.1.1.1 The proposed models of RNAi; Taken from Ramaswamy & Slack, 2002

3.1.3 Delivery of siRNA into cells

At present there are a number of strategies available for the carrying out siRNA mediated gene silencing in cells. All that is required is the cDNA sequence of the gene of interest and commercially available reagents with which to perform the synthesis of the siRNA.

The most rapid method for the production of siRNA is by using chemical synthesis. And due to the speed and high purity of siRNA's produced in this way it is becoming increasingly popular. An alternative to this is *in vitro* siRNA synthesis. In this technique T7 phage polymerase produces individual siRNA's sense and antisense strands which when annealed form siRNA's (Donze *et al*, 2002)

Alternatively, siRNA's can be produced using DNA plasmids or expression cassettes with a RNA polymerase III promoter. These have the benefit that once introduced into cells they produce multiple copies of the siRNA sustaining the PTGS.

3.1.4 pSilencer ™ 1.0-U6 siRNA expression vector

The work carried out in this thesis has made use of the pSilencer expression vector (Ambion). This method of producing siRNA's was chosen over others, as it is more economical for the production of multiple sequences, although it is more labour intensive.

With this vector based system a 19 nucleotide sequence is selected from the target gene which is used in the production of two DNA oligonucleotides each of approximately 55 bases which when annealed together form the insert for the pSilencer vector (figure 2.3.1) In the forward oligonucleotide the 19 nucleotide sense sequence is linked to the reverse complementary siRNA sequence by a 9 nucleotide spacer (TTCAAGAGA) and 5-6 T's are added at the 3' end of the oligonucleotide.

In the reverse oligonucleotide 4 nucleotide overhangs at *EcoR1* and *Apa1* recognition sites are added at the 5' and 3' end of the 54 nucleotide sequence. The resulting RNA transcript folds back and forms a stem loop structure known as shRNA comprising a 19 base pair stem and 9 nucleotide loop with 2 uridines at the 3' end as this has been found

to facilitate RNAi (Elbashire *et al*, 2001). The shRNA's are then processed in cells by Dicer into siRNA's (Paddison *et al*, 2002).

Work presented in this chapter serves to detail the production and validation of several RNAi constructs produced using the pSilencer ^{TM} 1.0-U6 expression plasmid. These constructs are used in functional experiments in later chapters of this thesis.

3.2 Results

3.2.1 Specificity and effectiveness of RNAi constructs against their targets.

3.2.1.1 Human Miz-1 Construct

A constant amount (0.5μg) of a human Miz-1-eGFP construct (cloned into N3 vector by A. Westwood) was transfected into the African green monkey kidney cell line, Cos-1, using LA as described in 2.2.5. Cells were co-transfected with the human Miz-1 RNAi construct, or as a control the pSilencer^{TM}1.0-U6 empty vector. Fluorescence levels were then detected using flow cytometry (Becton Dickinson Flow Facscaliber) and the mean fluorescence levels were recorded. Figure 3.3.1 shows the mean fluorescence readings for both titrations and these data demonstrated that this construct is active in knocking down its target mRNA. The mean fluorescence dropped from 237.9 fluorescence units with 0μg of RNAi to 139.95 with 1.0μg of Miz-1 RNAi, a knock down of over 40%. The specificity of this construct against its target is demonstrated by the separation between the data points for the RNAi construct and the control at both 0.8 and 1.0μg. This specificity is confirmed by co-transfection of the N3 Wild-type eGFP vector and the Miz-1 RNAi (3.3.2). This was again measured using flow cytometry, but in this instance untransfected cells were also analysed in order to detect background or auto-fluorescence in the cells. Background fluorescence was then gated out and the value 'percent gated' refers to those cells fluorescing above the background level. Figure 3.3.2a (A) shows that the N3 Wild-type eGFP empty vector was not affected by the Human Miz-1 RNAi construct even at levels as high as 2.0μg. However, the effect of the Miz-1 RNAi on the Miz-1 GFP could be seen clearly with a maximum knockdown of fluorescence at 1.4μg. At this concentration the percentage of cells gated falls from 9.05% at 0μg of RNAi to just 2.5%, a knockdown of over 70%.

3.2.3.2 Generation of a control RNAi construct

To avoid potential non-specific effects as a result of the introduction of double-stranded RNA into cells, a control RNAi construct was produced in the pSilencer^{TM} 1.0-U6 vector. This construct was originally designed against the IREG-1. However, flow cytometry data using this RNAi construct revealed that it was not effective at knocking down its

target. Cos-1 cells were co-transfected with a constant amount (0.5μg) of an pN3-eGFP IREG-1 construct and increasing amounts of either the IREG-1 RNAi or the pSilencer ^{TM}1.0-u6 vector. Untransfected cells were also analysed via flow cytometry to enable background fluorescence to be gated out. As shown in figure 3.3.3 there is very little difference between the values obtained using the RNAi construct and the empty vector control as compared to the Miz-1 RNAi construct in figure 3.3.1 which significantly knocked down its target at this concentration.

Confirmation of the inactivity of the IREG-1 construct allowed it to be used in subsequent experimental work in the place of the pSilencer ^{TM}1.0-u6 empty vector control, which had been used previously. However, the non-recombinant vector did not result in the production of siRNA molecules in cells and therefore the IREG-1 construct may be considered more appropriate for the potential non-specific effects caused by dsRNA.

Using this control RNAi as an alternative to the pSilencer ^{TM}1.0-u6 empty, vector Miz-1 RNAi/Miz-1 GFP co-transfections were repeated in Cos-1. Cells were transfected with a constant amount (0.5μg) of the Miz-GFP and 1.4 μg (Which had previously been shown to give the largest knockdown Figure 3.3.4B) of the Human Miz-1 or control (IREG-1) RNAi. Cells were analyzed 48 hours after transfection using Fluorescence microscopy (Nikon, Eclipse E8000) cells were scraped from the tissue culture dish that they had been transfected in and wet mounted in PBS.

Comparison of the brightfield and fluorescence images of cells co-transfected with Miz-1 GFP and the control RNAi (3.3.4) showed a transfection efficiency of over 50%. This level of expression within the cells is sufficient for the RNAi construct being used to have significant effects on the endogenous protein levels within the transfected cells. Co-transfection of the Miz-1GFP with the Human Miz-1 (3.3.4B) showed that at the same exposure time (2500m/s) the fluorescence levels are significantly reduced and are undetectable at this exposure.

3.2.3.3 Murine c-Myc construct

Production of an pN3-Myc eGFP construct allowed the effectiveness of a knockdown using a c-Myc target RNAi to be assessed. Cos-1 African green monkey kidney cells were co-transfected with a constant amount of the N3 Myc construct (0.5μg) and increasing amounts of either the c-Myc RNAi or the control (IREG-1) RNAi. These transfections were carried out in triplicate. As had been done previously un-transfected cells were also analyzed using flow cytometry thus allowing any background fluorescence to be gated out and the value 'percent gated' to be measured referring to cells fluorescing above the background. At the highest concentration of RNAi used (figure 3.3.5) there was a significant 'knockdown' of fluorescence using the c-Myc RNAi as compared to the control (IREG-1) RNAi. Comparison of these values using the students T-Test gave a P value of 0.026 showing that this difference is significant at the five percent level. However, levels of fluorescence in this cell type using the pN3-Myc eGFP construct were much lower than with the Miz-1 eGFP construct and this is likely to be due to problems associated with the accumulation of this particular transcription factor eGFP chimera in cells.

This work was then repeated using the ND7 Mouse-Rat chimera neuroblastoma cell line and the reagent LipofectAMINE (as described in 2.2.5). In this experiment cells were transfected using a either the N3 eGFP Wild-type vector or the N3 Myc construct (0.5μg) and 1.5μg of either the c-Myc or the control (IREG-1) RNAi (figure 3.3.6). Students T-tests show that there was no significant knockdown of the N3 eGFP Wild-type vector using the c-Myc RNAi compared to the control RNAi construct P=0.869, however when the N3 Myc construct was used a 2-fold reduction in gated cells was observed with c-Myc RNAi, P=0.020.

3.2.3.4 Murine Sp1 Construct

The efficacy of the Sp1 RNAi construct was tested against pMSp1Gal4 and the *Nramp1* promoter construct pL4Sp1MGal4 (Phillips *et al*, Unpublished). Compared with a control (IREG-1) RNAi construct, Sp1 RNAi provided 8-fold knockdown of the luciferase reporter gene (P=0.026). However, without pMSp1Gal4 there was no

significant difference between the levels of reporter genes with either RNAi construct (P=0.9).

In addition to these experiments Western blot analysis was also carried out in order to determine whether there was a significant knock down of endogenous Sp1 protein. ND7 cells were transfected (as described in 2.2.5) with 1.5μg/90mm dish of either Sp1 or control (IREG-1) RNAi. Following 48 hour incubation cells were harvested and nuclear and cytoplasmic extracts were produced as described in 2.2.11 Western blotting of these extracts using an Sp1 antibody (Upstate) at a concentration of 1:1000 (secondary 1:15,000) revealed that endogenous Sp1 protein levels were reduced following transfection with the Sp1 RNAi. Figure 3.2.8 shows two exposures. The upper film represents the shorter exposure time (30 seconds) the lower represents the longer exposure time (1 minute). Comparison of Sp1 present in the nuclear extracts can be seen at both exposures to be reduced in the cells treated with the Sp1 RNAi. In addition to this, closer inspection of the longer exposure reveals that a small amount of Sp1 is present in the cytoplasmic extract produced from the control cells. No Sp1 can be detected in the cytoplasm of the cells treated with Sp1 RNAi. Taken together these data indicate activity of the RNAi construct is directed against Sp1.

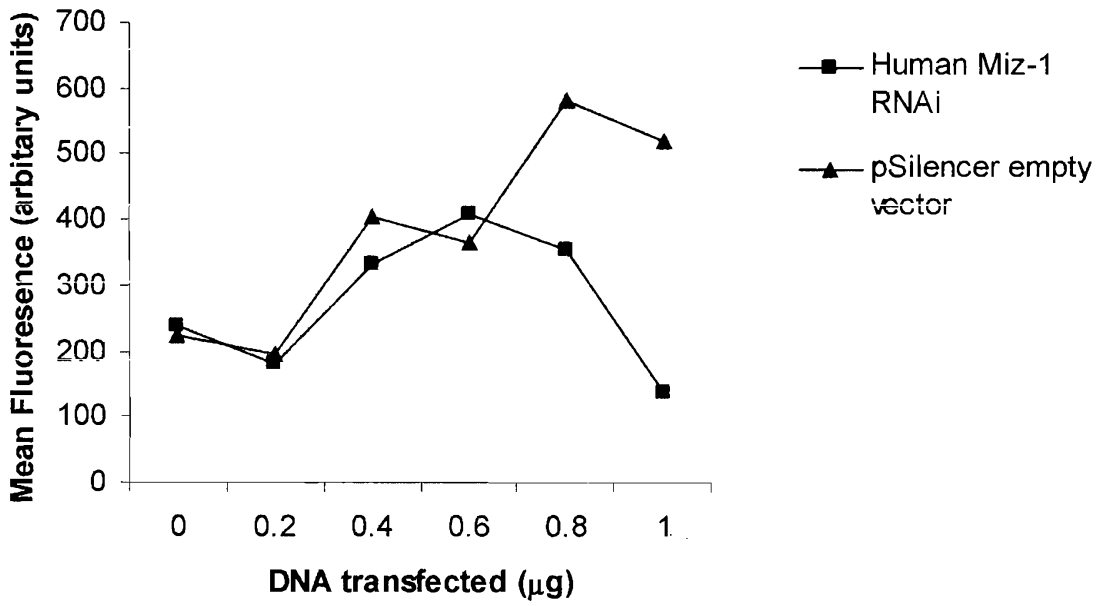
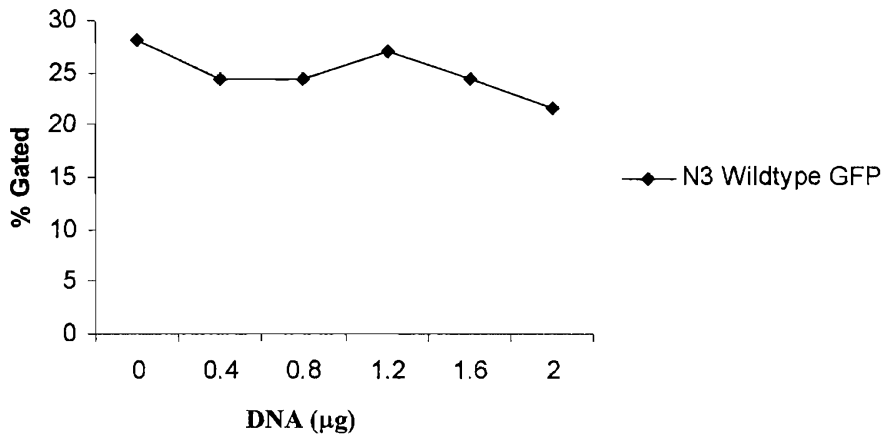


Figure 3.3.1 Co-transfection of Miz-1 GFP construct with either Human Miz-1 RNAi construct or empty vector control within the Cos-1 cell line. Cos-1 cells were transfected as described in 2.2.5 with 0.5µg of a chimeric Miz-GFP construct and increasing amounts of either the human Miz-1 RNAi construct or the empty vector control as indicated. Plasmid DNA was made up to 1.5µg using the pBABE empty vector. Levels of fluorescence were detected using flow cytometry. Values shown are the mean fluorescence level for 10,000 cells analyzed.

A)



B)

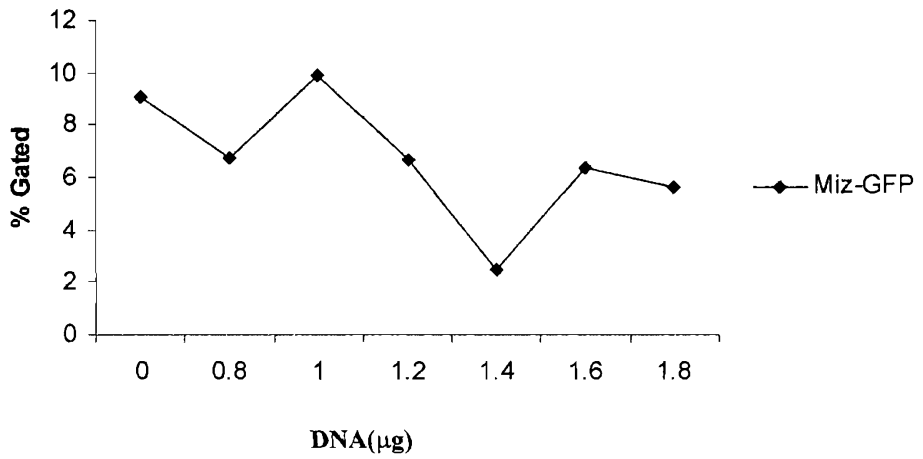


Figure 3.3.2 Co-transfection of either the N3 Wildtype eGFP vector (A) or the Miz-1 GFP construct (B) with the Human Miz-1 RNAi construct in the Cos-1 cell line. Cos-1 cells were co-transfected, as described in 2.2.5 with either 0.5µg of wild type N3 eGFP or the Miz-1 GFP construct and increasing amounts (as shown) of the human Miz-1 RNAi. DNA concentrations were made up to 2.5µg (A) or 2.3µg using the pSilencer empty vector. Levels of fluorescence were detected using flow cytometry. Values shown are the mean fluorescence level of 10,000 cells analyzed.

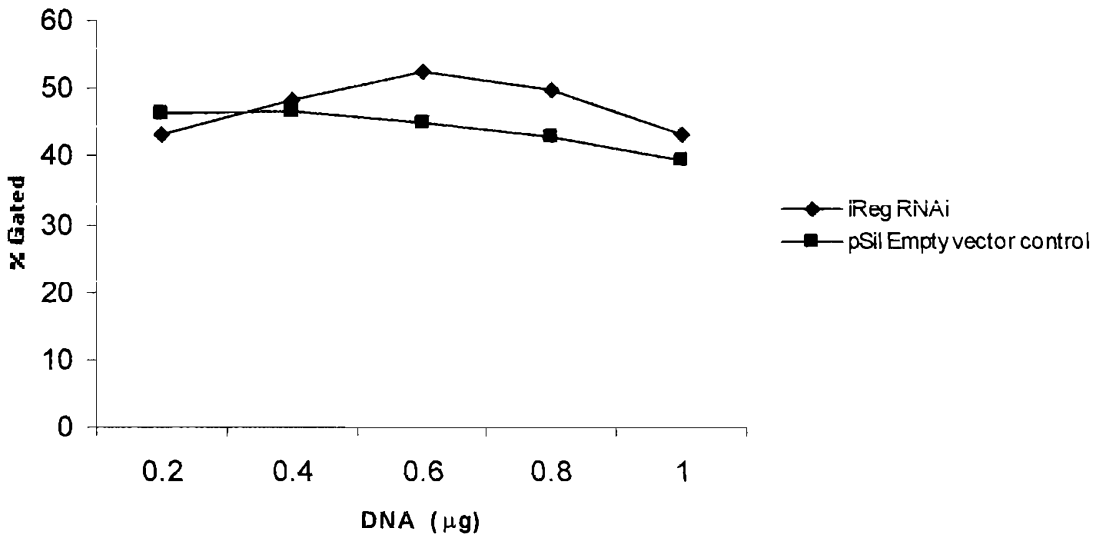
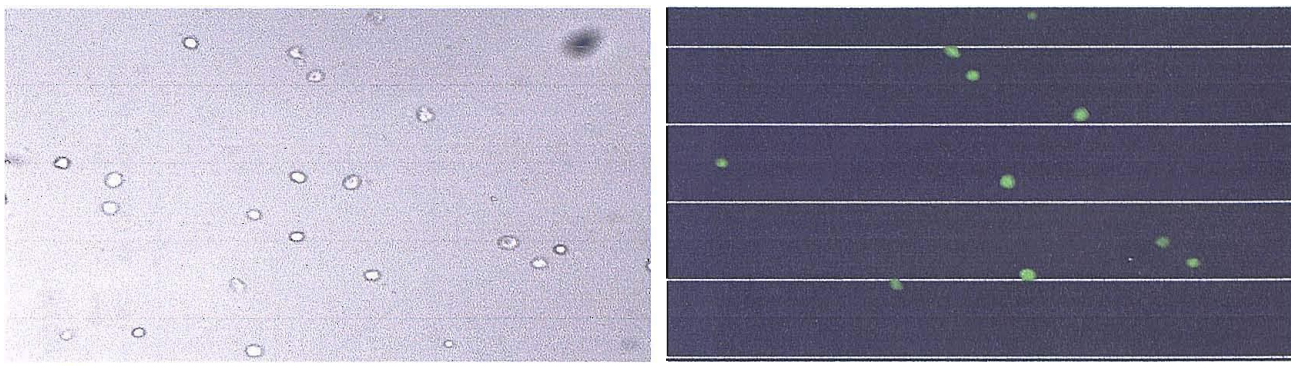


Figure 3.3.3 Co-transfection of IREG-1-GFP construct with either IREG-1 RNAi construct or empty vector control within the Cos-1 cell line. Cos-1 cells were transfected as described in 2.2.5 with 0.5µg of IREG-GFP construct and amounts of either the IREG-1 RNAi construct or the empty vector control as indicated. DNA concentrations were made up to 1.5µg using the pBABE empty vector. Levels of fluorescence were detected using flow cytometry. Values shown are the mean fluorescence level of 10,000 cells analyzed.

A)



B)

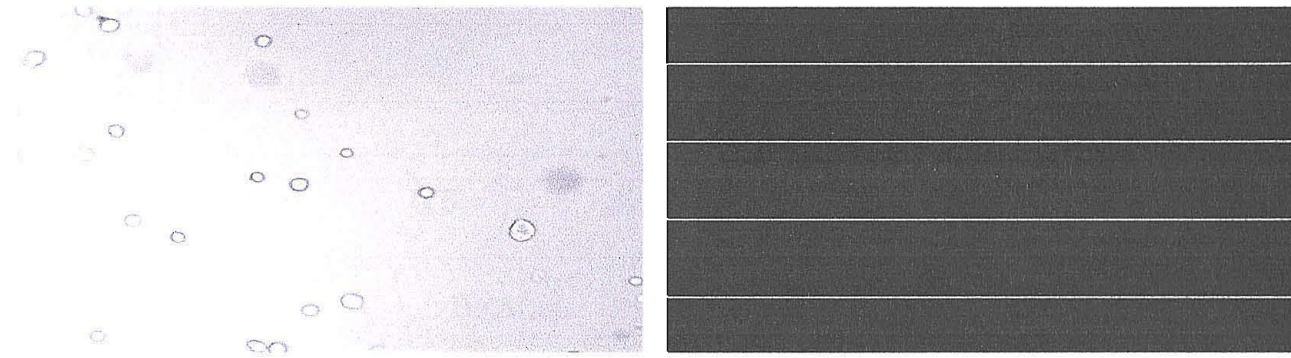


Figure 3.3.4 Co-transfection of Miz-1 GFP construct with either Human Miz-1 RNAi construct or a control RNAi within the Cos-1 cell line. Cos-1 cells were transfected as described in 2.2.5 with 0.5μg of Miz-1 GFP construct either 1.4μg of the human Miz-1 RNAi (A) or the 1.4μg of the control (IREG-1) RNAi construct. Following a 48 hour incubation cells were analyzed using the Leica Eclipse E8000 microscope (x10, 2500 m/s exposure time). Images on the left hand side show the cells under bright field. The image on the right hand side shows the same cells using fluorescence microscopy.

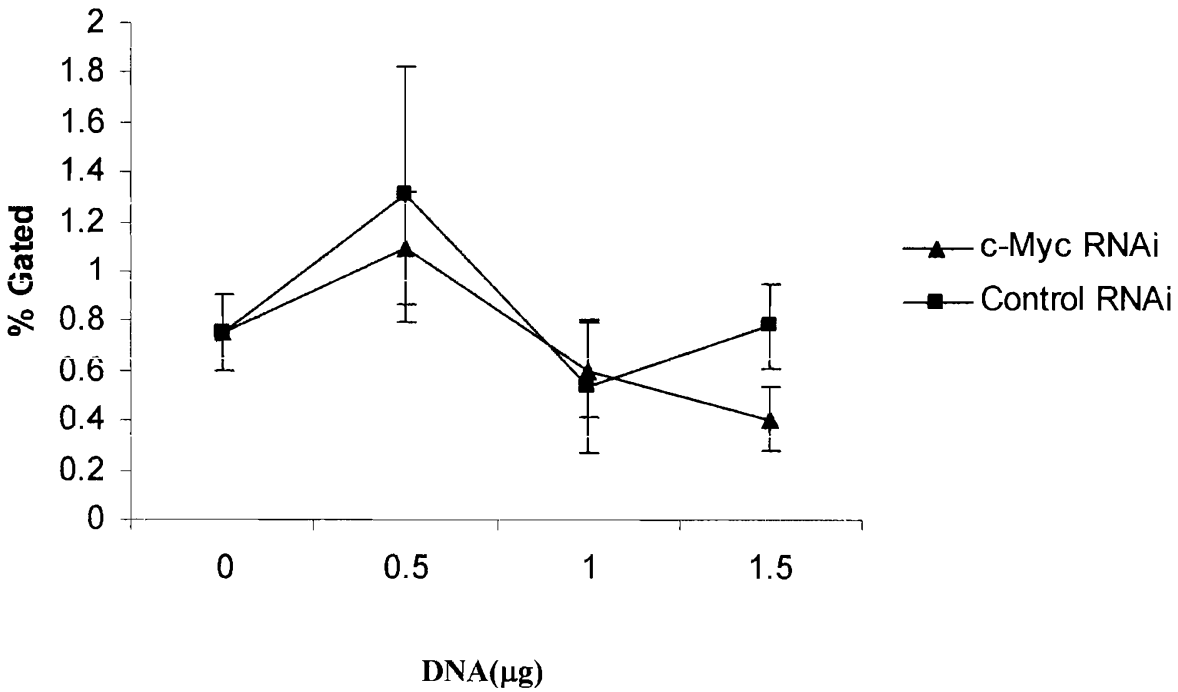


Figure 3.3.5 Co-transfection of c-Myc GFP construct with either murine c-Myc RNAi construct or control (IREG-1) RNAi construct within the Cos-1 cell line. Cos-1 cells were transfected in triplicate as described in 2.2.5 with 0.5µg of c-Myc-GFP construct and increasing amounts, as indicated of either the murine c-Myc RNAi construct or the control (IReg) RNAi construct. DNA concentrations were made up to 2.0µg using the pBABE empty vector. Levels of fluorescence were detected using flow cytometry.

Students T-tests comparing 1.5µg of RNAi construct P=0.026

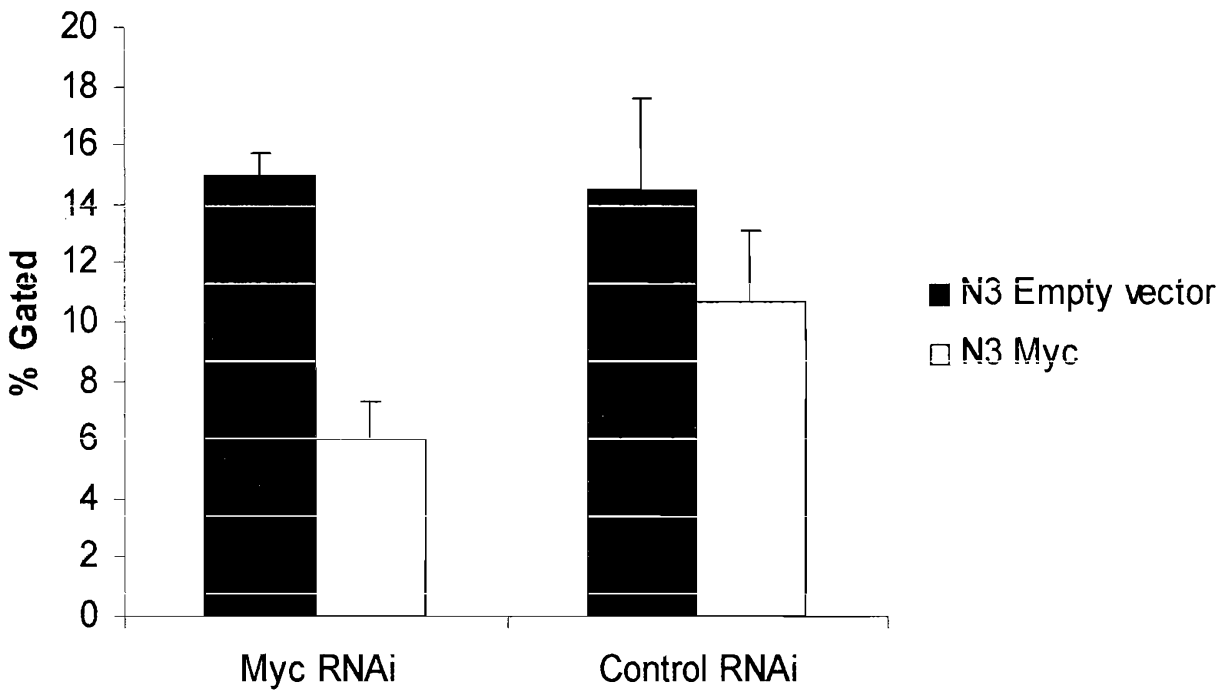


Figure 3.3.6 Co- transfection of ND7 cells with N3 wild type/ N3 Myc and Myc RNAi. ND7 cells were co-transfected as described in 2.2.5 with 0.5μg of either N3 wild type eGFP vector or the c-Myc GFP construct and 1.5μg of either Murine c-Myc RNAi or the Control (IREG-1) RNAi as shown.

Students T-Tests- c- Myc GFP+Myc RNAi/Control RNAi P=0.020
N3 Wild type eGFP +Myc RNAi/Control RNAi P=0.869

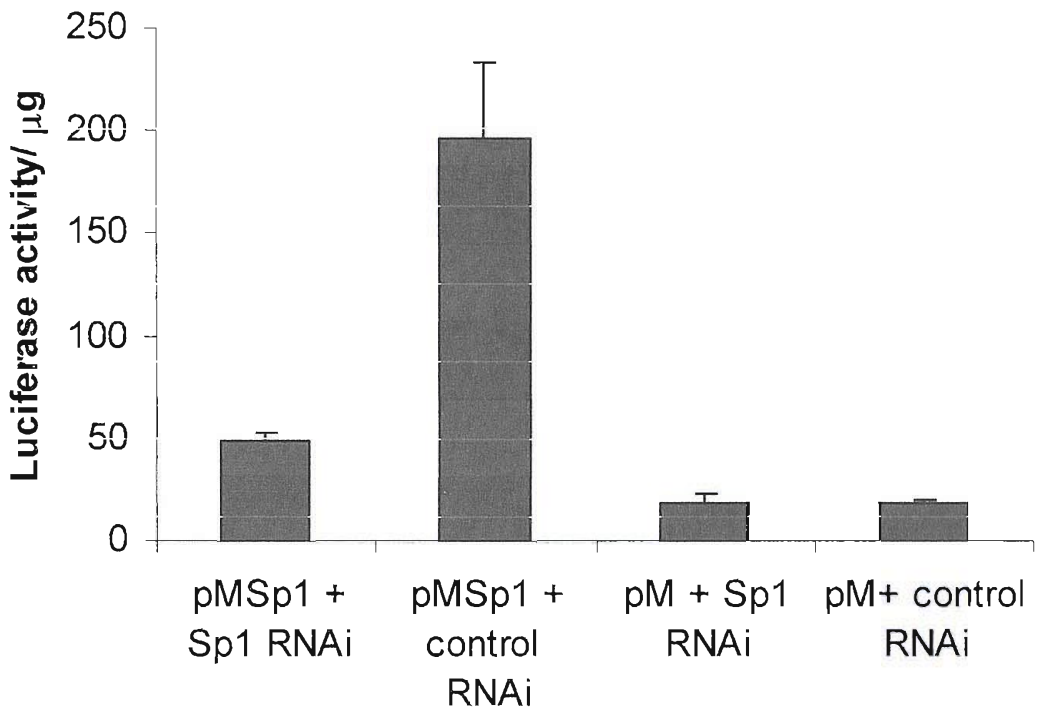
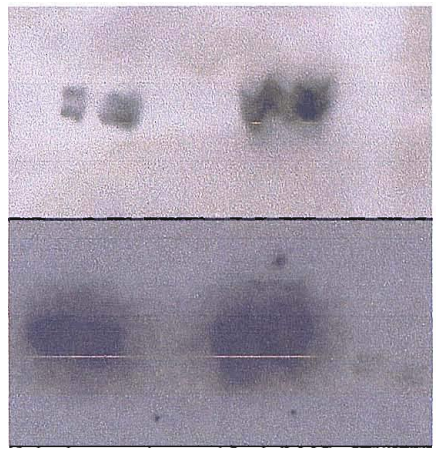


Figure 3.3.7 Validation of Sp1 RNAi construct using GAL4 Constructs RAW 267.4 cells were co-transfected as described in 2.2.5 with 1.0µg of the pL Sp1MGal4 construct and 1.0 µg of either the PM or PMSp1Gal4 and 1.0 µg of either the control or the SP1 RNAi construct as indicated

Students T-tests PMSp1 +/- Sp1 RNAi P=0.026, PM +/-Sp1 RNAi P=0.888

A) 1 2 3 4



B) 1 2 3 4

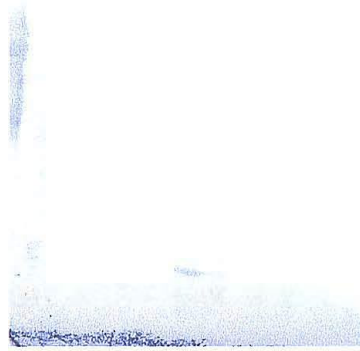


Figure 3.3.8 Western blot to show knock down of endogenous Sp1 protein using Sp1 RNAi construct. ND7 cells were transfected (as described in 2.2.6) with either a Sp1 or a control RNAi construct. Following a 48 hour incubation cells were harvested and Nuclear and Cytoplasmic extracts were made as described in 2.2.11. Extracts were western blotted (as described in 2.2.12). With a Sp1 antibody (Upstate) at a concentration of 1:1000 (Secondary 1:15,000).
A) Photographic film upper and lower films represent shorter and longer exposures respectively- 1) Sp1 RNAi, Nuclear (2) Sp1 RNAi, Cytoplasmic (3) Control RNAi nuclear (4) Control RNAi Cytoplasmic.
B) Amido black stainded PVDF membrane from A

3.4 Discussion

Data presented in this chapter demonstrated the efficacy of each of the RNAi constructs used and in each case demonstrate specificity against their target mRNA. Validation of the murine Miz-1 construct was not carried out as it has been for the other constructs as this construct was created using a published target sequence (Herold *et al*, 2002).

Although the length of dsRNA used for RNAi is below that which is required to induce an interferon response it has been reported that activation of interferon signaling and Interferon Stimulated Gene (ISG) expression can occur following the transfection of siRNA into cells or as a result of shRNA expression using vector based systems (Bridge *et al*, 2003, Sledz *et al*, 2003).

Bridge *et al* (2003) used microarray analysis to look at the effects on ISG's of a shRNA targeting MORF4L1 in human lung fibroblasts. In this study a large number of ISG's were found to be induced in a response which was shown to be unrelated to the silencing of MORF4L1. This study also revealed that shRNA's expressed using a U6 vector as compared to an H1 vector resulted in higher ISG induction. More recent studies looking at the expression of shRNA in cells (Pebernard & Iggo, 2004) have shown that this may be a consequence of the presence of an AA di-nucleotide repeat near the transcription start site.

Research into the effects of siRNA transfection into cells (Sledz *et al* 2003) has revealed that transfection of siRNA into cells can induce non-specific effects including the triggering of cytokine production. In these studies a range of unrelated siRNA's were shown to induce IFN mediated activation of the Jak-Stat pathway and a global up-regulation of ISG's. It is clear from these studies that off target or non-specific effects of RNAi must always be considered as a possible explanation for the results obtained.

The development of a control RNAi construct (IREG-1) enables the potential non-specific effects of transfecting double stranded RNA into cells to be controlled. This effect was not accounted for in early work which relied on the use of the pSilencer ™

1.0-U6 empty vector as a control. The use of a dsRNA control, in this case the IREG-1 RNAi made it possible to control for the more broad effects beyond the selective silencing of a gene that may occur when RNAi is used.

Fluorescence microscopy using the Miz-1 eGFP construct allowed assessment of transfection efficiency to be quantitated. The efficiency observed was sufficient (over 50%) to ensure that enough of the cells were transfected with the RNAi to cause a significant reduction in endogenous levels of the target protein.

Due to problems encountered in obtaining a murine Sp1 cDNA clone it has not been possible to create a Sp1 eGFP construct. However co-transfection of this construct with reporter genes known to be regulated by Sp1 as compared to a control RNAi has indicated that this construct is effective against its target and that its effects are specific. In addition to this the efficacy of the Sp1 RNAi construct was tested against pMSp1Gal4 and the *Nramp1* promoter construct pL4Sp1MGal4 (Phillips *et al*, Unpublished) Compared with a control (IREG-1) RNAi construct. Western blot analysis also confirms a drop in the levels of Sp1 present in cells following transfection with the Sp1 RNAi construct.

Problems were encountered with the c-Myc eGFP construct due to low levels of fluorescence in cells before the use of the RNAi. Previous data (fluorescence microscopy figure 3.3.4) has shown that the transfection efficiency of these cells using the LipofectAMINE (described in 2.2.5) is over fifty percent. Therefore it is likely that this low level of fluorescence is due to problems associated with high levels of c-Myc being present in cells. Over expression of c-Myc has been shown to trigger apoptosis in normal cells (Askew *et al*, 1991). It is believed that this pathophysiological response is due to c-Myc induced hyperproliferation which induces a 'stress' response that triggers apoptosis in order to eliminate the cell (Scherr *et al*, 2001). Therefore, it is possible that those cells expressing the c-Myc eGFP at high levels may have been eliminated by apoptosis.

Despite problems encountered, these data clearly showed that the c-Myc RNAi construct is effective at silencing its target. This is demonstrated by the inability of the control (IREG-1) RNAi to reduce levels of fluorescence and in further support, the inability of the c-Myc RNAi to reduce the levels of fluorescence using the wild-type eGFP. In addition to these findings ChIP analysis was able to reveal a loss of c-Myc bound at the NCAM promoter following transfection of cells with c-Myc RNAi.

Taken together the data presented in this chapter demonstrate that all of the RNAi constructs used in this thesis in later chapters are effective against their targets.

CHAPTER 4

Analysis of the *Nramp1* promoter

4.1. Introduction

4.1.1 Discovery of *Nramp1*

The phenotype for which *Slc11a1* was identified around thirty years ago and is more commonly referred to by its previous designation *Nramp1*, as it shall be referred to throughout this thesis. *Nramp1* was originally known as *Ity/Lsh/Bcg* for its role in regulating resistance or susceptibility to *Salmonella typhimurium*, *Leishmania donovani* and *Mycobacterium bovis* BCG infections in mice. *Nramp1* is expressed in the murine macrophage (Gros *et al*, 1983) and was the first mouse gene to be cloned using the technique of positional cloning (Vidal *et al*, 1993). *Nramp1* encodes a 548 residue polypeptide of molecular weight 59.7 KDa (Barton *et al*, 1994) which functions as a divalent cation transporter (Cellier *et al*, 1995). Nramp1 protein is localised at the membrane of cytoplasmic vesicles in both resting and activated macrophages derived from bone marrow (Atkinson et al, 1997) and is recruited to the phagosomal membrane upon macrophage activation (Gruenheid *et al*, 1997; Atkinson *et al*, 1997).

Nramp1 encodes the prototypic member of a family of highly conserved proteins of predicted transport function. At least three members of the family are found in the mouse; Nramp1, Nramp2 (Gruenheid *et al*, 1995) and Nramp r-s (Dosik *et al*, 1994). Nramp1 has also been identified in humans (Blackwell et al, 1995) where it has been shown to control infections with a number of antigenically unrelated pathogens and is thought to contribute to autoimmune and infectious disease susceptibility.

4.1.2 Biochemical function of *Nramp1*

At present there are two models regarding the biochemical function of *Nramp1* and the mechanism of bacterial growth restriction. The first proposes that *Nramp1* increases the concentration of intraphagosomal Fe^{2+} in order to provide a catalyst for the Haber-Weiss/Fenton reaction-

$$Fe^{3+} + \cdot O_2^- \longrightarrow Fe^{2+} + O_2;\ Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^-$$

This reaction results in the generation of hydroxyl radicals that are highly toxic and have bactericidal activity (Goswami *et al*, 2001; Kuhn *et al*, 1999). In this case Nramp1 would pump cations using the proton gradient to power transport i.e. antiport- where the transport of protons and cations occurs in opposite directions.

The second model proposes that Nramp1 serves to deplete the intraphagosomal lumen of divalent cations, thus depriving micro-organisms contained within the phagosome of Fe^{2+} and other cations critical for their growth (Gomes & Appelberg, 1998; Jabado *et al*, 2000). This is termed the bacteriostatic mechanism.

4.1.3 The role of the macrophage in iron metabolism

The reticuloendothelial system is also called the mononuclear phagocyte system and is made up of monocyte macrophages and bone-marrow precursor cells (Lydyard & Grossi, 1996). One of the common tasks of this system is the recycling of iron from senescent red cells and resident macrophages of the liver and spleen; a role fulfilled by splenic macrophages and Kupffer cells. Recruited macrophages must also fulfil a similar role to prevent toxicity from liberated haem during haemorrhagic injury. Iron is delivered to most cells following uptake by endocytosis of the plasma iron binding protein transferrin, bound to its cell surface receptor, TfR a dimer of 90KDa subunits which associates with a regulatory molecule called HFE. This allows the uptake of iron from transferrin (Reviewed, Richardson and Ponka 1997). Once the newly formed endocytic vesicle is acidified, iron is released from the transferrin/transferrin receptor complex, then the complex is recycled and the trivalent iron is reduced by ferrireductase then divalent iron is pumped into the cytosol by Nramp2. Iron levels are regulated post transcriptionally by two cytoplasmic iron regulatory proteins IRP1 and IRP2. These iron response proteins bind to IREs present in the mRNA of factors involved in iron metabolism, namely TfR and ferritin, a sequesterer of iron. If the cytoplasmic iron levels are low the IRPs bind to the IREs making the TfR mRNA more stable and decreasing the translation of ferritin. Conversely if the cytoplasmic iron levels are high the IRPs are unable to bind to the IREs

resulting in degradation of the TfR mRNA, expression of ferritin and thus iron storage is facilitated. The availability of cytosolic free or redox active iron is crucial for metabolic purposes and for cell proliferation, although the exact nature of this iron pool is poorly defined. However, it needs to be rigorously controlled, as it can be accessed by pathogens for growth and can participate in Fenton chemistry resulting in the formation of reactive oxygen species that can damage the cell.

In addition to having control over iron regulatory genes at a post-transcriptional level, work by Wu *et al*, 1999 has shown additional control by the proto-oncogene c-Myc at a transcriptional level. As detailed in this section, iron is crucial for cell growth and for any tumour to proliferate it is necessary that it can acquire its own supply of iron and at the cellular level this includes enhanced expression of genes associated with iron acquisition. Conversely genes that limit iron availability are suppressed. Wu and colleagues were able to show that c-Myc repressed the H-ferritin promoter in a dose-dependent manner by using a luciferase reporter gene driven by the H-ferritin promoter, co-transfected with c-Myc into monkey CV1 cells. This repression could not be induced when using c-Myc with mutations in Myc box 2, (needed for trans-repression) or the helix-loop-helix domain (needed to form a dimer with Max). It was also shown that mutations in the initiator element, a core promoter element of the H-ferritin promoter removed the ability of the wild type c-Myc to cause repression.

In this work researchers also investigated whether c-Myc controlled other genes involved in iron homeostasis. They observed that the over-expression of c-Myc in rat fibroblasts and CB33 cells was associated with increased levels of the IRP2. Combined with the H-ferritin results this indicated that c-Myc has a role in co-ordinately regulating the expression of genes controlling iron homeostasis, with complementary affects that serve to increase the intracellular iron pool. This is consistent with other data that show iron chelation leads to growth arrest (Broxmeyer *et al*, 1991).

These findings prompted Bowen *et al*, (2002) to investigate the role of c-Myc in the regulation of *Nramp1*, as previous work had provided data to indicate that *Nramp1*

expression correlated with a reduced availability of redox-active or "free" iron within the cytosol. Work by Bowen revealed that c-Myc repressed the *Nramp1* promoter via a mechanism dependent on the initiator elements present in the promoter region. The mechanism involved the recently discovered Miz-1 gene (Peukert *et al*, 1997) and in the absence of c-Myc, Miz-1 stimulated *Nramp1* transcription.

4.1.4 Analysis of the *Nramp1* promoter

Nramp1 is composed of 15 exons spanning 11.5Kb of genomic DNA (Vidal *et al*, 1993). Primer extension analysis and S1 nuclease mapping (Govani *et al*, 1995) identified several minor and one major transcription initiation sites. Nucleotide sequencing (Govani *et al*, 1995) revealed that *Nramp1* lacks classical TATA or CAAT elements. This finding corresponded to the precise lack of initiation site and multiple start sites. However, two alternative start sites 'initiator elements' were identified (Govani *et al*, 1995), located at positions -5 and -16 relative to the major transcriptional start site-

- $CA_{+1}CTCGCT$
- $TCCCA_{+1}CTCTT$ (A_{+1} is the transcription start site)

These two elements correspond to the consensus sequence for initiator elements found within mammalian genes (Smale and Baltimore, 1989)-

- Py-Py-A_{+1}-N-T/A-Py-Py

Work by Govani *et al* (1995), also identified several *cis*-acting sequences, elements associated with the binding of both ubiquitous and tissue specific transcription factors. These ubiquitous factors included binding sites for activator proteins, AP1, AP2 and AP3 located at positions, -6,-25 and -36 and -114bp respectively. The second of the AP2 sites was found to overlap an Sp1 binding motif, GC box located at position -26bp. *Cis*-acting motifs that are known to influence the response of the macrophage to induction by differing agents were identified in the region spanning -256 to +127bp, these included a binding site for the transcription factor PU.1, a member of the ETS family of transcription factors at -173bp. Also present in this region were several sequences associated with inducible gene expression by IFN-γ (-183,+70,+128,+118 and +127bp),

and bacterial LPS (-144 and -243bp) which are of importance due to their role in mediating macrophage differentiation or activation.

Work presented in this chapter makes use of RNAi, validated in chapter 3, transient transfection, and binding studies using recombinant protein, to analyze further the effects of the transcription factors c-Myc, Miz-1 and Sp1 upon *Nramp1* transcriptional regulation.

4.2 Results

4.2.1 Stimulatory role of Miz-1 in the regulation of *Nramp1* transcription

Use of RNAi to reduce endogenous Miz-1 levels reveals that Miz-1 is essential for *Nramp1* transcription (Figure 4.3.2a). Transient transfections were carried out using the wild-type promoter construct (pHB4) or a pHB4M6M, with its mutated E-Box and the human Miz-1 RNAi construct. Data are expressed as activities relative to control cultures set at 100%. As has been shown previously, the pHB4M6M construct is more active than pHB4, 74-fold (P=0.03). Transfection into Cos-1 cells with 0.5μg of Miz-1 RNAi construct and 0.5μg of promoter construct gave 3.1-fold and 10-fold reductions in promoter activity of pHB4 and pHB4M6M respectively.

RNAi has been used to knock-down endogenous Miz-1 in the murine Raw264.7 cell, with the murine Miz-1 RNAi construct (Herold *et* al, 2002). The effect of this murine Miz-1 RNAi construct alongside the human Miz-1 RNAi and the control (IREG-1) RNAi construct on *Nramp1* promoter activities from pL4 in the murine macrophage cell line RAW 267.4 is shown (Figure 3.4.2b). Co-transfection of the *Nramp1* promoter construct with the murine Miz-1 RNAi construct results in a ~90% diminution of transcription compared to the human Miz-1 or control RNAi constructs. These data also serve to demonstrate species specificity of the murine Miz-1 RNAi (Human Miz-1 RNAi / Control P=0.118, Murine Miz-1 RNAi / Control P=0.020).

The c-Myc mutant V394D (figure 4.3.3) confirms that repression of the *Nramp1* promoter by c-Myc is due to the interaction of the HLH domain of c-Myc with Miz-1 (Herold *et al,* 2002)and not via any other mechanism associated with Sp1, as has been described for p15 (Feng *et al* 2000,2002). c-Myc V394D does not interact with Miz-1 and fails to repress either of the *Nramp1* constructs tested as compared to the repression seen when co-transfection is carried out with the wild-type c-Myc expression plasmid. The pL4 construct shows greater than 12-fold reduction in promoter activity when co-

transfected with 2.5μg of c-Myc expression plasmid (P=0.003). No significant reduction in transcription is seen when co-transfection is carried out with the same concentration of the V394D mutant expression plasmid (P=0.669). The V394D mutant retains the ability of wild-type c-Myc to activate transcription from a Pax3 promoter (4-fold induction with 40ng of c-Myc) where it binds to a stimulatory E-box (Figure 4.3.4) (0/20ng V394D P=0.011). These data indicate that the mutant protein is expressed at a similar level to the wild type and also that the mutation is restricted to a discrete region of the protein and does not influence global structural changes. The values obtained using 40ng of the wild type c-Myc construct appear higher than those obtained using the wild-type construct. However, this does not achieve significance (P=0.05).

4.2.2 Analysis of the role of c-Myc in *Nramp1* promoter activity

To investigate the role of c-Myc in the control of *Nramp1* transcription, the murine c-Myc RNAi construct was used in co-transfections with three of the *Nramp1* promoter constructs, pL4, pL4M6M and pL4SP1M (Figure 4.3.5). With each of these three constructs significant reduction in promoter activity was observed using 1.5μg of Myc RNAi compared to control RNAi. Two-fold repression of the pL4 construct was seen (P=0.020) and slightly lower,1.7-fold repression was seen using both the pL4M6M and pL4Sp1M (P=0.04 for both constructs).

In order to investigate whether this slightly unexpected repression was due to an indirect effect of reduced endogenous c-Myc levels on Miz-1 expression or function, a 'rescue' experiment was carried out in ND7 cells (Figure 4.3.6). Cells were co-transfected with a constant amount of pL4, either c-Myc RNAi or control RNAi and indicated amounts of Miz-1 expression plasmid. Results show that the 3.4-fold knock down, seen with the c-Myc RNAi as compared to the control (P=0.011), can be overcome with transfection of a Miz-1 expression plasmid. In addition to just restoring promoter activity to that without the Myc RNAi, the increased Miz-1 levels due to the co-transfection of the Miz-1

expression plasmid served to increase promoter activity (0.5μg Miz-1 gave a 2.6-fold induction rising to over 3-fold with 1.5μg of Miz-1).

Observations made during co-transfections of the c-Myc RNAi and the *Nramp1* promoter construct and the Miz-1 rescue experiment raised the possibility that the reduction in the endogenous c-Myc levels was also leading to reduced Miz-1 function. To investigate this hypothesis further, cells were transfected with a constant amount (0.5μg) of the Miz-1 GFP construct and 1.5μg of either the control (IREG-1) or the c-Myc RNAi (Figure 4.3.7). Analysis using flow cytometry 48 hours after transfection revealed that the mean fluorescence was significantly lower when the c-Myc RNAi was used compared to the control RNAi. Mean fluorescence was only 2-fold higher for c-Myc RNAi-treated compared with un-transfected cells, compared to a 10-fold increase in mean fluorescence with the control RNAi. The fluorescence levels for the two RNAi treated cultures were significantly different (P=0.010).

In order to assess if the effect of the c-Myc RNAi of Miz-1 GFP fluorescence could be overcome by over expression of P300 the experiment was repeated with the addition of 1.0μg of P300 along with the c-Myc RNAi (figure 4.3.8). The addition of P300 did serve to slightly increase fluorescence levels of Miz-1 eGFP causing a rise in mean fluorescence from 3.5 to 3.7-fold with the c-Myc RNAi as compared to un-transfected cells however, this was still far lower than the 10-fold difference observed with the control RNAi as compared to untransfected cells (P=0.039).

The stability of the Miz1 GFP protein with and without the c-Myc RNAi expression plasmid was evaluated in the presence of cycloheximide. ND7 cells were co-transfected with a constant amount of the Miz-1 GFP expression plasmid and either the c-Myc or the control RNAi. Following 48 hour incubation, cells were treated with cycloheximide (10μg/ml) and mean fluorescence levels were detected using flow cytometry at timed intervals. The Miz-1GFP protein was less stable when co-transfected with the c-Myc

RNAi, as compared to the control (Figure 4.3.9). The most significant differences were seen between 2 and 4 hours (P=0.032) with fluorescence levels using the c-Myc RNAi down to 62% of starting levels after 4 hours as compared to using the control RNAi where fluorescence levels still remained at 85% of starting levels. These data confirm that the stability and steady state levels of Miz-1 are reduced when the levels of endogenous c-Myc in an ND7 cell are also reduced.

Miz-1 lacks its own NLS and exploits a surrogate NLS within another unidentified protein to gain entry to the nucleus. If this carrier protein is c-Myc then the shorter half life of the Miz-1 GFP using the c-Myc RNAi could also explain the effect of the reduced pL4 promoter activity with Myc RNAi. To test this hypothesis a Miz-1 GFP construct with an NLS was produced 'Miz NLS' (as described in 4.2.4). This construct was co-transfected into ND7 cells with either the c-Myc RNAi or the control RNAi, as before.

Data shown was following 24-hour incubation after transfection (Figure 4.3.10a). The Miz NLS construct accumulated much more compared to the wild-type Miz-1 GFP, (P=0.003), significant knockdown of fluorescence is apparent with the c-Myc RNAi as compared to the control (P=0.000). In this experiment the reduction in Miz-1 fluorescence seen as a result of the c-Myc RNAi construct was lower than that which has been seen in previous experiments (Fig 4.3.7-9). This is due to the fact that the incubation time following transfection was reduced from 48 to 24 hours in this instance as the increased accumulation of the Miz-1 NLS GFP construct appeared to cause cell death. Western blotting was used to confirm that the introduction of the NLS was indeed leading to increased transport into the nucleus. Analysis of nuclear and cytoplasmic extracts by Western blotting (Figure 4.3.10b) showed that the NLS construct is completely absent from the cytoplasm and is only found in the nucleus. This is as compared to the wild-type Miz-1 GFP that is present in both the nuclear and cytoplasmic extracts.

4.2.3 Demonstration of the role of Sp1 in *Nramp1* transcription.

The requirement for Sp1 in *Nramp1* transcription was demonstrated using RNAi. RAW 264.7 cells were co-transfected with a constant amount of the *Nramp1* promoter construct pL4 and increasing amounts (2.5, 3.0 and 3.5μg) of the murine Sp1 RNAi construct, or the control RNAi (IREG-1) construct. Analysis of promoter activity using a luciferase assay reveals between ~ 90-98% ($P<0.008$) knock down of *Nramp1* promoter activity using the Sp1 RNAi as compared to the control (IREG-1) RNAi construct (figure 4.3.11), thus demonstrating a requirement for Sp1 in *Nramp1* transcription.

In order to determine whether Miz-1 is able to overcome the effects of reduction in endogenous Sp1 levels due to Sp1 RNAi treatment, the Miz-1 expression plasmid was co-transfected into RAW 264.7 cells with either the control (IREG-1) or Sp1 RNAi (Figure 5.3.12). Miz-1 RNAi brings about a 2.3-fold reduction in promoter activity compared to the control RNAi ($P=0.026$). This is much lower than the ~90-fold reduction observed by the Sp1 RNAi ($P=0.005$). The Miz-1 expression plasmid stimulates *Nramp1* expression 2-fold compared with control RNAi ($P=0.013$). A doubling is seen with the Miz-1 expression plasmid and the Sp1 RNAi.

4.2.4 Recruitment and binding of transcription factors at the *Nramp1* promoter.

In order to determine whether the mechanism of Myc mediated repression of *Nramp1* is via HDAC recruitment, the HDAC inhibitor TSA was used. RAW 264.7 cells were co-transfected with the *Nramp1* reporter construct pL4 (0.5μg) and increasing amounts (2.5, 3.0 and 3.5μg) of the c-Myc expression plasmid. This experiment was performed in duplicate with one set treated with TSA (200ng/ml), added with the serum five hours after transfection and the other untreated (Figure 4.3.16). Cells were incubated in the normal manner for 24 hours. TSA treatment was found to have little or no effect on *Nramp1* promoter activity. *Nramp1* repression of 81% was observed without TSA treatment and this was significant at 2.5μg ($P=0.001$). Repression with TSA was significant at 2.5 and 3.0μg of c-Myc with 85 and 90% repression respectively ($P<0.008$).

At 3.0μg of Myc, repression is observed with both the TSA treated and TSA un-treated samples. There is no significant difference between these values.

ChIP assays were used to look at endogenous Miz-1 and c-Myc proteins bound at the *Nramp1* promoter. Included in this study alongside untreated cells were cells treated with TSA (200ng/ml) or serum starved for 24 hours. ChIP (Figure 4.3.14) revealed that both Miz-1 and c-Myc proteins were bound at the *Nramp1* promoter in-vivo and that neither serum starvation nor TSA treatment were able to relieve this binding.

As shown in Figure 4.3.1a/b *Nramp1* contains a tandem duplication of candidate Inr's (Govani *et al*, 1995). Recombinant Wild type Miz-1 and c-Myc protein produced in *E. coli* (as described in 2.2.10) were analyzed for binding to the *Nramp1* promoter by Pulldown assay. The following synthetic, biotinylated oligonucleotide was produced (Sigma Genosys, UK), corresponding to the region of the *Nramp1* promoter containing both Initiator elements and consensus Sp1 site (See figure 4.3.1a, -31 to +4)
(5'-TGG GAA GCC GCG TGG GTT CCC ACT CTT ACT CAC TCG G-3')
The corresponding antisense oligonucleotide (shown below) was not biotinylated-
(5'-CCG AGT GAG TAA GAG TGG GAA CCC ACG CCC TTC CCA-3')
The two oligonucleotides are annealed by placing equimolar amounts in a water bath at 94°c and allowed to cool to room temperature.

The western blots using the Miz-1 antibody and c-Myc antibody (Figure 4.3.15a) demonstrate that the Miz-1 protein alone is able to bind to the *Nramp1* core promoter. However c-Myc is only able to bind in the presence of Miz-1. It is also apparent that Miz-1 is able to bind in the absence of c-Myc, although with c-Myc there is cooperative Miz-1 binding. The negative controls of the protein in the absence of the biotinylated oligonucleotide confirm that the protein is binding to the *Nramp1* promoter sequence and not simply sticking to the streptavidin Dynal beads.

The use of competitor oligonucleotides demonstrated that the binding of the Miz-1 protein to the DNA was specific. The following pairs of un-biotinylated oligonucleotides were used:

Wild type-

NRP#3: 5'-GATGGGAAGGGCGTGGGTTCCCACTCTTACTCACTCGGACC-3'

NRP#4: 5'-CTGGTCCGAGTGAGTAAGAGTGGGAACCCACGCCTTCCCA-3'

Initiator mutant 1-

NRP#15: 5'-GATGGGAAGGGCGTGGGTTCCCACTCTTGCTCACGGGGACC-3'

NRP#16: 5'-CTCGTCCCCGTGAGCAAGAGTGGGAACCCACGCCCTTCCCA-3'

Initiator mutant 2-

NRP#17: 5'-GATGGGAGGGGCGTGGGTGCCCACGGGTACTCACTCGGACC-3'

NRP#18: 5'-CTGGTCCGAGTGAGTACCCGTGGGCACCCACGCCCTTCCCA-3'

Double Initiator mutant-

NRP#19: 5'-GATGGGAAGGGCGTGGGTGCCCACGGGTGCTCACGGGGACC-3'

NRP#20: 5'-CTGGTCCCCGTGAGCACCCGTGGGCACCCACGCCCTTCCCA-3'

These data (Figure 4.3.16) show that the wild type and both of the single Inr mutants are able to compete with the biotinylated oligonucleotides for the binding of Miz-1, and therefore the mutation of a single initiator element does not prevent Miz-1 from binding the DNA. However, the double initiator mutant did not compete for binding and demonstrated that Miz-1 is only able to bind DNA in the presence of an Inr element.

Nramp1 luciferase reporter constructs were produced containing these same Inr mutations (as described in 4.2.2) and used to determine whether there was functional redundancy of the Inr elements present in the *Nramp1* promoter. RAW 264.7 cells were co-transfected with either one of the four *Nramp1* promoter constructs and increasing amounts of either Miz-1 (Figure 4.3.17a) or c-Myc (Figure 4.3.17b) expression vectors.

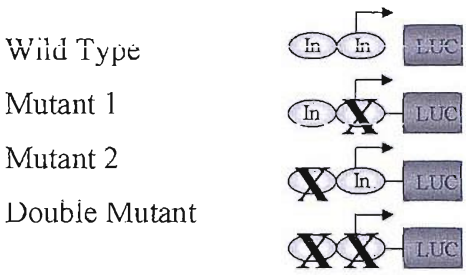


Figure 4.2.1 Schematic diagram of the *Nramp1* mutant promoter constructs.
The wild type construct consists of two Inr elements; each of the Mutants 1 & 2 has one of these sites mutated. The double mutant does not contain a functional Inr.

Mutant 1 was shown to have significantly reduced basal activity (P=0.009) with a 50% reduction in activity as compared to the wild type (Figure 4.3.17a). In subsequent experiments (Figure 4.3.17b) lower activity (29% reduction as compared to wild type) was also seen with this construct, although this failed to achieve significance. Mutant 2 was found to have significantly increased activity of between 60-70% P<0.048 (figures 4.3.17b-4.3.17a respectively). All of the constructs, with the exception of the double mutant were responsive to Miz-1 (Figure 4.3.17a). A 38-fold increase in promoter activity was achieved when the wild type promoter was co-transfected with 600ng of Miz-1 (P=0.008). This was significantly lower than the 71-fold increase seen when this was repeated using the Mutant 2 *Nramp1* promoter (P=0.000). A 23- fold increase was also observed when this experiment was performed using the Mutant 1 promoter, although this failed to achieve significance (P=0.075).

As for Miz-1, all of the constructs with the exception of the double mutant were found to be repressed by c-Myc (figure 4.3.17b). Using the maximum dose of 600ng of c-Myc 4, 5 and 2.4 fold repression was achieved with the wild type, mutant 1 and mutant 2 promoters respectively (P<0.031). These findings corresponded with the data obtained using transient ChIP technique (Figure 4.3.18). ND7 cells were used for this experiment, as the transfection efficiency achievable with this cell type is greater than that for the

RAW 264.7 cell. Cells were transfected as described in 2.2.5 with 1.5μg of each mutant *Nramp1* promoter construct, in duplicate. Following 24 hours incubation cells were harvested and ChIP analysis was carried out (as described in 2.2.16), using 2μl of the c-Myc antibody (Calbiochem) and a no antibody control for each construct. PCR was carried out on the recovered DNA using the following primer pair-

Sense: 5'-AGAAGGGGACAGATTGAG-3'

(Found in the synthetic oligonucleotide #5 (pair 2) which is common to all the mutant promoter constructs.)

Anti-sense: 5'-AACCAGGGCGTATCTCTT-3'(Present in the sequence of the vector pGL3 basic.)

Analysis of the PCR products recovered (Figure 4.3.18) revealed that c-Myc was bound *in-vivo* at both of the single mutant promoters (Mutant 1&2) but not at the double mutant promoter.

In order to discover whether Max is a part of the Miz-Myc complex involved in repression of the *Nramp1* promoter, the Myc and Max EG constructs were used (Amati *et al*, 1993). These constructs are unable to bind to native Max and Myc respectively. Results demonstrated (Figure 4.3.19) that repression of the *Nramp1* promoter could only be achieved with a functional Myc/Max complex. MycEG alone failed to repress the *Nramp1* promoter $P>0.05$ whereas MycEG and MaxEG together were able to significantly repress ($P=0.040$) the *Nramp1* promoter.

Recent studies (Herbst *et al*, 2005) have identified a conserved domain centrally located at amino acids 188-199 of Myc, termed MBIII. This domain is believed to be important for some aspects of Myc function, including repression. In order to determine whether this region of Myc is important for Myc mediated repression of the *Nramp1* promoter a MBIII mutant (Herbst *et al*, 2005) in which the MBIII region had been deleted or wild-type Myc in the same expression vector was co-transfected into RAW cells along with

the *Nramp1* reporter construct pL4. Loss of MBIII was seen to abolish the ability of Myc to repress the *Nramp1* promoter as compared to a wild-type Myc control construct which was able to significantly repress the *Nramp1* promoter at 400 and 600ng ($P<0.043$) (Herbst *et al*, 2005).

4.2.5 The role of the POZ domain in Miz-1 function

Miz-1 (see figure 1.6) contains a BTB/POZ domain at its N-terminus (Peukert *et al*, 1997). This domain is evolutionary conserved and is often found in transcription factors containing C_2H_2 Zinc fingers and permits homo- or hetero-dimer formation (Bardwell & Treisman, 1994). Analysis of the POZ domain from PLZF (Ahmad *et al*, 1998) revealed that the POZ domain is capable of forming an intertwined dimer of α-helices and short β-sheets. The N-terminal domain of each chain associates with the opposing polypeptide in the dimer. Several strongly conserved residues have been identified in the POZ domain of a number of proteins, including Keap1 a homologue of the drosophila Kelch protein (Zipper & Mulcahy, 2002). It has been shown that for Keap1 substitution of a highly conserved serine residue at position 104, with an alanine residue was found to abolish the ability of this protein to form a dimer. Alignment of the POZ domains of several proteins including the human and murine homologues of Miz-1 identified the corresponding residue in Miz-1 at position 46 (Figure 3.2.2).

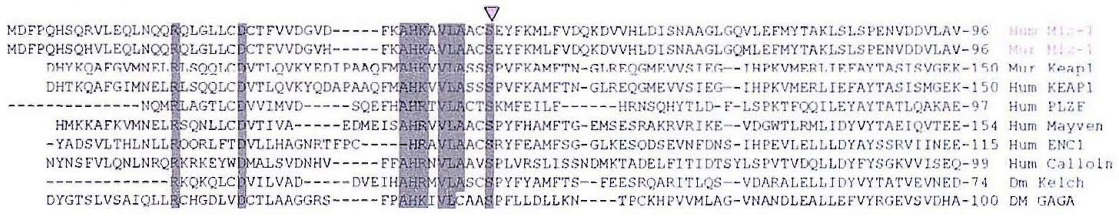


Figure 3.2.2 POZ domain alignment shows conserved residues

Shown above is a sequence alignment of the POZ domains of a number of proteins as indicated on the right hand side of the diagram. A number of conserved residues have been identified as indicated by the shading. The serine residue mutated in the Miz-1 S46A Miz-1 and S104A Keap1 is highlighted with a purple arrow.

The serine present in Miz-1 was substituted with an alanine residue using Quikchange The resulting recombinant protein was found to be expressed at equal or slightly higher levels than the wild type protein in *E.coli* (Appendix 5). Pull down assays using the biotinylated oligonucleotide corresponding to the *Nramp1* promoter (Figure 4.3.21a) demonstrated that the S46A mutant was able to bind to the promoter with equal or perhaps slightly greater affinity than the wild-type Miz-1 protein.

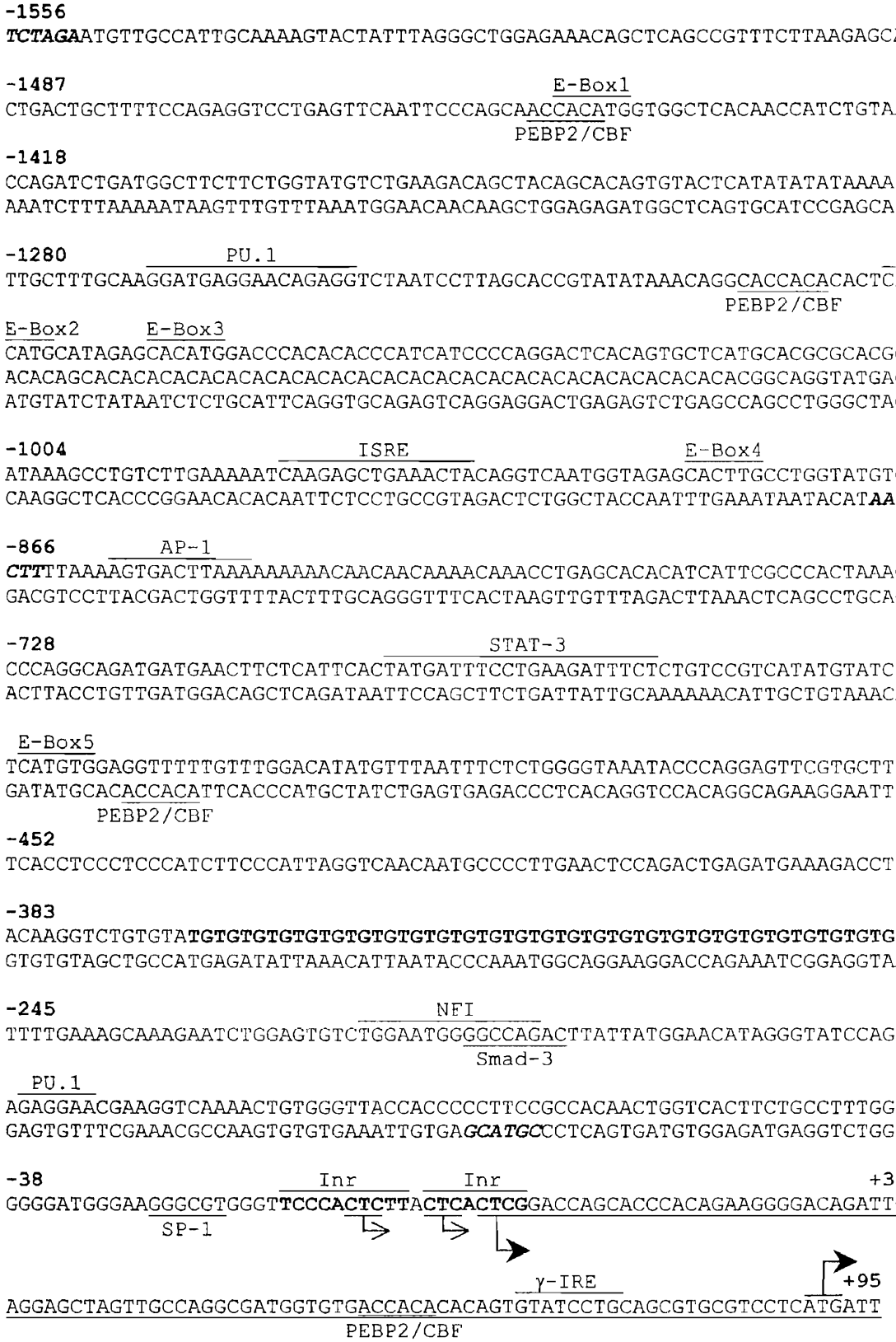
Use of the S46A and Δ33-60 Miz-1 mutants with the *Nramp1* promoter construct in RAW 264.7 cells, revealed that unlike the wild-type Miz-1 these mutants failed to activate (Figure 4.3.21b). Instead these mutants were found to significantly reduce promoter activity. A 400ng dose of S46A mutant was found to result in a 2.5-fold decrease in promoter activity (P=0.045). Similarly the deletion construct Δ33-60 caused a significant decrease in promoter activity of 1.5-fold with a 600ng dose (P=0.002). Subsequent experiments performed using these mutant constructs showed that they functioned as dominant negative for Miz-1 activity (Figure 4.3.21c). Co-transfection of these mutants with wild-type Miz-1 showed that the 93-fold (P=0.003) *Nramp1* promoter activation observed with Miz-1 alone could not be achieved when the wild type was co-transfected with either of the mutants (P>0.05).

The effect of the dominant negative allele Δ33-60 was examined with respect to transactivation of the *Nramp1* promoter by ICSBP and P300 in RAW 264.7 cells (Figure 4.3.22a&b). These interactions are of interest as they have both been found to be dependent upon interaction with Miz-1 (Staller et al, 2001; Alter-Koltunoff *et al,* 2003). In these experiments the wild type Miz-1 was seen to facilitate transactivation by both ICSBP and P300 as had previously been reported. However, the mutant Miz-1 was found to prevent this transactivation. In the presence of the wild-type Miz-1 a 1.0μg dose of ICSBP resulted in a 29-fold increase (P=0.041) of *Nramp1* promoter activity, whereas the mutant did not activate the promoter at any of the doses tested (P>0.05) (Figure 4.3.22b)

P300 was also found to be incapable of transactivation in the presence of the Δ33-60 mutant Miz-1. In the presence of wild type Miz-1 P300 was shown to significantly activate the *Nramp1* promoter at every concentration tested ($P<0.006$) with maximum activation of 115-fold at the highest (1.5μg) dose.

Taken together data produced using both the S46A and Δ33-60 Miz-1 indicate that these mutants are still capable of binding to the *Nramp1* promoter and function as dominant negatives that appear to be incapable of interaction with other co-factors.

A)


-1556
TCTAGAATGTTGCCATTGCAAAAGTACTATTTAGGGCTGGAGAAACAGCTCAGCCGTTTCTTAAGAGCA
-1487
E-Box1
CTGACTGCTTTTCCAGAGGTCCTGAGTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCTGTAA
PEBP2/CBF
-1418
CCAGATCTGATGGCTTCTTCTGGTATGTCTGAAGACAGCTACAGCACAGTGTACTCATATATATAAAAT
AAATCTTTAAAAATAAGTTTGTTTAAATGGAACAACAAGCTGGAGAGATGGCTCAGTGCATCCGAGCAT
-1280
PU.1
TTGCTTTGCAAGGATGAGGAACAGAGGTCTAATCCTTAGCACCGTATATAAACAGGCACCACACACTCA
PEBP2/CBF
E-Box2
E-Box3
CATGCATAGAGCACATGGACCCACACACCCATCATCCCCAGGACTCACAGTGCTCATGCACGCGCACGC
ACACAGCACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACGGCAGGTATGAC
ATGTATCTATAATCTCTGCATTCAGGTGCAGAGTCAGGAGGACTGAGAGTCTGAGCCAGCCTGGGCTAC
-1004
ISRE
E-Box4
ATAAAGCCTGTCTTGAAAAATCAAGAGCTGAAACTACAGGTCAATGGTAGAGCACTTGCCTGGTATGTG
CAAGGCTCACCCGGAACACACAATTCTCCTGCCGTAGACTCTGGCTACCAATTTGAAATAATACATAAG
-866
AP-1
CTTTTAAAAGTGACTTAAAAAAAAACAACAACAAAACAAACCTGAGCACACATCATTCGCCCACTAAAC
GACGTCCTTACGACTGGTTTTACTTTGCAGGGTTTCACTAAGTTGTTTAGACTTAAACTCAGCCTGCAG
-728
STAT-3
CCCAGGCAGATGATGAACTTCTCATTCACTATGATTTCCTGAAGATTTCTCTGTCCGTCATATGTATCC
ACTTACCTGTTGATGGACAGCTCAGATAATTCCAGCTTCTGATTATTGCAAAAAACATTGCTGTAAACA
E-Box5
TCATGTGGAGGTTTTTGTTTGGACATATGTTTAATTTCTCTGGGGTAAATACCCAGGAGTTCGTGCTTT
GATATGCACACCACATTCACCCATGCTATCTGAGTGAGACCCTCACAGGTCCACAGGCAGAAGGAATTT
PEBP2/CBF
-452
TCACCTCCCTCCCATCTTCCCATTAGGTCAACAATGCCCCTTGAACTCCAGACTGAGATGAAAGACCT-
-383
ACAAGGTCTGTGTATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGC
GTGTGTAGCTGCCATGAGATATTAAACATTAATACCCAAATGGCAGGAAGGACCAGAAATCGGAGGTAA
-245
NFI
TTTTGAAAGCAAAGAATCTGGAGTGTCTGGAATGGGGCCAGACTTATTATGGAACATAGGGTATCCAGG
Smad-3
PU.1
AGAGGAACGAAGGTCAAAACTGTGGGTTACCACCCCCTTCCGCCACAACTGGTCACTTCTGCCTTTGGT
GAGTGTTTCGAAACGCCAAGTGTGTGAAATTGTGAGCATGCCCTCAGTGATGTGGAGATGAGGTCTGGA
-38
Inr
Inr
+38
GGGGATGGGAAGGGCGTGGGTTCCCACTCTTACTCACTCGGACCAGCACCCACAGAAGGGGACAGATTG
SP-1
γ-IRE
+95
AGGAGCTAGTTGCCAGGCGATGGTGTGACCACACACAGTGTATCCTGCAGCGTGCGTCCTCATGATT
PEBP2/CBF

B)

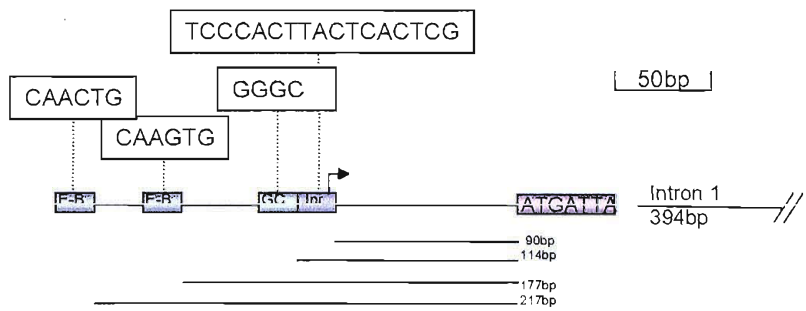


Figure 4.3.1 The sequence of the *Nramp1* Promoter Construct pHB4. (A) This sequence spans from the XbaI site at position -1556bp over a total of 1.665Kbp to the ATG translation initiation site at +95bp(indicated with an arrow above the sequence). The two initiator (Inr) elements are highlighted in bold type. The three translation initiation sites are indicated by the presence of arrows below the sequence with the larger arrow representing the major site. Also shown are the positions of the transcription factor binding sites as described in 4.1. Above the sequence are the sites present on the sense strand, below are those present on the anti-sense strand.
(Bowen *et al*, 2002)

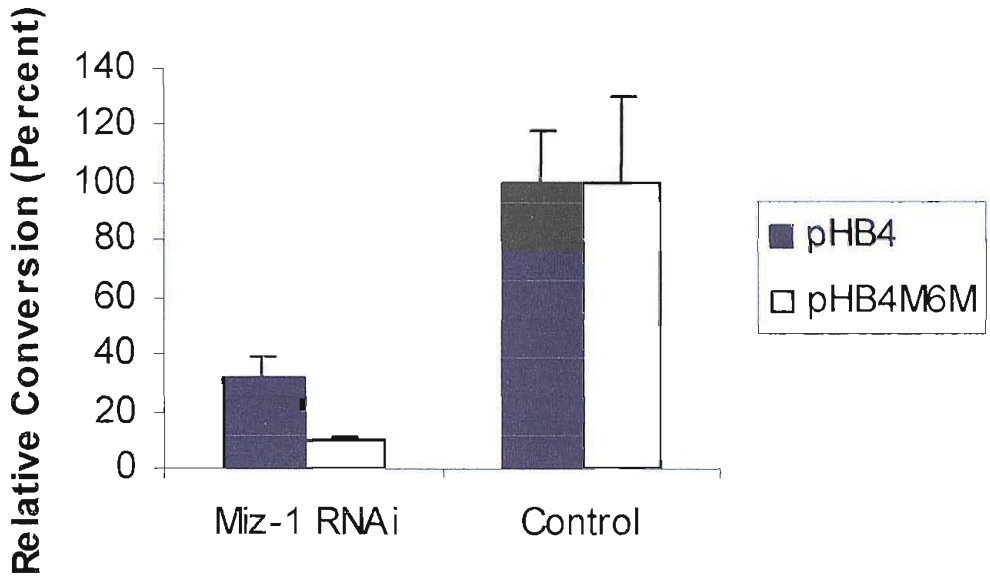
(B) A schematic diagram of the *Nramp1* promoter-The region containing the initiator and initiator like element is indicated. Also shown are the GC box and two E-boxes. The distance (in base pairs) between these sites is indicated below.

Figure 4.3.2 Use of Human and Murine Miz-1 RNAi constructs.

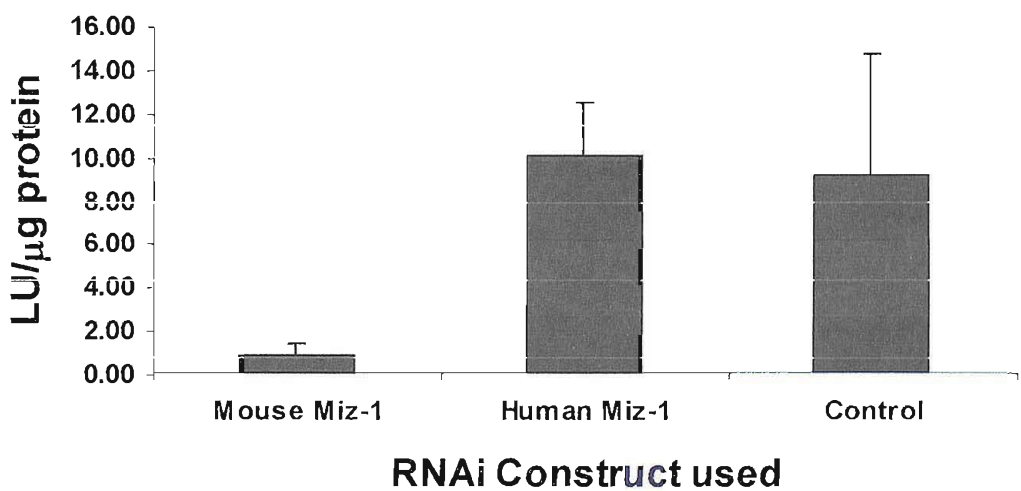
A) Reduced promoter activity of *Nramp1* promoter constructs pHB4 and pHB4M6M following co-transfection with the human Miz-1 RNAi construct. Cos-1 cells were co-transfected with the 0.5μg *Nramp1* promoter construct (either pHB4 or pHB4M6M) and 0.5μg of the human Miz-1 RNAi or control (IREG-1) RNAi. Expression of reporter gene was detected using a CAT assay (20μg protein/4hours). Data are expressed as activities relative to control cultures set to 100%.
Students T-Test- pHB4 +/-Miz-1 RNAi P=0.040, pHB4M6m +/- Miz-1 RNAi P= 0.029

B) Demonstration of the species specificity of the murine Miz-1 RNAi construct. RAW 264.7 cells were co-transfected as described in 2.2.5. With 1μg of the *Nramp1* promoter construct pL4 and 3.5μg of the murine Miz-1 RNAi construct, the human Miz-1 RNAi construct or the control (IREG-1) RNAi construct. Expression of the reporter gene was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein. Students T-tests - murine RNAi/control RNAi, P=0.020, Human RNAi/Control RNAi, P=0.118

A)


Relative Conversion (Percent)
140
120
100
80
60
40
20
0
Miz-1 RNAi
Control
pHB4
pHB4M6M

B)


LU/µg protein
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Mouse Miz-1
Human Miz-1
Control
RNAi Construct used

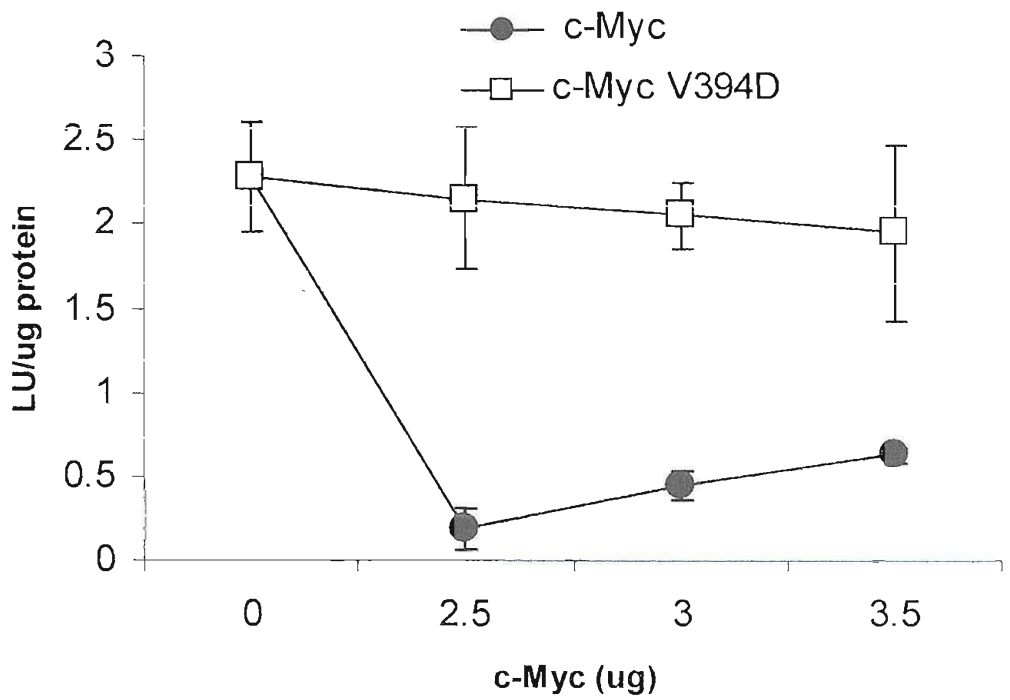


Figure 4.3.3 The Myc mutant V394D which is unable to interact with Miz-1 cannot repress the *Nramp1* promoter construct pL4. A) RAW 264.7 cells were co-transfected as described in 2.2.5 with a constant amount of the *Nramp1*promoter construct pL4 (0.5μg) and increasing amounts of the c-Myc expression plasmid or the c-Myc mutant V394D mutant as shown. DNA concentrations were normalized using the pcDNA3.1 empty vector control. Expression of the reporter gene was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.

Students T-tests-

Myc Mutant (V394D)- 0/2.5μg, 0/3.0μg P>0.05. c-Myc (Wild-type)- 0/2.5μg P=0.003, 0/3.0μg P=0.017.

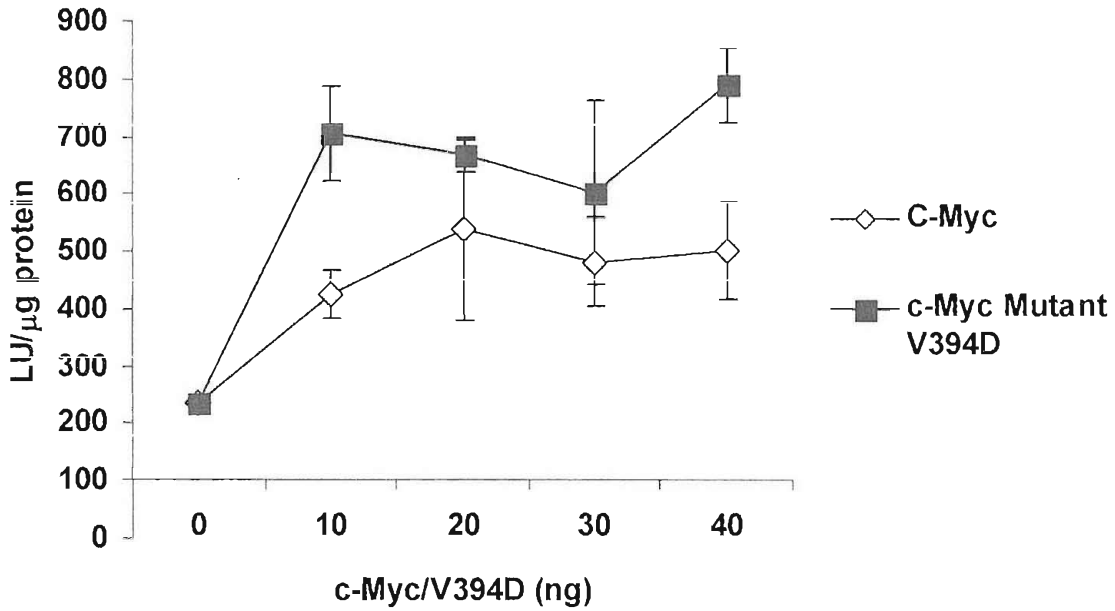


Figure 4.3.4 The c-Myc mutant V394D is still able to activate transcription of the Pax 3 promoter. ND7 cells were co-transfected with a constant amount (0.5µg) of the Pax3 promoter construct (Harris *et al*, 2002) and increasing amounts as shown of either the c-Myc expression plasmid or the V394D Myc mutant. DNA concentrations were normalized using the pcDNA 3.1 empty vector control.

Students T-tests-

Myc 0/20ng P=0.019, V394D mutant 0/20ng P=0.011

Page 111 missing

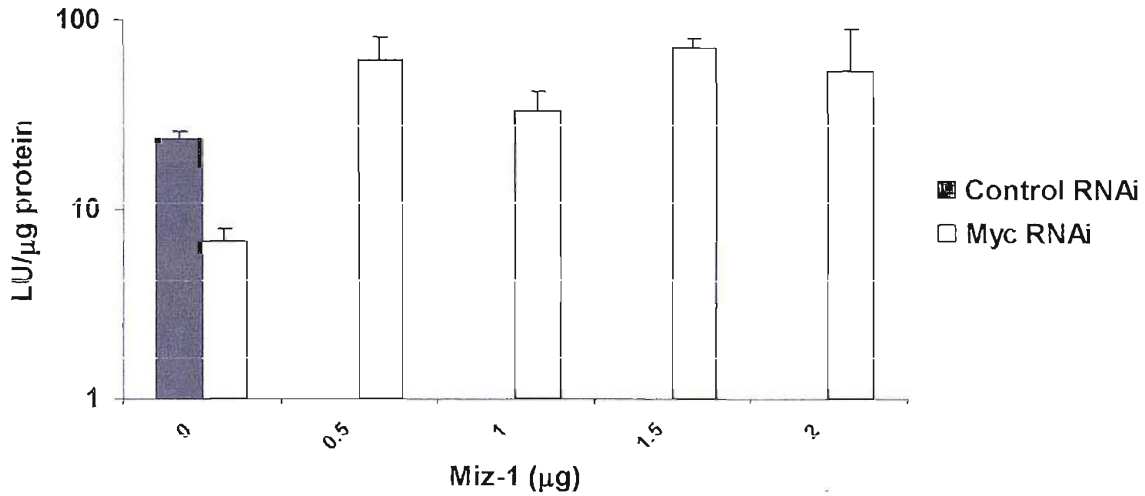


Figure 4.3.6 Miz-1 is able to 'rescue' the effect of the c-Myc RNAi in ND7 cells. ND7 cells were co-transfected, as described in 2.2.5 with 0.5µg of promoter construct and 1.5µg of either the murine c-Myc RNAi or the control (IREG-1) RNAi and increasing amounts of Miz-1 DNA as shown. DNA concentrations were made up to 4µg using the pBabe empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Test-

Control RNAi versus c-Myc RNAi P=0.011

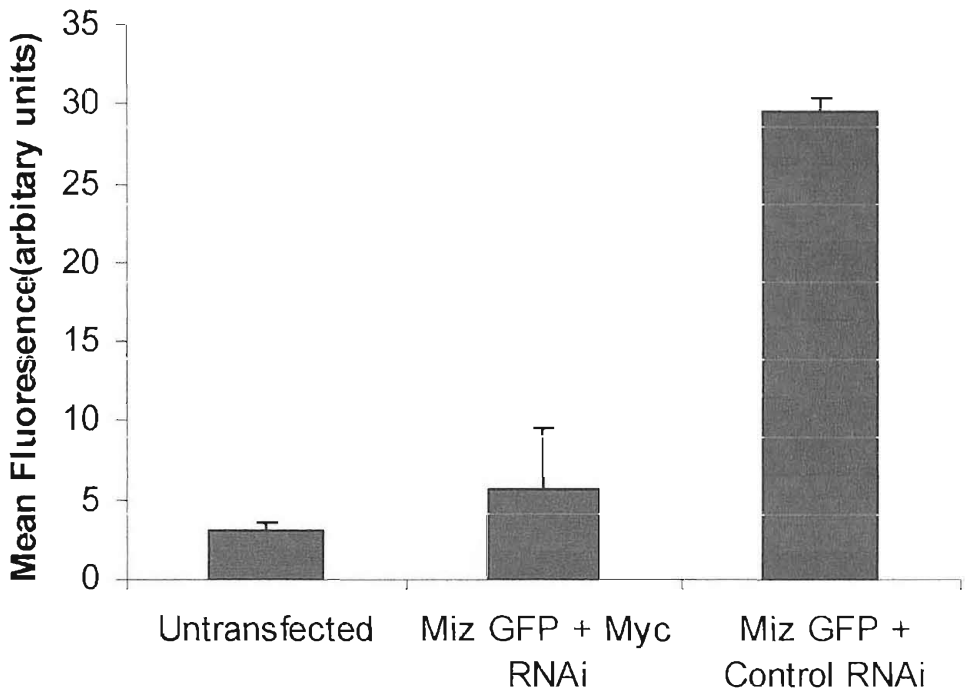


Figure 4.3.7 Myc RNAi reduces the accumulation of Miz-1 GFP construct in ND7cells. ND7 cells were co-transfected as described in 2.2.5 with a constant amount (0.5μg) of a Miz-1 GFP construct and 1.5μg of either the c-Myc or control (IREG-1) RNAi using Following 48 hour incubation the level of fluorescence in the cells was detected using flow cytometry.

Students T-Tests- Miz-1 GFP + Myc RNAi versus Miz-1 GFP + control RNAi P=0.010

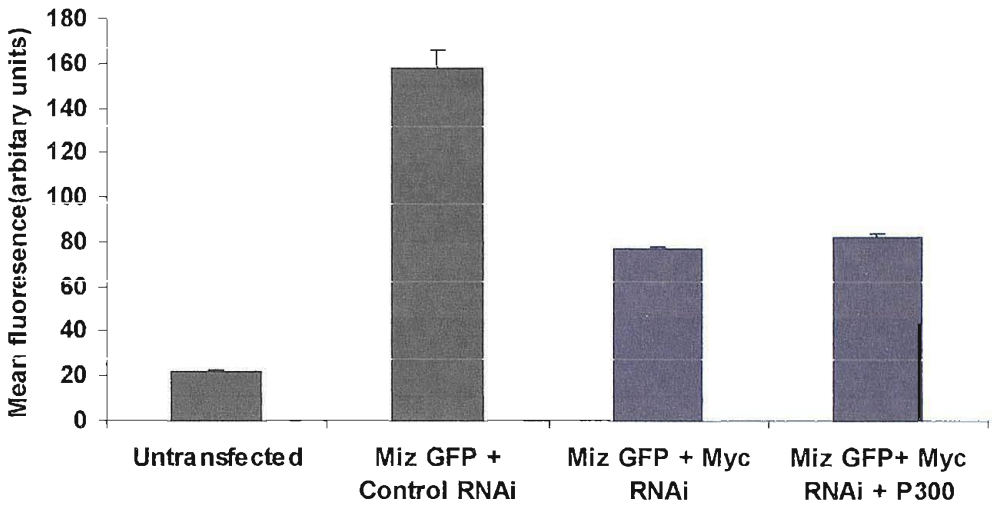


Figure 4.3.8 Myc RNAi effect cannot be rescued using p300 in ND7 cells. . ND7 cells were co-transfected as described in 2.2.5 with a constant amount (0.5μg) of a Miz-1 GFP construct and 1.5μg of either the c-Myc or control (IREG-1) RNAi. In addition to this 1μg of p300 or pBabe control plasmid was also used. Following 48 hour incubation the level of fluorescence in the cells was detected using flow cytometry.

Students T-test-

Miz GFP + control RNAi versus Miz GFP + c-Myc RNAi P=0.002. Miz GFP + control RNAi versus Miz GFP + c-Myc RNAi + p300 P=0.039.

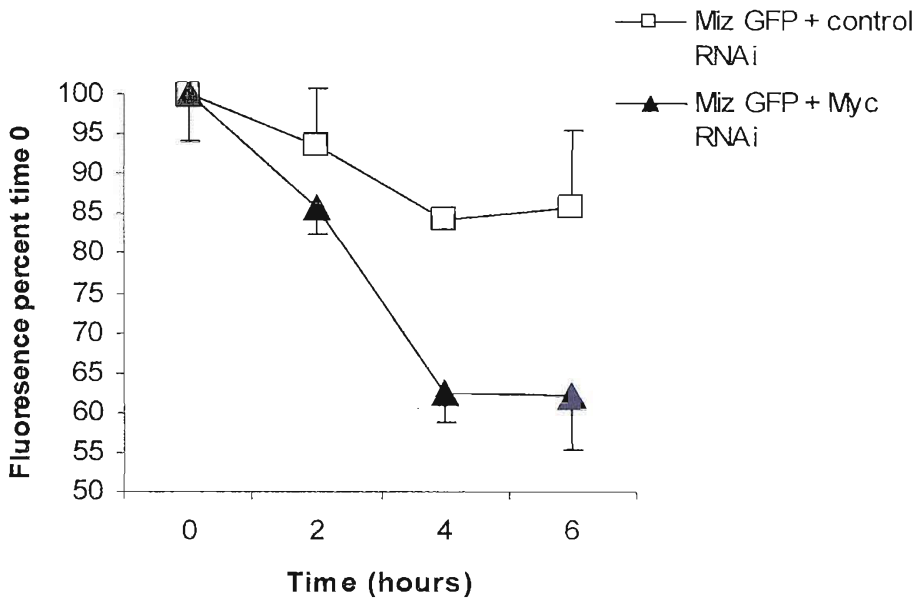
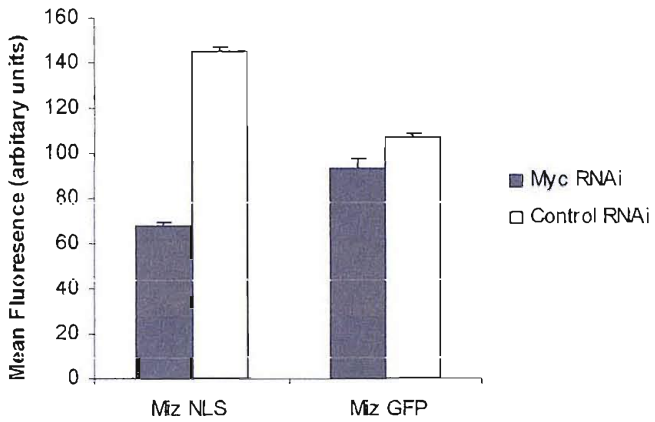


Figure 4.3.9 Miz GFP is more stable in the presence of c-Myc. ND7 cells were co-transfected, as described in 2.2.5 with a constant amount (0.5μg) of a Miz-1 GFP construct and 1.5μg of either the c-Myc or control (IREG-1) RNAi. Following 48 hour incubation cycloheximide was added at a concentration of 10μg/ml . The level of fluorescence in the cells at timed intervals was detected using flow cytometry.

Students T-test-

Control RNAi 2-4 hour time interval P=0.120, Myc RNAi 2-4 hour time interval P=0.032

A)



B)

Figure 4.3.10 A Miz-1 NLS construct is still 'Knocked down' by Myc RNA. A) ND7 cells were co-transfected, as described in 2.2.5 with a 0.5μg of a Miz-1 GFP or Miz-NLS construct and 1.5μg of either the c-Myc or control (IREG-1) RNAi. Following24 hour incubation the level of fluorescence in the cells was detected using flow cytometry.Students. T-test- Miz NLS +/- Myc RNAi P=0.000, Miz GFP +/- Myc RNAi P=0.026

B) ND7 cells were co-transfected, as described in 2.2.5 with a 0.5μg of a Miz-1 GFP or Miz-NLS construct and 1.5μg of either the c-Myc or control (IREG-1) RNAi. Following24 hour incubation cells were harvested and Nuclear (N) and Cytoplamic (C) extracts were made as described in 2.2.11. Extracts were western blotted as described in 2.2.13 with a GFP antibody (Roche) at a concentration of 1:1000 (secondary 1:2000).

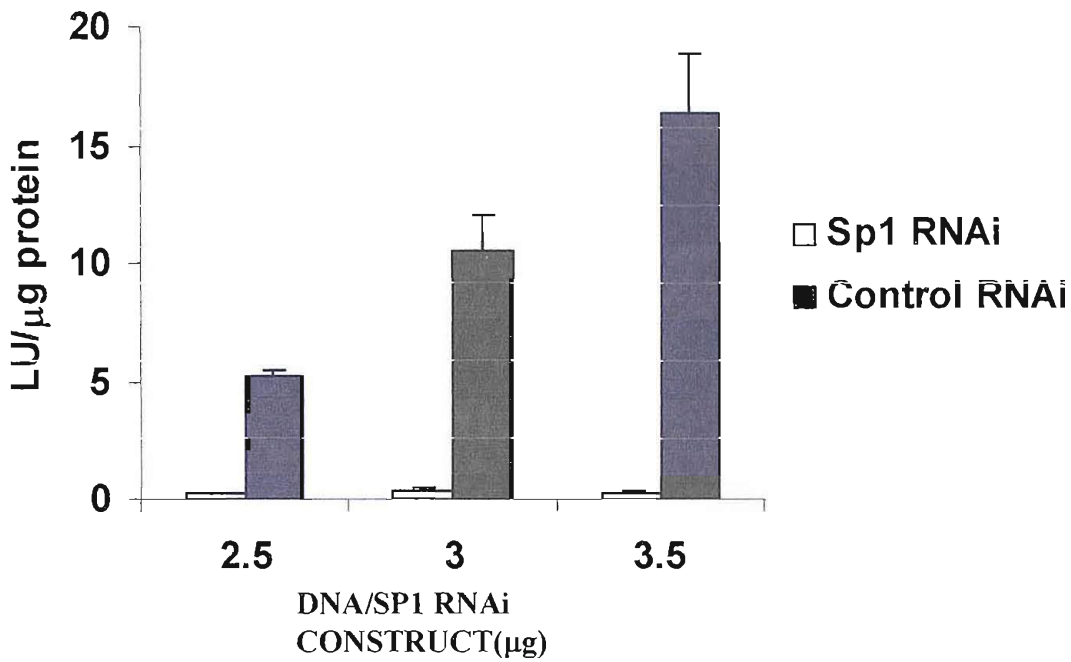


Figure 4.3.11 Use of Murine Sp1 RNAi causes repression of *Nramp1* promoter activity in RAW 264.7 cells. RAW 264.7 cells were co-transfected, as described in 2.2.5 with a constant amount (1.0µg) of the pL4 *Nramp*1 promoter construct and increasing amounts as shown, of either the murine Sp1 RNAi construct or the control (IREG-1). DNA concentrations were made up to 4.5µg using the pBabe empty vector control plasmid. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Tests- Sp1 RNAi compared to control (IREG-1) RNAi- 2.5µg P=0.001, 3.0µg P=0.008, 3.5µg P=0.008.

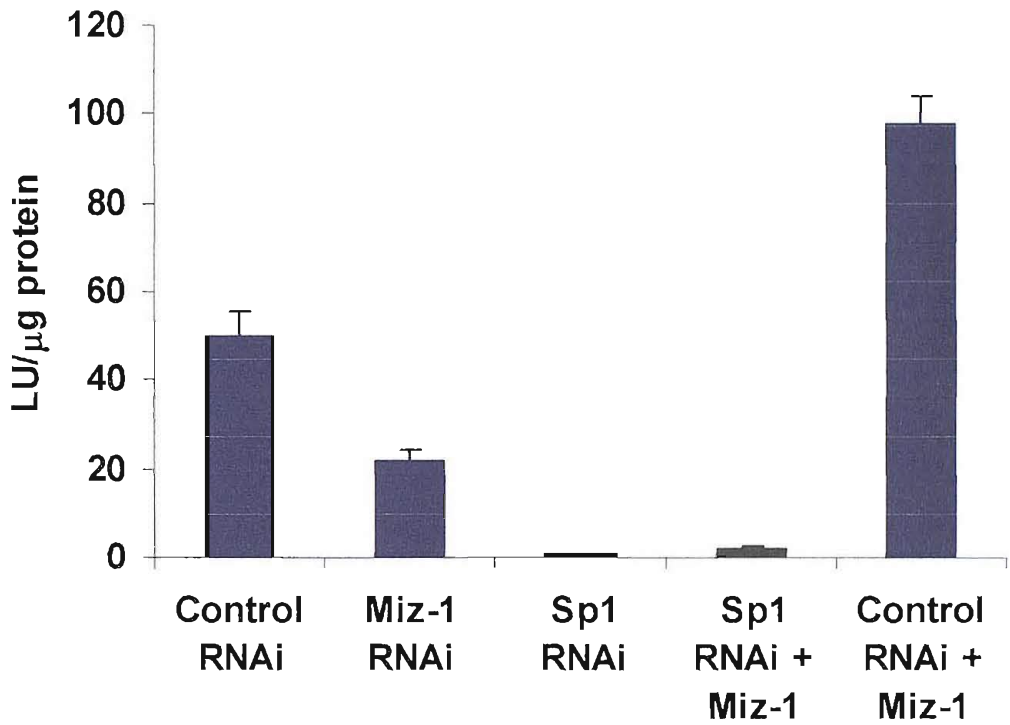


Figure 4.3.12 Miz-1 is unable to rescue to Sp1 RNAi effect in RAW 264.7 cells. RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (1.0µg) of the *Nramp1* promoter construct pL4 and 3.5µg of and RNAi construct (either Murine Sp1, Murine Miz-1 or control (IREG-1). 1.0µg of Miz-1 DNA or pBabe empty vector control was also used. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Tests

Control versus Miz-1 RNAi P=0.026, Control versus Sp1 RNAi P=0.005, Sp1 RNAi versus Sp1 RNAI +Miz-1 P=0.062, Control RNAi versus Control RNAi + Miz-1 P=0.013

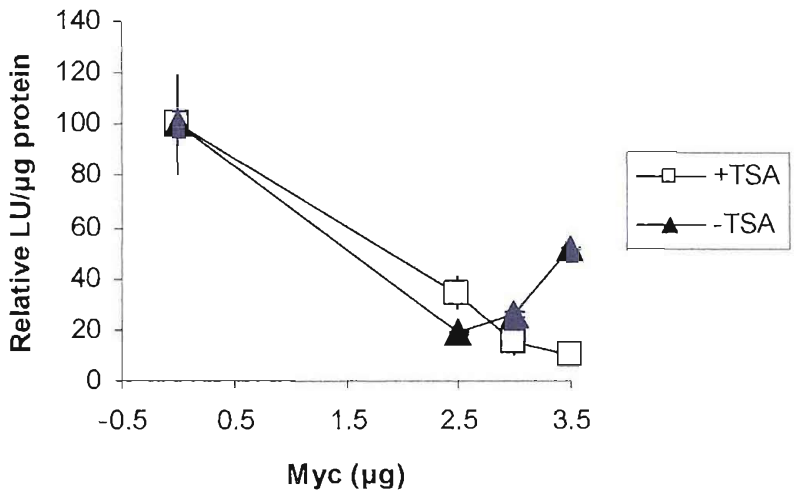


Figure 4.3.13 c-Myc repression of *Nramp1* does not require HDAC activity. RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the *Nramp1* promoter construct pL4 and increasing amounts as shown of the c-Myc expression vector. DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample. Data is shown as relative LU/µg with respect to c-Myc untreated cultures.

Students T-Tests-

TSA untreated cultures- 0/2.5µg Myc P=0.038. TSA treated cultures- 0/3.0µg Myc P=0.03, 0/3.8µg Myc P=0.020.

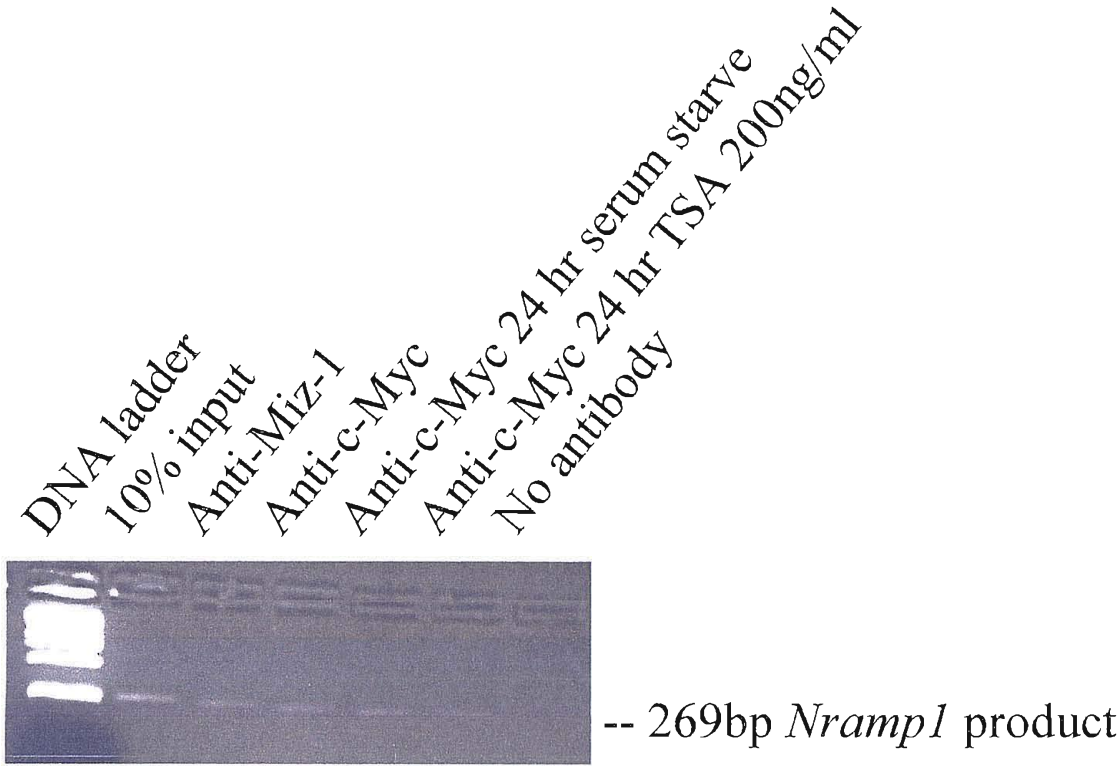


Figure 4.3.14 Miz-1 and c-Myc are bound to the *Nramp1* promoter *in-vivo*. RAW 264.7 cells were plated out at $1x10^6$/90mm dish and incubated either with or without serum/TSA as indicated above. Following 24 hour incubation cells were harvested and ChIP assays were carried out (as described in 2.2.16) using antibodies as indicated above. Products of PCR reactions were then loaded onto a 2% agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 volts for 30 minutes.

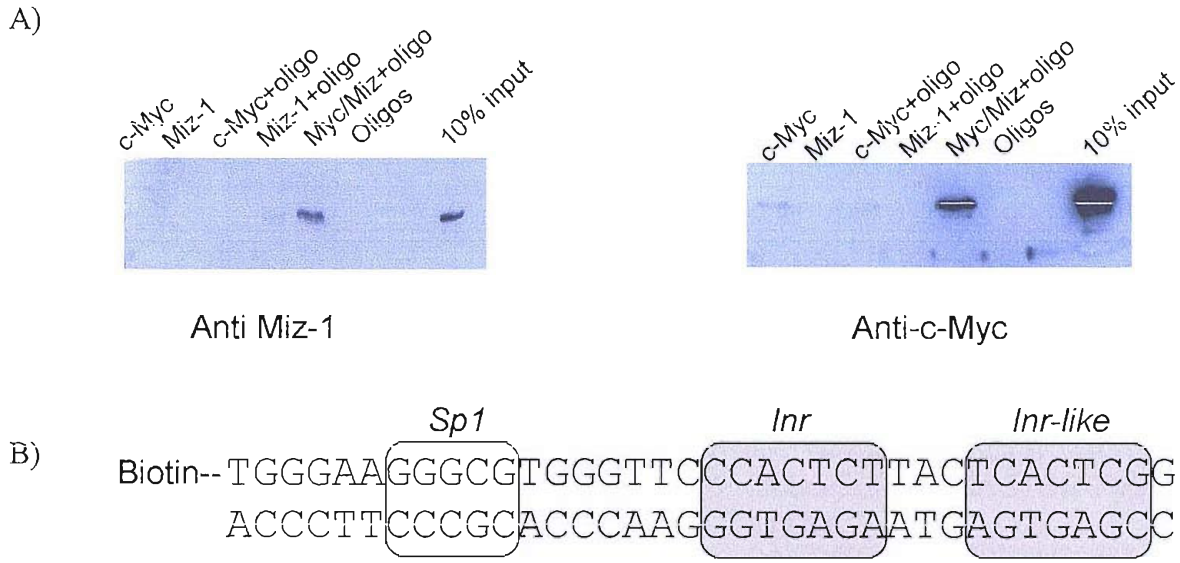
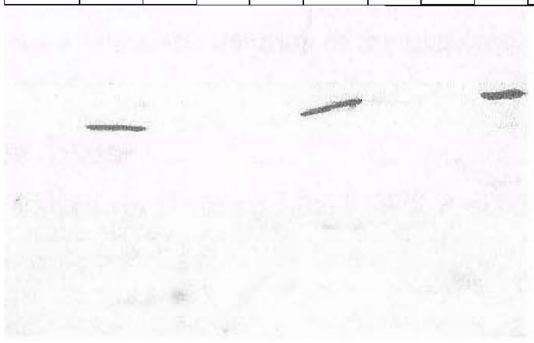


Figure 4.3.15 Oligonucleotide pull-down assay using Miz-1 and c-Myc Antibodies to detect recombinant protein bound to the *Nramp1* promoter. A Pulldown assay was performed using c-Myc and Miz-1 recombinant proteins and the biotinylated oligonucleotide to the *Nramp1* promoter (as shown in B). Samples were analyzed for the presence of bound proteins using either Miz-1 antibody (#38 bleed 2) at a concentration of 1:500 or c-Myc antibody (Santa-Cruz, UK) at a concentration of 1:200 (secondary, Goat-anti-Rabbit, 1:15,000) by western blotting (as described in 2.2.13).

		+							10 fold w/t competition
			+						10 fold mutant 1 competition
				+					10 fold mutant 2 competition
					+				10 fold double mutant compeition
+	+	+	+	+	+	-			Biotinylated inr oligo
-	+	+	+	+	+	+			Miz-1 protein
								+	10% Miz-1 input



Mutant 1
GATGGGAAGGGCGTGGGTGCC
CACGGGTACTCACTCGGACC
Mutant 2
GATGGGAAGGGCGTGGGTTCC
CACTCTTGCTCACGGGGACC
Double mutant
GATGGGAAGGGCGTGGGTGCC
CACGGGTGCTCACGGGGACC

Figure 4.3.16 Pulldown assay to show competition for Miz-1 binding to *Nramp1* Inr elements using non-biotinylated competitor oligos. An oligonucleotide pulldown assay was performed using Miz-1 and c-Myc recombinant proteins and the biotinylated oligonucleotide to the *Nramp1* promoter as well as non-biotinylated competitor oligos. (as described in 2.2.12). Competitor oligos used are as indicated on the lower right hand side of the figure. Samples were analyzed for the presence of Miz-1 protein using the Miz-1 antibody #38 at a concentration of 1:500 (secondary, Goat-anti-Rabbit, 1:15,000) to carry out a western blot (as described in 2.2.13).

Figure 4.3.17 Deletion of either Inr does not alter the Miz-1 or c-Myc response.

RAW 264.7 cells were co-transfected as described in 2.2.5 0.5μg of either the wild type or a mutant *Nramp1*promoter construct (as indicated) and increasing amounts of the Miz-1 (A) or c-Myc (B) expression plasmid. DNA concentrations were normalized using the pcDNA3.1 empty vector control. Expression of the reporter gene was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.

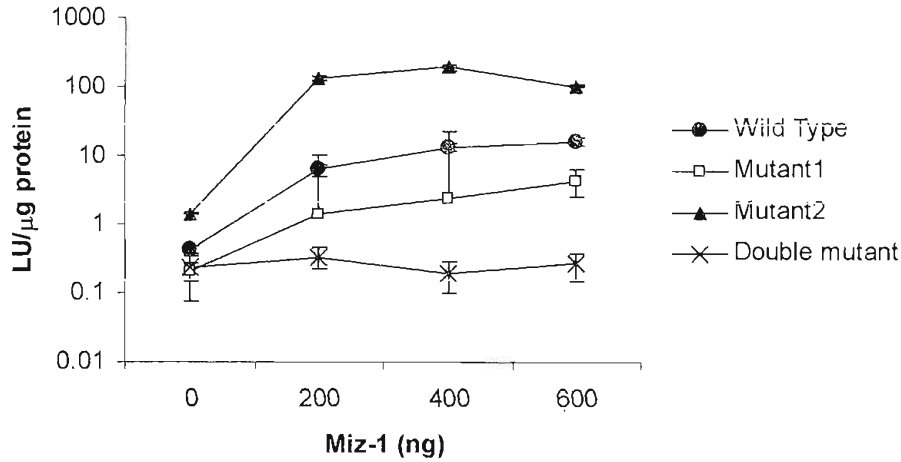
C) Shows a schematic diagram of the mutations made.

Students T-tests-

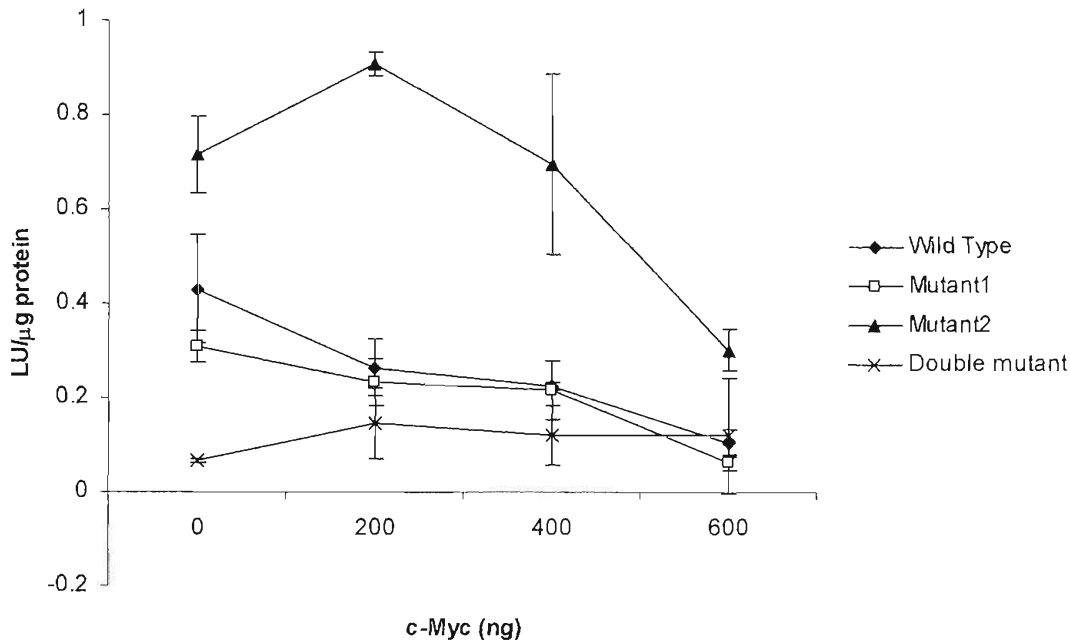
A) P-Values for 0/600ng Miz-1: WT P=0.007, Mutant 1 P=0.075 Mutant 2 P=0.000, Double mutant P>0.05.

B) P-Values for 0/600ng c-Myc: WT P=0.031, Mutant 1 P=0.001, Mutant 2 P=0.028, Double mutant P>0.05.

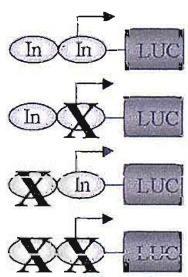
A)


1000
100
10
1
0.1
0.01
LU/μg protein
0
200
400
600
Miz-1 (ng)
Wild Type
Mutant1
Mutant2
Double mutant

B)


1
0.8
0.6
0.4
0.2
0
-0.2
LU/μg protein
0
200
400
600
c-Myc (ng)
Wild Type
Mutant1
Mutant2
Double mutant

C)


In
In
LUC
In
LUC
In
LUC
LUC

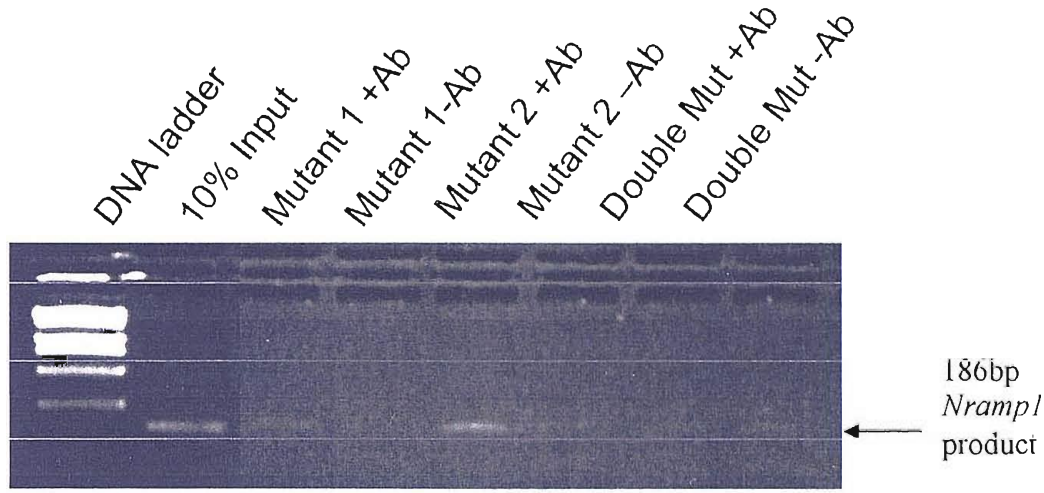


Figure 4.3.18 Myc is bound to both *Nramp1* single Inr mutant promoters but not the double Inr mutant promoter in-vivo. RAW 264.7 cells were plated out at $1x10^6$/90mm dish and transfected with 1.5μg of Nramp1 promoter construct. Following 24 hour incubation cells were harvested and ChIP assays were carried out (as described in 2.2.16) using 2μl of c-Myc antibody (Calbiochem), or no antibody controls. Products of PCR reactions were then loaded onto a 2% agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 volts for 30 minutes.

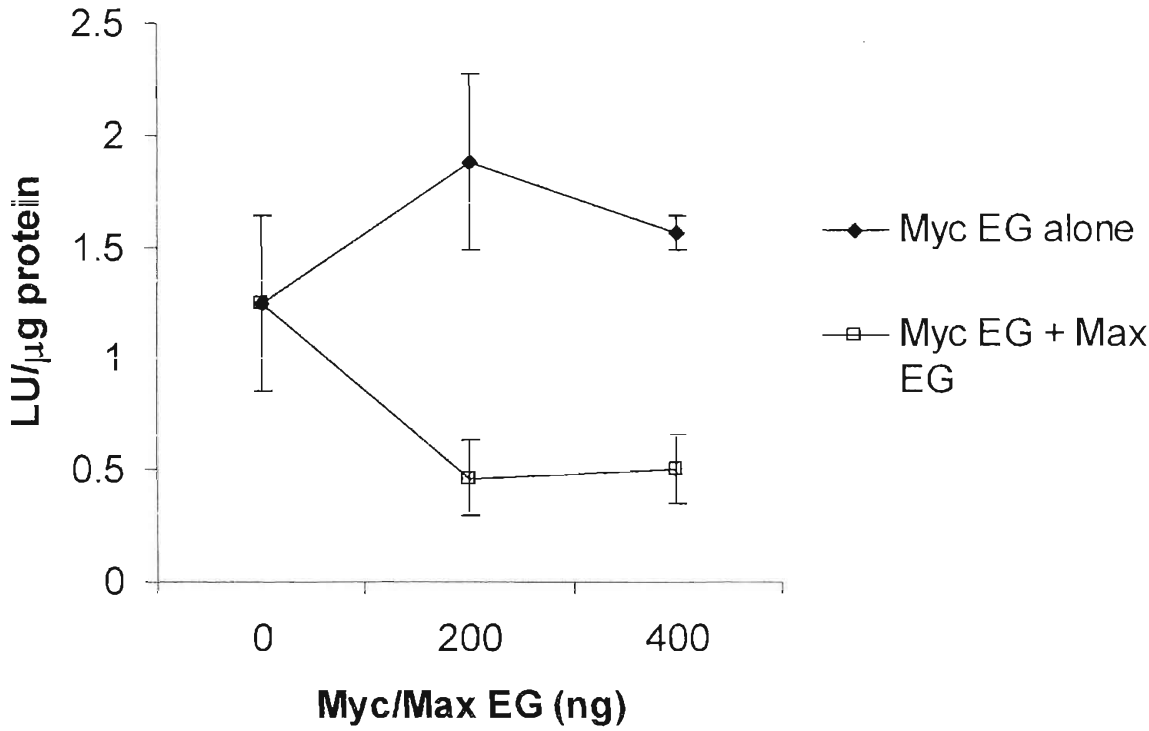


Figure 4.319 Max is required for Myc repression of the *Nramp1* promoter. RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the *Nramp1* promoter construct pL4 and increasing amounts of either the Myc EG construct or both the Myc and Max EG constructs together. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-test- 0/200ng MycEG/MaxEG P=0.040, 0/200ng MycEG alone P=>0.05.

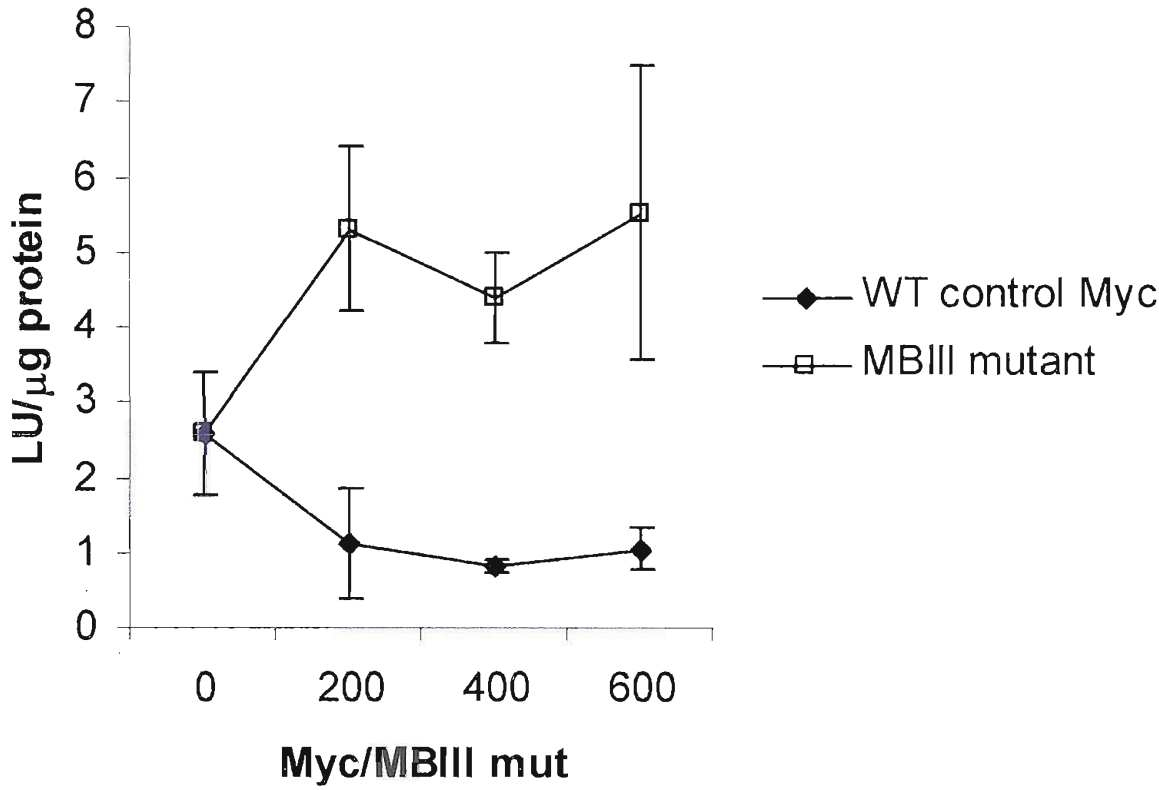


Figure 4.3.20 Integrity of MBIII is essential for repression of the *Nramp1* promoter. RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (0.5μg) of the *Nramp1* promoter construct pL4 and increasing amounts of either the Myc control or both the MBIII mutant construct. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.

Students T-test- 0/400ng WT control Myc P=0.018. 0/600ng WT control Myc P=0.043.

Figure 4.3.21 The integrity of the POZ domain is essential for Miz-1 activation of the *Nramp1* promoter but is not necessary for promoter binding.

A) A Pulldown assay was performed using wild type and mutant Miz-1 (point mutant S46A) recombinant proteins and the biotinylated oligonucleotide to the *Nramp1* promoter. Samples were analyzed for the presence of bound proteins using the Miz-1 antibody (#38 bleed 2) at a concentration of 1:500 (secondary, Goat-anti-Rabbit, 1:15,000) by western blotting (as described in 2.2.13).

B) RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (0.5μg) of the *Nramp1* promoter construct pL4 and increasing amounts of either the Miz-1 Wild type or the Miz-1 mutants (point mutant S46A or deletion mutant Δ33-60). DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.

Students T-test- 0/600ng WT Miz-1 P=0.007(activation), 0/600ng deletion mutant Miz-1 P=0.002 (repression), 0/400ng point mutant Miz-1 P=0.045 (repression).

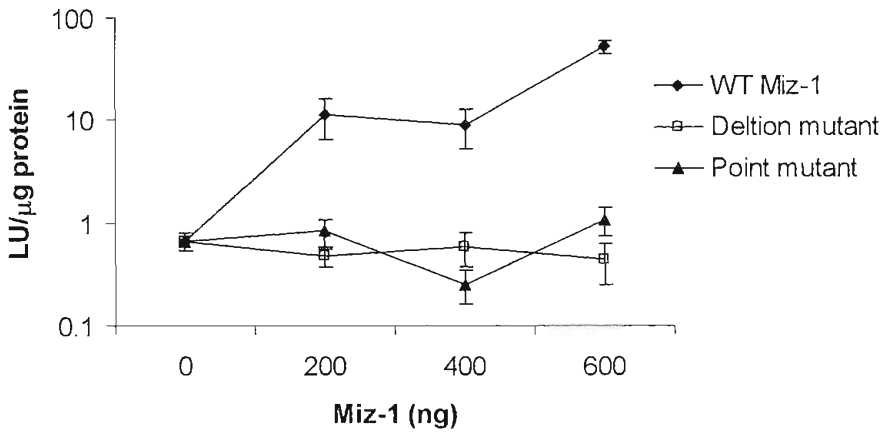
C) RAW 264.7 cells were transfected as described in 2.2.5, with a constant amount (0.5μg) of the *Nramp1* promoter construct pL4 and 0.5μg of either the Miz-1 Wild type or the Miz-1 mutants (point mutant S46A or deletion mutant Δ33-60) or both. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.

Students T-test- Control/WT Miz-1 P=0.003, control/mutants/mutants+WT Miz-1 P>0.05

A)


WT Miz-1 alone
Mutant Miz-1 alone
WT Miz-1 + oligo
Mutant Miz-1 +oligo

B)


100
10
1
0.1
LU/μg protein
0
200
400
600
Miz-1 (ng)
WT Miz-1
Deltion mutant
Point mutant

C)

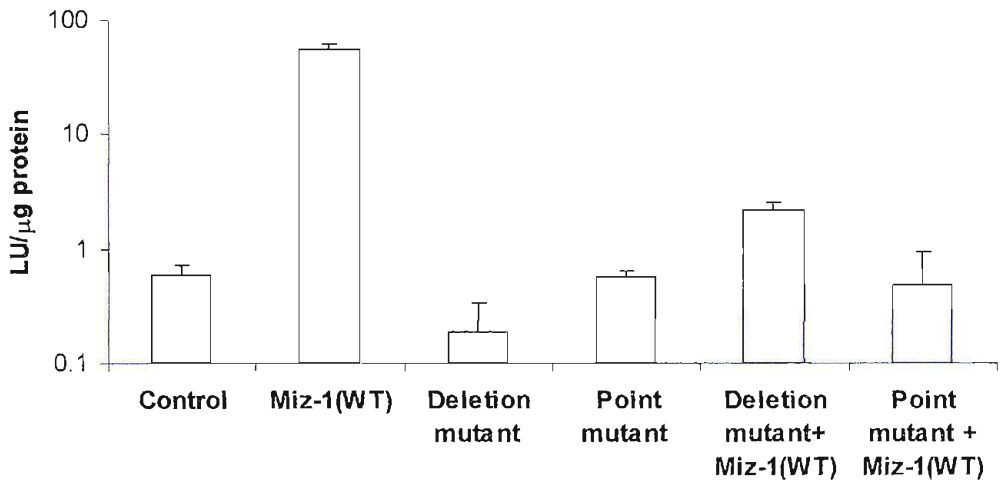
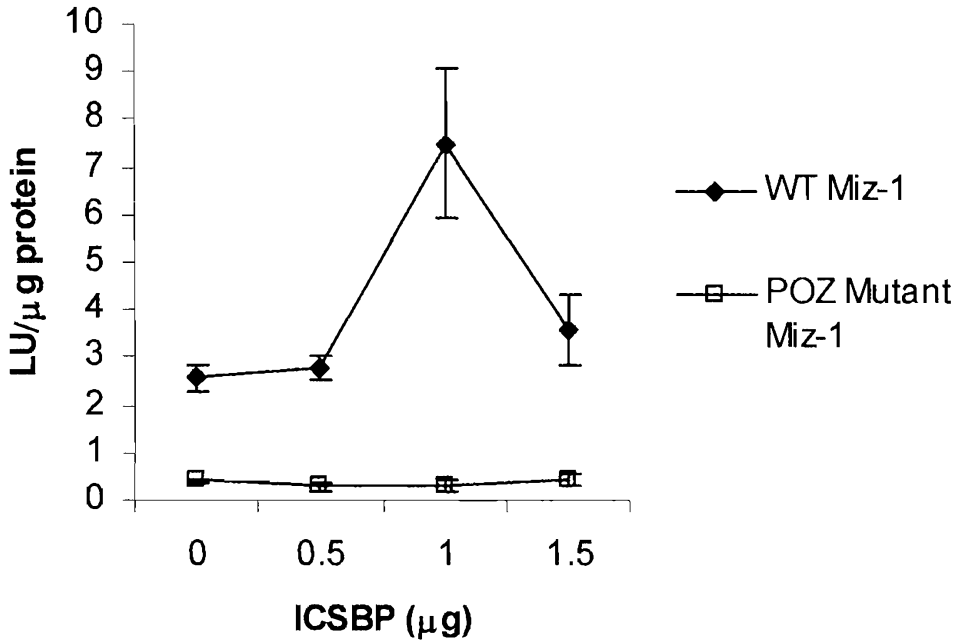

100
10
1
0.1
LU/μg protein
Control
Miz-1(WT)
Deletion mutant
Point mutant
Deletion mutant+ Miz-1(WT)
Point mutant + Miz-1(WT)

Figure 4.3.22 Integrity of the POZ domain is essential for activation of the *Nramp1* promoter with ICSBP and P300.

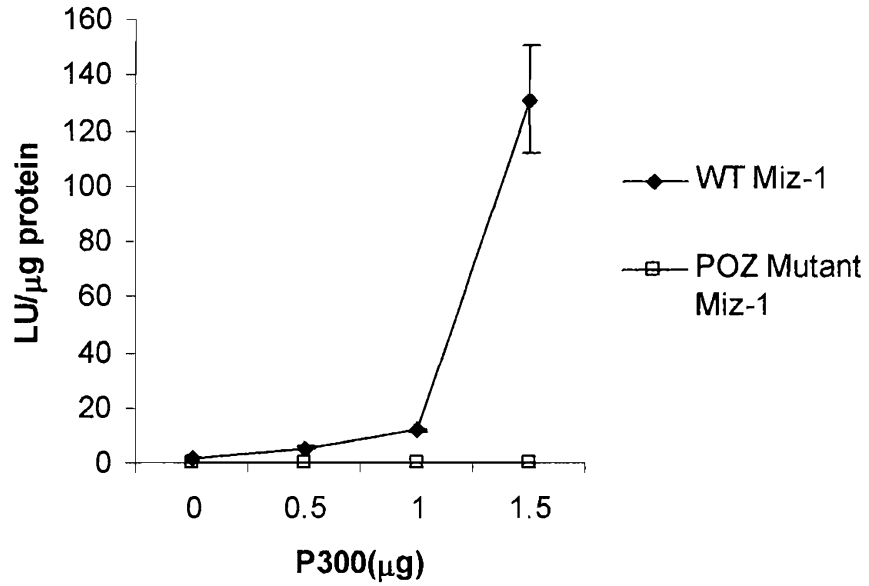
A) RAW 264.7 cells were transfected as described in 2.2.5, with constant amounts (0.5μg) of the *Nramp1* promoter construct pL4, either the Miz-1 Wild-type or deletion mutant-Δ33-60 (0.5μg) and increasing amounts of the ICSBP expression vector as shown. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.
Students T-test- Wild-type Miz-1 0/1.0μg ICSBP P=0.041. Mutant Miz-1 0/0.5, 0/1.0, 0/1.5μg ICSBP P>0.05.

B) RAW 264.7 cells were transfected as described in 2.2.5, with constant amounts (0.5μg) of the *Nramp1* promoter construct pL4, either the Miz-1 Wild-type or deletion mutant-Δ33-60 (0.5μg) and increasing amounts of the P300 expression vector as shown. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per μg of protein.
Students T-test- Wild-type Miz-1 0/0.5μg P300 P=0.006, 0/1.0μg P=0.001, 0/1.5μg P=0.007. Mutant Miz-1 0/0.5, 0/1.0, 0/1.5μg P300 P>0.05

A)


10
9
8
7
6
5
4
3
2
1
0
LU/μg protein
0
0.5
1
1.5
ICSBP (μg)
WT Miz-1
POZ Mutant Miz-1

B)


160
140
120
100
80
60
40
20
0
LU/μg protein
0
0.5
1
1.5
P300(μg)
WT Miz-1
POZ Mutant Miz-1

4.4 Discussion

The data presented in this chapter provide further evidence as to the role of c-Myc, Miz-1 and Sp1 in the regulation of the *Nramp1* promoter.

Use of both murine and human RNAi constructs has confirmed the requirement of Miz-1 for *Nramp1* promoter function. Using the constructs (as described in 4.2.1) pL4 and pL4M6M, with its higher basal activity and increased transactivation by Miz-1 (Bowen *et al,* 2003) reduction in endogenous Miz-1 levels using RNA interference was seen to significantly reduce *Nramp1* promoter activity.

In order to confirm that repression of the *Nramp1* promoter by c-Myc is mediated by interaction with Miz-1 as has been shown to be the case for several but not all c-Myc repressed promoters , $p15^{INK4B}$ (Seoane *et al,* 2001; Staller *et al,* 2001), $p21^{CIP1}$ (Wu,*et al,*2003; Herold *et al,* 2002; Seoane *et al,* 2002) and Mad4 (Kime & Wright, 2002) a point mutant of c-Myc V394D was used. c-Myc has also been shown to cause repression via direct interaction with Sp1 (Feng *et al,* 2000,2002) and in the case of TGF-β dependent induction of the $p15^{INK4B}$ promoter, via the formation of a Smad complex which interacts with Sp1 and is rendered transcriptionally inactive following c-Myc binding (Feng *et al,* 2002). This mechanism that is completely independent of Miz-1 and does not involve any direct DNA binding of c-Myc. Other promoters repressed by c-Myc have also been shown to be repressed without the involvement of Miz-1. GADD45a has been shown to be repressed by c-Myc and chromatin immunoprecipitation assays confirmed that Miz-1 was not bound to the promoter (Barsyle-Lovejoy *et al,* 2004). In addition to this the p27 promoter has been shown to undergo repression by c-Myc via direct interaction of a Myc-Max complex with the initiator element (Yang *et al,* 2001), although in this study the possibility that Miz-1 was a component of this complex was not investigated.

The V394D Myc mutant is unable to interact with Miz-1 but retains the ability to bind Max and activate transcription from E-box elements (Wu *et al,* 2003; Herold *et al,* 2002). Data generated using this construct clearly demonstrate that the ability to interact with

Miz-1 is crucial for c-Myc mediated repression of the *Nramp1* promoter construct pL4 and as the V394D mutant is unable to repress this construct.

c-Myc has been shown to repress *Nramp1* promoter activity (Bowen *et al*, 2002, 2003) as well as a number of other Initiator element containing constructs (reviewed in Gartel & Schcors, 2002), it was of interest to discover that a reduction in the endogenous c-Myc levels caused by RNAi resulted in repression of *Nramp1* promoter activity and not activation as was expected. These findings, along with the fact that co-transfection with Miz-1 could 'rescue' this effect raised the possibility that reduction in endogenous c-Myc levels was somehow causing a reduction in Miz-1 levels. Previous studies (Peukert *et al*, 1997) had suggested that as Miz-1 lacked its own nuclear localization signal (NLS) it may be dependent on c-Myc for its import into the nucleus. Indeed both endogenous and transfected Miz-1 has been shown to accumulate in the cytosol of cells.

We have developed a novel method to monitor the stability of proteins using a Miz-1 GFP chimera and flow cytometry. Levels of fluorescence can be determined following co-expression with RNAi constructs and half-lives of proteins determined following treatment with the translational blocker cycloheximide. Flow cytometry experiments using Miz-1 GFP provided clear evidence that levels of transfected Miz-1 GFP were significantly reduced upon reduction of endogenous c-Myc levels as a result of RNAi. It has been proposed that in the absence of c-Myc other proteins such as P300 (Salghetti *et al*, 1999) may facilitate the entry of Miz-1 into the nucleus but flow cytometry experiments in which P300 was co-transfected in order to 'rescue' the c-Myc RNAi effect failed to restore the Miz-1 GFP levels to that of cells transfected with a control RNAi.

Cycloheximide treatment of cells co-transfected with the Miz-1 GFP and the RNAi constructs revealed that the Miz-1 GFP protein was significantly less stable and with a shorter half life when endogenous c-Myc levels were reduced. This finding links to previous work (Salghetti *et al*, 1999) which has shown that in the absence of Miz-1, c-Myc is less stable and so it is possible that the Miz-1/c-Myc interaction serves to stabilize both partners.

The production of a MIZ NLS construct with its own nuclear localization signal was able to discount the theory that the lack of an NLS and dependency on interaction with c-Myc for translocation into the nucleus was the reason for reduced Miz-1 GFP levels in cells co-transfected with the c-Myc RNAi construct. Flow cytometry clearly showed the Miz NLS construct was still present at much lower levels in cells that had been co-transfected with the c-Myc RNAi construct as compared to the controls, and so the fact that Miz-1 lacks its own NLS and is dependent on other proteins for translocation into the nucleus can not explain the reduced *Nramp1* promoter activity observed with reduced cellular c-Myc levels.

Studies on the *Nramp1* promoter (Bowen *et al*, 2003), have shown that a GC box juxtaposed to the two inrs in the *Nramp1* promoter (figure 4.3.1a&b) is not necessary for c-Myc mediated repression of the *Nramp1* promoter as both deletion experiments and mutation of this GC box have shown that the initiator elements (inr) alone are sufficient to mediate c-Myc repression. However, these studies did reveal that this site is important for Miz-1 activation as both the Sp1 mutant and Sp1 deletion mutant constructs were rendered unable to be activated by Miz-1. This is of interest as it is clear that Miz-1 must still be competent of binding to the *Nramp1* promoter in order for c-Myc repression to occur, as data presented in this thesis using the point mutant of c-Myc V394D (figure 4.3.6) which is unable to bind Miz-1, show that without Miz-1 binding c-Myc repression cannot occur.

Current work (Phillips *et al*, unpublished) has shown that *Nramp1* is positively regulated by and provides protection against oxidative stress. The proposed mechanism for this is thought to be through sequestering iron and limiting the formation of hydroxyl species. Sp1 is a factor that senses oxidant stress and maybe subjected to altered glycosylation through enhanced hexosamine pathway flux. There is controversy here regarding the precise role of glycosylation and Sp1 activity as some studies support an inhibitory role and others a stimulatory role. Glycosylation at specific sites may inhibit interaction with co-factors alternatively glycosylation may stabilize the protein and induce nuclear translocation.

In the RAW 267.4 cells an increased dose of Sp1 led to increased transcriptional activity of the *Nramp1* promoter. (Phillips *et al*, unpublished). These studies demonstrate the requirement of Sp1 for *Nramp1* promoter activity by reducing endogenous Sp1 levels via the use of an RNAi based approach. Results suggest a necessary role for Sp1 in regulating basal *Nramp1* transcription, although it is difficult to assess if Sp1 is required for oxidant stimulated transcription given its necessary role in controlling basal activity.

HDAC recruitment by c-Myc has been investigated by a number of researchers using the potent HDAC inhibitor TSA. TSA treatment blocks Myc repression of the PDGFR–β gene, indicating that c-Myc mediated repression in this instance is via recruitment of HDAC's (Mao *et al*, 2004). In contrast treatment had no effect on Myc repression of the $p21^{Waf1/cip1}$ (Gartel *et al*, 2001, Mao *et al* 2001) or the GADD45A (Gartel *et al*, 2001) promoters. TSA was used in these studies in order to assess the role of HDAC's in Myc mediated repression of the *Nramp1* promoter. The *Nramp1* promoter was found to behave in a similar manner to both the $p21^{Waf1/cip1}$ and the GADD45a promoter with TSA treatment having no effect on Myc repression. Therefore these data show that *Nramp1* repression is occurs independently of HDAC recruitment. ChIP analysis indicated that Myc recruitment and binding to the *Nramp1* promoter is more stable than for other promoters. Myc is known for its short half life and instability (Reviewed Grandori *et al*, 2000). However serum starvation and TSA treatment failed to alleviate endogenous c-Myc binding to the *Nramp1* promoter *in-vivo*. These findings may indicate that Myc binding proteins present at the *Nramp1* promoter are serving to increase its stability as previous studies (Salghetti et al, 1999) have shown that Miz-1 binding stabilises Myc preventing its destruction by ubiquitin mediated proteolysis.

Inspection of the *Nramp1* promoter sequence (Figure 4.3.1a) reveals that *Nramp1* contains two Inr elements in tandem (Govani *et al*, 1995). Functional redundancy of these two Inr's was demonstrated by both *in-vivo* and *in-vitro* binding studies and also by functional experiments. Pull down assays using non-biotinylated competitor oligos with either one or both of the initiator elements mutated demonstrate firstly, that Miz-1 is

binding directly to the initiator elements and secondly that Miz-1 is able to bind to either one of these initiator elements, with seemingly equal affinity. Transient ChIP assays on the mutant *Nramp1* promoter constructs confirm these findings with c-Myc shown to bind to either of the Inr elements *in-vivo*. In addition pull down assays show that c-Myc is only able to bind the *Nramp1* promoter in the presence and not the absence of Miz-1. Therefore the binding of c-Myc to the initiator elements of the *Nramp1* promoter must be via an interaction, or as part of a complex with Miz-1 and not directly to DNA as suggested by (Feng *et al*, 2000, 2002).

This has been demonstrated for Myc mediated repression of a number of other initiator containing promoters, including $p21^{CIP1}$ (Wu *et al*, 2003; Herold *et al*, 2002), $p15^{INK4B}$ (Seoane *et al*, 2001, Staller *et al*, 2001) *Mad4* (Kime and Wright, 2002) and *GADD 153* (Barsyte-Loveljoy *et al*, 2004). Interestingly the presence of c-Myc seems to enhance the binding of the Miz-1 protein to the DNA. It is possible that this is due to enhanced stability of the Miz-1 protein in the presence of c-Myc, as it has already been shown (Salghetti *et al*, 1999) that interaction with Miz-1 is able to stabilize the c-Myc protein, alternatively c-Myc may drive a conformation of Miz-1 that is more appropriate for direct DNA binding.

Data from functional analysis of Inr mutant constructs supports the findings of the binding studies. Mutation of either Inr was not found to extinguish promoter activity, as compared to the double Inr mutant. In addition both single Inr mutants were found to retain their responsiveness to both Miz-1 and c-Myc.

These findings taken with the lack of repression of the *Nramp1* promoter with the V394D c-Myc mutant which is unable to interact with Miz-1, confirm that c-Myc mediated repression of the *Nramp1* promoter is via an interaction with Miz-1 at either initiator element, and not via an alternative mechanism such as that involving the Sp1 site as that proposed for the $p15^{INK4B}$ promoter (Feng *et al*, 2000, 2002).

Although several researchers have indicated that hetero-dimerization with Max is not necessary for Myc repression (Gartel et al, 2001; Mao et al, 2004) it has been demonstrated in these studies that repression of *Nramp1* by c-Myc requires a functional Myc/Max complex. Using the Myc and MaxEG constructs (Amati *et al*, 1993) which are unable to bind to native Max and Myc respectively, MycEG was shown to be incapable of repressing the *Nramp1* promoter in the absence of MaxEG. These findings support those of Staller (Staller *et al*, 2001) who showed that Max was present in the Myc/Miz-1 complex bound at the $p15^{ink4b}$ promoter and was therefore necessary for repression.

The recently identified Myc Box III is highly conserved among Myc family members and is localised centrally (amino acids 188-199). Studies have shown that this region is important for Myc function, contributing to cellular transformation and downmodulating the ability of Myc to induce apoptosis (Herbst *et al*, 2005). MBIII deletion mutants show that this region is essential for repression of all Myc repressed target genes tested (Herbst *et al*, 2005). In agreement with these findings *Nramp1* could not be repressed by a MBIII mutant, confirming that this region is essential for *Nramp1* repression.

N-terminal BTB/POZ mutants of Miz-1 (S46A and Δ33-60) revealed that the integrity of this domain is essential for Miz-1 function. This is in accordance with the findings of Peukert *et al* (1997) who demonstrated that deletion of this domain resulted in loss of function of Miz-1. Studies on the POZ domains of several other BTB/POZ domain containing proteins have indicated that this domain mediates Homo- and Hetero-dimer formation and that the conserved serine residue located at position 104 in Keap1 and 46 is Miz-1 is required to mediate these interactions (Ahmad *et al*, 1998; Zipper & Mulcahy, 2002).

Findings presented in this thesis support the idea that this residue is essential for dimer formation and provides evidence to support the findings of Zeigelbauer *et al* (2004) who propose that Miz-1 functions as a dimer or multimer. Both POZ/BTB domain mutants were found to function as dominant negatives and to prevent transactivation with co-

factors such as P300 and ICSBP. These dominant negative Miz-1 mutants could serve as useful tools in understanding the genome wide role of Miz-1.

CHAPTER 5

Analysis of the NCAM promoter

5.1 Introduction

5.1.1 NCAM structure and function

NCAM was the first cell-cell adhesion molecule to be purified and sequenced (Thiery *et al*, 1977). NCAM is expressed by a variety of cell types including most nerve cells. In the mouse alternative splicing has been shown to produce several variants of NCAM, including 180, 140 and 120KDa isoforms. However, the predominant forms are the 180 and 140KDa forms which each contain three domains, an extra cellular domain, a membrane spanning domain and a cytoplasmic domain. The smallest, 120kDa splice variant of NCAM consists solely of an extra cellular domain covalently bound to a membrane lipid (Alberts *et al*, 1994). In all splice variants of NCAM the extra cellular domain consists of a polysaccharide chain folded into five IgG like domains.

Unlike other cell-cell adhesions molecules such as the cadherins NCAM does not require calcium for its cell binding. NCAM molecules on adjacent cells interact with each other homophillically to construct bonds between cells. NCAM is only able to mediate cell-cell adhesion in other cells containing NCAM on their surface (Rutishauser *et al*, 1982).

The cell-cell adhesion properties of NCAM are modulated by the levels of polysialic acid, which is a negatively charged sugar molecule. This sugar changes in amount and form during developments and the presence of longer chains in observed during cell migration where large quantities of negative charge hinders cell-cell adhesion (Hoffman and Edleman, 1983).

5.1.2 NCAM promoter analysis

The gene for NCAM was cloned and sequenced in the late 1980's (Cunningham *et al*, 1987), and revealed the basis for the different splice variants of NCAM. All of the major RNA transcripts were found to utilize a common constitutive 5' exon indicating that they arise from a common transcriptional control region.

Characterization of the NCAM promoter region revealed that it lacked a classical TATA or CAAT element and that it contained two major transcription initiation start sites

(Figure 7.3.1) (Barton *et al*, 1990). Sequence analysis also revealed the presence of a GC box (Sp1 site) in the promoter region.

5.1.3 Transcriptional regulation of the NCAM promoter

The expression of NCAM has been shown to be precisely regulated in terms of cell type specificity and developmental control (Cunningham *et al*, 1987). However, the role of extra cellular factors that may be involved in this control is still not fully understood. Previous studies (Roubin *et al*, 1990) have shown a role for TGF-β in modulating NCAM expression by stimulating the activity of the NCAM promoter.

TGF-β belongs to a family of over 35 related pleitrophic cytokines in vertebtates and functions through binding to a Type II Serine /Threonine receptor kinase. This binding leads to the phosphorylation of the type I Serine/Threonine receptor to form an activated complex (Dijke & Hill, 2004). The activated receptor then phosphorylates Smad 2 and 3 enabling them to form complexes with Smad 4 and translocate to the nucleus (Nakao *et al*, 1997). Once in the nucleus these Smad complexes are able to regulate the transcription of TGF-β target genes.

Transactivation of TGF-β target genes is by the binding of the N-terminal MH1 of Smad3 to a Smad Binding Element (SBE) present in the promoter of target genes. The MH1 domain of Smad3 is tethered by a protein rich linker to the MH2 which contains a transactivation domain capable of binding co-activators such as CBP/P300 (Jankecht *et al*, 1998).

The proto-oncogene n-Myc has been shown to cause repression of NCAM promoter activity (Akeson & Bernards, 1989) and this could provide an alternative explanation for the effects of TGF-β on the NCAM promoter. It has been shown that TGF-β down-regulation of c-Myc expression is required for the transactivation of the CDK inhibitors $p15^{Ink4B}$ and $p21^{CIP1}$ (Claassen *et al*, 2000; Seoane *et al*, 2001). TGF-β mediated repression is less well understood than transactivation, although the mechanism is beginning to be elucidated.

A TGF-β inhibitory element (TIE) was first identified in the TGF-β repressed gene *Stromelysin* 1 (Kerr *et al*, 1990). This element was shown to bind a nuclear protein complex whose recruitment led to repression of *Stromelysin* 1. A TIE has also been identified in the *c-Myc* promoter and has recently been characterized and shown to be essential for TFG-β mediated repression of *c-Myc* (Chen *et al*, 2001). More recent studies (Frederick *et al*, 2004) have shown that transcriptional repression of *c-Myc* by TGF-β is dependent of the binding of Smad3 to an element present within the TIE of the *c-Myc* promoter which they have termed a 'Repressive Smad Binding Element'.

Data presented in this chapter aims compare the transcriptional repression by c-Myc of the NCAM promoter with that of the *Nramp1* promoter. This is of interest as the NCAM promoter, like the *Nramp1* promoter is TATA box deficient. However, unlike the *Nramp1* promoter which contains both an initiator and an initiator like element, the NCAM promoter contains two overlapping initiator elements.

5.2 Results

5.2.1 Myc mediated repression of the NCAM promoter

The NCAM promoter construct, NCAM-pGL3 basic, was co-transfected into ND7 cells with increasing amounts of either the Myc expression plasmid or the V394D Myc mutant which is unable to interact with Miz-1 (Herold *et al*, 2002). Figure 5.3.2 shows that both of the Myc constructs tested are able to repress the NCAM promoter, although the V394D Myc mutant seems to repress much more strongly than the wild type. At a DNA concentration of 120ng the wild type Myc caused a 3.2-fold repression (P=0.001) compared to the mutant, which at the same concentration gave 25-fold repression (P=0.001) compared to the activity of the promoter alone. These data indicate that the Myc mediated repression of the NCAM promoter is not due to an interaction of Myc with Miz-1, and must instead be through an alternative mechanism. In order to confirm that this result was a feature of the NCAM promoter and not of the different cell types used the *Nramp1* promoter construct was introduced into ND7 cells (figure 5.3.3). Repression of *Nramp1* promoter function was observed with wild type c-Myc but not the V394D mutant (P=0.004 for 400ng), confirming that Miz-1 is functional in the ND7 cell and is capable of binding to the *Nramp1* promoter.

5.2.2 c-Myc but not Miz-1 is bound at the NCAM promoter *in-vivo*

ChIP assays were carried out in ND7 cells to identify factors bound at the NCAM promoter in-vivo. Miz-1 binding to the NCAM promoter could not be detected *in-vivo*, whereas c-Myc was shown to be bound in proliferating cells but not serum starved or TSA treated cells (figure 5.3.4). Figure 5.3.5 shows that transfection of ND7 cells with either Sp1 or c-Myc RNAi leads to loss of both these factors at the NCAM promoter. Loss of c-Myc was also observed following serum starvation and time course experiments (figure 5.3.6) reveal that this loss occurs rapidly with c-Myc binding at the NCAM promoter lost after 30 minutes.

5.2.3 Demonstration of a role for c-Myc and Sp1 in NCAM promoter function.

The NCAM promoter has been found to contain an Sp1 site (GC box) (figure 5.3.1a&b). The murine Sp1 RNAi construct is used to assess the effect of reduced endogenous Sp1 levels on the activity of the NCAM promoter construct NCAM-pGL3 basic. Increasing amounts of the murine Sp1 RNAi construct were co-transfected into ND7 cells with a constant amount of the NCAM promoter construct NCAM-pGL3 basic. Figure 5.3.7 shows that even at the lowest concentration tested (0.5μg), the use of this construct results in a 10.6-fold reduction (P=0.016) in *NCAM* transcription. This confirms that the level of Sp1 present in a cell can influence the level of *NCAM* transcription.

Despite Myc repression of the NCAM promoter being clearly demonstrated, NCAM promoter activity is seen to be reduced when the endogenous Myc levels are reduced using the Myc RNAi construct (figure 5.3.8). At a concentration of 1.5μg a five fold reduction in promoter activity was observed as compared to co-transfection carried out using the control RNAi construct, (P=0.001). This effect could not be rescued by co-transfection of the Myc RNAi construct with the Miz-1 expression plasmid (figure 5.3.9). A significant reduction in promoter activity is still observed with the Myc RNAi as compared to the control at every concentration of Miz-1 tested.

5.2.4 Identification of the mechanism of c-Myc repression.

Use of the potent HDAC inhibitor TSA revealed that c-Myc mediated repression of the NCAM promoter requires HDAC activity. 50 fold activation of the basal transcription level of NCAM was observed following TSA treatement (200ng/ml for 24 hours). c-Myc repression of NCAM was found to be sensitive to TSA treatment with no concentration of Myc tested achieving significant repression (figure 5.3.10). In contrast with untreated cultures significant repression was observed at 10ng Myc (P=0.035). These results suggest that in the case of the NCAM promoter Myc may be recruiting via the Sp1 consensus site either an HDAC or another factor with HDAC activity.

As Miz-1 was shown to have no role in Myc mediated repression of the NCAM promoter, a Sp1 mutant NCAM promoter construct was used (figure 5.3.11). This enabled elucidation of a possible role for Sp1 in Myc repression as reported for other Sp1 site containing Inr promoters (Feng *et al*, 2002; Gartel *et al*, 2001). The NCAM Sp1 mutant construct was found not to be repressed by c-Myc at any concentration relative to controls using wild type c-Myc ($P<0.01$ for concentrations of wild type c-Myc).

Substitution of the Sp1 site of the NCAM promoter with the binding site for the yeast GAL4 transcription factor allowed the Sp1 activity to be re-constituted using a Sp1-GAL4 chimera, pMSp1 with the empty vector pM as control. The NCAM Sp1GAL4 promoter construct was found to have significantly reduced activity following re-constitution of Sp1 activity ($P=0.003$ at 200ng pMSp1) as shown in figure 5.3.12a. In addition reconstitution of Myc repression was observed when pMSp1 was added but not pM ($P=0.039$) figure 5.3.12b.

Although a role for Sp1 in c-Myc repression of the NCAM promoter has been clearly demonstrated, the use of Myc constructs which contain central region deletions still repress the NCAM promoter ($P<0.04$ for all constructs) (figure 5.3.13). This is of interest as the central region of c-Myc has been shown to interact with Sp1 (reviewed in Gartel *et al*, 2002). Neither of the mutants used contain an entire central region deletion as this would result in loss of the NLS also present in this region. Therefore it is possible that neither of the deletions made completely obliterate Myc Sp1 binding which may be via a two pronged interaction.

In order to discover whether Max is a part of the complex involved in repression of theNCAM promoter, the Myc and Max EG constructs were used (Amati *et al*, 1993). These constructs are unable to bind to native Max and Myc respectively. Results demonstrated (Figure 5.3.14) that repression of the NCAM promoter could only be achieved with a functional Myc/Max complex. MycEG alone failed to repress the

NCAM promoter P>0.05 whereas MycEG and MaxEG together were able to significantly repress (P<0.025 at 20 & 40ng) the NCAM promoter.

MBIII, a conserved domain centrally located at amino acids 188-199 of Myc (Herbst *et al*, 2005) is believed to be important for some aspects of Myc function, including repression. In order to determine whether this region of Myc is important for Myc mediated repression of the NCAM promoter a MBIII mutant (Herbst *et al*, 2005) in which the MBIII region had been deleted was co-transfected into ND7 cells along with an NCAM promoter construct. Loss of MBIII was seen to abolish the ability of Myc to repress the NCAM promoter as compared to a wild-type Myc control construct which was able to significantly repress the NCAM promoter (P=0.002 AT 40ng) (Herbst *et al*, 2005).

Figure 5.3.1 (A) Annotated sequence of the *NCAM* promoter from Pst site at position -1198 to the ATG translation initiation site at +194 Base pairs. Transcription factor binding sites present in the sense strand of the promoter have been identified and are shown in bold. The initiator element which is present at and around +1 is also shown in bold and the major transcription initiation sites are indicated with an arrow below the sequence. The ATG translation initiation sequence is shown in bold and is indicated with an arrow above the sequence.

(B) A schematic diagram of the NCAM promoter-The region containing the two overlapping initiator elements is indicated. Also shown are the GC box and two E-boxes. The distance (in base pairs) between these sites is indicated below.

A)

-1198
CTGCAGCCTGGGCGACAGAGCGAGGCTCCATCTCAAAAAAACAAAACAGAAACAAAACAAAACAAAACAAAAAACCTGCTACAG

-1109 LEF-1 LEF-1
GAGTGGGGAGGCCGA**C*CTTTGAA***GAAAAACGGAGTACCCGGTAACATTAGTGCTTTAATGC***CTTTGAA***CTTATGCAGACTTCCTCTGTT
AGAGGGTTTCAGTGTTCTAGGCTAATGGG

-991 EMS#1
TTAACCTGACATCTAGAACACCTTTCTCACATTAGTTCCTTACATACCCAAGCCTT**CAGGTG**CTGAGACATGATTCTTTTCACCCCGCT

-902 EMS #2
TTCTCCACCCCCTACTTTTGAAAA**CACGGG**T*GGAATTTTAATTAAA*GCCTATTGTGTT*GGTACC*TCAGTAATATTATACATTAATATCT
TTAAGAATTAAGGTCACGTCCCCATGTAAGAAAATATTATTTAATGACGCTTCTATATCATAATACCTATATAAAAGCCTGGCTATTTT
AATAAAGAGACCACAGATTTCAGAATTTATAAACAGGAAAACATTTTCTTCGGGTTATTTCTGGAAATCTCTTCCAAACATCGGAGTTT
TCTTCTAACTTAAGTCTCTTCCCACCTCCTTCCCAGGGGATCTGCTGAAGGGTGTTGTATGTCTCTCTGTGGGAGAGCAAACTTCACAG
TTAGGATAAAACAAACAAACAAACAATATCCAAACAACACCCAGCAACGGCAACCCCCATCCCTCTCCAAAGTTCTAATTTCCCGCACT
TAAAGTCCTGGGCTATCCTTGTGTTGCAAGGATCTTAGAATCGAAATGGAGGGATTTGACAACTTTACCTAACCAAATCTAAAATTTTG
CTTTTATTATTT*ACTAGT*TATCAAAATATGCAAACTGCTGATTAAGGAAGGCTGGGTAGCAGGAGCGCCTGCGAAGGCGTAGGGTAGAA
GTGTGAAAAGAAATCCCAGCTCTCCCAGGGAGACTGCGTGTGAAAGAGCCCGGCTCCCCCAAAAGCTCCAGGCCGCGTTTTGCAGGCTT

-190 EMS #3
CCG**CATCTG**CCTCCCTGTCTCTCT*TACCTCCTTGATGTTCGGCACTATTTG*TGGCCGGCGTGGTGGAAGGACACAGTGAGGTTCTCACC

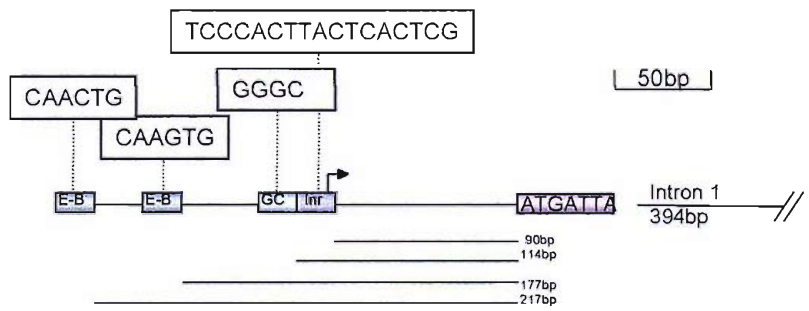
Sp1
C**CCGCC**CCCCGCTCCTCGCTCCCATCCCAGTTCCATCAAAACGAACCCGGGCCAGCGCAAGGATCTCCGAGTTGCGAGTGTGCTGAGGC

-8 Inr
TGGGACTG**TCACTCATT**CTCCGATCAGCGCGTGAACGCAGCTCGGCTG**CC**GCTGGCAGGAAACAATTCTGCAAAAATAATCATACTCAG

+81
CCTGGCAATTGTCTGCCCCTAGGTCTGTCGCTCAGCCGCCGTCCACACTCGCTGCAGGGGGGGGGGCACAGAATTTACCGCGGCAAGAC

ATCCCTCCCAGCC***ATG***CTGCCAAACTAAGGATTTCAT*TTGGACTTTGTTTTTCC*TGGGAA**CT**GCAG
+195

B)



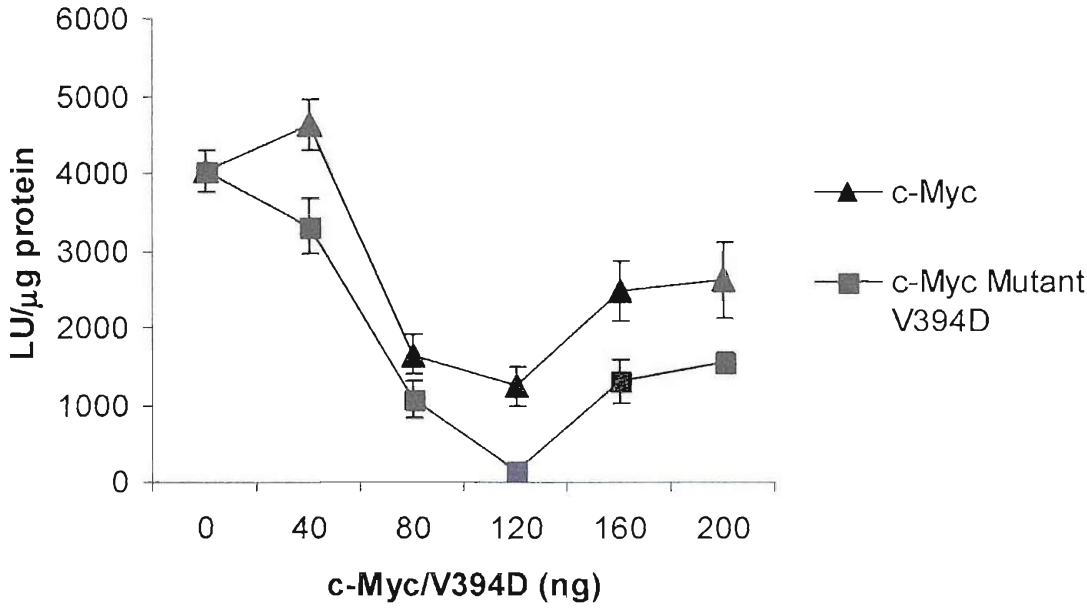


Figure 5.3.2 The Myc mutant V394D is able to repress the NCAM promoter. ND7 cells were co-transfected as described in 2.2.6 with a constant amount (0.5µg) of the NCAM promoter construct and increasing amounts of the Myc or V394D Myc mutant expression plasmid, as shown. DNA concentrations were normalized using the pCDNA3.1 empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Test-

Myc 0/120ng P=0.001, V394D 0/120ng P=0.001

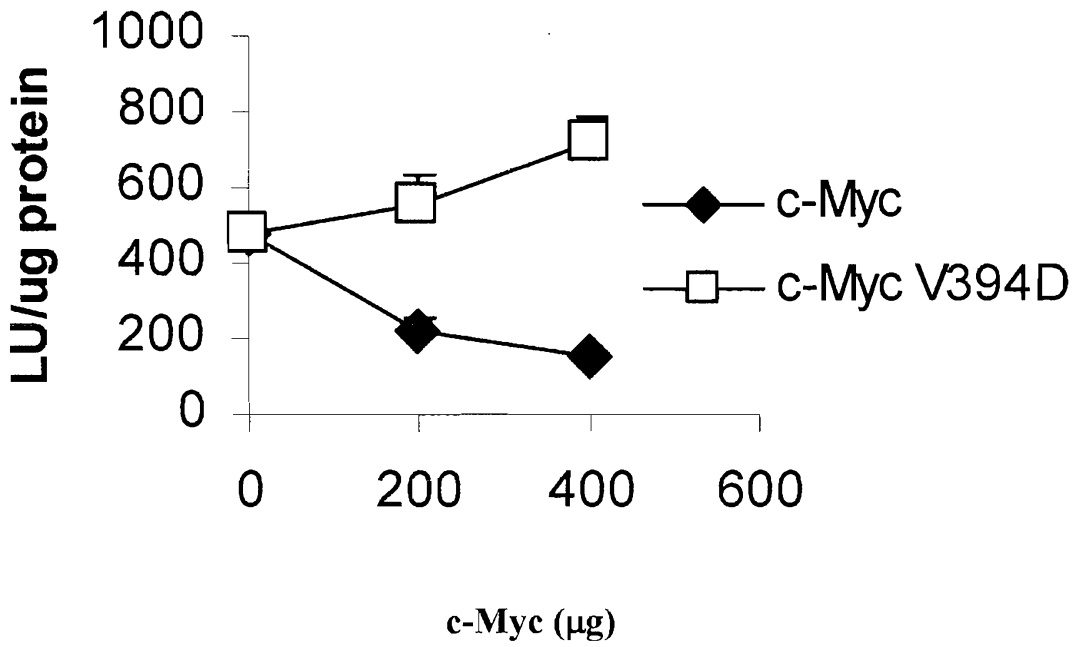


Figure 5.3.3 Miz-1 is still functional in ND7 cells. ND7 cells were co-transfected as described in 2.2.6 with a constant amount (0.5µg) of the *Nramp1* promoter construct and increasing amounts of the Myc or V394D Myc mutant expression plasmid, as shown. DNA concentrations were normalized using the pCDNA3.1 empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Test-
0/400ng WT c-Myc P=0.004.

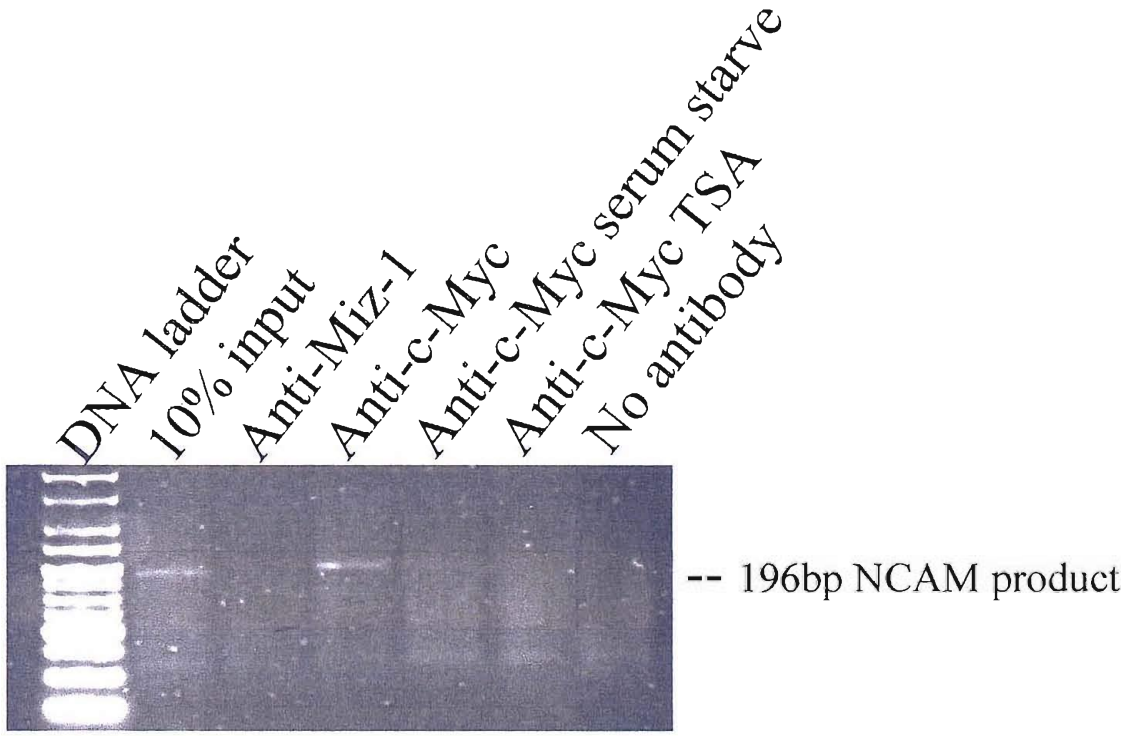


Figure 5.3.4 c-Myc and not Miz-1 is bound to the NCAM promoter *in-vivo*. ND7 cells were plated out at $1x10^6$/90mm dish and incubated either with or without serum/TSA as indicated above. Following 24 hour incubation cells were harvested and ChIP assays were carried out (as described in 2.2.16) using antibodies as indicated above. Products of PCR reactions were then loaded onto a 2% agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 volts for 30 minutes.

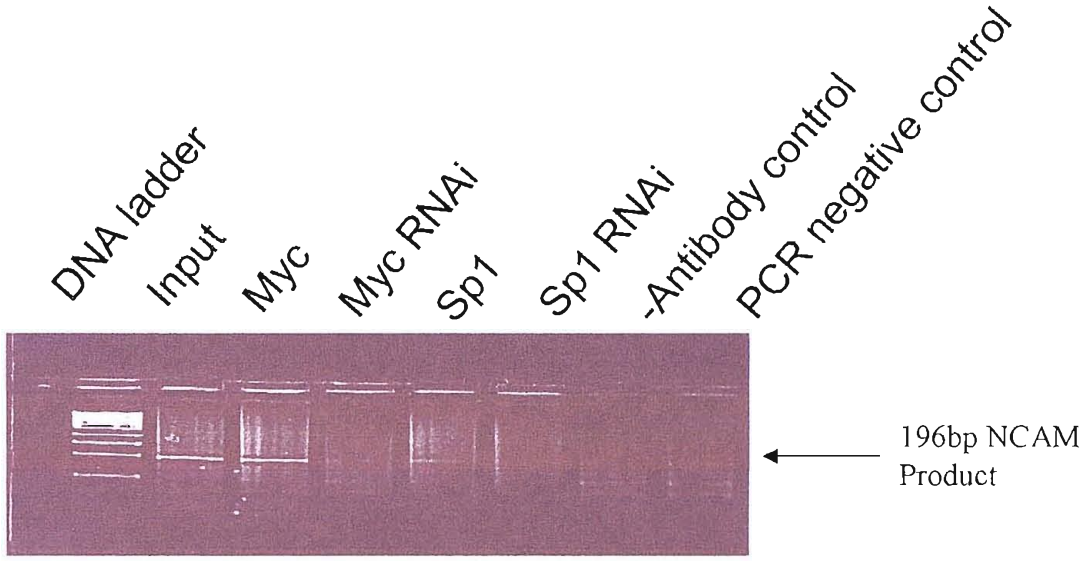


Figure 5.3.5 ChIP assay to demonstrate knock down of endogenous protein at NCAM promoter.

ND7 cells were transfected with either a control RNAi expression plasmid (IREG-1) or an RNAi construct directed against a protein of interest (as indicated). ChIP assays were carried out (as described in section 2.2.16), using antibodies as detailed above. Products of PCR reactions were loaded onto a 2% Agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 Volts for 30 minutes

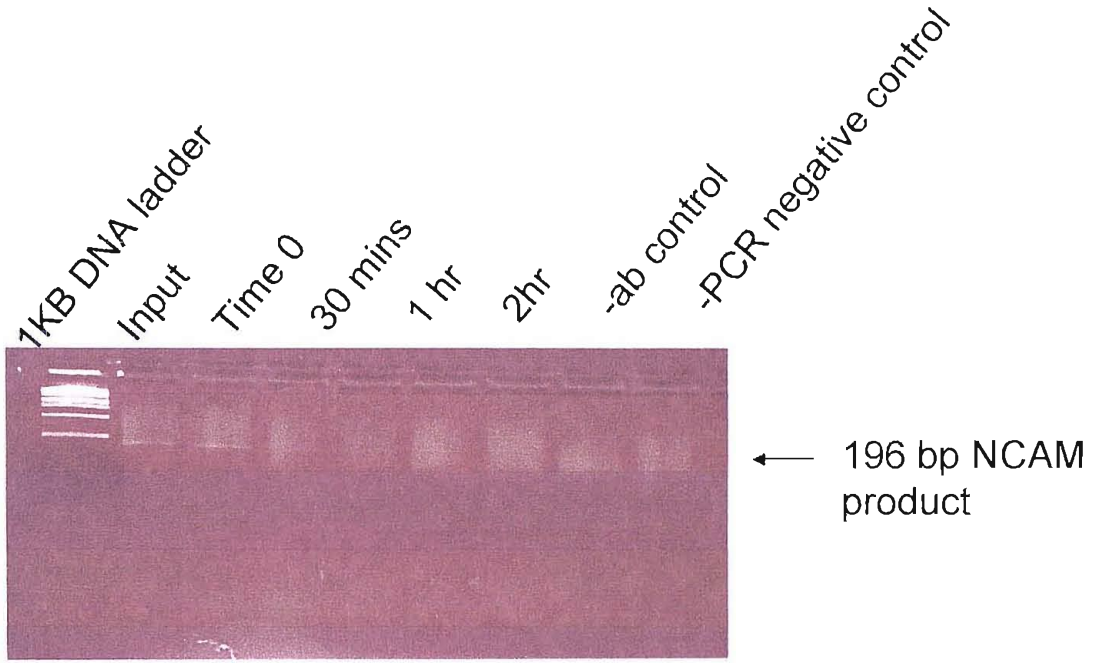


Figure 5.3.6 c-Myc is lost from the NCAM promoter upon serum starvation. ND7 cells were plated out at $1x10^6$/90mm dish and incubated either with or without serum as indicated above. Following 24 hour incubation cells were harvested and ChIP assays were carried out (as described in 2.2.16) using antibodies as indicated above. Products of PCR reactions were then loaded onto a 2% agarose TBE gel containing 0.01% Ethidium Bromide and run at 120 volts for 30 minutes.

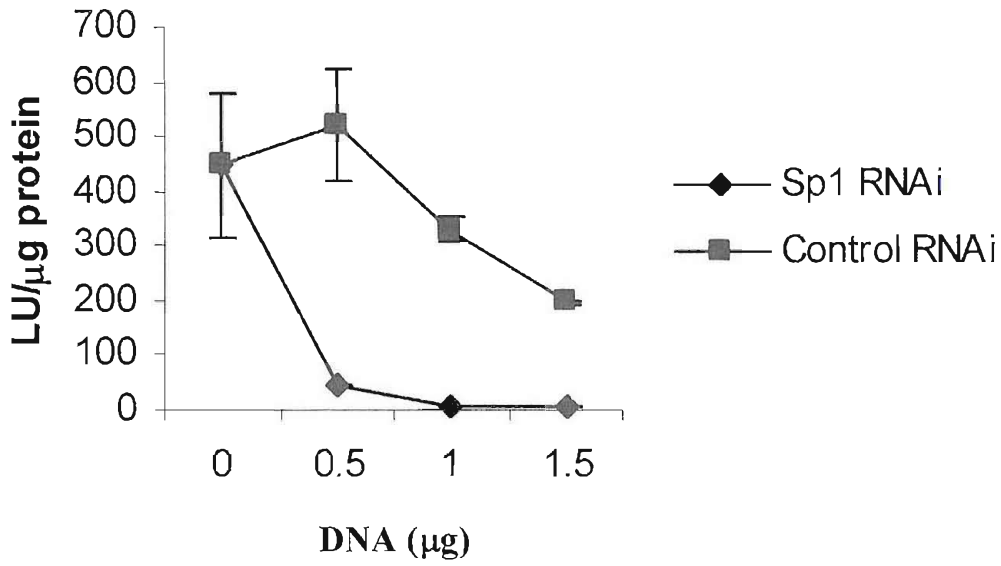


Figure 5.3.7 Demonstration of a role for Sp1 in NCAM promoter activity. ND7 cells were co-transfected (as described in 2.2.6) with a constant amount of NCAM promoter construct (0.5µg) and increasing amounts of the either the Sp1 or Control RNAi constructs. (0,0.5,1.0& 1.5µg)). DNA concentration was normalized using the pBABE empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students-T-test

Sp1 RNAi/Control RNAi at all concentrations $P<0.016$.

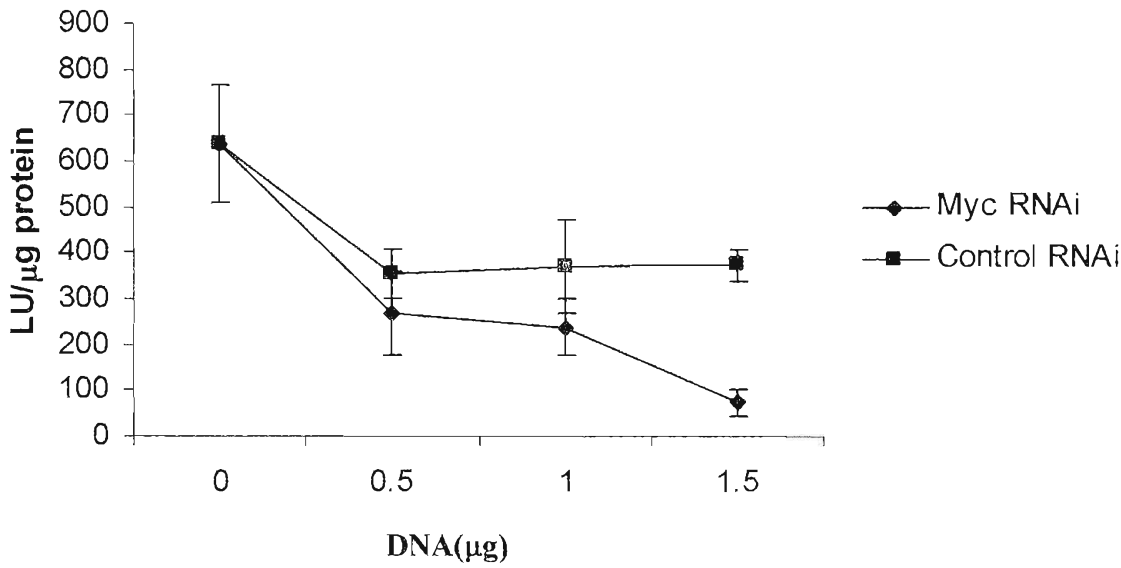


Figure 5.3.8 Demonstration of a role for Myc in NCAM promoter activity. ND7 cells were co-transfected (as described in 2.2.6) with a constant amount of NCAM promoter construct (0.5µg) and increasing amounts of the either the Myc or Control RNAi constructs. (0,0.5,1.0& 1.5µg)). DNA concentration was normalized using the pBABE empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students-T-test

Myc RNAi/Control RNAi at 1.5µg P=0.001

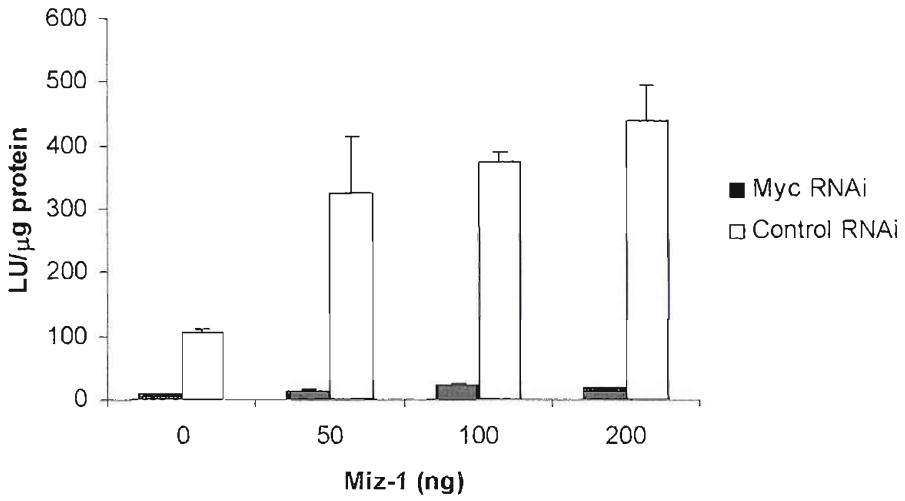


Figure 5.3.9 Miz-1 is unable to rescue the Myc RNAi effect in ND7 cells. ND7 cells were co-transfected as described in 2.2.6 with a constant amount (0.5µg) of the NCAM promoter construct and 1.5µg of either the control or the Myc RNAi construct. In addition to this increasing amounts of the Miz-1 expression plasmid were titrated (0, 50,100 & 200ng). DNA concentrations were normalized using the pBABE empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-test

Myc RNAi/ Control RNAi $P<0.027$ for all concentrations of Miz-1

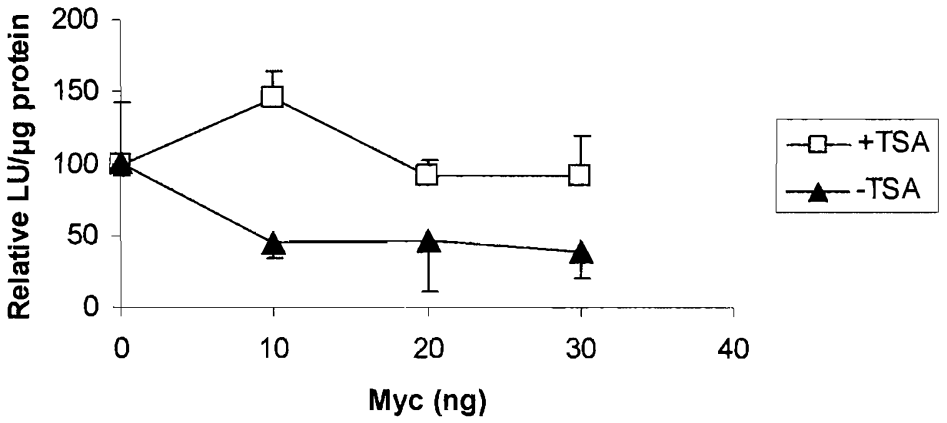


Figure 5.3.10 c-Myc repression of NCAM requires HDAC activity. ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the NCAM promoter construct and increasing amounts as shown of the c-Myc expression vector. DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample. Data is shown as relative LU/µg with respect to c-Myc untreated cultures.

Students T-Tests- (-TSA) 0/10ng c-Myc P=0.035

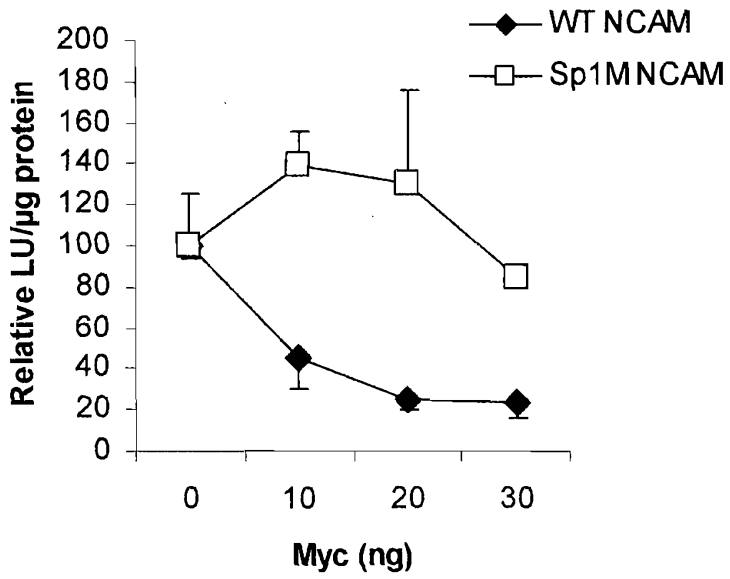


Figure 5.3.11 The Sp1 binding site is essential for repression of NCAM by c-Myc. ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the wild type or Sp1 mutant NCAM promoter construct and increasing amounts as shown of the c-Myc expression vector. DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample. Data is shown as relative LU/µg with respect to c-Myc untreated cultures.

Students T-Tests- Wild type NCAM promoter P=<0.01 for all concentrations of c-Myc.

Figure 5.3.12 Use of a GAL4 SP1 Chimera to determine the mechanism of Myc mediated repression of the NCAM promoter.

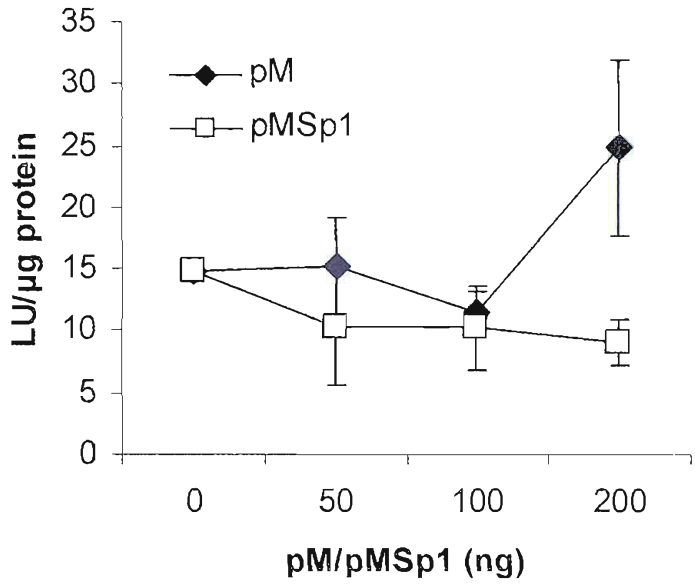
A) ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5μg) of the NCAM Sp1 GAL4 promoter construct and increasing amounts as shown of the empty vector pM or the pMSp1 GAL4 chimera. DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample.

Students T-Tests- 0/200ng pMSp1 P=0.003.

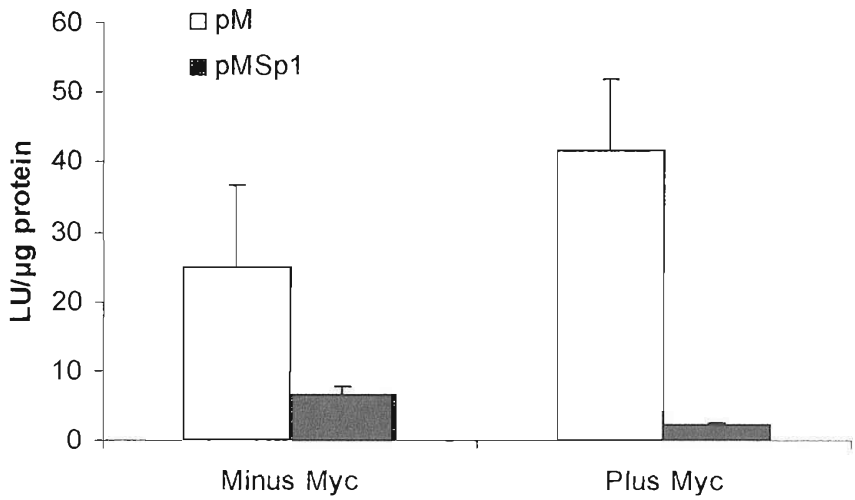
B) ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5μg) of the NCAM Sp1 GAL4 promoter construct and 200ng of either the empty vector pM or the pMSp1 GAL4 chimera with and without 40ng c-Myc. DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample .

Students T-Tests- pMSp1 +/- c-Myc P=0.039.

A)


35
30
25
20
15
10
5
0
LU/µg protein
pM
pMSp1
0
50
100
200
pM/pMSp1 (ng)

B)


60
50
40
30
20
10
0
LU/µg protein
pM
pMSp1
Minus Myc
Plus Myc

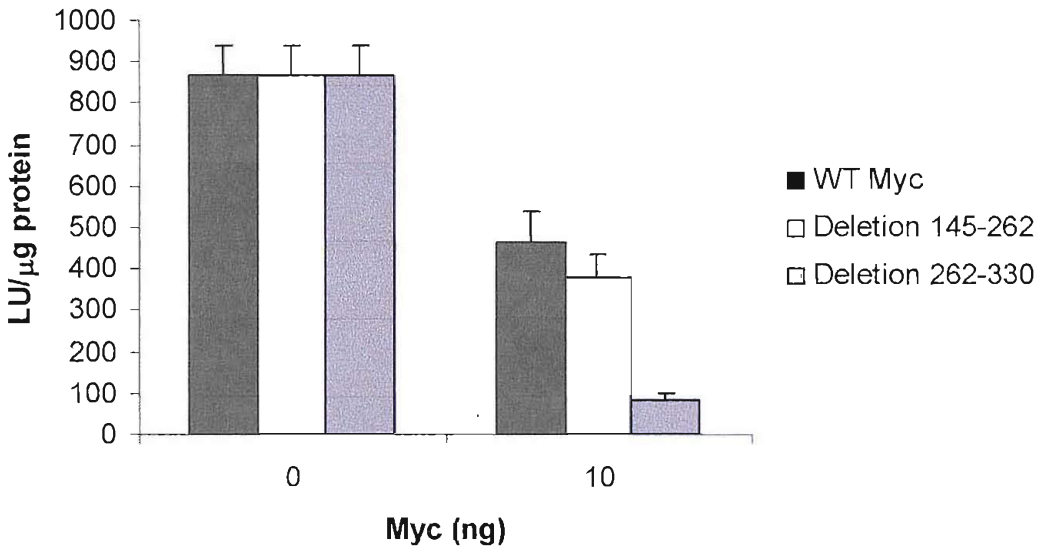


Figure 5.3.13 Central region deletions of c-Myc do not alter its ability to repress the NCAM promoter. ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the NCAM promoter construct and 10ng of wild type or deletion mutant c-Myc as shown . DNA concentrations were normalised using pCDNA3.1 empty vector. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Tests- For all constructs 0/10ng $P<0.004$

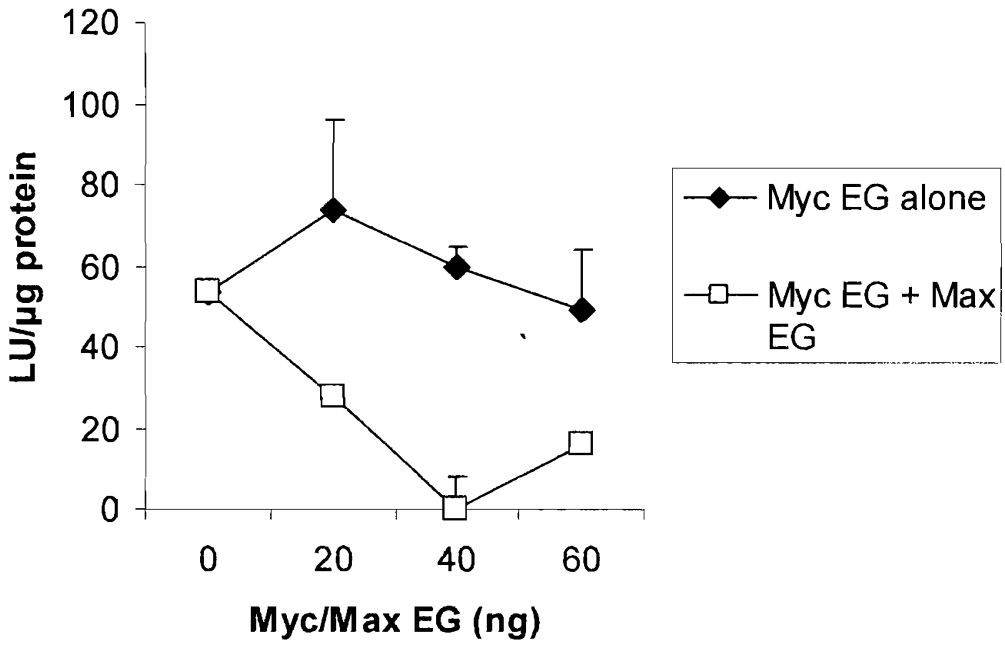


Figure 5.3.14 Max is required for Myc repression of the NCAM promoter. ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the NCAM promoter construct and increasing amounts of either the Myc EG construct or both the Myc and Max EG constructs together. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-Test- 0/20 & 0/40ng MycEG/MaxEG $P<0.025$ MycEG alone all concentrations $P=>0.05$

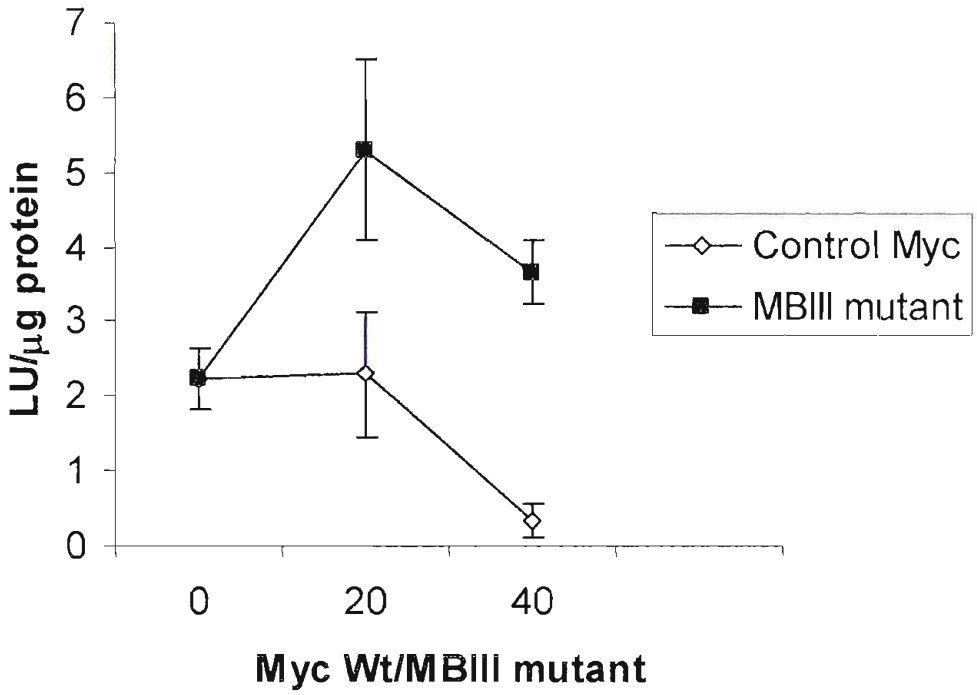


Figure 4.3.15 Integrity of MBIII is essential for repression of the NCAM promoter. ND7 cells were transfected as described in 2.2.5, with a constant amount (0.5µg) of the NCAM promoter construct and increasing amounts of either the Myc control or both the MBIII mutant construct. DNA concentrations were normalised using the pCDNA3.1 empty vector control. Expression of the reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample to give a final value of luciferase activity per µg of protein.

Students T-test- 0/40ng WT control Myc P=0.002. 0/40ng MBIII Mutant Myc P=0.060.

5.4 Discussion

These data serve to confirm previous findings (Akeson & Bernards, 1989) that Myc represses the NCAM promoter. Failure of the Myc mutant V394D to repress the NCAM promoter construct NCAM-pGL3 basic indicates that the Myc repression in not through interaction with Miz-1, a finding supported by ChIP analysis which revealed that Miz-1 was not bound at the NCAM promoter. This is in contrast with other c-Myc repressed promoters including the cyclin dependent kinase inhibitor $p21^{CIP1}$, (Wu *et al*, 2003: Herold *et al*, 2002) and also current studies on *Nramp1* transcription, which were not seen to be repressed with this mutant of Myc, due to its inability to bind Miz-1

Data presented in this chapter support the hypothesis that the Sp1 site upstream of the initiator element (figure 5.3.1) is involved in Myc-mediated repression of NCAM transcription. It has been proposed that interaction with Miz-1 is not the only mechanism through which Myc can cause repression. Work by Feng *et al*, (2000, 2002) has uncovered two mechanisms which do not involve Miz-1 binding and instead depend on Sp1 which is known to bind to the central region of Myc. The first of these is through c-Myc binding to the Sp1 transcription factor bound at the Sp1 site, the second involves Smad mediated activation of the Sp1 transcription factor by Myc. It is proposed that this activation of Sp1 may convert it to a repressor, or that the presence of a Smad-Myc-Sp1 complex may prevent the recruitment of other activators to the promoter (Feng *et al*, 2002).

Results obtained using the Sp1 RNAi construct show that reducing the endogenous Sp1 levels in the cell result in reduced NCAM promoter activity which is of interest as if Myc repression were through interaction with Sp1 it would be likely that reduction in endogenous Sp1 levels would lead to NCAM promoter activation due to reduced Myc binding. It is also of interest to note that central region deletions of Myc (Stone *et al*, 1987) did not prevent repression. However this observation could be explained by the fact that neither of the deletion constructs used had the entire central region deleted due to the fact that this region of Myc contains the NLS. Therefore it is possible that the

interaction of Myc with Sp1 could only be abolished if the entire region were to be deleted.

Results obtained using the Myc RNAi construct were unexpected in that the lowering of endogenous Myc levels actually led to a reduction in NCAM promoter activity. Unlike results obtained using the *Nramp1* promoter constructs, this effect could not be rescued with Miz-1 indicating that the reduced promoter activity is unlikely to be due to a reduction in Miz-1 levels highlighting another difference between the regulation of the two promoters.

Despite these findings data presented in this thesis are supportive for a mechanism involving Sp1 for c-Myc recruitment to the NCAM promoter and subsequent transcriptional repression. Mutation of the Sp1 site was shown to prevent c-Myc repression of NCAM. This repression was found to return upon reconstitution of the site using a GAL4 Sp1 chimera.

c-Myc binding to the NCAM promoter was found to be sensitive to TSA treatment. This indicated that c-Myc mediated repression of NCAM is via an active mechanism involving HDAC recruitment. c-Myc binding was also shown to be sensitive to serum starvation with ChIP analysis revealing an loss of c-Myc from the NCAM promoter after only 30 minutes of serum starvation, consistent with the findings of others in the field that Myc is a very unstable protein with a very short half life (reviewed Grandori *et al*, 2000).

It has been demonstrated in these studies that repression of NCAM by c-Myc requires a functional Myc/Max complex. Using the Myc and MaxEG constructs (Amati *et al*, 1993) which are unable to bind to native Max and Myc respectively, MycEG was shown to be incapable of repressing the NCAM promoter in the absence of MaxEG. These findings support those of Staller (Staller *et al*, 2001) who showed that Max was present in the Myc/Miz-1 complex bound at the $p15^{ink4b}$ promoter and was therefore necessary for repression.

The recently identified Myc Box III is highly conserved among Myc family members and is localised centrally. Studies have shown that this region is important for Myc function, contributing to cellular transformation and downmodulating the ability of Myc to induce apoptosis (Herbst *et al*, 2005).

The MBIII deletion mutant (Δ188-199) was used to confirm that this region is essential for repression of NCAM, in agreement with the observation (Herbst *et al*, 2005), that this region is essential for all Myc repressed target genes.

CHAPTER 6

A Comparison of the NCAM and *Nramp1* promoters

6.1 Discussion

Activation of target genes via c-Myc is well understood with a clearly defined mechanism (Grandori *et al*, 2000). However, the mechanism by which c-Myc represses target genes is less well understood and has been the subject of much investigation. Amongst the most intensively studied of the c-Myc repressed target genes is the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$. These studies have revealed that c-Myc is capable of employing a number of pathways in order to repress transcription of target genes. These mechanisms can be broadly categorised as Miz-1 dependent and Miz-1 independent. In all studies a proximal promoter region (~100bp) has been shown to be essential (reviewed in Wanzel *et al*, 2003; Gartel & Shchors, 2003), with an Inr element rather than a TATA box present at the start site of transcription (Gartel *et al*, 2000,2001; Peukert *et al*, 1997).

Miz-1 binding at the Inr element of genes results in activation of transcription via interaction with the co-activator P300 (Peukert *et al*, 1997; Schneider *et al*, 1997; Staller *et al*, 2001; Seoane *et al*, 2001). The Myc interacting domain of Miz-1 overlaps with the P300 binding domain in such a way that binding of Myc to Miz-1 displaces P300 leading to transcriptional repression (Peukert *et al*, 1997). Proposed Miz-1 independent mechanisms are thought to act via a number of different pathways.

Among the most intensively studied of these Miz-1 independent pathways is that involving the transcription factor, Specificity protein 1 (Sp1). Sp1 was originally identified as a transcription factor which binds to and activates transcription from G-rich elements known as GC boxes, GGG GCG GGG (Dynan & Tjian, 1983). Since its discovery in the early 1980's other proteins able to act through GC boxes or so called 'Sp1 sites' have been identified. The family of proteins that is able to bind currently consists of four members, Sp1, Sp2, Sp3 and Sp4 – the so called 'Sp' family of transcription factors (reviewed in Suske, 1999).

The human Sp1 protein consists of 778 amino acids and contains three zinc fingers close to the C-terminus as well as two glutamine rich domains, shown to act as strong activation domains, adjacent to serine threonine stretches in the N-terminus (Courey & Tjian, 1988). More recently (Murala *et al,* 1994) an inhibitory domain has been identified in the N-terminus. Studies have also shown that Sp1 can be both phosphorylated (Jackson *et al,* 1990) and glycosylated (Jackson & Tjian, 1988). However, the role glycosylation plays, either enhancing or inhibiting activity is an area of controversy.

The importance of Sp1 binding sites adjacent to Inr elements has been well documented (reviewed in Smale *et al*, 1997). A role for this transcription factor in Myc mediated repression has been proposed for a number of genes including $p15^{INK4B}$. Studies on the $p15^{INK4B}$ promoter (Feng *et al,* 2002, 2003) show that Myc repression can be mediated by either direct binding of Myc to the Sp1 transcription factor bound at the Sp1 site, or via Smad mediated activation of the Sp1 transcription factor by Myc. In this case Sp1 may be converted to a repressor, or a Smad- Myc-Sp1 complex may prevent the recruitment of other activators to the promoter.

An initiator independent mechanism of Myc repression which does not involve Miz-1 has also been identified in the case of the Growth arrest and DNA damage gene, GaDD 45. In this case mapping studies have identified a GC rich region containing an Sp1 site which is responsible for the inhibition of GaDD 45 transcription (Adumson *et al*, 1998). Studies on the *Nramp1* promoter (Bowen *et al,* 2003), have shown that a GC box juxtaposed to the two inrs in the *Nramp1* promoter (figure 4.3.1) is not necessary for c-Myc mediated repression of the *Nramp1* promoter as both deletion experiments and mutation of this GC box have shown that the initiator elements (Inr) alone are sufficient to mediate c-Myc repression. However, these studies did reveal that this site is important for Miz-1 activation as both the Sp1 mutant and Sp1 deletion constructs were rendered unable to be activated by Miz-1. Data presented in this thesis provide a comparison of

Miz-1 dependent and independent mechanisms of Myc mediated repression of the *Nramp1* and NCAM promoters respectively.

Work by Bowen *et al* (2002), identified a role for c-Myc in repression of *Nramp1*. This discovery was made following the publication by Wu *et al* (1999), in which a role for c-Myc in the regulation of a number of iron regulatory genes, including H-ferritin, was shown. This work highlighted the importance of c-Myc in iron regulation making it likely that *Nramp1*, encoding a divalent cation transporter, could be a possible target gene. Subsequently Bowen *et al* (2002, 2003) demonstrated a role for Miz-1 in activation of the *Nramp1* promoter, and mapped these effects to a core promoter region containing two Inr elements and a consensus Sp1 binding site.

Studies on the NCAM promoter were initially undertaken in order to provide another model with which to study the activity of Miz-1. The NCAM gene was selected as it carries two overlapping consensus Inr elements and a consensus Sp1 binding site (Li *et al*, 1994). Like *Nramp1* this promoter is TATA box deficient and is repressed by Myc (Akeson & Bernards, 1990).

Initial studies revealed only modest stimulatory effects of Miz-1 in contrast to the very large fold inductions observed with *Nramp1*. Subsequent studies indicated that these effects were likely to be indirect, as data obtained from ChIP assays demonstrated an absence of this transcription factor bound at the NCAM promoter. In addition the use of the V394D Myc mutant, incapable of binding to Miz-1, demonstrated that Myc repression of NCAM was independent of Miz-1 with this mutant retaining the ability to repress NCAM. This was in contrast to *Nramp1* which was not repressed by the V394D Myc and NCAM was therefore deemed to be repressed by Myc in a Miz-1 dependent manner, a finding supported by ChIP assay data demonstrating Miz-1 bound at the *Nramp1* promoter *in-vivo*, but not NCAM. These responses were not tissue specific as

Nramp1 was still capable of being repressed by Myc in ND7 cells, indicating that functional Miz-1 is present in this cell type.

Indirect effects on the NCAM promoter such as those observed with Miz-1 have also been seen in studies with Pax-3 (Chalepakis *et al*, 1994), in which stimulatory effects were found to be indirect as binding of Pax-3 to the promoter could not be demonstrated.

The differential effects of the S46A and Δ33-60 dominant negative alleles of Miz-1 are also supportive of a role for Miz-1 in the regulation of *Nramp1*, but not NCAM (Figure 6.1.1). Neither of the dominant negative alleles of Miz-1 was found to have any significant effect on the activity of the NCAM promoter compared with the wild-type Miz-1. In contrast these mutants were shown to act as dominant negatives for *Nramp1*. Data presented in this thesis are supportive of a mechanism of achieving dominant negative status by loss of the ability to interact with known Miz-1 co-factors, P300 and ICSBP. Data presented in the literature on other POZ domain containing proteins (Zipper & Mulcahy, 2002) suggest that proteins containing these domains function as either Homo- or Heterodimers and that mutation of highly conserved serine residues, such as S46 are sufficient to prevent dimerization and lead to loss of function.

The *Nramp1* and NCAM genes, repressed by Myc in Miz-1 dependent and independent mechanisms respectively, provide good models with which to study Myc repression. The work presented in this thesis demonstrates that the mechanism of action or the role of a particular transcription factor in regulation of a gene cannot be demonstrated by sequence analysis or inspection alone. In this study the use of the V394D Myc mutant coupled with the results of the ChIP assay was able to provide a definitive answer as to the role of Miz-1 in Myc repression in contrast to the work of other groups. Studies on the GaDD45a and 135 genes (Barsyte-Lovejoy *et al*, 2004) used the V394D Myc mutant to implicate that both of these genes were repressed in a Miz-1 dependent manner, with the Myc mutant unable to repress these promoters. However, these findings conflicted with

in-vivo binding studies with ChIP assays only able to identify a low-level of Miz-1 binding at the GaDD45a and no binding at the GaDD135 promoter. The significance of the Myc V394D result was attributed to Myc repression not being entirely dependent on Miz-1.

Work by Herold *et al*, (2002) showed that V394D Myc mutant lost the ability to repress $p21^{WAF1/CIP1}$, and proposed a mechanism for Myc repression of this gene that was dependent on Miz-1. However, this has been the subject of much controversy with some groups suggesting that $p21^{WAF1/CIP1}$ may not be a bone-fide target of Miz-1(Zeigelbauer *et al*, 2004), as micro-array analysis of cells treated with Miz-1 RNAi failed to identify it as a target gene. This same study also failed to identify $p15^{INK4B}$ as a Miz-1 target gene (although it is possible that certain transcripts such as $p21^{WAF1/CIP1}$ and $p15^{INK4B}$ were below the detection limit of this analysis).

Subsequent studies on the $p21^{WAF1/CIP1}$ promoter identified a number of Miz-1 independent pathways, such as those involving the transcription factor Sp1 (Vaque *et al*, 2005) and an active repression mechanism of Dnmt3a, a methyltransferase co-repressor recruited by Myc (Brenner *et al*, 2005). In addition studies on PDGF-β promoter (Mao *et al*, 2004) have highlighted a third mechanism involving HDAC recruitment by Myc, although this mechanism has been shown not to be relevant for $p21^{WAF1/CIP1}$ repression.

Data presented in this thesis are supportive of a mechanism of Myc mediated repression of NCAM, involving Sp1. This is in contrast to the Miz-1 dependent passive mechanism employed by Myc in repression of *Nramp1*. Mutation of the Sp1 site present in the NCAM promoter was not found to ablate the activity of the promoter, as has been shown to be the case for other Inr promoters (Smale & Baltimore, 1989) including *Nramp1* (Phillips & Barton, unpublished). It is possible that this is due to the difference in spacing of the Sp1 site relative to the initiator elements. The Sp1 site in *Nramp1* is juxtaposed to the Inr elements, compared to an 80bp gap between Inr element and Sp1

site in the NCAM promoter. Mutation of the Sp1 site in the NCAM promoter prevented Myc repression. It was not possible to replicate this experiment for *Nramp1* due to the low activity of the Sp1 mutant promoter, although Bowen demonstrated in non-macrophage cells that the Sp1 site mutation was still subjected to Myc repression. Re-constitution of the Sp1 site in the NCAM promoter using a GAL4 Sp1 chimera resulted with a significant reduction in promoter activity, consistent with the ability of Myc to bind. In addition, re-constitution of the site correlated with the ability of transiently transfected Myc to repress.

Another difference in Myc binding to these two promoters was the sensitivity to TSA. Myc binding, and therefore repression was TSA sensitive in the case of the NCAM promoter. However, TSA treatment was found to have no effect on Myc repression of the *Nramp1* promoter. This would indicate that Myc repression of the NCAM promoter is via an active mechanism involving HDAC recruitment, similar to the PDGF-β promoter (Mao *et al*, 2004). These results correlated with the *in-vivo* binding data obtained via ChIP assays carried out on TSA treated and untreated cells. TSA treatment resulted in a loss of Myc binding at the NCAM promoter, but in the case of *Nramp1* Myc remained bound. These differential responses mirror those seen upon serum starvation. Myc is lost rapidly from the NCAM promoter (Myc binding not detectable following ~30minutes serum starvation), but remains bound at the *Nramp1* promoter even after 24 hours serum starvation. It is possible that these results could be explained by differential stability of Myc depending on how it is bound at the promoter. Binding of Myc to Miz-1 increases the stability of Myc (Salghetti *et al*, 1999), which is widely reported to be a very unstable protein with short half life (reviewed Grandori *et al*, 2000).

Use of the Myc and Max-EG constructs (Amati *et al*, 1993) demonstrated that a Myc/Max complex is required for repression of both promoters tested. Myc-EG alone was unable to repress either promoter. The requirement of a Myc/Max heterodimer for transactivation by Myc is well documented, although the requirement of Max in

transcriptional repression is more controversial. Most recently Max binding at the $p21^{WAF1/CIP1}$ has been demonstrated by ChIP assay (Mao *et al*, 2003); this is in contrast to the earlier findings of Gartel *et al* (2001) who indicated that this was not the case. In the case of another Myc repressed gene $p15^{INK4B}$, Max was shown by EMSA to be part of a functional repressor complex bound at the core promoter (Staller *et al*, 2001). Therefore it is possible that Max may not be essential for Myc repression, but is often present as a component of a repressive complex with Myc bound at the core promoter and may contribute to the stability of Myc within cells.

The requirement for Max in Myc mediated repression is not the only similarity in the repression mechanism of the two promoters. The recently identified Myc Box III, localised centrally (amino acids 188-199), was essential for repression of both promoters, in agreement with the observation (Herbst *et al*, 2005) that this region is essential for all Myc repressed target genes.

Data presented in this thesis provide models for the study of the pathways involved in Myc mediated repression of Inr containing promoters. This is an area which until recently has received little attention, but which now is emerging as a major function of this intensively studied transcription factor. Initial sequence analysis of these two promoters would suggest that due to the conservation of transcripton factor binding sites, the mechanism of repression would be the same. However this was shown not to be the case, with detailed promoter analysis revealing both passive and active mechanisms of repression of the *Nramp1* and NCAM promoters respectively. This highlights a need for caution in the use of sequence inspection alone in determining the role of a particular transcription factor in the context of a particular promoter.

Repression of the NCAM promoter by c-Myc is an important area for further study. Decreased NCAM expression has been observed in a subset of tumours including colon carcinoma and pancreatic cancer. In addition loss of NCAM correlates with poor

prognosis (Fogar *et al*, 1997; Tezel *et al*, 2001). It is only recently that the mechanisms by which reduced NCAM expression results in tumour progression has begun to be understood. Early clues come from data obtained using Rip1Tag2 mice, a transgenic model of pancreatic β cell carcinogenesis. In these animals loss of NCAM leads to metastatic dissemination of β tumour cells to the regional lymph nodes (Perl *et al*, 1999). This is due to the fact that loss of NCAM function leads increased VEGF-C and D expression which in turn leads to increased tumour lymphangeogenesis.

In human neuroblastoma amplification of N-Myc is associated with the progression of Neuroblastoma. Expression of N-Myc results in reduced NCAM levels (Akeson & Bernards, 1990) and is thought to be a key factor in the increased metastatic potential of N-Myc amplified neuroblastoma.

The transcriptional regulation of the *Nramp1* promoter is also an important area for further study. *Nramp1* plays an important role in the control of resistance or susceptibility to infectious disease in the mouse, depending on the presence or absence of a disease causing mutation within the open reading frame. In the human, resistance or susceptibility to infection and the susceptibility to autoimmune disease has been linked to a functional polymorphism within the promoter and it is assumed that the short tandem repeat polymorphism alters basal expression and responsiveness to the pro-inflammatory cytokine IFN-γ, although studies to definitively demonstrate this are lacking (Blackwell *et al*, 1995).

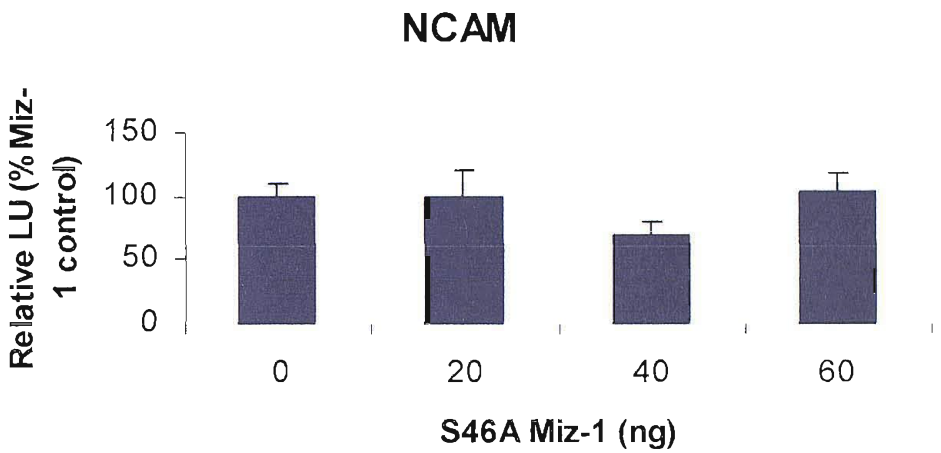
Until recently, very little was known of the mechanisms that control the expression of mouse or human *Nramp1*/NRAMP1. Studies by Bowen *et al* (2002/2003) identified a role for c-Myc and Miz-1 in the regulation of *Nramp1*. These studies serve to identify the precise mechanistic basis for this regulation. It is hoped that these findings will be of relevance in the study of human NRAMP1 promoter regulation. The contrast in mechanism of Myc repression between the *Nramp1* and NCAM promoters also

highlights the importance of the role of Miz-1 in *Nramp1* regulation and makes it clear that this transcription factor is only of relevance for a subset of Inr containing promoters.

Figure 6.1.1 The S46A dominant negative allele of Miz-1 has no effect on the NCAM promoter. A) ND7 cells were co-transfected as described in 2.2.6 with a constant amount (0.5μg) of the NCAM promoter construct and increasing amounts of the Miz-1 S46A dominant negative control, as shown. DNA concentrations were normalized using the pCDNA3.1 empty vector control. Expression of reporter gene activity was detected using a luciferase assay and normalized to the protein concentration of each sample. Data is presented as percentage of wild type Miz-1 control.
Students T-test- 20-60ng S46A $P>0.05$

B) RAW 264.7 cells were co-transfected as described in 2.2.5 with a constant amount of the *Nramp1*promoter construct pL4 (0.5μg) and increasing amounts of the c-Myc expression plasmid or the c-Myc mutant V394D mutant as shown. DNA concentrations were normalized using the pcDNA3.1 empty vector control. Expression of the reporter gene was detected using a luciferase assay and normalized to the protein concentration of each sample. Data is presented as percentage of wild type Miz-1 control.
Students T-test- 200-600ng S46A $P<0.03$

A)


NCAM
Relative LU (% Miz-1 control)
150
100
50
0
0
20
40
60
S46A Miz-1 (ng)

B)

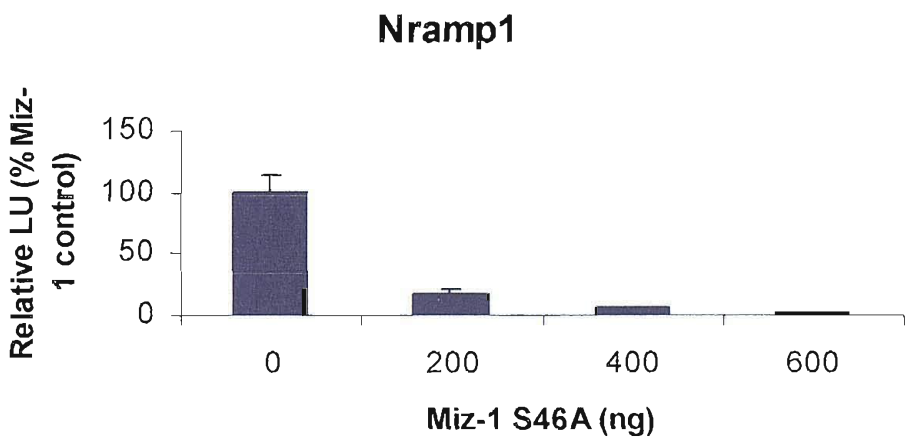
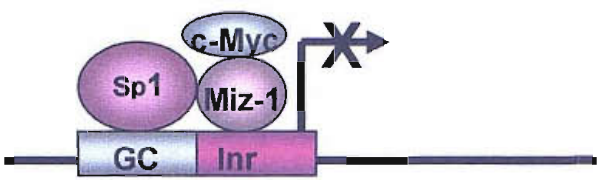

Nramp1
Relative LU (% Miz-1 control)
150
100
50
0
0
200
400
600
Miz-1 S46A (ng)

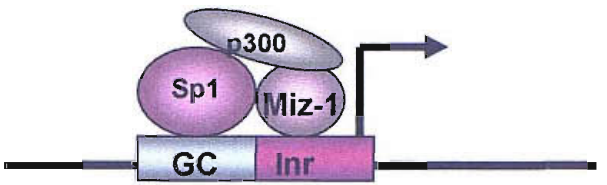
Figure 6.1.2 Schematic diagram showing the putative protein complexes bound to *Nramp1* and NCAM promoters in c-Myc repressed and derepressed states

***Nramp1*:** In the derepressed state interaction of Miz-1 with its co-activator P300 bound at the Inr leads to activation of transcription. However binding of Myc displaces P300 resulting in transcriptional repression.

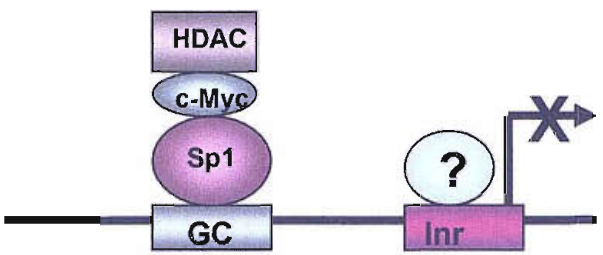
NCAM: Repression of NCAM is via interaction of c-Myc with the transcription factor Sp1 at a GC box present in the NCAM core promoter and subsequent HDAC recruitment. Derepression occurs when Myc binding is lost from the core promoter. The involvement of Inr binding proteins other than Miz- 1 has not been ruled out.

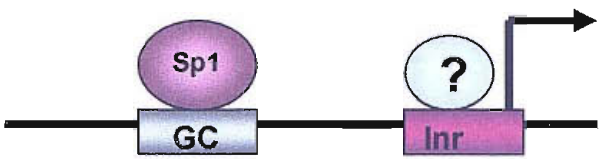
Nramp1


c-Myc
Sp1
Miz-1
GC
Inr


p300
Sp1
Miz-1
GC
Inr

NCAM


HDAC
c-Myc
Sp1
?
GC
Inr


Sp1
?
GC
Inr

REFERENCES

Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell. 2002;9(2):423-32.

Atchley WR, Fitch WM. Myc and Max: molecular evolution of a family of proto-oncogene products and their dimerization partner. Proc Natl Acad Sci U S A. 1995;92(22):10217-21.

Amundson SA, Zhan Q, Penn LZ, Fornace AJ Jr. Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents. Oncogene. 1998 Oct 29;17(17):2149-54.

Ahmad KF, Engel CK, Prive GG. Crystal structure of the BTB domain from PLZF. Proc Natl Acad Sci U S A. 1998;95(21):12123-8.

Akeson R, Bernards R. N-myc down regulates neural cell adhesion molecule expression in rat neuroblastoma. Mol Cell Biol. 1990 ;10(5):2012-6.

Alberts B, Bray B, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell (3rd Edition). Garland Publishing, 1997.

Alter-Koltunoff M, Ehrlich S, Dror N, Azriel A, Eilers M, Hauser H, Bowen H, Barton CH, Tamura T, Ozato K, Levi BZ. Nramp1-mediated innate resistance to intraphagosomal pathogens is regulated by IRF-8, PU.1, and Miz-1. J Biol Chem. 2003;278(45):44025-32.

Amati B, Brooks MW, Levy N, Littlewood TD, Evan GI, Land H. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell.1993 ;72(2):233-45.

Amati B, Dalton S, Brooks MW, Littlewood TD, Evan GI, Land H. Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature. 1992;359(6394):423-6.

Amati B, Alevizopoulos K, Vlach J. Myc and the cell cycle.Front Biosci. 1998;3:250-68.

Amundson SA, Zhan Q, Penn LZ, Fornace AJ Jr. Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents. Oncogene. 1998;17(17):2149-54.

Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene. 1991;6(10):1915-22.

Atkinson PG, Blackwell JM, Barton CH. Nramp1 locus encodes a 65 kDa interferon-gamma-inducible protein in murine macrophages. Biochem J. 1997 1;325 (Pt 3):779-86.

Augenlicht LH, Wadler S, Corner G, Richards C, Ryan L, Multani AS, Pathak S,Benson A, Haller D, Heerdt BG. Low-level c-myc amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. Cancer Res. 1997;57(9):1769-75.

Ayer DE, Lawrence QA, Eisenman RN. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell. 1995;80(5):767-76.

Ayer DE, Kretzner L, Eisenman RN. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity.Cell. 1993;72(2):211-22.

Bardwell VJ, Treisman R. The POZ domain: a conserved protein-protein interaction motif. Genes Dev. 1994;8(14):1664-77.

Barsyte-Lovejoy D, Mao DY, Penn LZ. c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism. Oncogene. 2004 ;23(19):3481-6.

Barton CH, Mann DA, Walsh FS. Characterization of the human N-CAM promoter. Biochem J. 1990 ;268(1):161-8.

Barton CH, White JK, Roach TI, Blackwell JM. NH2-terminal sequence of macrophage-expressed natural resistance-associated macrophage protein (Nramp) encodes a proline/serine-rich putative Src homology 3-binding domain. J Exp Med. 1994;179(5):1683-7.

Bernards R, Dessain SK, Weinberg RA. N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma.Cell. 1986;47(5):667-74.

Bernstein E, Denli AM, Hannon GJ. The rest is silence. RNA. 2001 ;7(11):1509-21

Blackwell JM, Searle S. Genetic regulation of macrophage activation: understanding the function of Nramp1 (=Ity/Lsh/Bcg). Immunol Lett. 1999;65(1-2):73-80.

Blackwell JM, Barton CH, White JK, Searle S, Baker AM, Williams H, Shaw MA. Genomic organization and sequence of the human NRAMP gene: identification and mapping of a promoter region polymorphism .Mol Med. 1995 ;1(2):194-205.

Blackwood EM, Eisenman RN. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science. 1991 ;251(4998):1211-7.

Breathnach R, Chambon P. Organization and expression of eucaryotic split genes coding for proteins. Annu Rev Biochem. 1981;50:349-83.

Brough DE, Hofmann TJ, Ellwood KB, Townley RA, Cole MD. An essential domain of the c-myc protein interacts with a nuclear factor that is also required for E1A-mediated transformation. Mol Cell Biol. 1995;15(3):1536-44.

Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, Reed S, Sicinski P, Bartek J, Eilers M. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27.EMBO J. 1999 ;18(19):5321-33.

Bowen H, Biggs TE, Baker ST, Phillips E, Perry VH, Mann DA, Barton CH. c-Myc represses the murine Nramp1 promoter .Biochem Soc Trans. 2002 ;30(4):774-7.

Bowen H, Biggs TE, Phillips E, Baker ST, Perry VH, Mann DA, Barton CH. c-Myc represses and Miz-1 activates the murine natural resistance-associated protein 1 promoter.J Biol Chem. 2002 ;277(38):34997-5006.

Bowen H, Lapham A, Phillips E, Yeung I, Alter-Koltunoff M, Levi BZ, Perry VH, Mann DA, Barton CH. Characterization of the murine Nramp1 promoter: requirements for transactivation by Miz-1. J Biol Chem. 2003 ;278(38):36017-26.

Brenner C, Deplus R, Didelot C, Loriot A, Vire E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. Myc represses transcription through recruitment of DNA methyltransferase corepressor.EMBO J. 2005 Jan 26;24(2):336-46

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet. 2003;34(3):263-4.

Broxmeyer HE, Cooper S, Levi S, Arosio P. Mutated recombinant human heavy-chain ferritins and myelo suppression in vitro and in vivo: a link between ferritin ferroxidase activity and biological function. Proc Natl Acad Sci U S A. 1991;88(3):770-4.

Burke TW, Willy PJ, Kutach AK, Butler JE, Kadonaga JT. The DPE, a conserved downstream core promoter element that is functionally analogous to the TATA box. Cold Spring Harb Symp Quant Biol. 1998;63:75-82.

Burke TW, Kadonaga JT. The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila. Genes Dev. 1997;11(22):3020-31.

Carcamo J, Maldonado E, Cortes P, Ahn MH, Ha I, Kasai Y, Flint J, Reinberg D. A TATA-like sequence located downstream of the transcription initiation site is required for expression of an RNA polymerase II transcribed gene. Genes Dev. 1990;4(9):1611-22.

Cellier M, Prive G, Belouchi A, Kwan T, Rodrigues V, Chia W, Gros P. Nramp defines a family of membrane proteins. Proc Natl Acad Sci U S A. 1995 ;92(22):10089-93.

Chalepakis G, Jones FS, Edelman GM, Gruss P. Pax-3 contains domains for transcription activation and transcription inhibition. Proc Natl Acad Sci U S A. 1994 Dec 20;91(26):12745-9.

Chen CR, Kang Y, Massague J. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program.Proc Natl Acad Sci U S A. 2001;98(3):992-9.

Claassen GF, Hann SR. Myc-mediated transformation: the repression connection. Oncogene. 1999 13;18(19):2925-33.

Claassen GF, Hann SR. A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. Proc Natl Acad Sci U S A. 2000;97(17):9498-503.

Ciechanover A, DiGiuseppe JA, Bercovich B, Orian A, Richter JD, Schwartz AL,Brodeur GM. Degradation of nuclear oncoproteins by the ubiquitin system in vitro.Proc Natl Acad Sci U S A. 1991;88(1):139-43.

Crnic I, Strittmatter K, Cavallaro U, Kopfstein L, Jussila L, Alitalo K, Christofori G. Loss of neural cell adhesion molecule induces tumor metastasis by up-regulating lymphangiogenesis. Cancer Res. 2004;64(23):8630-8.

Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science. 1987;236(4803):799-806.

Courey AJ, Tjian R. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell. 1988;55(5):887-98.

Dang CV, Lee LA.C-Myc transrepression and cell transformation. Curr Top Microbiol Immunol. 1997;224:131-5

Datto MB, Hu PP, Kowalik TF, Yingling J, Wang XF. The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. Mol Cell Biol. 1997;17(4):2030-7.

Davis AC, Wims M, Spotts GD, Hann SR, Bradley A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev. 1993;7(4):671-82.

ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. Trends Biochem Sci. 2004;29(5):265-73

Donze O, Picard D. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. Nucleic Acids Res. 2002;30(10)

Dosik JK, Barton CH, Holiday DL, Krall MM, Blackwell JM, Mock BA.
An Nramp-related sequence maps to mouse chromosome 17. Mamm Genome. 1994;5(7):458-60.

Dynan WS, Tjian R. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell. 1983 Nov;35(1):79-87.

Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. 1994;8(8):869-84.

Eisenman RN. The Max network: coordinated transcriptional regulation of cell growth and proliferation. Harvey Lect. 2000-2001;96:1-32

Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods. 2002;26(2):199-213.

Emami KH, Jain A, Smale ST. Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization.Genes Dev. 1997;11(22):3007-19.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM,Penn LZ, Hancock DC. Induction of apoptosis in fibroblasts by c-myc protein.Cell. 1992;69(1):119-28.

Feng XH, Liang YY, Liang M, Zhai W, Lin X. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(Ink4B).Mol Cell. 2002;9(1):133-43.

Feng XH, Lin X, Derynck R. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. EMBO J. 2000;19(19):5178-93

Feng XH, Liang YY, Liang M, Zhai W, Lin X. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(Ink4B).Mol Cell. 2002;9(1):133-43.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C.,. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 1998, 391, pp. 806–811.

Fogar P, Basso D, Pasquali C, De Paoli M, Sperti C, Roveroni G, Pedrazzoli S, Plebani M. Neural cell adhesion molecule (N-CAM) in gastrointestinal neoplasias. Anticancer Res. 1997;17(2B):1227-30.

Frederick JP, Liberati NT, Waddell DS, Shi Y, Wang XF. Transforming growth factor beta-mediated transcriptional repression of c-myc isdependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol Cell Biol. 2004 ;24(6):2546-59.

Freytag SO, Geddes TJ. Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. Science. 1992;256(5055):379-82.

Gallant P, Shiio Y, Cheng PF, Parkhurst SM, Eisenman RN. Myc and Max homologs in Drosophila. Science. 1996;274(5292):1523-7.

Gartel AL, Shchors K. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. Exp Cell Res. 2003;283(1):17-21.

Goldberg HJ, Whiteside CI, Fantus IG. The hexosamine pathway regulates the plasminogen activator inhibitor-1 gene promoter and Sp1 transcriptional activation through protein kinase C-beta I and-delta. J Biol Chem. 2002;277(37):33833-41.

Gomes MS, Appelberg R. Evidence for a link between iron metabolism and Nramp1 gene function in innateresistance against Mycobacterium avium.Immunology. 1998;95(2):165-8.

Gomez-Roman N, Grandori C, Eisenman RN, White RJ. Direct activation of RNA polymerase III transcription by c-Myc. Nature. 2003;421(6920):290-4.

Goswami T, Bhattacharjee A, Babal P, Searle S, Moore E, Li M, Blackwell JM. Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter.Biochem J. 2001;354(Pt 3):511-9.

Govoni G, Vidal S, Cellier M, Lepage P, Malo D, Gros P. Genomic structure, promoter sequence, and induction of expression of the mouse Nramp1 gene in macrophages. Genomics. 1995;27(1):9-19.

Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol. 2000;16:653-99.

Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, White RJ. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol.7(3):311-8.

Grant PA, Schieltz D, Pray-Grant MG, Yates JR 3rd, Workman JL. The ATM-related cofactor Tra1 is a component of the purified SAGA complex. Mol Cell. 1998;2(6):863-7.

Gros P, Skamene E, Forget A. Cellular mechanisms of genetically controlled host resistance to Mycobacterium bovis (BCG). J Immunol. 1983;131(4):1966-72.

Gruenheid S, Cellier M, Vidal S, Gros P. *Identification and characterization of a second mouse Nramp* gene.Genomics. 1995;25(2):514-25.

Gruenheid S, Pinner E, Desjardins M, Gros P. Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. J Exp Med. 1997;185(4):717-30.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF. Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett. 2001 ;509(2):309-16.

Hann SR, Eisenman RN. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. Mol Cell Biol. 1984;4(11):2486-97.

Hannon GJ. RNA interference .Nature. 2002;418(6894):244-51

Harris RG, White E, Phillips ES, Lillycrop KA. The expression of the developmentally regulated proto-oncogene Pax-3 is modulated by N-Myc. J Biol Chem. 2002;277(38):34815-25.

Hayward WS, Neel BG, Astrin SM. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature. 1981 ;290(5806):475-80.

Herbst A, Hemann MT, Tworkowski KA, Salghetti SE, Lowe SW, Tansey WP. A conserved element in Myc that negatively regulates its proapoptotic activity. EMBO Rep. 2005;6(2):177-83.

Herold S, Wanzel M, Beuger V, Frohme C, Beul D, Hillukkala T, Syvaoja J,Saluz HP, Haenel F, Eilers M. Negative regulation of the mammalian UV response by Myc through association with Miz-1.Mol Cell. 2002;10(3):509-21.

Henriksson M, Luscher B. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res. 1996;68:109-82.

Hoang AT, Lutterbach B, Lewis BC, Yano T, Chou TY, Barrett JF, Raffeld M, Hann SR, Dang CV. A link between increased transforming activity of lymphoma-derived MYC mutant alleles, their defective regulation by p107, and altered phosphorylation of the c-Myc transactivation domain.Mol Cell Biol. 1995;15(8):4031-42

Hoffman S, Edelman GM. The mechanism of binding of neural cell adhesion molecules.Adv Exp Med Biol. 1984;181:147-60.

Hueber A-O, Z¨ornig M, Lyon D, Suda T, Nagata S, Evan GI.. Requirement for the CD95 receptor-ligand pathway in c-Mycinduced apoptosis. Science 1997 278:1305–9

Hurlin PJ, Queva C, Koskinen PJ, Steingrimsson E, Ayer DE, Copeland NG, Jenkins NA, Eisenman RN. Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. 1995;14(22):5646-59

Hurlin PJ, Queva C, Eisenman RN. Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. Genes Dev. 1997;11(1):44-58

Iritani BM, Eisenman RN. c-Myc enhances protein synthesis and cell size during B lymphocyte development.Proc Natl Acad Sci U S A. 1999;96(23):13180-5.

Jabado N, Jankowski A, Dougaparsad S, Picard V, Grinstein S, Gros P. Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane.J Exp Med. 2000;192(9):1237-48.

Javahery R, Khachi A, Lo K, Zenzie-Gregory B, Smale ST. DNA sequence requirements for transcriptional initiator activity in mammalian cells. Mol Cell Biol. 1994 Jan;14(1):116-27.

Jackson SP, MacDonald JJ, Lees-Miller S, Tjian R. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell. 1990 ;63(1):155-65.

Jackson SP, Tjian R. O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation.Cell. 1988;55(1):125-33.

Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300.
Genes Dev. 1998;12(14):2114-9

Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. Drosophila myc regulates cellular growth during development. Cell. 1999;98(6):779-90.

Kato GJ, Barrett J, Villa-Garcia M, Dang CV. An amino-terminal c-myc domain required for neoplastic transformation activates transcription.Mol Cell Biol. 1990;10(11):5914-20.

Kerr LD, Miller DB, Matrisian LM. TGF-beta 1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence.Cell. 1990;61(2):267-78.

Kime L, Wright SC. Mad4 is regulated by a transcriptional repressor complex that contains Miz-1 and c-Myc. Biochem J. 2003 ;370(Pt 1):291-8.

Kretzner L, Blackwood EM, Eisenman RN. Transcriptional activities of the Myc and Max proteins in mammalian cells. Curr Top Microbiol Immunol. 1992;182:435-43 .

Kuhn DE, Baker BD, Lafuse WP, Zwilling BS. Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1Gly169 or Nramp1Asp169.J Leukoc Biol. 1999;66(1):113-9.

Langenau DM, Traver D, Ferrando AA, Kutok JL, Aster JC, Kanki JP, Lin S, Prochownik E, Trede NS, Zon LI, Look AT._Myc-induced T cell leukemia in transgenic zebrafish. Science. 2003;299(5608):887-90.

Lee LA, Dolde C, Barrett J, Wu CS, Dang CV. A link between c-Myc-mediated transcriptional repression and neoplastic transformation. J Clin Invest. 1996;97(7):1687-95

Lee TC, Li L, Philipson L, Ziff EB. Myc represses transcription of the growth arrest gene gas1. Proc Natl Acad Sci U S A. 1997;94(24):12886-91.

Lewin B, Genes V. Oxford University Press, 1994.

Li LH, Nerlov C, Prendergast G, MacGregor D, Ziff EB. c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J. 1994;13(17):4070-9.

Lieu PT, Heiskala M, Peterson PA, Yang Y. The roles of iron in health and disease. Mol Aspects Med. 2001 Feb-Apr;22(1-2):1-87.

Lindeman GJ, Gaubatz S, Livingston DM, Ginsberg D. The subcellular localization of E2F-4 is cell-cycle dependent. Proc Natl Acad Sci U S A. 1997;94(10):5095-100.

Lipardi C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell. 2001;107(3):297-307.

Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature. 1983;306(5939):194-6.

Malo D, Vidal S, Lieman JH, Ward DC, Gros P. Physical delineation of the minimal chromosomal segment encompassing the murine host resistance locus Bcg.Genomics. 1993 ;17(3):667-75.

Malo D, Vidal SM, Hu J, Skamene E, Gros P. High-resolution linkage map in the vicinity of the host resistance locus Bcg. Genomics. 1993 ;16(3):655-63.

Malo D, Vogan K, Vidal S, Hu J, Cellier M, Schurr E, Fuks A, Bumstead N,Morgan K, Gros P. Haplotype mapping and sequence analysis of the mouse Nramp gene predictsusceptibility to infection with intracellular parasites. Genomics. 1994 ;23(1):51-61.

Mao DY, Barsyte-Lovejoy D, Ho CS, Watson JD, Stojanova A, Penn LZ.
Promoter-binding and repression of PDGFRB by c-Myc are separable activities. Nucleic Acids Res. 2004;32(11):3462-8.

Mao DY, Watson JD, Yan PS, Barsyte-Lovejoy D, Khosravi F, Wong WW, Farnham PJ, Huang TH, Penn LZ. Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. Curr Biol. 2003;13(10):882-6.

Mariani-Costantini R, Escot C, Theillet C, Gentile A, Merlo G, Lidereau R,Callahan R. In situ c-myc expression and genomic status of the c-myc locus in infiltrating ductal carcinomas of the breast. Cancer Res. 1988;48(1):199-205.

Mateyak MK, Obaya AJ, Adachi S, Sedivy JM. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ. 1997 Oct;8(10):1039-48.

Martinez E, Kundu TK, Fu J, Roeder RG. 1998. A human Spt3-Tafii31-Gcn5-l acetylase complex distinct from transcription factor IID. J. Biol. Chem. 273:23781–85

Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 2002;110(5):563-74.

McArthur GA, Eisenman RN. 1997. The Maxtranscription factor network: Mad1 inhibition of cell cycle progression is inhibited by the human papilloma virus E7 protein.
In American Society for Clinical Oncology Educational Book, ed. MC Perry, pp. 42–47.
Washington, DC: Am. Soc. Clin. Oncol.

Murata Y, Kim HG, Rogers KT, Udvadia AJ, Horowitz JM. Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain.J Biol Chem. 1994 ;269(32):20674-81.

McMahon SB, Wood MA, Cole MD. The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol Cell Biol. 2000 ;20(2):556-62.

McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell. 1998;94(3):363-74.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, et al. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell. 1989;58(3):537-44.

Murre C, McCaw PS, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell. 1989;56(5):777-83.

Nair SK, Burley SK. X-ray structures of Myc-Max and Mad-Max recognizing DNA. Molecular bases of regulation by proto-oncogenic transcription factors. Cell. 2003 Jan 24;112(2):193-205.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J. 1997;16(17):5353-62.
Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer.Oncogene. 2003 ;22(56):9007-21.

Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter.Nature. 1997;388(6641):482-8.

Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell. 2001;107(3):309-21.

O'Shea-Greenfield A, Smale ST. Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription. J Biol Chem. 1992;267(2):1391-402.

Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. 2002; 16(8):948-58.

Patikoglou GA, Kim JL, Sun L, Yang SH, Kodadek T, Burley SK. TATA element recognition by the TATA box-binding protein has been conserved
throughout evolution. Genes Dev. 1999;13(24):3217-30.

Pebernard S, Iggo RD. Determinants of interferon-stimulated gene induction by RNAi vectors. Differentiation. 2004 ;72(2-3):103-11.

Pelengaris S, Khan M. The c-MYC oncoprotein as a treatment target in cancer and other disorders of cell growth. Expert Opin Ther Targets. 2003;7(5):623-42.

Penn LJ, Brooks MW, Laufer EM, Land H. Negative autoregulation of c-myc transcription. EMBO J. 1990;9(4):1113-21.

Perl AK, Dahl U, Wilgenbus P, Cremer H, Semb H, Christofori G. Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic beta tumor cells. Nat Med. 1999;5(3):286-91.

Peukert K, Staller P, Schneider A, Carmichael G, Hanel F, Eilers M. An alternative pathway for gene regulation by Myc. EMBO J. 1997 ;16(18):5672-86.

Philipp A, Schneider A, Vasrik I, Finke K, Xiong Y, Beach D, Alitalo K, Eilers M. Repression of cyclin D1: a novel function of MYC. Mol Cell Biol. 1994;14(6):4032-43.

Queva C, Hurlin PJ, Foley KP, Eisenman RN. Sequential expression of the MAD family of transcriptional repressors during differentiation and development. Oncogene. 1998;16(8):967-77.

Ramaswamy G, Slack FJ. siRNA. A guide for RNA silencing. Chem Biol. 2002 Oct;9(10):1053-5.

Richardson DR, Ponka P. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells.Biochim Biophys Acta. 1997 ;1331(1):1-40

Roubin R, Deagostini-Bazin H, Hirsch MR, Goridis C. Modulation of NCAM expression by transforming growth factor-beta, serum, and autocrine factors.J Cell Biol. 1990 ;111(2):673-84.

Roy AL, Du H, Gregor PD, Novina CD, Martinez E, Roeder RG. Cloning of an inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. EMBO J. 1997;16(23):7091-104

Roy AL, Meisterernst M, Pognonec P, Roeder RG. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature. 1991;354(6350):245-8.

Roy AL, Carruthers C, Gutjahr T, Roeder RG. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature. 1993;365(6444):359-61

Rutishauser U, Hoffman S, Edelman GM. Binding properties of a cell adhesion molecule from neural tissue.Proc Natl Acad Sci U S A. 1982 ;79(2):685-9.

Ryu H, Lee J, Olofsson BA, Mwidau A, Deodeoglu A, Escudero M, Flemington E, Azizkhan-Clifford J, Ferrante RJ, Ratan RR. Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1 dependent pathway. Proc Natl Acad Sci U S A. 2003;100(7):4281-6

Saleh A, Collart M, Martens JA, Genereaux J, Allard S, Cote J, Brandl CJ. TOM1p, a yeast hect-domain protein which mediates transcriptional regulation through the ADA/SAGA coactivator complexes. J Mol Biol. 1998;282(5):933-46.

Salghetti SE, Kim SY, Tansey WP. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J. 1999; 18(3):717-26.

Sawadogo M, Roeder RG. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell. 1985;43(1):165-75.

Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 1995;9(10):1149-63.

Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003;115(2):199-208

Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature. 2002 17;419(6908):

Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat Cell Biol. 2001;3(4):400-8

Seto E, Shi Y, Shenk T. YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro. Nature. 1991;354(6350):241-5.

Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. Cell. 1991;67(2):377-88.

Shrivastava A, Saleque S, Kalpana GV, Artandi S, Goff SP, Calame K. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. Science. 1993 262(5141):1889-92.

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs .Nat Cell Biol. 2003;5(9):834-9.

Smale ST, Kadonaga JT. The RNA polymerase II core promoter.Annu Rev Biochem. 2003;72:449-79.

Smale ST, Baltimore D. The "initiator" as a transcription control element. Cell. 1989 7;57(1):103-13.

Smale ST. Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochim Biophys Acta. 1997;1351(1-2):73-88.

Spotts GD, Patel SV, Xiao Q, Hann SR. Identification of downstream-initiated c-Myc proteins which are dominant-negative inhibitors of transactivation by full-length c-Myc proteins. Mol Cell Biol. 1997;17(3):1459-68.

Stach JL, Delgado G, Strobel M, Millan J, Lagrange PH. Preliminary evidence of natural resistance to Mycobacterium bovis (BCG) inlepromatous leprosy.Int J Lepr Other Mycobact Dis. 1984;52(2):140-6.

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T,Bartek J,Massague J, Hanel F, Eilers M. Repression of p15INK4b expression by Myc through association with Miz-1.Nat Cell Biol. 2001;3(4):392-9.

Steiner P, Philipp A, Lukas J, Godden-Kent D, Pagano M, Mittnacht S, Bartek J, Eilers M. Identification of a Myc-dependent step during the formation of active G1 cyclin-cdk complexes. EMBO J. 1995;14(19):4814-26.

Stone J, de Lange T, Ramsay G, Jakobovits E, Bishop JM, Varmus H, Lee W. Definition of regions in human c-myc that are involved in transformation and nuclear localization. Mol Cell Biol. 1987;7(5):1697-709.

Suske G. The Sp-family of transcription factors.Gene. 1999 ;238(2):291-300. Review.

Thange NBL, Man Chan H, p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci. 2001;114, 2363-2373.

Tezel E, Kawase Y, Takeda S, Oshima K, Nakao A. Expression of neural cell adhesion molecule in pancreatic cancer. Pancreas. 2001;22(2):122-5.

Thiery JP, Brackenbury R, Rutishauser U, Edelman GM. Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J Biol Chem. 1977;252(19):6841-5.

Turner PC, McLennan AG, Bates AD, White MRH. Instant notes in Molecular Biology (2nd Edition). BIOS Scientific Publishers Ltd, 2000.

Unkles SE, Hawker KL, Grieve C, Campbell EI, Montague P, Kinghorn JR. crnA encodes a nitrate transporter in Aspergillus nidulans. Proc Natl Acad Sci U S A. 1991 ; 88(1):204-8.

Vidal S, Tremblay ML, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E,
Localisation of Nramp1 in macrophages: modulation with activation and infection.J Cell Sci. 1998;111 (Pt 19):2855-66.

Vidal S, Gros P, Skamene E. Natural resistance to infection with intracellular parasites: molecular genetics identifies Nramp1 as the Bcg/Ity/Lsh locus.J Leukoc Biol. 1995;58(4):382-90.

Vidal SM, Malo D, Vogan K, Skamene E, Gros P. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell. 1993; 73(3):469-85.

Wanzel M, Herold S, Eilers M. Transcriptional repression by Myc. Trends Cell Biol. 2003;13(3):146-50.

Weis L, Reinberg D. Accurate positioning of RNA polymerase II on a natural TATA-less promoter is independent of TATA-binding-protein-associated factors and initiator binding proteins. Mol Cell Biol. 1997;17(6):2973-84.

Wong JM, Bateman E. TBP-DNA interactions in the minor groove discriminate between A:T and T:A base pairs.Nucleic Acids Res. 1994 25;22(10):1890-6.

Wu KJ, Polack A, Dalla-Favera R. Coordinated regulation of iron-controlling genes, H ferritin and IRP2, by c-MYC. Science. 1999; 283(5402):676-9.

Wu S, Cetinkaya C, Munoz-Alonso MJ, von der Lehr N, Bahram F, Beuger V,
Eilers M, Leon J, Larsson LG. Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter . Oncogene. 2003;22(3):351-60 .

Yang W, J. Shen, M. Wu, M. Arsura, M. FitzGerald, Z. Suldan, D.W. Kim, C.S. Hofmann, S. Pianetti, R. Romieu-Mourez, L.P. Freedman and G.E. Sonenshein , Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* **20** (2001), pp. 1688–1702

Ziegelbauer J, Shan B, Yager D, Larabell C, Hoffmann B, Tjian R. Transcription factor MIZ-1 is regulated via microtubule association. Mol Cell. 2001;8(2):339-49.

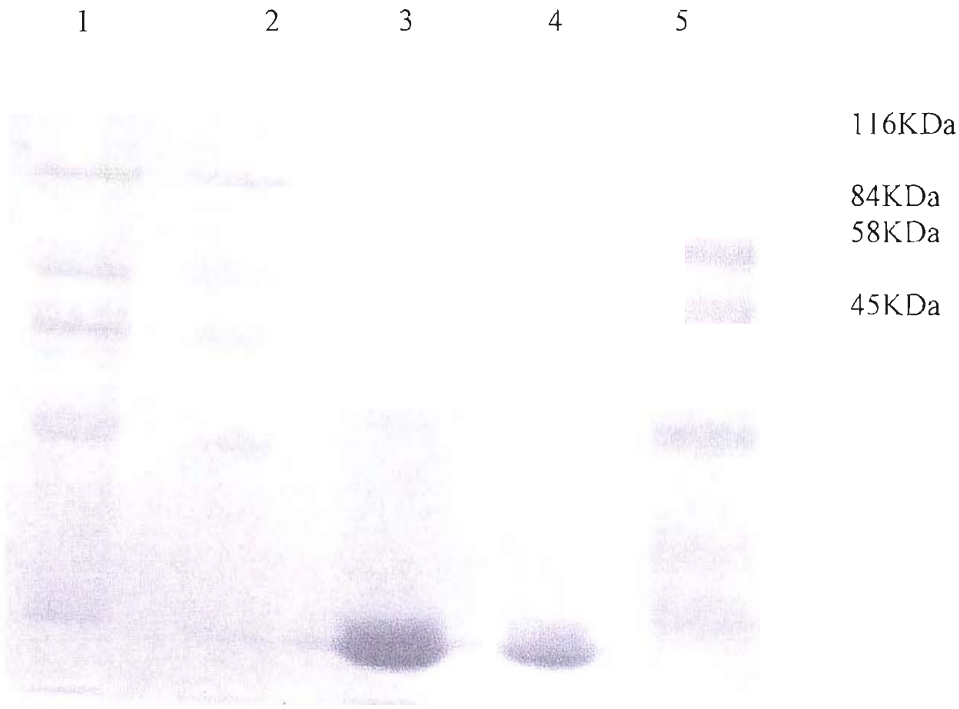
Ziegelbauer J, Wei J, Tjian R. Myc-interacting protein 1 target gene profile: a link to microtubules, extracellular signal-regulated kinase, and cell growth. Proc Natl Acad Sci U S A. 2004;101(2):458-63.

Zervos AS, Gyuris J, Brent R. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell. 1993;72(2):223-32

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization.Genes Dev. 1998; 12(15):2424-33.

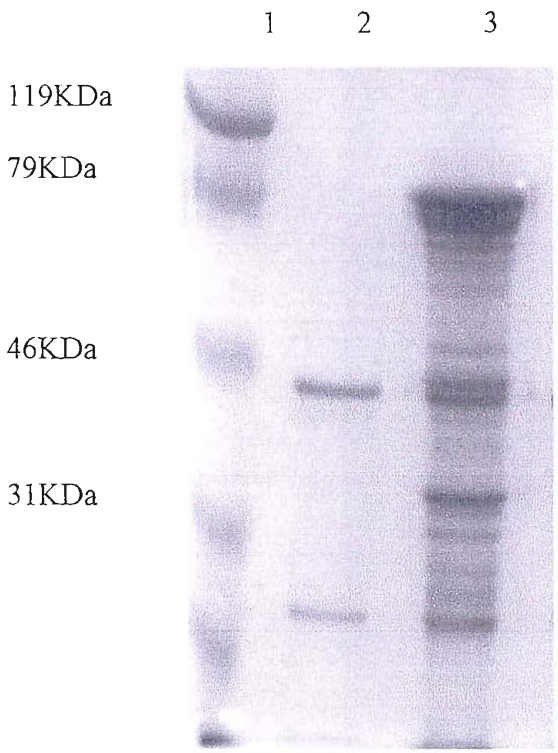
Zipper LM, Mulcahy RT. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm.J Biol Chem. 2002;277(39):36544-52.

Zwilling BS, Kuhn DE, Wikoff L, Brown D, Lafuse W. Role of iron in Nramp1-mediated inhibition of mycobacterial growth. Infect Immun. 1999 ;67(3):1386-92.



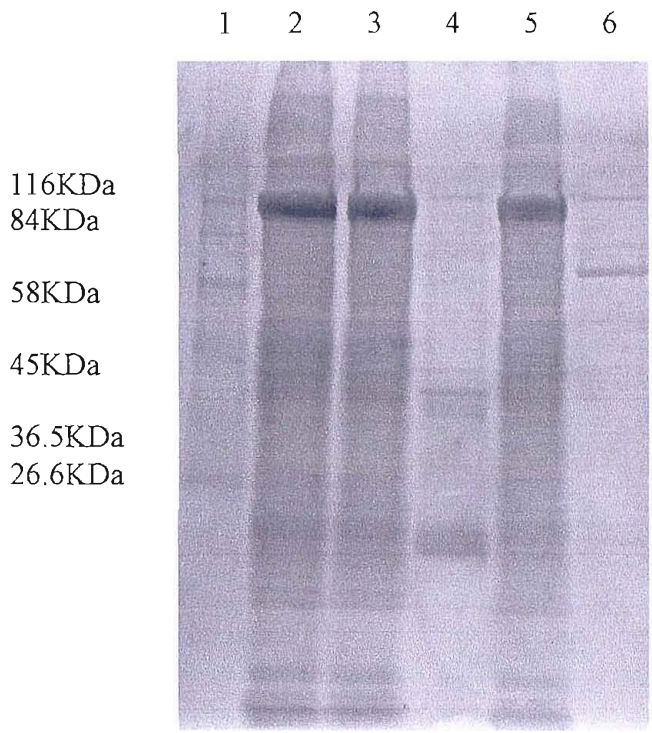
Appendix 1 Miz-1 protein-small scale purification using GST beads. 9ml pre-warmed LB was inoculated with 1ml of an overnight culture of pGex/Miz-1 Clone 1 or the pGex-2 empty vector control as described in 2.2.10 following incubation at 37° for 1hour samples were induced with IPTG to a final concentration of 0.1mM and incubated for further four hours before protein was purified, as described in 2.2.10. Samples were run on a 10% SDS gel at 30mA for 45 minutes.

(1) pGex/Miz-1 Clone 1-Induced, (2) pGex/Miz-1 Clone 1-Not induced, (3) pGex-2-Induced, (4) pGex-2- Not induced. (5) Molecular weight markers, (Sigma, UK).



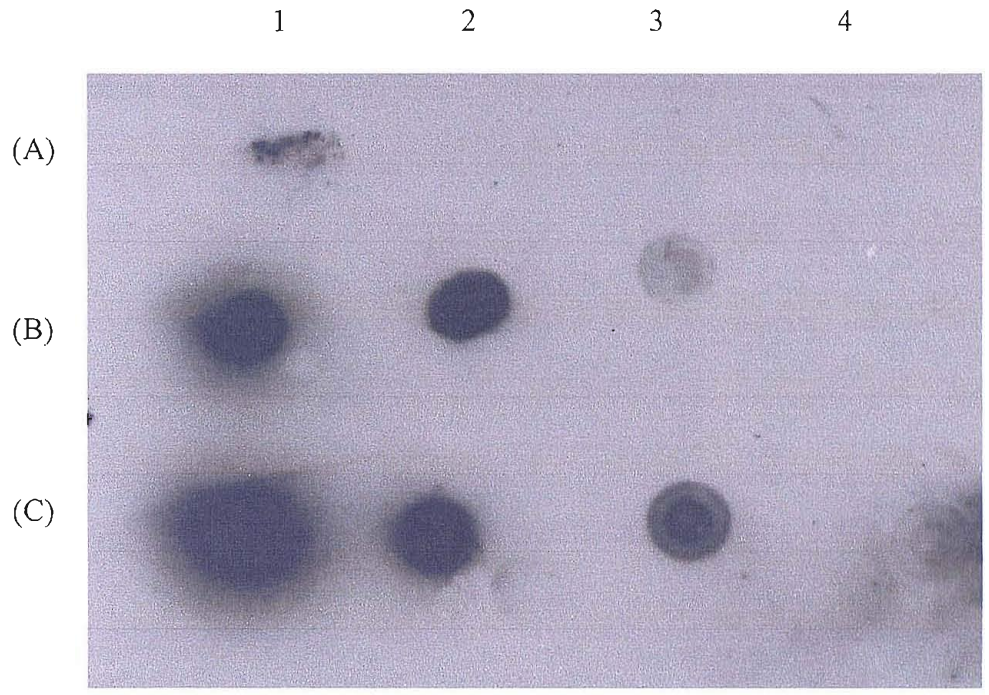
Appendix 2 c-Myc protein-small scale purification using GST beads. 9ml pre-warmed LB was inoculated with 1ml of an overnight culture of pGex/c-Myc or the pGex-2 empty vector contol as described in 2.2.10 following incubation at 37° for 1hour samples were induced with IPTG to a final concentration of 0.1mM and incubated for further four hours before protein was purified. Samples were run on a 10% SDS gel at 30mA for 45 minutes.

(1) Molecular weight markers, (Biorad, UK), (2) pGex/c-Myc-Induced, (3) pGex/c-Myc-Not induced.



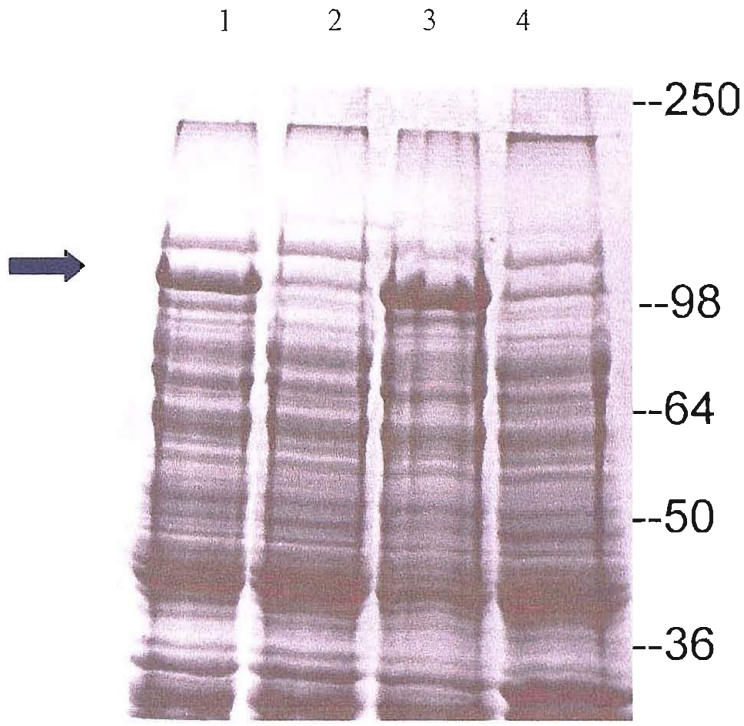
Appendix3 Large scale Miz-1 protein purification. A large scale Miz-1/pGex sample was prepared as described in 2.2.10 and purified using a GST column 100μl samples were removed at each stage (i.e. prior to loading, flow through from column and eluted) and boiled for 5 minutes in SDS loading dye. 20μl of each sample was run on a 10% SDS gel at 30mA for 45 minutes.

(1) Molecular weight markers, (Sigma), (2) Sample prior to loading onto column, (3) Flow through from colum-1, (4) Eluate from column-1, (5) Flow through from column-2, (6) Eluate from column-2.



Appendix4 Dot Blot of Miz-1 Antibody raised against synthetic Miz-1 peptide.

A dot blot, as described in 2.2.12 was carried out using the Miz-1 antibody #38 (Bleed 2) at a 1:500 dilution (secondary, Goat-anti-Rabbit, 1:15,000) against (A) BSA, (B) The synthetic peptide to which the antibody was raised, (C) GST Miz-1 fusion protein created using the Miz-1/pGex construct. The numbers shown above the gel correspond to; 1-5µl of neat solution, 2-5µl of 1/10 solution, 3- 5µl of 1/100 solution, 4-5µl of 1/1000 solution.



Appendix. 5 Small scale purification of wild-type and S46A Miz-1.

9ml pre-warmed LB was inoculated with 1ml of an overnight culture of pGex/Miz-1 or the pGex/S46A Miz-1 as described in 2.2.10 following incubation at 37° for 1hour samples were induced with IPTG to a final concentration of 0.1mM and incubated for further four hours before protein was purified. Samples were run on a 10% SDS gel at 30mA for 45 minutes.(1) Wild type Miz-1 + IPTG (2) Wild type Miz-1 IPTG (3) S46A Miz-1 + IPTG (4) S46A Miz-1 IPTG (Size markers are as indicated on the right hand side)