COORDINATE REGULATION OF VEGF SIGNALLING GENES BY THE PROLINE RICH HOMEODOMAIN PROTEIN.

by

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Abstract.

PRH is a transcriptional repressor that regulates development of the haematopoietic and vascular systems. VEGF is a mitogen that stimulates cell survival via cell surface receptors including VEGFR1 and VEGFR2. This thesis identifies Vegf, Vegfr1, and Vegfr2 as bona fide PRH target genes and shows PRH can control cell survival through the modulation of VEGF and VEGF receptor signalling. CK2 is a stress-activated protein kinase with pleiotropic activity and CK2 phosphorylation of PRH inhibits its DNA binding activity. Here I show that CK2 can antagonise PRH induced cell death and transcriptional regulation. Furthermore I show that CK2 reduces PRH stability and decreases nuclear retention of PRH. The oncogenic BCR-ABL fusion protein increases CK2 activity. I show that Inhibition of BCR-ABL in CML cells results in decreased PRH phosphorylation and the down-regulation of PRH target genes. Reestablishment of gene control by PRH is partly responsible for the therapeutic effects of BCR-ABL inhibition in CML and that the reestablishment of PRH function could be valuable for the treatment of leukaemias with elevated CK2 activity. These data show that PRH is a key regulator of the VEGF family and loss of PRH transcriptional activity, through elevated CK2, has a role in leukaemogenesis.

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3C	Chromatin Conformation Capture
AA	Amino acid
ABL	V-abl Abelson murine leukemia viral oncogene homolog 1
ADE	Anterior definitive endoderm
ADP	Adenosine diphosphate
AGM	Aorta gonad mesonephros
ALL	Acute lymphoblastic leukaemia
AML	Acute myelogenous leukaemia
APC	Allophycocyanin
APL	Acute promyelocytic leukaemia
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
AVE	Anterior VE
BCL2	B-cell lymphoma 2
BCR	Breakpoint cluster region
bFGF	Basic fibroblast growth factor
BL-CFC	Blast colony-forming cells
bp	Base pair
BSA	Bovine serum albumin
CAP	Co-factor activated phosphorylation
CBF	Core binding factor
CcN	CK2/cell cycle-dependent kinase 2/nuclear localization sequence
cdc2	p34cdc2 kinase
CDK	Cyclin dependent kinase

cDNA	Coding DNA
CFSE	Carboxy-fluorescein diacetate succinimidyl ester
CFU	Colony forming unit
ChIP	Chromatin immuno-precipitation
CIAP	Calf intestinal alkaline phosphatase
CK2	Protein kinase casein kinase 2
CML	Chronic myelogenous leukaemia
COMPASS	Complex proteins associated with set1
CON	Control
CREB	cAMP response element binding protein
CrKL	v-CRK avian sarcoma virus CT10-homolog-like
CTD	C-terminal domain
DCE-MRI	Dynamic contrast enhanced magnetic resonance imaging
dH2O	Distilled water
Dll	Distal-less
DMAT	2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
dpc	Days post coitum
DPE	Downstream promoter element
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECL	Electro-chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescence protein

EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
Eh-1	Engrailed homology domain 1
eIF4E	Eukaryotic translation initiation factor 4E
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
ERG	Ets related gene
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
esm-1	Endothelial specific molecule-1
EVC	Empty vector control
EVI-1	Ecotropic viral integration site-1
FACS	Fluorescence activated cell sorting
FACT	Factilitates chromatin transcription
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FLI1	Friend leukemia virus integration 1
FLT3	FMS-like tyrosine kinase 3
FSC	Forward scatter
GAB2	GRB2-associated binding protein 2
GATA-1	GATA binding factor-1
GATA-2	GATA binding factor-2
GEF	Guanine-nucleotide exchange factor
GFP	Green fluorescence protein
GRB2	Growth factor receptor-bound protein 2

GRIP1	Glutamate receptor-interacting protein 1
GTF	General transcription factor
GTP	Guanosine-5'-triphosphate
HATs	Histone acetyltransferases
HC8	Proteasome alpha-subunit C8
HCK	Hemopoietic cell kinase
HD	Homeodomain
HDACs	Histone deacetylases
HESR1	Hairy and enhancer of split-related 1
HEX	Haematopoietically expressed homeobox
HHEX	Human HEX
HIF	Hypoxia inducible factor
HNF-1α	Hepatic nuclear factor 1 alpha
HRE	Hypoxia response elements
HSCs	Haematopoietic stem cells
HSPGs	Heparan sulphate proteoglycans
HUVECs	Human umbilical vein endothelial cells
IFN	Interferon
IgH	Immunoglobulin heavy chain
Inr	Initiator
IP	Immuno-precipitation
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KD	Knockdown
kDa	Kilodaltons
L-PK	L-type pyruvate kinase

LB	Luria-Bertani medium
LMO2	LIM domain only 2
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAPK	Mitogen activated protein kinase
MCL1	Induced myeloid leukemia cell differentiation protein 1
MDS	Myelodysplastic syndrome
MeCP2	Methyl CpG binding protein 2
MED	Mediator
MEK	MAPK/ERK kinases
MEPs	Multi-potent progenitors
MM	Multiple Myeloma
***μ*M**	Micromolar
mRNA	Messenger RNA
MTT	Thiazolyl blue formazan [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan]
MVD	Microvessel density
N-CoR	Nuclear receptor co-repressor
NB	Nuclear body
NF-κB	Nuclear Factor kappa B
NLS	Nuclear localization sequence
nm	Nanometre
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthase
NPC	Nuclear pore complex
NUP98	Nucleoporin 98
OD	Optical density

P/CAF	p300/CBP associated factor
PBS	Phosphate buffer solution
PC-HC	Pericentromeric heterochromatin
PCI	Phenol:Chloroform:Isoamylalcohol
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEST	Sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
Ph	Philadephia
PHD	Pleckstrin homology domain
PHD2	Prolyl hydroxylase 2
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PIC	Pre-initiation complex
PIP3	Phosphatidyl inositol 3 phosphate
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PML	Promyelocytic leukaemia
PMSF	Phenylmethylsulphonyl Fluoride
PNBs	PML nuclear bodies
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
pPRH	Phosphorylated PRH
PRH KD	PRH Knockdown
PRH	Proline Rich Homeodomain

PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene Fluoride
pVHL	Von Hippel-Lindau protein
pXRCC1	Phosphorylated x-ray repair complementing defective repair in Chinese hamster cells 1 protein
qPCR	Quantitative PCR
RA	Retinoic acid
RAC	Ras-related C3 botulinum toxin substrate
RAR	Retinoic acid receptor
Ras	Rat sarcoma viral oncogene
rDNA	Ribosomal DNA
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAP	RNA polymerase
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
SAG	S-Antigen
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAG	Sodium dodecyl sulfate polyacrylamide gel
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
SF-1	Steroidogenic factor 1
SH2	Src homology domain 2
SHP-2	SH2-containing tyrosine protein phosphatase
shRNA	Short hairpin RNA

SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
snRNP	Small nuclear ribonucleoproteins
SOS	Son of sevenless
SOX13	SRY-related HMG-box protein 13
SRC	Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SRF	Serum responsive factor
SSC	Side scatter
STAT	Signal transducers and activators of transcription
T-ALL	T-cell acute lymphoblastic leukaemia
TAFs	TBP-associated factors
TBB	4,5,6,7-tetrabromobenzotriazole
TBL	Transducin beta-like protein
TBLR	Transducin beta-like related protein
TBP	TATA-binding protein
tcr	T-cell receptor
TEMED	N,N,N',N'- Tetramethylethylenediamine
TF	Transcription factor
TGF-β	Transforming growth factor beta
tk	Thymidine kinase
TLE	Transducin-like Enhancer of Split
TN	Tinman
TNF	Tumor necrosis factor
TSAd	T-cell adapter
TSS	Transcription start site
UBX	Ultrabithorax
UV	Ultraviolet

VE	Visceral endoderm
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMCs	Vascular smooth muscle cells
VSP	VEGF signalling pathway
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1 protein

Introduction

1 Introduction.

1.1 Transcription.

1.1.1 Introduction to Transcription.

Humans, and other higher eukaryotes, are complex organisms that are formed from several specialized cell types. These cell types are composed of a specific combination of proteins that characterize each cell and allow it to perform specific functions within a multi-cellular organism. The specific combination of proteins in a particular cell is tightly regulated by numerous endogenous and exogenous signals that influence both the regulation of gene expression and protein turnover. Regulation of gene expression is a critical process that conveys the cellular response to extracellular stimuli. Gene expression is a multi-step process, consisting of gene transcription, mRNA processing, mRNA export, protein translation and correct protein folding. Gene expression can be regulated at many stages in this process.

Regulation of the transcriptional process is very important in the control of gene expression and protein concentration. The process of transcription is complex and involves multiple levels of regulation and organization that will be outlined in detail over the next section. The RNA that is produced during transcription is a temporary copy of the DNA sequence which can either perform a structural role, or be transported and processed for protein translation, or act to regulate transcription and mRNA stability. The protein product then fulfills its role in the cell, influencing the function or phenotype of the cell.

1.1.2 Overview of Transcription.

Transcription is the process of synthesizing an RNA sequence transcript from DNA nucleotide sequence information. The process of transcription requires a multi-subunit enzyme called RNA polymerase (RNAP), which catalyses the joining of RNA nucleotides from 5' to 3' using DNA as the template sequence.

Eukaryotic cells have three nuclear RNA polymerases (RNAP I-III). RNAP I and III act on a limited profile of genes that encode ribosomal RNA, transfer RNA and small nuclear RNAs. RNAP II can potentially transcribe more than 20,000 genes that code for proteins (Lander et al., 2001). Transcription of protein coding genes is a highly regulated process, with more than 60 polypeptides making up the 3MDa RNAP II holoenzyme complex that is required for transcription initiation (Krishnamurthy and Hampsey, 2009). The complexity of the RNAP II holoenzyme provides multiple points at which the rate of transcription initiation (and elongation) can be influenced. Activation occurs through interactions between activator proteins, such as transcription factors, and other proteins within the RNAP II holoenzyme, which increase the affinity of the RNAP II holoenzyme with the DNA template. Alternatively transcription can be inhibited, through blocking access to the DNA template by the RNAP II holoenzyme. This is often achieved by inhibitory interactions between repressor proteins and specific subunits of the RNAP II holoenzyme.

The DNA template is divided into a coding region and non-coding regulatory regions. Promoter DNA consists of multiple features that assist in the binding of components of the RNAP II transcription complex. Examples of these include the TATA box (located ~25bp from transcription start site), the initiator (Inr) (at the transcription start site) and the downstream promoter element (DPE) (+30 upstream from the transcription start site) (reviewed by (Krishnamurthy and Hampsey, 2009)). A single promoter can include one or more of these elements however no single promoter

element is essential for transcription. These promoter elements assist the formation of the transcription pre-initiation complex (PIC) and allow association of the RNAP II holoenzyme. Formation of the PIC is nucleated by the TATA-binding protein (TBP), a subunit of TFIID (which includes a further 14 TBP-associated factors [TAFs]) that binds to TATA elements. If no TATA element is present then other members of the TFIID complex can bind alternative promoter elements (such as the Inr or DPE) (Krishnamurthy and Hampsey, 2009).

The RNAP II transcription complex then assembles in a stepwise mechanism *in vitro* (figure 1.1). TFIIB associates with the DNA-TBP complex and binds to the DNA surrounding the TATA element. This allows docking of RNAP II and TFIIF, through interactions with TFIIB. TFIIE binds to RNAP II and facilitates TFIIH binding to complete the PIC (reviewed by (Buratowski, 1994; Lee and Young, 2000)).

TFIIH, as part of PIC, melts approximately 14 bp of promoter DNA surrounding the transcription start, providing access to the DNA template (Wang et al., 1992). Another multi-protein complex, Mediator (MED), provides a bridge between the RNAP II complex and transcription factors bound to upstream enhancer elements, which enhances the interaction of RNAP II complex with the promoter DNA (Kornberg, 2005) (figure 1.2). It had been proposed that the RNAP II holoenzyme, consisting of MED and the RNAP II transcription complex exists as a pre-assembled complex at transcription initiation sites (Greenblatt, 1997). However, this has not been fully accepted because subsequent ChIP data demonstrated that recruitment of MED precedes RNAP II at gene promoters, which supports a stepwise assembly of the RNAP II holoenzyme (Cosma et al., 1999; Bhoite et al., 2001; Kornberg, 2005). Once the RNAP II holoenzyme is assembled at the gene promoter RNA synthesis begins. RNAP II normally fails to achieve productive RNA synthesis for several cycles releasing short RNA products (2-10 nucleotides in length) (reviewed by (Holstege et

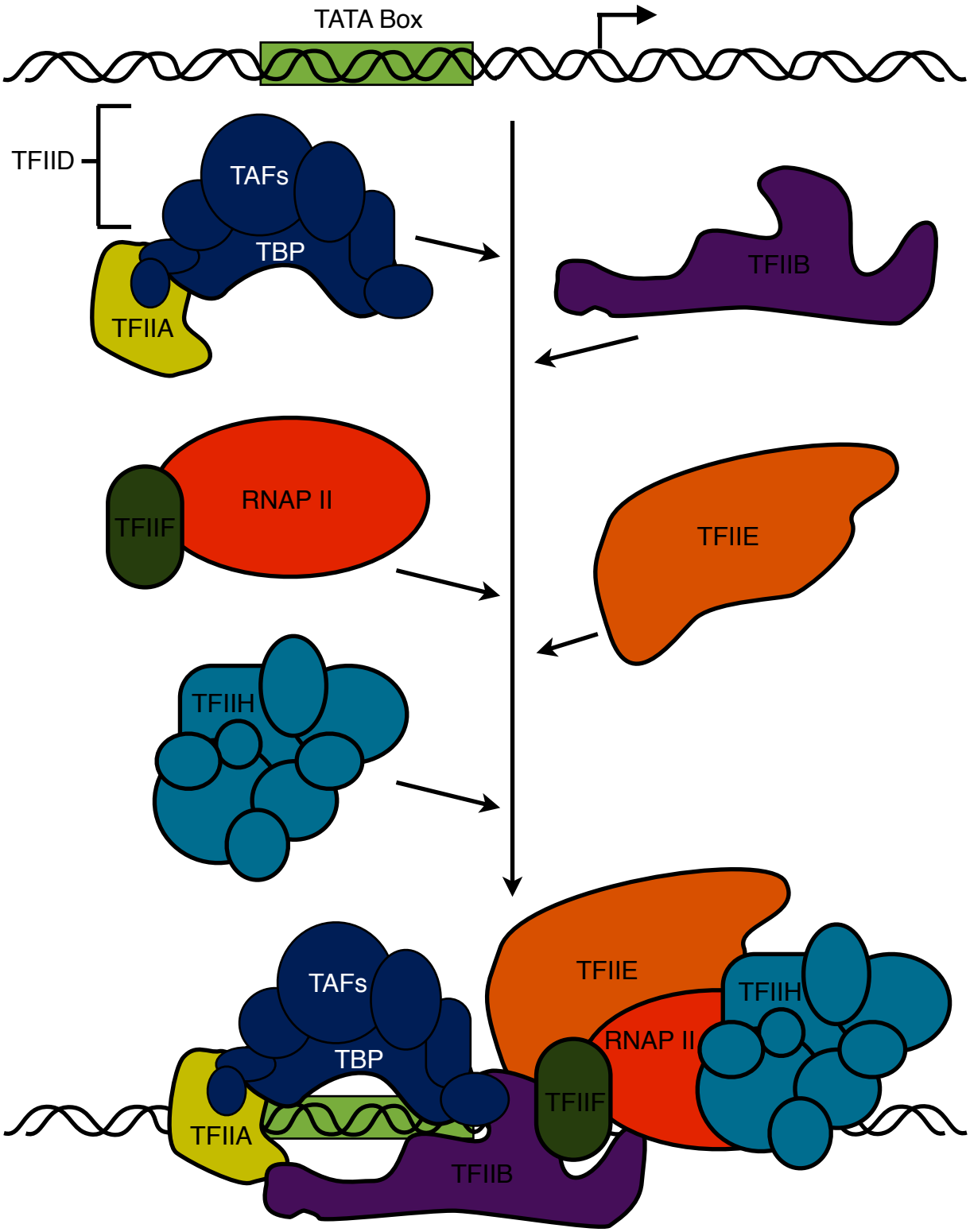


Figure 1.1 - Stepwise mechanism of the assembly of the basal transcription machinery.

The diagram illustrates the step by step process required for the nucleation of the basal transcription machinery around the core promoter. TFIID, including TBP, bind to the TATA box with TFIIA. Then TFIIB associates with the promoter, recruiting TFIIF and RNAP II. Finally, TFIIE and TFIIH complete the RNAP holoenzyme complex and transcription is initiated.

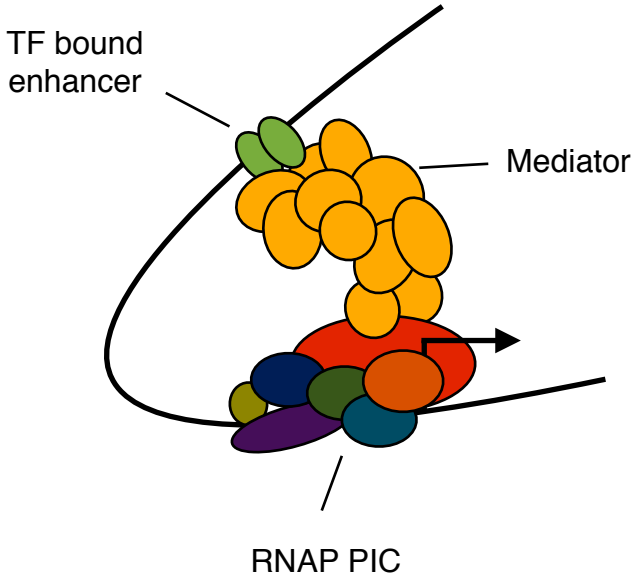


Figure 1.2 - Model of transcriptional activation through Mediator enhancer interactions.

The diagram illustrates the contribution of Mediator for transcriptional activation. Mediator promotes the association of the RNAP PIC to promoter DNA through interactions with transcription factors (TF) bound to upstream enhancer elements.

al., 1997)). Once RNAP II succeeds in synthesizing an RNA product greater than 11 nucleotides in length it will break its interaction with initiation factors and promoter DNA to begin translocating along DNA, processively synthesizing RNA (Holstege et al., 1997). An RNAP II-DNA elongation complex with stably bound nascent RNA is now formed. The C-terminal domain (CTD) of RNAP II is phosphorylated by TFIIH subunit and contact with the other GTFs is lost so that transcription elongation can continue. The phosphorylated CTD can recruit other factors that are required for efficient elongation and mRNA processing (reviewed by (Li et al., 2007; Buratowski, 2003)). These include; Rad4, a ubiquitin ligase; COMPASS, a Set1 methyltransferase complex; FACT, a histone chaperone; and Set2, a methyltransferase (Li et al., 2007). Each of these factors play a role in transcription elongation by providing access to the

DNA template by remodeling the chromatin. After transcriptional initiation and elongation, termination of transcription follows to give a messenger RNA product.

1.1.3 Chromatin Structure.

The human genome consists of just over 3 billion base pairs (bp) of DNA and each base pair of DNA is 3.4Å thick. Since the average cell is 10μm in length (1μm = 10000Å) this presents a packaging problem. To overcome this, eukaryotic cells have developed a way to increase the amount of DNA that can be packaged into a single cell and still allow efficient gene regulation to take place. Approximately 146 bp of DNA is wrapped around a histone octamer to form a DNA-protein particle known as a nucleosome (Luger et al., 1997). Nucleosomes condense DNA via nucleosome-nucleosome interactions to form higher order compaction into nucleosome arrays. Nucleosome arrays have been proposed to further fold into a 30nm diameter fiber, which could be further folded into large-scale configurations of nucleosomes (Tremethick, 2007). In interphase, there are regions of the chromatin that are transcriptionally active. Transcriptionally active DNA consists of more accessible DNA and less nucleosome-nucleosome interactions to provide space for the components of the general transcription machinery (reviewed by (Dehghani et al., 2005)). However, the most condensed form of DNA is found during the metaphase of the cell cycle, where DNA is further condensed into the transcriptionally inactive metaphase chromosome (Dehghani et al., 2005).

1.1.4 Nucleosomes and Chromatin Remodeling.

Nucleosomes are made up of a histone octamer, consisting of two of each histone subunit H2A, H2B, H3 and H4, and these subunits maintain multiple contact points with the DNA template (Razin et al., 2007). Since nucleosomes play a key role in compaction of DNA, the regulation of nucleosome-nucleosome interactions are critical for transcription factor accessibility. Protruding from each nucleosome

structure are the N-terminal tails of each of the histones. Histones can be post-translationally modified by methylation, acetylation, ubiquitination, ADP-ribosylation, sumoylation and phosphorylation (reviewed by (Kouzarides, 2007)). The protruding N-terminal tails of histones provide an accessible region of the histone subunits that can be modified. When modified these tails can influence chromatin compaction. There are three major roles of histone modification:

1. All histone modifications, except for methylation, alter the net charge of the nucleosomes and reduce the interaction between DNA and histones.
2. The post-translational modifications can be recognized by other proteins and could control recruitment of transcription factors (e.g. acetylation of histones can recruit bromo-domain containing transcription factors and cofactors).
3. Some modifications can influence higher-order chromatin structure (e.g. H4 K16 inhibits the formation of compact 30nm fibers).

Promoter DNA for active genes have a low nucleosome compaction, providing access for transcription factors (Lee et al., 2004). However, there are also examples of transcription factors binding to nucleosomal DNA (Adams and Workman, 1995; Taylor et al., 1991), prior to nucleosome displacement (Adkins et al., 2004). Once a transcription factor is bound to a promoter, the transcription factor can recruit co-activator complexes, such as histone modifying enzymes that acetylate or methylate histone tails. In fact, acetylation of promoter histones H3 and H4 directly correlates with the rate of transcription initiation (Pokholok et al., 2005). In some cases transcription factors can also recruit additional ATP-dependent chromatin remodeling complexes, through direct interactions with the transcription factor or through binding to acetylated histones (Hassan et al., 2001). These ATP-dependent chromatin remodeling complexes can further impact on the nucleosome density at a promoter by altering histone-DNA interactions, by displacing H2A-H2B dimers or by sliding

nucleosomes along DNA to provide access for further transcription factors (Kassabov et al., 2003). These chromatin remodeling complexes are a vital part of transcription initiation as they facilitate greater access to DNA for the multi-protein RNAP II holoenzyme. However, a large number of promoters could have a partial PIC already associated with the promoter, which includes TFIIA, TFIID, TFIIB, TFIIE, and TFIIF subunits, but not the TFIIH subunit or RNAP II itself (Zanton and Pugh, 2006). This suggests that chromatin remodeling does not always regulate the accessibility of general transcription factors at a promoter, but instead regulates initiation of RNAP transcription (Sharma et al., 2003).

1.1.5 Nuclear Architecture.

The nucleus is a complex organelle and consists of several compartments that possess specific functions. Many of these functions are not related to RNAP II dependent gene transcription, such as the nucleolus which is assembled around rDNA genes and is important for coordinating rRNA synthesis. Other characterized nuclear compartments include, Cajal Bodies, Clastosome, Cleavage Bodies, Nuclear speckles and Paraspeckles although these only occupy a small fraction of the volume of the nucleus. These compartments have roles in; snRNP biogenesis, nuclear proteasomal activity, mRNA cleavage, splicing factor storage, and RNA-binding protein storage respectively (reviewed by (Spector, 2003)).

Few nuclear compartments have been implicated in transcription, likely because the majority of the nucleus is used for transcription. However, nuclear organization is required for efficient transcription of specific genes encoded in the genome at specific points during differentiation. For example, up-regulation of the β-globin genes is required during erythrocyte differentiation, however, they need to be inactive up until this point (Ragoczy et al., 2006). Therefore, tight control of active and inactive genes is required. Interestingly, active genes may cluster together for transcription, as there

are more active RNAP II molecules than there are sites of active transcription (Iborra et al., 1996). It is thought that up to 20 active RNAP II proteins are clustered together at specific sites of transcription. These clusters are termed transcription factories (Carter et al., 2008). It is proposed that these 'transcription factories' nucleate several loops of chromatin, and these may involve several genes being transcribed together, or even from regions of different chromosomes (Lomvardas et al., 2006). The nucleus has two types of chromatin, euchromatin and heterochromatin. Euchromatin is located within the centre of the nucleus and contains the active RNAP II 'transcription factories' (Spector, 2003; Bolzer et al., 2005). Inactive genes are associated with heterochromatin that is found towards the nuclear periphery (Spector, 2003; Bolzer et al., 2005).

Genes associated with the nuclear periphery are generally thought to be repressed or less active. In fact recent studies have shown that tethering of genes to the nuclear periphery can robustly reduce transcriptional activity (Reddy et al., 2008). However, there has been significant variation (20%-75%) in the amount of transcriptional repression executed by localization to the nuclear periphery (Reddy et al., 2008; Finlan et al., 2008). This suggests that certain genes maybe more susceptible than others to repression complexes located at the nuclear periphery. It has also been observed that the β-globin gene locus moves from the nuclear periphery to the nuclear core when activated (Ragoczy et al., 2006). This movement has suggested that the transcription factories maybe relatively immobile and predominantly occupied at the nuclear core and that therefore nuclear periphery localization is a physical barrier to transcription. However, localization to the nuclear periphery is not solely associated with transcriptional repression, as active gene transcription has been observed to occur at regions associated with the nuclear pore complex (NPC) (Brown and Silver, 2007).

PML nuclear bodies (PNBs) have also been described as a nuclear body (NB) associated with transcriptional regulation. PNBs are small nuclear domains that are 0.1μM-1.0μM in diameter, and PML is a major constituent (Lallemand-Breitenbach and de Thé, 2010). PML forms the outer shell of PNBs, with the interior consisting of PML partner proteins (Lallemand-Breitenbach and de Thé, 2010). There are now around 100 proteins that are known to associate with PNBs, but PML is still thought to be the key organizer of PNBs. Sumoylation of PML or the partner protein is thought to have a significant role in the recruitment of many of these PNB-associated proteins (Lallemand-Breitenbach and de Thé, 2010). PNBs are attached to the nuclear matrix (Chang et al., 1995), a nuclear meshwork of insoluble proteins, and have been found to correlate with transcriptional repression in some model systems (Tsukamoto et al., 2000). However, PNBs are not restricted to playing a roles in transcription, a subset of PNBs have a granular core under the electron microscope, which is typical of ribonucleoproteins, and PNBs have also been found associated with telomeres, with a role in maintaining telomere length (Lallemand-Breitenbach and de Thé, 2010).

1.1.6 Transcriptional Regulation.

As illustrated in 1.1.2 the gene promoter region is key in the regulation of a specific gene. There are also several other important DNA elements that regulate transcription initiation, including enhancers, silencers and insulators. Enhancer regions are regulatory sequences that act at a distance (Maston et al., 2006). Enhancers can be located several thousand base pairs upstream or downstream of the transcription start site (TSS), but they can be shorter distances away as well. As with proximal promoter elements these sequences regulate transcription through transcription factor association (Maston et al., 2006) and it has been proposed that chromatin is looped to cluster these enhancer regions with the core promoter (Cook,

2003). In contrast, silencer regions repress transcription in an orientation and distance-independent manner and can be considered the opposite of enhancer DNA sequences (Maston et al., 2006). Insulator regions are a further level of complexity which block the influence of enhancers and silencers by forming insulator bodies at the nuclear periphery (Fourel et al., 2004). These insulator bodies collate insulator elements preventing association of enhancers or silencers from associating with the core promoter (Fourel et al., 2004). These DNA regulatory elements dictate which transcription factors that are capable of binding and influencing transcription initiation by RNAP II (Orphanides and Reinberg, 2002).

For transcriptional activation, transcription factors bind to defined binding motifs within these DNA sequences. Once bound they can recruit further transcription co-factors, ATP dependent chromatin remodeling complexes or histone acetyl-transferase complexes to increase the stability of the interaction between the PIC and DNA (Orphanides and Reinberg, 2002). Ultimately, transcriptional activation can be altered by:

1. Increased nuclear concentration of the specific transcription factors and certain co- factors or accessory proteins.
2. Post-translational modifications to the gene specific transcription factor that allows interaction of other necessary proteins.
3. Reduction of inhibitory/competing transcription factors.

For example, Nuclear Factor -κB (NF-κB), an important inflammatory response transcription factor, is found in the cytoplasm in unstimulated cells. However, upon cytokine stimulation, a phosphorylation cascade targets IκB, the inhibitory subunit of NF-κB, for degradation, unmasking the nuclear localization sequence for NF-κB (Israël, 2010). This results in a rapid accumulation of nuclear NF-κB that is able to activate transcription. In addition NF-κB can be further modified by phosphorylation

and acetylation to increase transcriptional activation of specific genes (Chen and Greene, 2004). The method used for transcriptional activation is transcription factor and gene promoter dependent.

Like transcriptional activation, transcriptional repression is a vital process for tight control of gene expression. Transcriptional repressor proteins associate with their target genes directly through a DNA-binding domain or indirectly through other DNA-bound proteins. Selective transcriptional inhibition by a transcriptional repressor protein can be mediated by:

1. Displacement or blocking of a transcription factor from binding to the gene promoter.
2. Masking the activation domain on a gene specific transcriptional activator.
3. Blocking the interaction of a transcription factor with other components of the transcriptional machinery.
4. De-stabilization of the RNAP II holoenzyme.

Transcriptional repression can be transient, through transcription factor binding to promoter elements, or stable gene silencing, by methylation of CpG islands. Transient transcriptional repression can allow rapid activation or repression of a gene. For example, thyroid-hormone and retinoic acid receptors bound to their promoter regulatory binding motifs recruit the nuclear receptor co-repressor (N-CoR) protein, in their ligand-independent form (Hörlein et al., 1995). N-CoR, a co-repressor, forms a complex with TBL1 and TBLR1 and recruits HDAC3 to de-acetylate histones and induce transcriptional repression (Yoon et al., 2003). This repression is transient and is distrupted upon ligand binding to the nuclear receptor (Perissi et al., 2010). However, stable gene silencing can provide a mechanism for longer term silencing of genes, such as silencing of genes during differentiation. CpG methylation by DNA methyl-transferases can lead to the formation of repressive

complexes that also reduce access to promoter DNA elements. Methyl-CpG binding domain containing proteins, such as MeCP2, bind to these methylated regions and again recruit a co-repressor complex that includes HDACs, which maintains chromatin in an inaccessible state (Bowen et al., 2004). MeCP2 also competes with histone H1 and encourages 30nm chromatin fiber formation, which could be important for its transcriptional repression (Ghosh et al., 2010).

1.1.7 Regulation of Transcription Factor Activity.

Controlled gene expression is critical to maintain cellular homeostasis. Regulation of transcription factor activity is controlled through specific post-translational modification of the transcription factors. These post-translational modifications can influence DNA binding, co-factor association, sub-cellular localization and stability of the transcription factor.

Transcription factor activity relies on sequence specific DNA binding to control target gene expression. For example, p53 is a tumour suppressor protein that is activated in response to DNA damage and is tightly regulated by post-translational modifications. Upon exposure of cells to UV light or ionizing radiation, p53 is phosphorylated at serine residues 33 and 37, by DNA-PK (Sakaguchi et al., 1998). These N-terminal post-translational modifications increase site directed acetylation of the C-terminal domain of p53 by p300 and P/CAF (Sakaguchi et al., 1998). Acetylation of lysine 320, by P/CAF, and lysine 382, by p300, increase p53 sequence specific DNA binding to activate specific target genes (Gu and Roeder, 1997; Sakaguchi et al., 1998). Phosphorylation and acetylation are the most common post-translational modifications to activate transcription factor activity, and these occur in response to most inter- or intra-cellular signalling cascades and can promote or inhibit DNA binding.

Transcription factor association with a co-factor can not only direct transcriptional activity (activation versus repression), but can also limit its transcriptional activity. Often accessory proteins are required for transcriptional activation or repression, transcription factor binding alone is not sufficient for transcriptional activity. Post-translational modifications can alter these associations. For example, the orphan nuclear receptor, steroidogenic factor 1 (SF-1), is phosphorylated by mitogen activated protein kinase (MAPK) in a ligand independent manner (Hammer et al., 1999). This phosphorylation of serine 203, within the activation function 1 domain, stimulates SF-1 transcriptional activity in a luciferase promoter reporter assay (Hammer et al., 1999). Transcriptional activation is dependent on co-factor, GRIP1 or SMRT, association with SF-1 (Hammer et al., 1999). Mutation of the phosphorylation site inhibits GRIP1 or SMRT binding to SF-1 and inhibits transcriptional activation (Hammer et al., 1999). Post-translational modifications can alter protein conformation to facilitate protein-protein interactions, conversely they can also sterically hinder these associations to regulate transcription.

In addition to DNA binding and co-factor association, regulation of nuclear localization is an important determinant of transcription factor activity. The signal transducers and activators of transcription (STAT) family, of which there are 7 family members, is a good example of how post-translational modification can effect nuclear localization. Interferon (IFN) stimulation induces expression of immediate-early response genes through STATs (Bromberg, 2001). IFN induces a signal transduction pathway whereby Janus kinase (JAK) kinase activity is activated (Bromberg, 2001). JAK phosphorylates a single C-terminal tyrosine residue of a STAT protein, this induces homo- or hetero-dimerization and nuclear translocation (Darnell, 1997). Phosphorylated nuclear STATs are transcriptionally active.

As transcription factors induce temporal and spatial regulation of gene expression, inactivation of transcription factor activity can also be required. Post-translational modification of transcription factors are often signals for targeted degradation by the proteasome. GATA-1 is a critical haematopoietic transcription factor and tight regulation of its transcriptional activity is required for differentiation of haematopoietic cells (Orkin, 2000). Acetylation of GATA-1 is necessary for transcriptional activity, however, subsequent phosphorylation at multiple residues by MAPK induces poly-ubiquitination and proteasome dependent degradation (Hernandez-Hernandez et al., 2006). This targeted degradation of GATA-1 is also induced by erythropoietin (EPO), a mitogen that drives erythrocyte maturation (Hernandez-Hernandez et al., 2006).

For efficient and responsive changes in gene expression, intra- and extra-cellular signals need to alter transcription factor activity. This section has demonstrated examples of altered transcription factor DNA binding, co-factor association, sub-cellular localization and stability by post-translational modifications in response to signal transduction. Each of these plays a significant role in cellular homeostasis.

1.2 Proline Rich Homeodomain (PRH).

PRH is a transcription factor that belongs to the homeodomain super-family of DNA binding proteins (Soufi and Jayaraman, 2008). It is essential for complete development of the embryo, with critical roles in the development of the forebrain and foregut maintenance. PRH is also important for the haematopoietic compartment, influencing differentiation and growth.

1.2.1 Homeodomain (HD) Proteins.

The homeobox is a conserved sequence of DNA present within homeotic genes in *Drosophilia melanogaster* and encodes a protein domain termed the homeodomain that binds DNA or RNA (Scott and Weiner, 1984; McGinnis et al., 1984). Further homologs of this domain have been identified across species and in humans 299

homeodomain-containing genes are listed in the homeodomain resource database (Moreland et al., 2009). Homeodomain proteins are a large family of DNA binding transcription factors that have roles in many developmental processes. For example the Hox genes encode Hox homeodomain proteins and control morphology on the main body axis of most metazoans (Lemons and McGinnis, 2006).

The structure of the homeodomain is well-characterized. It is a DNA binding domain composed of 60 amino acids that form a helix loop helix turn helix secondary structure. The N-terminal arm of the homeodomain binds DNA and contacts base pairs in the minor groove, whilst the loop contacts the phosphate backbone of DNA (figure 1.3). The recognition helix (helix III) lies in the major groove and interacts through contacts from the side chain groups of this helix (Billeter, 1996). Helices I and II do not appear to make contacts with the DNA but provide the necessary structure

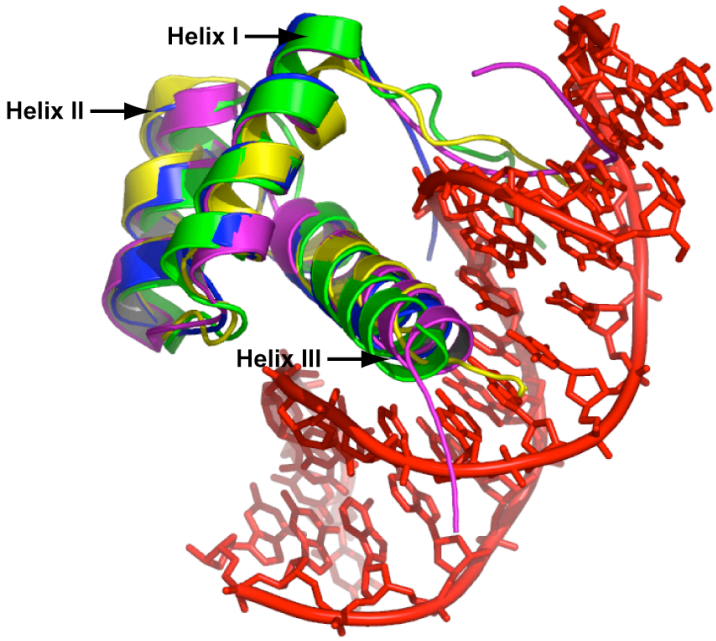


Figure 1.3 - A model of the PRH homeodomain bound to DNA.

The solution NMR structure of the PRH (magenta) homeodomain superimposed on the Engrailed (blue), and Oct-2 (yellow) DNA bound structures. The figure was taken from (Soufi and Jayaraman, 2008).

for interaction (Billeter, 1996; Neidle and Goodwin, 1994). These interactions provide the specificity for recognizing the core ATTA sequence found in many homeodomain binding sites.

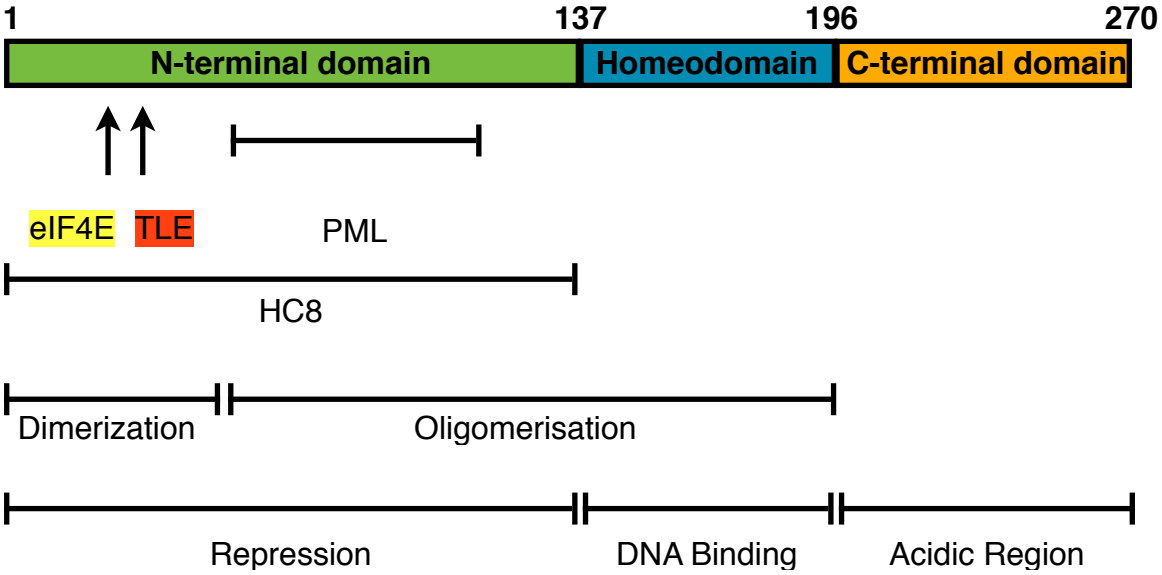
1.2.2 Structural Properties of PRH and Interaction Motifs.

The human PRH protein consists of 270 amino acids and has a calculated molecular weight of 30 kDa, although it shows retarded mobility on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAG) and runs at 36kDa. It is a modular protein composed of 3 major regions, a 20% proline-rich N-terminal domain (AAs 1–136), a central homeodomain (AAs 137–196), and an acidic C-terminal region (AAs 197–270) (figure 1.4) (Crompton et al., 1992).

1.2.2.1 PRH N-terminal Domain

The N-terminal domain of PRH has been extensively studied, and this has revealed key characteristics that may determine the activity of PRH within the nucleus. The N-terminal domain allows self-association and is vital for PRH to act as a transcriptional repressor (Soufi et al., 2006; Guiral et al., 2001). The composition of the 136 amino acids of the N-terminal domain of PRH includes 20% proline residues, 15% alanine and 10% glycine residues. The N-terminal domain does not appear to have typical β-sheet or α-helical structures. Proline residues are known to disrupt α-helix/β-sheet secondary structure formation, and as a result this domain probably has a more extended structure, which facilitates dimer formation (Soufi et al., 2006). The N-terminal domain of PRH also appears to be split into 2 regions that have separate roles. The first 50 amino acids are vital for PRH dimerization. The second region, C-terminal to the first 50 amino acids of PRH, are important for associating with a partner PRH homeodomain (AAs 132-270) to promote oligomerisation (figure 1.4) (Soufi et al., 2006). As well as facilitating oligomerisation, the N-terminal domain can repress transcription independently of the homeodomain when it is fused to another

[A]



[B]

PRH Amino Acid Sequence.

1 MQYPHPGPAA GAVGVPLYAP TPLLQPAHPT PFYIEDILGR
41 GPAAPTPAPT LPSPNSSFTS LVSPYRTPVY EPTPIHPAFS
81 HHSAAALAAA YGPGGFGGPL YPFPRTVNDY THALLRHDPL
121 GKPLLWSPFL QRPLHKRKGG QVRFSNDQTI ELEKKFETQK
161 YLSPPERKRL AKMLQLSERQ VKTWFQNRRA KWRRLKQENP
201 QSNKKEELES LDSSCDQRQD LPSEQNKGAS LDSSQCSPSP
241 ASQEDLESEI SEDSDQEVDI EGDKSYFNAG

Figure 1.4 - Schematic diagram of the modular structure of PRH.

[A] There are three distinct regions of PRH; the N-terminal domain, the Homeodomain, and the C-terminal domain. Regions with key properties and known interaction sites are marked. The diagram has been adapted from (Soufi and Jayaraman, 2008). [B] PRH amino acid sequence, showing the N-terminal domain (green highlight), homeodomain (blue highlight), and C-terminal domain (golden highlight). The eIF4E (yellow) and TLE (red) interaction domains are also shown.

DNA binding domain (Guiral et al., 2001).

An enduring theme of most transcriptional processes is the formation of a transcriptional complex for activation or repression, with the recruitment of co-factors to provide binding specificity and functional output. Similarly PRH has been shown to bind with other proteins that could affect the functional activity of PRH. For example PRH contains an Eh-1 motif (FXIXXIL) (Amino acids 31-39 (TFYIEDILG)) (figure 1.4), that is also found in the homeodomain proteins Engrailed and Goosecoid. This motif is the site of interaction between these homeodomain-containing proteins and a family of co-repressor proteins, known as the Groucho/TLE proteins. In PRH, the Eh-1 motif mediates the interaction with TLE proteins, the human homologs of Groucho. Mutation of the Eh-1 motif results in the loss of association between PRH and TLE proteins (Swingler et al., 2004). The N-terminal domain of PRH also interacts with the proteosome subunit HC8 (Bess et al., 2003), although the significance of this interaction is not fully understood. Proteasome activity does not appear to affect the ability of PRH to repress transcription (Bess et al., 2003). There are other proteins that also interact with the N-terminal domain of PRH and these include; SOX13 (Marfil et al., 2009), which will be discussed later in section 1.2.5; the PML protein (interacts with PRH AAs 50-115) (Topcu et al., 1999) and the translation factor eIF4E (interacts with PRH AAs 18-24) (Topisirovic et al., 2003a), which will both be discussed in section 1.2.6.

1.2.2.2 PRH Homeodomain.

PRH belongs to the NK subclass of homeodomain proteins that contain a Tinman (TN) motif located N-terminal to the homeodomain (Banerjee-Basu and Baxevanis, 2001). PRH contains a motif that resembles this TN decapeptide sequence but lacks a conserved arginine residue at position 5 in the homeodomain that all other members of the NK family possess and additionally lacks an NK-specific domain

downstream of the homeodomain. Therefore PRH can be considered to have its own subclass. Its closest homolog is the Hlx homeodomain protein, which only shares 56% identity with PRH within the homeodomain region (Crompton et al., 1992).

Homeodomain containing proteins are able to bind to AT-rich DNA sequences *in vitro*. The PRH consensus binding sequence has been determined to be 5'-C/TA/TATTAAA/G-3' using SELEX experiments but this study only used a C-terminal fragment of PRH containing the homeodomain and C-terminal domain of PRH (Crompton et al., 1992). Electrophoretic mobility shift assay (EMSA) studies using the isolated homeodomain have shown PRH can bind to 5'-ATTAA-3' and 5'-CAAG-3' sequences, suggesting a very poor binding specificity (Pellizzari et al., 2000). To date, no SELEX studies have been conducted to characterize the PRH consensus binding sequence using the whole PRH protein. Therefore full length PRH binding specificity is not well defined, although it is likely PRH binds elements with the core sequence ATTA or TAAT.

Recently PRH has been shown to bind with high affinity to a cluster of five ATTA sites in the goosecoid gene promoter (Williams et al., 2008). Moreover high concentrations of PRH result in widespread protection of promoter DNA from DNase I treatment *in vitro* (Williams et al., 2008). Binding of PRH to the *Goosecoid* promoter induces DNA distortion and it has been suggested that transcriptional repression by PRH may occur through the compaction of DNA wrapped around a PRH oligomer (Williams et al., 2008; Soufi et al., 2006). These studies indicate that it may be the number and spacing of potential binding motifs that is important for PRH binding, with co-operativity involved in the PRH-DNA association. Thus PRH may have a flexible consensus sequence, that only requires a few core ATTA like sequences to initiate binding.

1.2.2.3 PRH C-terminal Domain.

The C-terminal domain of PRH is a highly acidic region with four times the number of acidic residues than basic residues. PRH can activate or repress gene transcription depending on the cell type and promoter environment. The C-terminal domain of PRH appears to be necessary for transcriptional activation. The C-terminal domain can interact with other transcription factors or co-activators to facilitate gene activation. This includes interactions with hepatic nuclear factor 1 alpha (HNF-1α) (Tanaka et al., 2005) and serum responsive factor (SRF) (Oyama et al., 2004), these interactions will be discussed in detail in section 1.2.5.

1.2.2.4 Oligomerisation of PRH.

Proline residues are often found in protein-protein interaction regions (Kay et al., 2000), and many of the known protein interactions for PRH are located in the proline-rich N-terminal domain. Ultracentrifugation experiments have shown that PRH can form octamers and larger oligomers for which the proline rich N-terminal domain of PRH is critical (Soufi et al., 2006). A model for oligomer formation has been proposed (figure 1.5) (Soufi et al., 2006). PRH has a dimerisation domain within the N-terminal domain (AAs 1-50; 'P' domain), as well as two other domains that interact with each other. These two domains are the transcriptional repression domain (AAs 51-132; 'R' domain) and the homeodomain-C-terminal domain (AAs 133-270; 'H' domain). The P-P domain interactions occur between monomers and R-H domain interactions occur between PRH dimers, giving the potential for higher oligomeric fibres or the association of four PRH dimers to form a donut-like 'closed' structure (figure 1.5) (Soufi et al., 2006).

Recently it has been shown that PRH can bind to long DNA fragments and form protein-DNA fibres. This study also demonstrated that PRH oligomers are capable of compacting DNA without accessory proteins although this compaction is not sufficient

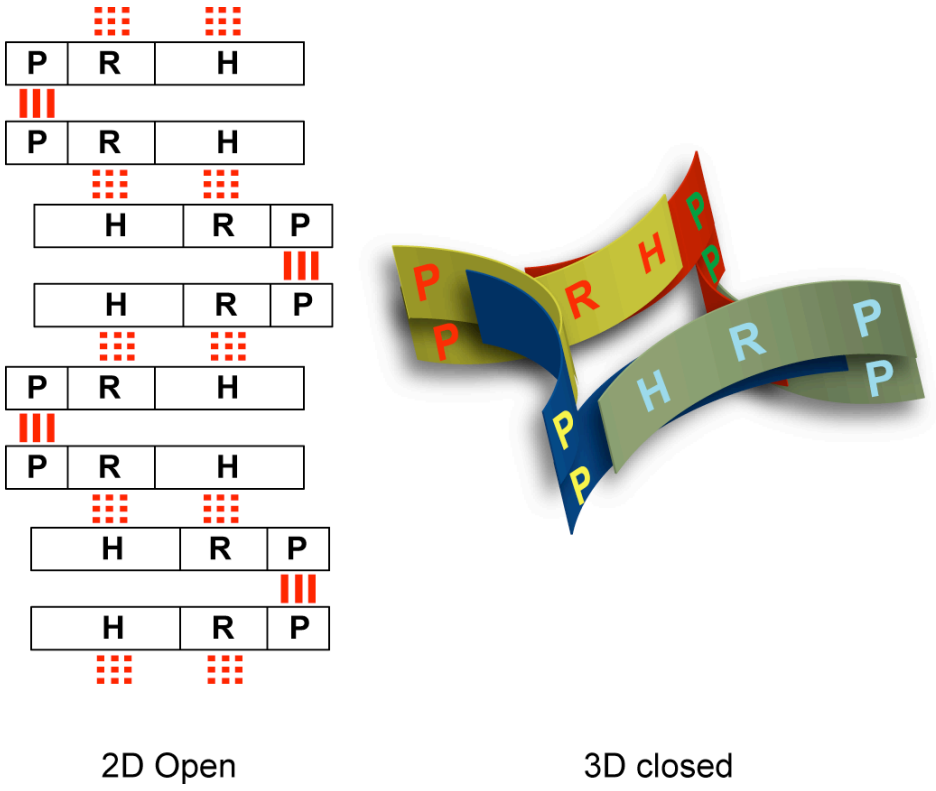


Figure 1.5 - A model for PRH oligomerization.

The diagram represents a model for the association of PRH monomers into oligomeric chains (2D open) or closed donut-like tetramers (3D closed). The P region represents the first 50 amino acids, the R region represents amino acids 50-137 and the H region represents amino acids 137-270. The figure was taken from (Soufi et al., 2006).

for transcriptional repression (Soufi et al., 2010). This suggests that PRH could be functionally and structurally related to the bacterial and archaea proteins, the Lrp/ AsnC family, which were not previously thought to have eukaryotic equivalents (Soufi et al., 2010).

Interestingly uncontrolled aggregation of PRH proteins does not occur and this could in part be due to the rich glycine content in the PRH N-terminal domain as this amino acid has been reported to discourage aggregation and key glycine residues can be conserved for this purpose (Parrini et al., 2005).

1.2.2.5 Post-translational Modifications of PRH.

PRH can be phosphorylated within the homeodomain at the serine residues 163 and 177 by protein kinase CK2 (CK2) (Soufi et al., 2009). CK2 promotes survival through multiple mechanisms, which will be discussed in more detail later (see section 1.5). PRH can interact with the β subunit of CK2, which facilitates the phosphorylation of PRH by the CK2 α subunit (Soufi et al., 2009). *In vitro* experiments demonstrated that phosphorylation of the PRH homeodomain inhibits the ability of PRH to bind DNA (Soufi et al., 2009). Phosphorylation of PRH by CK2 is therefore very likely to be an important mechanism of regulation of PRH transcriptional activity.

1.2.3 The Role of PRH in Embryonic Development.

PRH expression is critical for embryonic development. Although *Prh* +/- mice are normal and fertile, intercrosses between heterozygous mice give no live offspring. *Prh* -/- embryos show no significant difference to heterozygous littermates until 8.5 days post coitum (dpc), where a reduction in forebrain is observed. *Prh* -/- embryos begin to die at 11.5 dpc and no live embryos are observed by 15.5 dpc (Martinez Barbera et al., 2000). PRH expression is first detected in the primitive endoderm at 4.5 dpc. PRH expression is then observed in the visceral endoderm (VE) prior to the formation of the primitive streak and gastrulation (Thomas et al., 1998). During embryonic development the VE differentiates into the anterior VE (AVE), an early signalling centre that plays a pivotal role in establishing anterior-posterior polarity of the embryo. VE cells expressing PRH are morphologically distinct (Rivera-Pérez et al., 2003) and they actively migrate to form the AVE (Srinivas et al., 2004). In addition to the role of AVE in axis formation, AVE also has roles in neural patterning by signalling to the epiblast before and during gastrulation (Beddington and Robertson, 1998; Beddington and Robertson, 1999).

After gastrulation, *Prh* is expressed in the anterior definitive endoderm (ADE). The ADE develops from the primitive streak and the node signalling centres after gastrulation (Wells and Melton, 1999). The ADE replaces the AVE and this is an important step for correct head development. PRH expression in the ADE and the AVE is required for normal forebrain development (Martinez Barbera et al., 2000). Subsequent PRH expression is required for the maintenance of foregut identity and liver and pancreas development (McLin et al., 2007).

Development of the haematopoietic and vascular systems originate from a common mesodermal progenitor, the haemangioblast (Choi et al., 1998). Blood islands of the yolk sac contain haemangioblasts and these consist of haematopoietic stem cells (HSCs) and angioblasts (Durand and Dzierzak, 2005). The HSCs form the inner regions of these blood islands and the outer cells are the angioblasts (Durand and Dzierzak, 2005). Haematopoiesis then occurs within the embryo proper, in the aorta gonad mesonephros (AGM) region and the fetal liver (Durand and Dzierzak, 2005). Finally, at birth HSCs from the fetal liver migrate and colonize the bone marrow. Mature blood cells are produced in the bone marrow in the adult system (Baron, 2003; Park et al., 2005). The presence of PRH is vital for the development of HSCs and is also important for angioblast formation (Guo et al., 2003). Although PRH is required for angioblast formation, PRH is not expressed in the angioblast nor is there expression in any mature precursor cells of the endothelial lineage (Thomas et al., 1998). In contrast, PRH is expressed in early haematopoietic progenitors of all linages except T-cell derived lineages (figure 1.6) (Bedford et al., 1993; Manfioletti et al., 1995; Jayaraman et al., 2000). PRH expression is then down-regulated during terminal differentiation cell of haematopoietic cells and is lost in all mature cells, with the exception of granulocytes where PRH expression is maintained (figure 1.6) (Manfioletti et al., 1995; Jayaraman et al., 2000). Therefore PRH is likely to control

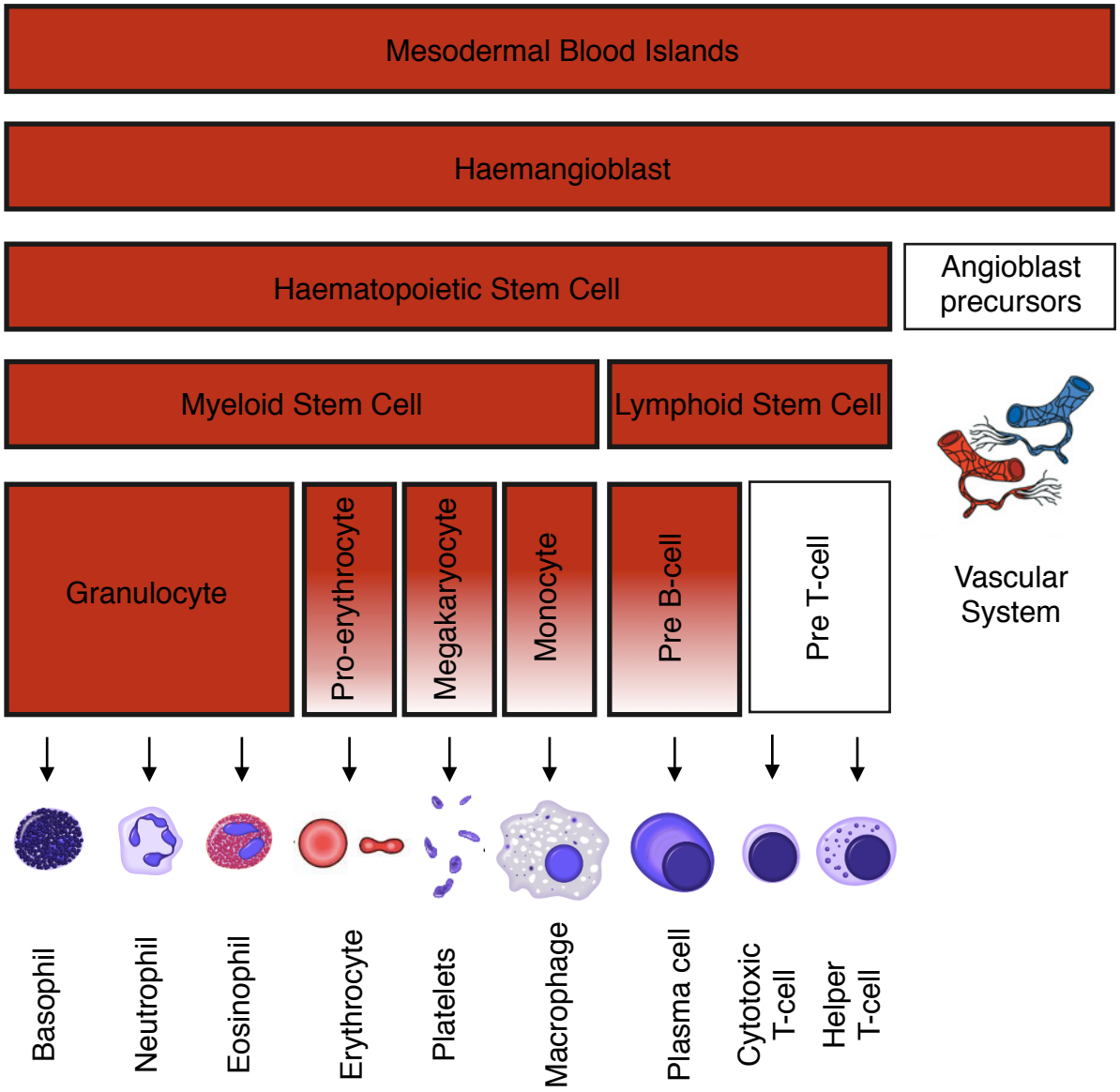


Figure 1.6 - PRH expression during haematopoiesis.

The diagram illustrates the process of haematopoiesis, with each box representing a distinctive cell type. PRH expression is shown in red. When PRH expression is lost in a lineage this is represented in white, for example pre T-cells do not express PRH and PRH expression is lost during monocyte differentiation to a mature macrophage. The diagram has been adapted from (Soufi and Jayaraman, 2008).

key steps within haematopoietic cell development.

1.2.4 Expression of PRH in the Adult System.

In the adult system PRH has a limited expression profile. PRH expression was first observed in avian and human haematopoietic cells (Crompton et al., 1992). PRH is often referred to as the human haematopoietically expressed homeobox (HHEX) in the literature (Bedford et al., 1993), but later it was observed that PRH is expressed in multiple embryonic tissues and also in the thyroid, lung and liver, as well as in the haematopoietic compartment (Soufi and Jayaraman, 2008). In adult liver cells, PRH expression is found in adult differentiated liver cells, but not in undifferentiated hepatocytes or in undifferentiated liver cell lines (Tanaka et al., 1999). This suggests that PRH is important for the maintenance of differentiated liver cells. PRH is expressed in adult thyroid cells and is present in both the nuclear and cytoplasmic compartments (Bogue et al., 2000). PRH expression is also observed in thyroid adenomas, and carcinomas but the localization of PRH is mostly cytoplasmic (D'Elia et al., 2002). Furthermore, PRH is expressed in the adult pancreas but its role is poorly understood and although PRH has roles in the development of the heart and skin its role in these tissues in the adult system is yet to be characterized (Hromas et al., 1993; Bogue et al., 2000; Hallaq et al., 2004; Foley and Mercola, 2005; Obinata et al., 2002).

1.2.5 Transcriptional Regulation by PRH.

PRH is primarily thought of as a transcriptional repressor (Williams et al., 2008; Swingler et al., 2004; Bess et al., 2003; Guiral et al., 2001), however, the number of tissues PRH is expressed in and the variety of functions that have been reported for PRH suggests that it is likely to act in a context dependent fashion. In fact PRH can also activate transcription at some genes (Kasamatsu et al., 2004; Oyama et al., 2004; Sekiguchi et al., 2001). The mechanisms utilized by PRH for transcriptional

regulation are also promoter and context dependent. There are four mechanisms by which PRH has been described to regulate gene expression (reviewed by (Soufi and Jayaraman, 2008)):

1. PRH can act as a transcriptional repressor that directly associates with gene promoter regions.
2. PRH can activate transcription through binding to gene promoter regions.
3. PRH can also repress transcription by indirectly disrupting the activity of another transcriptional regulator.
4. PRH can activate transcription indirectly through a mechanism other than binding to a gene promoter.

Examples of these four mechanisms will be illustrated below.

Direct transcriptional repression by PRH is best understood for PRH regulation of the *Goosecoid* gene. Goosecoid is a protein that is expressed during gastrulation in a group of cells, called the Spemann Organizer (Niehrs et al., 1993). PRH was first described to regulate this gene in *Xenopus laevis* (Brickman et al., 2000). Recent DNase I and chemical footprinting studies have shown that PRH binds directly to the promoter of the *Goosecoid* gene at multiple sites, covering long stretches of the promoter and induces DNA distortion (Williams et al., 2008). This study proposes that PRH could bind and repress transcription as an oligomer that wraps DNA around its oligomeric core (Williams et al., 2008). Another direct target of PRH is the endothelial specific molecule 1 (*esm-1*) gene, here the authors used chromatin immunoprecipitation (ChIP) assays to show PRH binds to the *esm-1* gene promoter (Cong et al., 2006). Electrophoretic mobility shift assays (EMSA) were used to determine that the PRH binding sites for the *esm-1* gene are conserved 5' C/TA/ TATTAAA/G 3' sequences (Cong et al., 2006).

Although the exact mechanism of PRH induced repression is not fully understood, some molecular interactions have been detailed that provide an insight into possible modes of action. As described above PRH can interact with proteins of the Groucho/ TLE family of transcriptional co-repressors (Swingler et al., 2004). The TLE family of proteins have no DNA binding capacity and rely on DNA binding transcription factor partners, such as PRH, for localization of their gene targets, this will be discussed in more detail later in section 1.3. Recruitment of TLE by PRH has been shown to be necessary for transcriptional repression of an artifical promoter, however, the significance of this interaction has not yet been shown *in vivo* at an endogenous gene (Swingler et al., 2004). Furthermore, it is not clear whether TLE is a general requirement for transcriptional repression by PRH or whether it is important for just specific target genes. Also, PRH DNA binding specificity is yet to be accurately characterized, as discussed above (see section 1.2.1), this is further highlighted by the fact that PRH has been described to bind to TATA-box sequences and induce transcriptional repression. PRH represses the artifical *thymidine kinase* (*tk*) reporter construct, which contains a TATA-box but no other sequences that resemble PRH binding sites (Guiral et al., 2001). Truncations of PRH containing the C-terminal and the homeodomain were used in EMSA experiments to show that PRH can bind directly to the *tk* TATA-box (Guiral et al., 2001). This suggests that PRH may be recruited to TATA-box containing genes and block TBP and formation of the PIC (Guiral et al., 2001). Therefore this might be another mode of transcriptional repression by PRH .

Indirect transcriptional repression by PRH has also been described for a number of genes. GATA-2 is a positive regulator of the *Vegfr2* gene and binds to specific binding elements at the *Vegfr2* promoter. PRH can inhibit the GATA-2 dependent activation of *Vegfr2* through a specific interaction with GATA-2 that disrupts GATA-2 DNA binding

(Minami et al., 2004). Furthermore, interactions between the PRH homeodomain and the N-terminal domain of jun can decrease jun-dependent transcriptional activation of basic fibroblast growth factor (bFGF) (Schaefer et al., 2001). A number of other genes are also known to be influenced by PRH expression, including *Chordin* (Brickman et al., 2000), *Thyroglobulin* (Pellizzari et al., 2000), *Vegfr1*, *Vegfr2*, *Tie-1*, *Tie-2*, *Neuropillin-1* (Nakagawa et al., 2003) and *Integrin* (Kubo et al., 2005; Mack et al., 2002). However, it is not yet known whether these genes are directly or indirectly influenced by PRH.

PRH can act as a direct transcriptional activator in certain contexts at particular genes. PRH has been shown to activate transcription of the *ntcp* gene through direct binding to the gene promoter (Kasamatsu et al., 2004). For this activity the C-terminal domain of PRH is reported to be required and truncations of PRH lacking the C-terminal domain are unable to induce transcriptional activation (Kasamatsu et al., 2004). PRH also directly activates the SMemb/NMHC-B gene in vascular smooth muscle cells (VSMCs) (Sekiguchi et al., 2001). A promoter element at -481 bp from the transcription start site (TSS) is thought to be critical for PRH mediated transcriptional activation of this gene (Sekiguchi et al., 2001).

Indirect transcriptional activation by PRH has also been described. For example, in hepatocytes PRH can interact with HNF-1α and stimulate expression of the L-type pyruvate kinase (L-PK). In this case, the PRH homeodomain is required to bind to HNF-1α and the C-terminal domain is also necessary to enhance L-PK gene expression (Tanaka et al., 2005). Similarly, in murine embryonic fibroblasts the SM22α gene is activated by SRF and activation is elevated by PRH independent of its DNA binding capacity (Oyama et al., 2004). Whereas, during embryogenesis PRH blocks SOX13-dependent repression (Marfil et al., 2009). SOX13, with its binding partner TCF1, is a repressor of Wnt/TCF signaling. PRH blocks SOX13-dependent

repression of Wnt/TCF activity by removing SOX13 from the SOX13/TCF1 complex (Marfil et al., 2009). Therefore, PRH acts as an antagonist of Wnt/TCF activity. This de-repression was shown to be dependent on the association of the PRH N-terminal domain and the LZ-Q domain of SOX13 (Marfil et al., 2009).

1.2.6 Regulation of PRH Activity.

The mechanisms that control the activity of PRH as a transcriptional repressor and a transcriptional activator are not fully understood. It is likely PRH is regulated *in vivo* through post-translational modifications, CK2 phosphorylation is known to inhibit DNA binding by PRH *in vitro* (Soufi et al., 2009). Phosphorylation of PRH could block direct regulation and simultaneously promote indirect gene regulation by enhancing interactions with other transcription factors.

1.2.7 The Role of PRH in Haematopoietic Disease.

Since PRH has key roles in the differentiation and proliferation of haematopoietic progenitor cells, it is not surprising that dysregulation of PRH expression or activity can lead to the progression of malignant diseases such as leukaemia. However, in the literature there is conflicting evidence regarding the role of PRH in haematopoietic disease. Loss of PRH function or up-regulation of PRH activity can lead to the progression of a tumourigenic phenotype. There also appears to be a divide in the role of PRH between disease of myeloid or lymphoid origin. In this section I will illustrate the conflicting roles of PRH by outlining the current knowledge of PRH function in disease of myeloid leukaemias and lymphoid tumours.

Leukaemias of myeloid origin, which have dysregulated PRH, have reduced PRH function (Topisirovic et al., 2003b; Topisirovic et al., 2003a; Jankovic et al., 2008). In at least one patient disruption of PRH activity has been demonstrated to result in Acute Myelogenous Leukaemia (AML). The patient carries a cytogenetic abnormality (t(10;11)(q23;p15) that results in the formation of a NUP98-HHEX(PRH) fusion

protein. This fusion protein replaces the N-terminal domain of PRH with that of NUP98 but retains the DNA binding activities of PRH. The fusion protein can function as a trans-dominant negative over wild-type PRH in repression assays and it has been suggested that the fusion protein can compete with endogenously expressed PRH and disrupt its cellular roles (Jankovic et al., 2008). A recent study has shown that the nucleoporin proteins, including NUP98, are important for the activation of some developmental and cell cycle genes. In fact NUP98 is able to activate gene expression away from the nuclear pore complex, within the nucleoplasm (Kalverda et al., 2010). The region of NUP98 present in the NUP98-Hex fusion protein corresponds to the activation domain in NUP98 (Kalverda et al., 2010). Therefore it is likely that the NUP98-HHEX fusion protein present in the patient with AML, has lost the N-terminal repression domain from PRH and gained a transcriptional activation module in its place resulting in activation of genes normally repressed by PRH in myeloid precursor cells. This fusion protein is not sufficient for the development of leukaemia as a further mutagenic event is required for full transformation. However, it demonstrates that dysregulation of PRH can initiate a malignant phenotype in cells of the myeloid lineage.

Previous work has shown that in cells, from human primary AML and chronic myelogenous leukaemia (CML) patients, PRH levels are aberrantly low or PRH is mis-localized from the nucleus to the cytoplasm (Topisirovic et al., 2003b). The mis-localization of PRH may be associated with disruption of key regulatory interactions with nuclear proteins within nuclear bodies (Topisirovic et al., 2003a). PRH is associated with PNBs (Topisirovic et al., 2003a; Topcu et al., 1999), through a direct interaction with PML. Amino acid residues 50-115 of the PRH N-terminal domain interacts with the RING domain of PML (Topcu et al., 1999). PRH also interacts with the eukaryotic translation initiation factor 4E (eIF4E), which is a translation factor that

has a role in the nucleo-cytoplasmic transport of specific gene transcripts including cyclinD1 (Topisirovic et al., 2003a). This interaction between PRH and eIF4E inhibits eIF4E dependent mRNA transport (Topisirovic et al., 2003a). When this interaction is altered by the loss of PRH from the nucleus, increased eIF4E-dependent mRNA transport of cyclinD1 is observed. This ultimately leads to an increase in cyclinD1 protein levels, accelerating progression through the G1 cell cycle check-point and increasing cell proliferation (Topisirovic et al., 2003a). eIF4E is increased in AML and CML leukaemias, where PRH activity is reduced (Topisirovic et al., 2003b). Interestingly, PRH and eIF4E co-localize in PNBs (Topisirovic et al., 2003a), suggesting that PNB localization of PRH and eIF4E may be important for this regulation. Clearly, negative regulation of eIF4E nucleo-cytoplasmic transport of cyclinD1 mRNA suggests disruption of this regulation would have a role in myeloid leukaemia.

In contrast, the role of PRH appears to be oncogenic in lymphoid tissues. The use of AKXD recombinant inbred mice, which develop leukaemias and lymphomas as a result of retroviral mediated activation of proto-oncogenes, identified PRH as a potential cause of B-cell leukaemias. PRH was found to be significantly up-regulated in these leukaemias (Hansen and Justice, 1999). In a follow up study, bone marrow cells transduced with retrovirus containing the PRH coding region, and injected into mice, lead to the development of tumours originating from T-cell precursor. These neoplasms are thought to develop due to inhibition of differentiation to mature T-cells (George et al., 2003). Furthermore, constitutive expression of the LMO2 oncogene in the thymus of mice induces the development of overt T-cell acute lymphoblastic leukaemia (T-ALL) (McCormack et al., 2010). Over-expression of PRH is sufficient to promote self-renewal of thymocytes and McCormack et al suggested this was important for the accumulation of subsequent mutations that allow for leukaemic

transformation (McCormack et al., 2010). This work is supported by data from Oram et al, who demonstrated that LMO2 forms a triad with ERG and FLI1 to bind the +1 enhancer of the PRH gene promoter (Oram et al., 2010).

This body of work presents the possibility that the role of PRH is dependent upon the environment; hence in certain contexts the loss of PRH results in the loss of repression and the elevation of oncogenic genes. Whereas in other environments loss of PRH expression may be vital for proper differentiation. Therefore, it is most likely that the determining factor controlling the balance is the time point at which the mutation or over-expression occurs during cell maturation. For example, if a mutation occurred in the stem cell population it may have a different phenotype compared to the same mutation in a myeloid progenitor cell, which may also give a different phenotype from the mutation arising in a lymphoid progenitor cell.

1.3 TLE.

Transducin-like Enhancer of split (TLE) proteins constitute a family of transcriptional co-repressors that do not bind DNA directly, but associate with DNA binding transcription factors (Fisher and Caudy, 1998). TLE dependent repression of gene expression requires oligomerization of TLE (Chen et al., 1998). TLE recruitment of HDACs (Chen et al., 1999) and its ability to bind to hypo-acetylated histones (Palaparti et al., 1997), are also thought to be important for transcriptional repression. The TLE family are a widely expressed family of proteins that have critical roles in embryonic development and they are involved in a variety of signalling pathways in the adult system (Buscarlet and Stifani, 2007).

1.3.1 Role of TLE in Haematopoiesis.

The TLE protein family are regulators of gene expression during haematopoiesis and there are four homologs of the mammalian TLE protein (Eberhard et al., 2000; Linderson et al., 2004; Ren et al., 1999; Levanon et al., 1998; Imai et al., 1998). As

stated in section 1.2, TLE proteins interact with a regulator of haematopoiesis, PRH (Swingler et al., 2004), although to date no gene targets have been identified that are co-regulated by PRH and TLE proteins. However, TLE proteins can bind to many other important haematopoietic factors and regulation by TLE proteins are important for lineage commitment (Levanon et al., 1998; Ren et al., 1999; Eberhard et al., 2000; Linderson et al., 2004). TLE proteins appear to have significant roles in B-cell development through interactions with the vital B-cell linage commitment factors, Pax5 and PU.1 (Eberhard et al., 2000; Linderson et al., 2004). These interactions are important for transcriptional repression of *IgH* (*immunoglobulin heavy chain*) and *Joining (J)-chain* genes, which both have B-cell specific cis elements at their promoters (Linderson et al., 2004). Interestingly, the TLE homolog Grg4 that was investigated in these studies is down-regulated after B-cell activation, suggesting a role for TLE proteins in maintaining B-cell precursors (Linderson et al., 2004). Furthermore, TLE proteins interact with the PRDI-BFI/Blimp-1 protein, another B-cell specific factor, to function as a co-repressor of *ifn-β* expression (Ren et al., 1999). TLE proteins are not limited to roles in the B-cell lineage. TLE also binds to AML1 and AML2 (Levanon et al., 1998). AML1 binds TLE proteins, through association with the VWRPY motif and C-terminal sequences of TLE, recruiting TLE proteins to AML1 DNA binding sites within the *t-cell receptor* (*tcr*) enhancers α and β (Levanon et al., 1998). AML1 transactivates *tcr* gene expression in Jurkat T-cells, however, recruitment of TLE1 inhibits this activity (Levanon et al., 1998). TLE1 can also counteract LEF-1:β-catenin-dependent transcription of *tcr* (Levanon et al., 1998), demonstrating a role for TLE-1 regulation of gene expression in T-cells as well. As a binding partner and negative regulator of AML1 transactivation it is likely that TLE-1 also has roles in the regulation of myeloid specific genes (Imai et al., 1998; Levanon et al., 1998), however none have yet been characterized.

1.3.2 Structure of the TLE Family.

Understanding the structural features of the TLE family is critical to understanding, how these proteins function. TLE proteins are split into two sub-groups of proteins; the GRO subgroup, which have five distinct domains (Q, GP, CcN, SP and WD-repeat domains), and the AES subgroup, which only contains the Q domain (Chen and Courey, 2000). Members of the AES subgroup act as trans-dominant negatives over members of the GRO subgroup, which are functional co-repressors of transcription. This section will characterize the key structural properties of TLE.

WD-repeat domains are characterized by specifically located tryptophan and aspartate residues within ~40 amino acid tandem repeats. This repetitive motif forms a β-propeller structure composed of seven β-blades, with each blade consisting of a four-stranded anti-parallel β-sheet (Li and Roberts, 2001). This structure is not unique to TLE proteins and is found in many proteins with a plethora of cellular functions including, signal transduction, vesicular trafficking and transcriptional regulation (Stirnimann et al., 2010). Functionally this region is recognized to facilitate protein-protein binding and in TLE proteins it functions as a surface for DNA-bound transcription factors to bind to TLE (Paroush et al., 1994; Copley, 2005; Jiménez et al., 1999). For example, the transcription factors Hairy and Engrailed both bind to TLE through the WD-repeat domain (Paroush et al., 1994; Jiménez et al., 1997).

The Q domain is highly conserved between TLE proteins, it is thought to form coiled-coil structures with two amphipathic α-helices (Miyasaka et al., 1993; Pinto and Lobe, 1996). These structural elements are vital for the formation of hetero- and homo-oligomers of the TLE family and point mutations within the α-helices of the Q domain disrupt oligomerization (Chen et al., 1998).

The central region of TLE proteins contains a CcN motif; it is a loosely conserved region of ~60 amino acids. Within this region there is a nuclear localization sequence

(NLS) and putative phosphorylation motif for CK2 and $p34^{cdc2}$ kinase (cdc2) (Stifani et al., 1992). These phosphorylation sites are important for regulation of TLE activity and will be discussed below (section 1.3.4).

The other two central regions are the GP and SP domains and these regions are poorly conserved through the TLE family. The GP domain has a high content of glycine and proline residues, whereas the SP domain is rich in serine and proline residues.

1.3.3 Mechanism of TLE-mediated Transcriptional Repression.

Although a definitive mechanism of action for TLE induced transcriptional repression is yet to be elucidated it is thought that its structural properties are vital for its activity. Homo- or hetero-oligomerization of TLE, via Q domain interactions, is important for transcriptional repression, and it has been suggested that TLE can form oligomeric complexes that associate with chromatin influencing the availability of binding sites for transcriptional activators (Chen et al., 1998). Oligomerization of TLE may also repress transcription by spreading over long distances (Martinez and Arnosti, 2008) and may contribute to chromatin compaction (figure 1.7) (Sekiya and Zaret, 2007). Once recruited to promoter regions TLE can bind to HDACs and induce deacetylation of histones (Chen et al., 1999). This interaction requires the SP domain of TLE (Chen et al., 1999). Generally, histone deacetylation is considered to repress transcription by decreasing transcription factor accessibility and compaction of DNA (Fuks, 2005). Moreover, TLE can bind to hypo-acetylated histone H3 via the N-terminal domain of TLE (Palaparti et al., 1997) and possibly further compacts the chromatin structure. A role for TLE proteins in masking the availability of phosphorylation, acetylation and methylation sites on histone H3 has been suggested (Palaparti et al., 1997). In addition the GP domain has a role in transcriptional repression, although the mechanism through which this occurs has not been characterized.

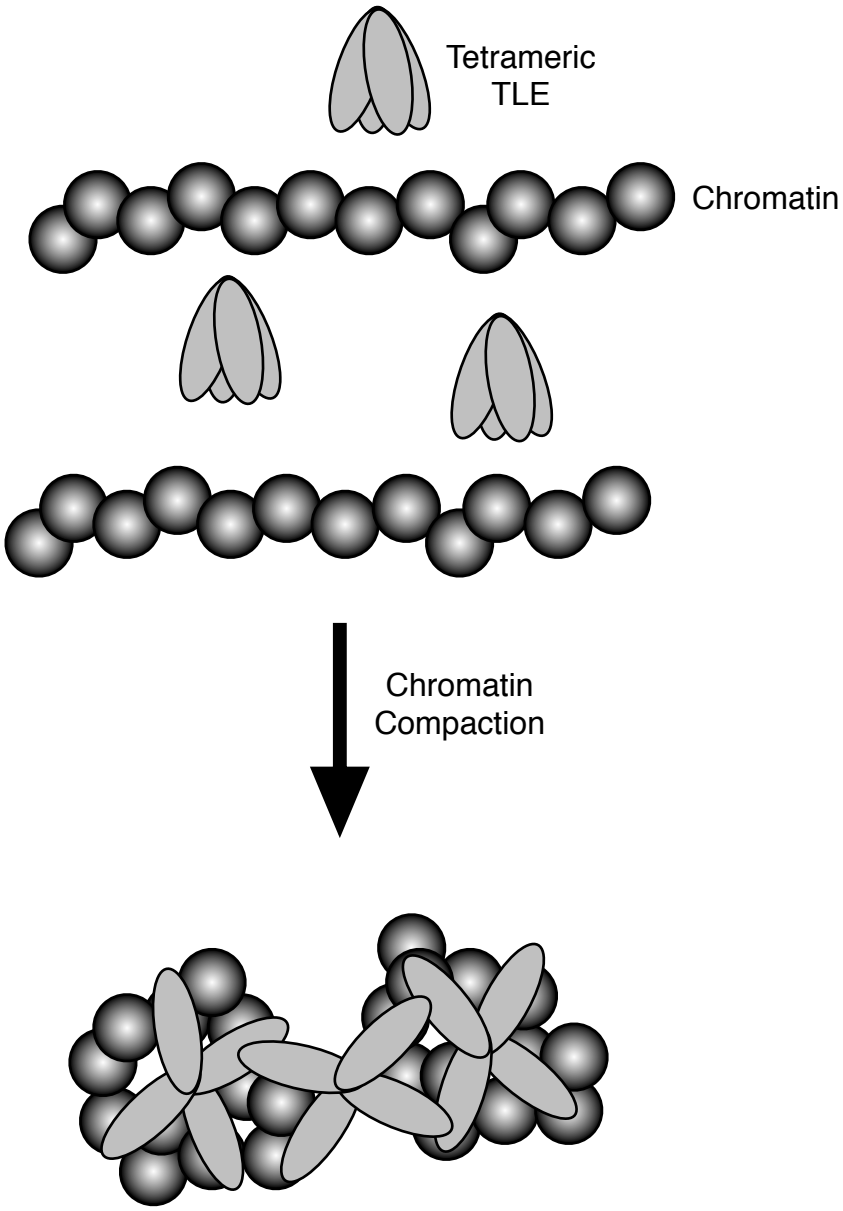


Figure 1.7 - A model for TLE compaction of chromatin.

The diagram illustrates a model for TLE compaction of chromatin. TLE can bind to hypo-acetylated histones and oligomerize to compact chromatin structure. This is likely one of the mechanisms of TLE induced repression. The diagram has been adapted from (Sekiya and Zaret, 2007).

Interestingly, the AES subgroup of TLE proteins only contain the Q domain and these proteins can oligomerize with the TLE proteins from the GRO subgroup (Ren et al., 1999). AES proteins have trans-dominant negative activity over the full-length GRO proteins and it has been suggested that AES proteins sequester GRO proteins and inhibit their ability to bind to DNA-bound transcription factors or associate with other co-factors necessary for transcriptional repression (Ren et al., 1999). This suggests that the ratio of GRO proteins compared to AES proteins will have a role in balancing the repression activity of TLE at target genes.

1.3.4 Phosphorylation of TLE.

Phosphorylation of TLE regulates its nuclear retention and its functional activity as a co-repressor (Buscarlet et al., 2009; Hasson et al., 2005; Nuthall et al., 2004; Nuthall et al., 2002b; Nuthall et al., 2002a; Eberhard et al., 2000; Husain et al., 1996). TLE is phosphorylated following association with a DNA binding partner. Studies by Nuthall et al revealed that over-expression of the transcription factor Hes-1 or AML1 (also known as RUNX-1), both DNA binding transcription factors that interact with TLE, induce hyper-phosphorylation of TLE (Nuthall et al., 2002a). This co-factor activated phosphorylation (CAP) of TLE increases the retention of TLE to the chromatin fraction and increases transcriptional repression (Nuthall et al., 2002a). Furthermore, TLE hyper-phosphorylation is dependent on constitutive phosphorylation of TLE proteins by CK2 (Nuthall et al., 2002a). TLE has two CK2 consensus sequences, within the CcN domain, at serine residues 239 and 253. Mutation of either or both of these serine residues significantly reduces or abolishes CAP of TLE (Nuthall et al., 2004). S239 phosphorylation is not dependent on association of TLE with a DNA binding transcription factor, however phosphorylation of S239 is vital for transcriptional repression (Nuthall et al., 2004). Nuthall et al proposed that S239 is

constitutively phosphorylated by CK2 after translation, priming TLE for subsequent hyper-phosphorylation (Nuthall et al., 2004).

TLE transcriptional activity can also be controlled by receptor tyrosine kinase (RTK) signalling through MAPK (Hasson et al., 2005; Cinnamon et al., 2008). There are two potential MAPK phosphorylation sites one within each of the SP and WD40 domains (Hasson et al., 2005). In the study by Hasson et al they showed that upon EGFR stimulation TLE is phosphorylated, reducing its co-repressor activities (Hasson et al., 2005). In addition they demonstrate the ability of EGFR signalling to antagonize Notch induced transcriptional output (Hasson et al., 2005). A follow up study identified general activation of MAPK, sufficient to reduce co-repression activity (Cinnamon et al., 2008). However, MAPK phosphorylation of TLE does not reduce nuclear localization, it only inhibits the transcriptional activity of TLE (Cinnamon et al., 2008).

TLE is also hyper-phosphorylated, in the CcN and SP domains, by cdc2 (Nuthall et al., 2002b). Cdc2 phosphorylation of TLE occurs during the G2/M phase of the cell cycle and reduces the nuclear retention of TLE (Nuthall et al., 2002b). Clearly, phosphorylation of TLE is important in determining the transcriptional activity and nuclear retention properties of TLE (Husain et al., 1996; Nuthall et al., 2004; Nuthall et al., 2002b; Nuthall et al., 2002a). There are at least four phosphorylation sites on TLE1 that affect its activity and therefore multiple combinations of phosphorylated residues can arise; it is likely that different combinations of phosphorylated residues have a distinct functional output. This sort of combinatorial regulation suggests TLE is tightly regulated to respond to internal and external stimuli as required.

1.4 Vascular Endothelial Growth Factor.

The Vascular Endothelial Growth Factor (VEGF) and its receptors VEGFR1 and VEGFR2 are important factors for new blood vessel formation, in the process of

angiogenesis (Murukesh et al., 2010). As a result these factors are dis-regulated in some diseases such as von Hippel-Lindau syndrome, where they have a role in neovascularization of growing tumors (Harris, 2000).

1.4.1 VEGF.

VEGF belongs to the PDGF superfamily and is a potent mitogen and survival factor for endothelial cells (Ferrara and Henzel, 1989; Gerber et al., 1998). However it also has other roles, for example it is thought to be a survival factor for haematopoietic stem cells (Gerber et al., 2002) and can stimulate monocyte migration (Barleon et al., 1996). VEGF is secreted as a covalently linked homo-dimer and exists in six isoforms; VEGF 121, 145, 165, 183, 189 and 206; with VEGF 121, 165, 183, and 189 all expressed across a wide range of cell types (Robinson and Stringer, 2001). In addition to these isoforms there are another 5 VEGF like mitogens; VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PlGF; that signal through the VEGF receptors, however, these will not be discussed further in this thesis (Robinson and Stringer, 2001).

1.4.2 VEGF Receptors: VEGFR1 and VEGFR2.

The VEGF receptors VEGFR1 and VEGFR2, are receptor tyrosine kinases that can bind and be activated by VEGF. The downstream pathways of these receptors involve a complex network of signalling cascades that include the p38MAPK, PI3K/Akt and PLCγ pathways (Roskoski, 2007; Matsumoto and Mugishima, 2006). VEGFR1 has a stronger binding affinity for VEGF than VEGFR2 (Waltenberger et al., 1994), yet VEGFR2 signalling is thought to be more important for endothelial cell proliferation and survival (Gille et al., 2001; Bernatchez et al., 1999). The physiological role of VEGFR1 described in the literature suggests that it predominantly regulates cell migration of both endothelial cells (Kanno et al., 2000) and monocytes (Barleon et al., 1996). However, it has also been described to play a

role in survival signalling through the Akt pathway (Nishi et al., 2008) and can increase cell survival in response to chemotherapeutic agents (Tsuchida et al., 2008).

1.4.3 Other Receptors for VEGF.

VEGFR1 and VEGFR2 are the most prominent VEGF receptors in the literature, but there is also a third receptor, VEGFR3, which is important for lymphatic endothelial cell development and function (Olsson et al., 2006). However, VEGFR3 does not bind VEGF, but binds VEGF-C and VEGF-D (Ferrara et al., 2003). Additionally there are co-receptors that can bind VEGF, neuropilin-1, neuropilin-2, and heparan sulphate proteoglycans (HSPGs). These appear to have no catalytic activity but can increase VEGF binding for other VEGF receptors (Soker et al., 1998). Neuropilins are also vital for proper vascular development (Kawasaki et al., 1999).

1.4.4 VEGF Signal Transduction.

VEGF induced signal transduction through its receptors VEGFR1 and VEGFR2 mediate different responses depending on the cellular environment. VEGFR2 signalling has been well studied and is thought to be primarily responsible for endothelial cell proliferation, survival and migration (Gerber et al., 1998; Bernatchez et al., 1999). It is also involved in a similar role in many other cell types (Ferrara et al., 2003). VEGFR1 in endothelial cells is widely reported as a decoy receptor, with no or few downstream targets (Hiratsuka et al., 1998; Meyer et al., 2006), resulting in less VEGFR2 signal transduction. However, in monocytes VEGFR1 signalling induces a strong chemotactic response (Barleon et al., 1996). Also, it has been shown that VEGFR1 is capable of similar signal transduction responses as those induced by VEGFR2 in endothelial cells, when sufficient stimulus is supplied (Knight et al., 2000). The following section will review the signalling pathways induced through VEGFR2 and VEGFR1 (figure 1.8).

1.4.4.1 VEGFR2 Signal Transduction.

VEGFR2 signal transduction is initiated by the binding of VEGF to the Ig-like domain 3 of the extracellular region of VEGFR2 (Lu et al., 2000). Upon ligand binding, receptor dimerization is induced and the receptor is activated through auto-phosphorylation (Dougher and Terman, 1999). There are several important tyrosine

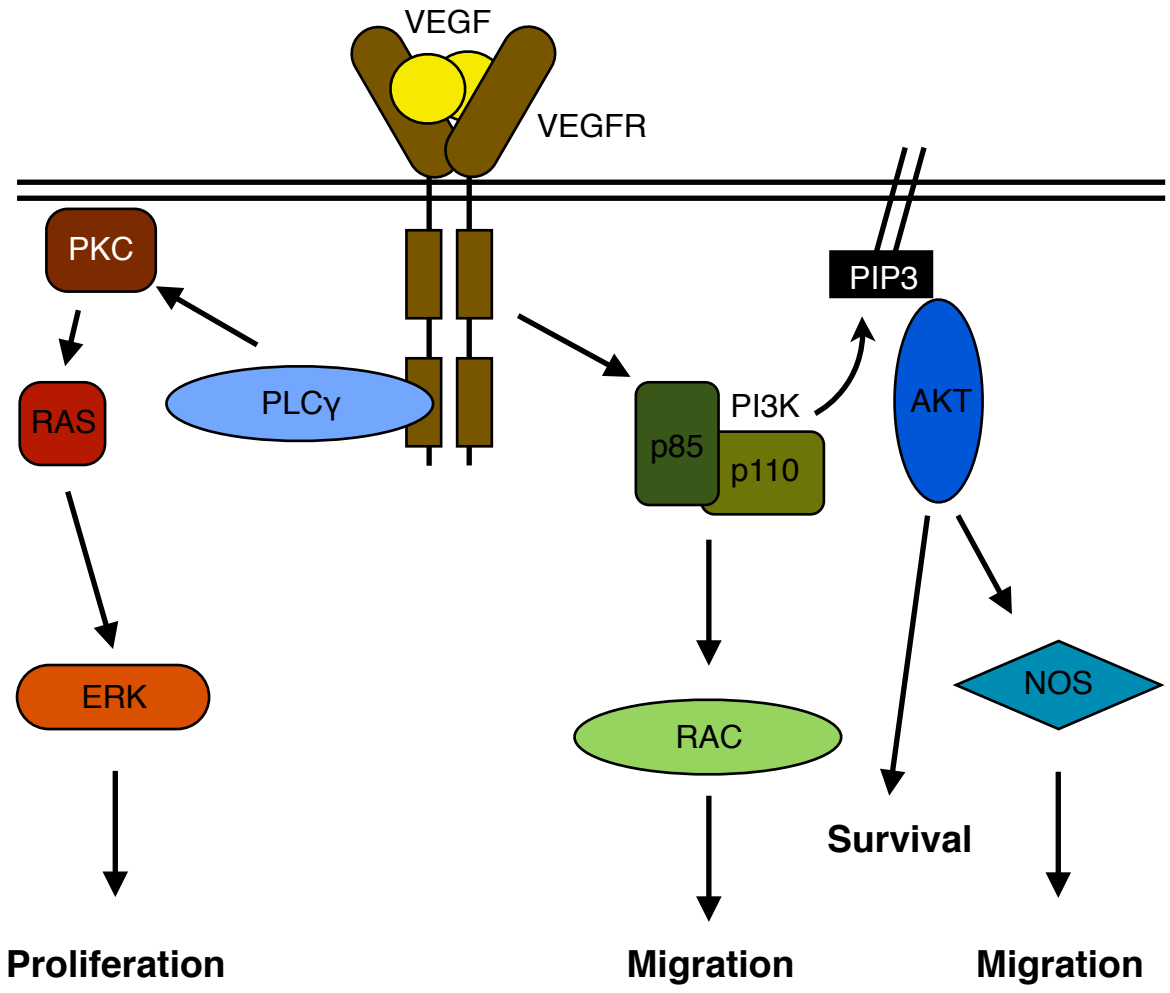


Figure 1.8 - VEGF signal transduction.

The diagram illustrates the pathways involved in VEGF signal transduction. The VSP induces proliferation, survival and migration, through the PKC, AKT, NOS and RAC pathways respectively.

phosphorylation sites in the cytoplasmic region of VEGFR2, including phosphorylation of residue 951 in the kinase-insert domain (Matsumoto et al., 2005), residues 1054 and 1059 in the kinase domain (Kendall et al., 1999), and residues 1175 and 1214 in the C-terminal tail of VEGFR2 (Takahashi et al., 2001). These phosphorylated tyrosine kinase residues provide a binding platform for SH2 domain containing accessory proteins, which mediate signal transduction. Phosphorylation of tyrosine 951 is vital for binding of the T-cell specific adapter (TSAd) protein, this interaction is critical for VEGF induced actin reorganization and cell migration but not for VEGF induced cell proliferation of endothelial cells (Matsumoto et al., 2005).

The 1054 and 1059 residues have regulatory roles for VEGFR2 and phosphorylation of these residues is required for receptor tyrosine kinase activity (Kendall et al., 1999). Phosphorylation of tyrosine 1175 provides a docking site for phospholipase C γ1 (PLCγ1), once bound PLCγ1 is phosphorylated and activated (Takahashi et al., 2001). Activation of PLCγ1 ultimately leads to Ca^{2+} influx and activation of the calcium-dependent protein kinase C (PKC) (Berridge and Irvine, 1989; Noh et al., 1995). PKC initiates a signal transduction cascade that results in the activation of the extracellular signal-regulated kinase (ERK). Phosphorylated tyrosine 1175 also binds the adapter molecules Shb and Sck (Holmqvist et al., 2004; Warner et al., 2000). Shb mediates the activation of the phosphoinositide 3-kinase (PI3K) pathway that regulates VEGF induced cell migration and survival (Holmqvist et al., 2004; Gerber et al., 1998). Activation of PI3K increases phosphatidyl inositol 3 phosphate (PIP3) and activates several important intracellular molecules, including Akt and the small GTP-binding protein Rac. The Akt pathway is fundamental for cell survival (Gerber et al., 1998).

1.4.4.2 VEGFR1 Signal Transduction.

Activation of VEGFR1 signalling is very similar to VEGFR2, with the first three Ig-like domains required for VEGF binding and receptor dimerization (Barleon et al., 1997). The major phosphorylation sites on the VEGFR1 cytoplasmic region are the tyrosine residues 1213 and 1242 (Ito et al., 1998). Residues 1327 and 1333 are also auto-phosphorylated (Ito et al., 1998). PLCγ binds to the phospho-tyrosine residues 1213 and 1333 of VEGFR1. Other signalling mediators include Grb2, the SH2-containing tyrosine protein phosphatase (SHP-2) (Ito et al., 1998) and PI3K (Yu et al., 2001) which all bind to 1213. Similar to VEGFR2 signalling, VEGFR1 signalling through the PI3K/Akt pathway can be vital for cell survival (Lee et al., 2007b). VEGFR1 signalling also appears to be important for Nitric Oxide Synthase (NOS) expression (Thirunavukkarasu et al., 2007).

Clearly VEGF can signal via multiple pathways through several receptors to mediate its effects. It is likely that the different receptors have alternative effects depending on the relative level of expression of these receptors and the relative concentration of downstream proteins. In addition, the regulation of VEGF mediated signalling is further complicated by cross-talk between several signalling pathways (Olsson et al., 2006) and heterodimerization between receptors and co-receptors (Waltenberger et al., 1994).

1.4.5 The Role of VEGF in Disease.

1.4.5.1 General Role of VEGF in Cancer.

As a regulator of angiogenesis, VEGF is a key molecule in neo-vascularization of solid tumours. As stated by Hanahan and Weinberg in their ‘The hallmarks of cancer’ review:

> "The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100μm of a capillary blood vessel." (Hanahan and Weinberg, 2000)

This underlines the importance of angiogenesis to a newly developing tumour because as the size of the tumour increases the availability of nutrients and oxygen is depleted, therefore inhibiting growth and survival signals they rely upon. For this reason VEGF expression has been proposed as a prognostic marker for many tumour types (Poon et al., 2001). Inhibition of VEGF function is currently an area of intense research. The development of Bevacizumab, a monoclonal antibody that recognizes VEGF, was widely thought to be a big step in cancer therapy however its efficacy as a therapy in isolation has shown little clinical benefit (Robert et al., 2009). Yet, in combination with chemotherapy or other targeted therapies Bevacizumab has proven to be significantly beneficial (Miller et al., 2007; Robert et al., 2009). Small molecule inhibitors of the VEGF receptors are also in clinical trials and have had varying success depending on the cancer type and the combinatorial therapies used (Murukesh et al., 2010).

1.4.5.2 Role of VEGF in Normal Haematopoiesis and Haematopoietic Disease.

VEGF is a critical factor for haematopoiesis. In gene knockout experiments for VEGF and VEGFR2, blood-island formation was impaired or completely failed, suggesting that VEGF is necessary for haematopoiesis (Ferrara et al., 1996; Shalaby et al., 1995). Expression of VEGF is observed in bone marrow cells (Bautz et al., 2000; Janowska-Wieczorek et al., 2001). Whereas, VEGFR2 and VEGFR1 are found on a subset of multipotent haematopoietic stem cells (Shalaby et al., 1995; Ratajczak et al., 1998). VEGFR1 expression, but not VEGFR2 expression, is up-regulated in more mature haematopoietic cells (Ratajczak et al., 1998). Autocrine signalling by VEGF through both VEGFR2 and VEGFR1 regulates haematopoietic stem cell survival

(Gerber et al., 2002). Autocrine signalling is defined as signalling induced by a ligand secreted from the same cell compared to paracrine signalling which is where the ligand is secreted by one cell and bound by a receptor on a nearby cell.

There is a significant amount of literature relating to the role of VEGF and its receptors in leukaemia and its roles in regulating leukaemic cell growth and survival. VEGF autocrine (Dias et al., 2000) and paracrine (Fiedler et al., 1997) signalling has been demonstrated to promote cell survival in leukaemic cells through the induction of Bcl-2 (Dias et al., 2002) and VEGF can protect against chemotherapeutic agents (Katoh et al., 1998) and radiotherapy (Katoh et al., 1995). The treatment of AML patients with an inhibitor of VEGFR1 and VEGFR2, PTK787/ZK222584, showed increased apoptosis and inhibited cell proliferation, with an additive effect when used with conventional chemotherapy (Barbarroja et al., 2009). Moreover, decreased cell growth is observed when VEGF expression is knocked down in CML cells (Ruan et al., 2004) and CML patients that express high levels of VEGFR2 have a worse survival prognosis (Verstovsek et al., 2003). Additionally, the use of VEGF neutralizing antibodies in myelodysplastic syndrome (MDS) has been shown to reduce cytokine production and colony-forming unit (CFU)-leukaemia formation in the majority of patient samples (Bellamy et al., 2001). These studies therefore suggest the therapeutic benefit of targeting VEGF signalling.

1.4.5.3 Role of Angiogenesis in Haematopoietic Disease.

Interestingly, increased angiogenesis of the bone marrow is an associated pathology of AML (Padró et al., 2000; Hussong et al., 2000; Rimsza et al., 2002; Matuszewski et al., 2007; Lee et al., 2007a; Hatfield et al., 2009; Shih et al., 2009), CML (Aguayo et al., 2000), acute lymphoblastic leukemia (ALL) (Perez-Atayde et al., 1997), multiple myeloma (MM) (Vacca et al., 1999) and MDS (Aguayo et al., 2000). It is thought that the increased angiogenesis of the bone marrow is associated with the

up-regulation of angiogenic factors such as VEGF secreted by leukaemic cells (Janowska-Wieczorek et al., 2002). For example, in mice transplanted with VEGF knock-down CML cells, reduced tumour microvessel density (MVD) is observed compared to controls (Ruan et al., 2004). Recently it has been shown that bone marrow angiogenesis imaged by dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) can predict high-risk patients that may benefit from anti-angiogenic therapies (Shih et al., 2009). Therefore, therapies are being trialled to target VEGF directly or to indirectly reduce VEGF expression (Li et al., 2008).

1.4.6 Transcriptional Regulation of VSP Genes.

1.4.6.1 Regulation of the *Vegf* Gene.

The *Vegf* gene in humans is located on chromosome 6p21.3 (Vincenti et al., 1996) and has a coding region spanning 14kb with eight exons (Tischer et al., 1991). Regulation of *Vegf* gene expression is complex (figure 1.9 [A]). Activation of *Vegf* gene transcription in response to hypoxia, is the most thoroughly studied mechanism of activation of *Vegf* in the adult system. The transcription factor, Hypoxia-inducible factor 1α (HIF-1α) is a potent regulator of *Vegf* expression (Forsythe et al., 1996). Under normal oxygen conditions conserved proline residues of HIF-1α are hydroxylated by prolyl hydroxylase 2 (PHD2) (Berra et al., 2003). Hydroxylation of HIF-1α provides a binding platform for the von Hippel-Lindau protein (pVHL) (Jaakkola et al., 2001), an E3 ubiquitin ligase that targets HIF-1α for degradation by the proteasome (Ohh et al., 2000; Maxwell et al., 1999). In low oxygen conditions HIF-1α is stabilized, due to a lack of oxygen for hydroxylation, and it binds to hypoxia response elements (HRE) within the proximal promoter region of the *Vegf* gene to induce expression of *Vegf* mRNA. *Vegf* expression can also be activated by HIF-2α in a similar manner (Takeda et al., 2004). Transcription of the *Vegf* gene can also be directly regulated by a number of other transcription factors that respond to numerous

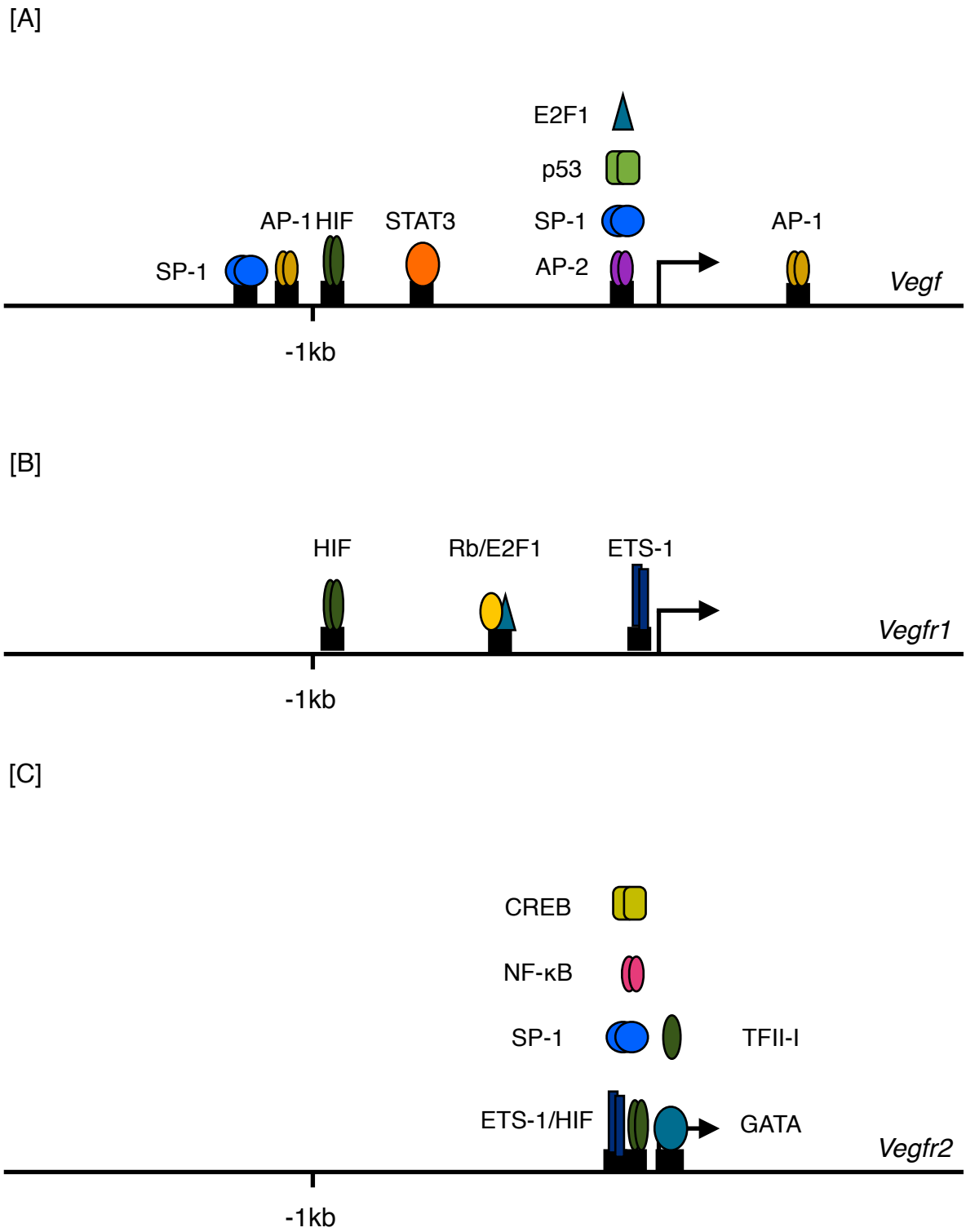


Figure 1.9 - Schematic diagram of transcription factor binding sites for the VSP genes.

The diagram illustrates the relative transcription factor binding sites for *Vegf* [A], *Vegfr1* [B], and *Vegfr2* [C]. The black boxes represent the binding sites relative to the TSS. The coloured shapes represent transcription factors that can bind to a specific gene promoter. Stacked coloured shapes represent binding of these factors to the same, or proximal to that, region of the promoter.

stimuli. The Sp-1 transcription factor is another important regulator of *Vegf* gene transcription. Sp-1 binds to a cluster of GC boxes within the *Vegf* promoter in response to Ras signal transduction, mediated through Erk phosphorylation of Sp-1 (Tischer et al., 1991; Milanini-Mongiat et al., 2002). However, there are also consensus binding sites for other transcriptional activators in this promoter region including AP-1, AP-2, and Stat3. Of these transcription factors, AP-1 is important for *Vegf* expression in response to hypoxia and through TGF-β signalling (Damert et al., 1997; Chua et al., 2000). In addition, there are several reported negative regulators of *Vegf* expression, for example p53 has been reported to bind to the *Vegf* gene promoter and may require Sp-1 for repression (Zhang et al., 2000). p53 also binds to and is required for E2F1 dependent repression of *Vegf* expression (Qin et al., 2006). Negative regulators of *Vegf* expression also include Smad4 (Schwarte-Waldhoff et al., 2000), p16 (Zhang et al., 2007), and RB2/p130 (Claudio et al., 2001) but the mechanisms for negative regulation of *Vegf* expression by these proteins are unknown and may be indirect. Thus negative reguation of *Vegf* expression is relatively poorly characterized.

1.4.6.2 Regulation of the *Vegfr1* Gene.

The *Vegfr1* gene locus is located on chromosome 13q12.13 and is more than 200kb in size containing 30 exons (Shibuya et al., 1989). Regulation of the *Vegfr1* gene is less well understood than the *Vegf* gene but *Vegfr1* has recently been targeted for investigation (figure 1.9 [B]). Expression of *Vegfr1* can be induced by low oxygen conditions, in a similar fashion to *Vegf* expression, as the proximal promoter of the *Vegfr1* gene contains HRE sites. Although the *Vegf* gene is regulated by HIF-1α and HIF-2α, as described above, the *Vegfr1* gene requires HIF-2α for hypoxia-induced expression (Takeda et al., 2004). Interestingly, *Vegfr1* expression can also be activated in response to EGF/FGF2 signalling, which induces recruitment of Ets-1

and HIF-2α in a hypoxia-independent manner (Dutta et al., 2008). Whereas *Vegf* expression is repressed by E2F1 through interactions with p53, E2F1 also binds to the promoter regions of the *Vegfr1* and *Vegfr2* genes and correlates with increased acetylation of nucleosomes at these promoters (Pillai et al., 2010). This binding of E2F1 leads to up-regulation of mRNA expression (Pillai et al., 2010). Daxx acts as a negative regulator of the *Vegfr1* gene (Murakami et al., 2006), by interacting with Ets-1 (Li et al., 2000), but it is unknown whether Daxx acts as a co-repressor or acts indirectly by inhibiting DNA binding of Ets-1.

1.4.6.3 Regulation of the *Vegfr2* Gene.

The *Vegfr2* gene locus is 190kb in size with a coding region spanning more than 47kb and contains 28 exons (Yin et al., 1998). The *Vegfr2* gene has been extensively studied and a variety of transcription factors have been reported to bind to the promoter of the *Vegfr2* gene (figure 1.9 [C]). The *Vegfr2* gene is induced by low oxygen conditions through binding of HIF to the HRE within the gene promoter, in a similar fashion to HIF binding at the *Vegf* and *Vegfr1* genes. HIF-2α is thought to mediate hypoxic induction of *Vegfr2* as HIF-2α binds and activates *Vegfr2* expression in an Ets-1-dependent manner, whereas HIF-1α does not activate transcription in a number of cell types (Elvert et al., 2003). Yet in patient samples for cerebral arteriovenous malformations, HIF-1α expression is significantly correlated with high *Vegfr2* expression, suggesting that HIF-1α might also play a role in some environments (Ng et al., 2005). In addition to the hypoxic regulation of *Vegfr2* expression, *Vegfr2* is also regulated by a number of other factors. Within the proximal promoter of the *Vegfr2* gene there are Sp-1 binding sites, which Sp-1 can bind to and then activate *Vegfr2* expression (Patterson et al., 1997). Sp-3 can act as a negative regulator of *Vegfr2* expression by inhibiting Sp-1 binding to the promoter (Hata et al., 1998). The ratio of Sp-1/Sp-3 correlates to expression of *Vegfr2* in endothelial and

non-endothelial cells (Hata et al., 1998). Furthermore, PPARγ1 can physically interact with the Sp-1 and Sp-3 proteins, via the ligand binding site of PPARγ1 (Sassa et al., 2004). This association can enhance Sp-1 promoter binding, yet interaction of PPARγ1 ligand to PPARγ1 inhibits the association of Sp-1 to the *Vegfr2* promoter (Sassa et al., 2004). Within the 5'-untranslated region of the *Vegfr2* gene there are GATA binding sites through which GATA-1 and GATA-2 can activate *Vegfr2* expression (Minami et al., 2001). Yet, TGF-β signalling, through TGF-β type I and type II receptors, can inhibit *Vegfr2* expression even in the presence of over-expressed GATA-1 or GATA-2 by preventing GATA binding, although the exact mechanism is still yet to be determined (Minami et al., 2001). GATA-2 nuclear localization and transcriptional activation of the *Vegfr2* gene is increased in response to mechanical signals of endothelial cells conveyed by increased extra-cellular matrix elasticity (Mammoto et al., 2009). The transcription factor TFII-I negatively regulates *Vegfr2* in this system, where siRNA knockdown of TFII-I under low elasticity increases *Vegfr2* expression (Mammoto et al., 2009). The *Vegfr2* gene promoter also contains Nuclear factor -κB (NF-κB) and cAMP response element binding protein (CREB) binding sites (Illi et al., 2000). NF-κB can recruit histone acetyl-transferases (HATs), p300/CBP and p300/CBP associated factor (P/CAF), to the *Vegfr2* promoter to activate transcription, but CREB represses *Vegfr2* expression (Illi et al., 2000). FoxH1 also represses *Vegfr2* transcription through a consensus binding site approximately 5kb upstream from the TSS (Choi et al., 2007). HESR1 is a transcriptional repressor that acts downstream of Notch signalling and is thought to suppress *Vegfr2* expression through binding to Sp-1-like factors (Holderfield et al., 2006).

1.5 Protein Kinase CK2.

1.5.1 Structure of Protein Kinase CK2.

Protein kinase CK2 is a ubiquitiously expressed serine/threonine protein kinase with the minimal recognition consensus sequence determined to be Ser-Xaa-Xaa-Acidic, where the acidic residue can be glutamic acid, asparagine, phospho-serine or phospho-tyrosine (Meggio et al., 1994). Although even this loose specificity is not required for some substrates (Meek et al., 1990). The presence of a consensus sequence within a protein does not guarantee phosphorylation (Meggio et al., 1994). Structurally CK2 exists as a tetrameric complex consisting of two catalytic CK2α subunits, and two regulatory CK2β subunits. There are three distinct CK2 catalytic subunits, CK2α, CK2α' (Lozeman et al., 1990) and CK2α'' (Shi et al., 2001), but only one regulatory CK2β subunit. CK2 complexes can be formed with two identical catalytic subunits or two non-identical subunits (Gietz et al., 1995). CK2α has substrates that are independent from substrates for the tetrameric CK2, for example calmodulin (Marin et al., 1999). Therefore, there maybe a role for regulated disassembly of the tetrameric structure, which drives CK2α and CK2β independent functions, although currently none have been described.

In the literature there are few distinctions made between CK2α and CK2α', even though they are encoded by separate genes, suggesting that they may have distinct biological functions. CK2α and CK2α' are 90% identical in the catalytic domain but the C-terminal region has no sequence homology (Litchfield, 2003). This suggests that the C-terminal regions of these proteins are important for their biological function, especially as these C-terminal regions are highly conserved through evolution. In support of this protein phosphatase 2A (PP2A) has been shown to specifically bind to CK2α, but the binding site for this protein is not conserved in CK2α' (Hériché et al.,

1997). Other proteins including, CKIP-1 (Bosc et al., 2000) and Pin1 (Messenger et al., 2002) are also CK2α specific interacting proteins.

CK2β is also highly conserved through evolution, with no amino acid sequence changes between birds and mammals and just a single conservative amino acid substitution between mammals and *Xenopus laevis* (Litchfield, 2003). CK2β has several key structural elements that are important for the regulation of CK2 activity. Amino acid residues 55-64 of CK2β (DLEPDEELED) is similar to auto-inhibitory sequences found in other protein kinases (Soderling, 1990). This sequence also regulates CK2 activity. Binding of polyamines to this sequence activates CK2 activity *in vitro* (Leroy et al., 1997). Point mutations of this sequence can also increase the basal activity of CK2 and inhibit polyamine binding, suggesting it has an auto-inhibitory role (Leroy et al., 1997). At the C-terminus of CK2β there is a zinc finger structure containing 4 cysteine residues. This structure mediates the dimerization of CK2β subunits (Canton et al., 2001). It is thought that the formation of CK2β dimers are a prerequisite for CK2α binding (Canton et al., 2001). The amino acid residues 181-203 of the C-terminal domain of CK2β encompasses a positive regulatory domain that is vital for increasing and stabilizing CK2α activity (Marin et al., 1997). In addition to these distinct features of CK2β, CK2β is also partly responsible for the selectivity of CK2 substrates. CK2β can act as a docking module for many proteins that are phosphorylated by CK2, such as p53 (Appel et al., 1995), and regulators of CK2 activity, such as FGF-2 (Bonnet et al., 1996).

1.5.2 Activation of Protein Kinase CK2.

Relatively little is currently known about the mechanisms behind CK2 activation under normal physiological conditions. However, CK2 activity can be modulated through phosphorylation of the C-terminal domain of CK2α in a cell-cycle dependent manner (Bosc et al., 1995). This phosphorylation increases the association with Pin1

and subsequently inhibits the CK2 phosphorylation of Topoisomerase IIα (Messenger et al., 2002).

Recent work has also identified CK2 as a downstream target for EGFR signalling (Ji et al., 2009). ERK2, which is activated by EGFR signalling, binds to the CK2α subunit and phosphorylates CK2α in the C-terminal domain at residues threonine 360 and serine 362, which enhances CK2 activity for α-catenin (Ji et al., 2009).

The best described mode of CK2 activation is in response to stress signals. This can occur through an allosteric interaction of CK2 with p38 MAP kinase and leads to the activation of p53, through serine 392 phosphorylation by CK2 (Sayed et al., 2000). Activation of p53 by CK2 is observed to occur in response to TNF-α signalling (Sayed et al., 2000). Furthermore, UV treatment can activate CK2 through a p38 MAP kinase dependent pathway (Kato et al., 2003). CK2 phosphorylation mediates the degradation of PML via the proteasome, and this activity is up-regulated by activation of the p38 MAP kinase pathway as well (Scaglioni et al., 2008).

1.5.3 The Role of Protein Kinase CK2 in Disease.

Despite relatively few studies describing CK2 activation, the role of CK2 is strongly correlated with tumourigenesis across most tumour types. In a significant proportion of malignant cells, CK2 levels have been found to be elevated compared to normal cells of the same type (Tawfic et al., 2001; Trembley et al., 2009). Notably, CK2 is known to control the activity of several tumour suppressors (figure 1.10). p53, the 'guardian of the genome' is a known target of CK2, and CK2 phosphorylation of MDM2, a negative regulator of p53, can influence the rate of turnover of p53 (Allende-Vega et al., 2005). PML is another tumour suppressor that is often mutated in cancers and phosphorylation by CK2 will target PML for degradation by the proteasome (Scaglioni et al., 2006). CK2 can also target the tumour suppressor PTEN, which is a key negative regulator of the Akt survival pathway, and

phosphorylation of PTEN by CK2 down-regulates PTEN activity (Silva et al., 2008). As well as negatively regulating tumour suppressor proteins, CK2 can also regulate oncogenes such as c-Myc. In this case, CK2 phosphorylation of c-Myc stabilizes the protein, reducing its turnover and enhancing c-Myc function (Channavajhala and Seldin, 2002). As a result of regulating these pro-survival and pro-proliferation proteins, the level of CK2 expression can be indicative of the disease prognosis, with high levels of expression often associated with reduced patient survival (Gapany et al., 1995; Lin et al., 2010; Kim et al., 2007; Eddy et al., 2005; O-charoenrat et al., 2004). It has been proposed that tumours can become 'addicted' to high CK2 expression due to the number of cellular proteins that are CK2 targets (Ruzzene and Pinna, 2010). Interestingly, MM (Piazza et al., 2006), AML (Kim et al., 2007) and T-ALL (Silva et al., 2008) cells are all susceptible to apoptosis after treatment with CK2 inhibitors. CK2 has pleiotropic effects on cellular processes but the general trend of CK2 action is through the promotion of survival pathways and inhibition of apoptotic processes (Trembley et al., 2009).

CK2 is an intriguing protein kinase, unlike most of the kinome, CK2 does not have a set of targets in a hierarchical pathway system based on a specific stimuli, but can influence several signal transduction pathways at multiple points within the hierarchy (Ruzzene and Pinna, 2010). The three key pathways that CK2 regulates to promote survival are the NF-κB pathway, the Wnt pathway and the Akt pathway (reviewed by (Ruzzene and Pinna, 2010)).

CK2 activity can further influence survival through the phosphorylation of key components of the apoptosis machinery, such as ARC (a caspase inhibitor) (Li et al., 2002), Bid (Desagher et al., 2001), and caspase 9 (where phosphorylation inhibits caspase 9 activation, protecting it from cleavage by caspase 8) (McDonnell et al.,

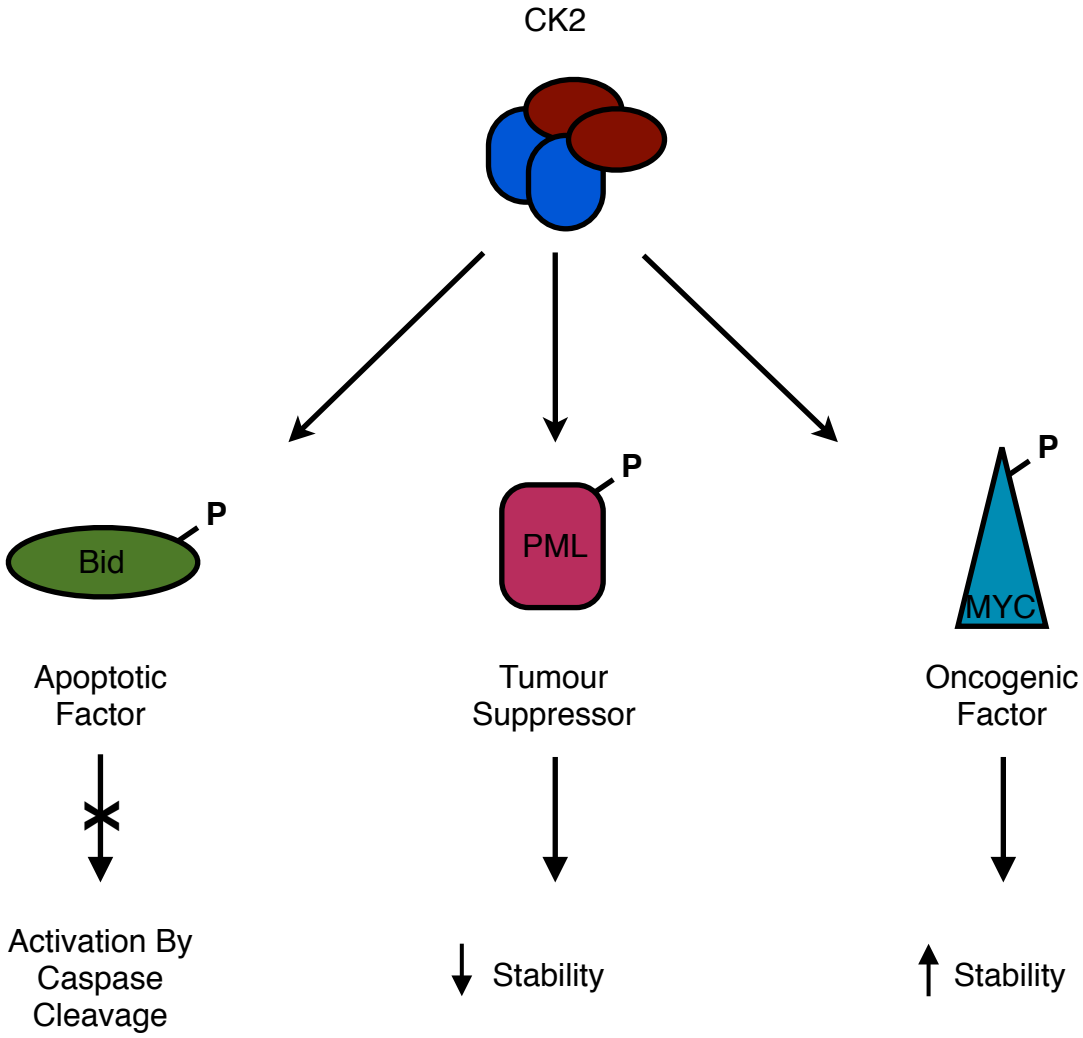


Figure 1.10 - CK2 action in disease.

The diagram illustrates how CK2 drives proliferation and survival when it is inappropriately expressed or activated, by promoting proliferation and survival whilst protecting from apoptosis. These three examples demonstrate the effect of CK2 phosphorylation on protein stability and protecting from caspase cleavage.

2008). The ability of CK2 to alter the activity of these factors further underlines why activation of CK2 is so important for tumour cells.

1.6 Tumour of Haematopoietic Tissues.

In this thesis my focus will be on the role of PRH in the myeloid compartment and diseases derived from these cells. Therefore, this next section will briefly discuss some of the molecular underpinnings of select haematopoietic diseases that are relevant to this thesis.

1.6.1 Acute Myelogenous Leukaemia (AML).

AML is a heterogeneous disease and can arise from a variety of somatic genetic alterations, gene mutations, aberrant gene expression and other molecular alterations (such as microRNA variation). There is no single underlying cause for AML and disruption of a number of genes can lead to AML progression. However, perturbed transcription factor activity is a feature for a subset of AML. Common transcription factor mutations include the RUNX/AML proteins 1-3 (Grimwade and Hills, 2009; Peterson and Zhang, 2004; Elagib and Goldfarb, 2007), PML (Grignani et al., 1993; Khan et al., 2001), and EVI-1 (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Byrd et al., 2002; Vinatzer et al., 2003; Hirai et al., 2001), amongst others. Interestingly, several of these factors are transcriptional repressors and demonstrate that perturbed activity of transcriptional repressors has a role in AML.

1.6.2 Chronic Myelogenous Leukaemia (CML).

CML is a myeloproliferative disorder that is characterised by increased proliferation of the granulocytic lineage without the loss of the capacity to differentiate. CML normally progresses through three clinical phases; an initial chronic phase; the accelerated phase; and finally blast crisis, which is very aggressive and usually fatal. Patients in the initial chronic phase have mild symptoms. Once patients progress to the blast crisis phase they have high numbers of blast cells (in excess of 30% of total blood cells) in the bone marrow and the peripheral blood, with reduced red blood cells and

platelets. About 90% of patients with CML have the Philadelphia (Ph) chromosome, which results from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11), and encodes the BCR-ABL1 fusion oncogene.

To understand how the BCR-ABL1 fusion protein can initiate leukaemogenesis it is important to understand the structural characteristics of the BCR and ABL1 proteins, and how the BCR-ABL1 fusion protein forms. Most patients with CML express the p210 BCR-ABL1 protein and this is formed through breakpoints around the exons Ia and Ib of the *Abl1* gene and exons 12-16 of the *Bcr* gene (Melo, 1996).

ABL1 is a modular protein containing a central kinase domain preceded by src-homology domain-2 (SH2) and SH3 domains. The C-terminal end of ABL1 has DNA-binding and actin-binding domains, with three nuclear localization signal motifs and a nuclear export signal motif. ABL1 has two isoforms ABL1a and 1b. ABL1b is highly expressed and has an N-terminal 'cap' domain that is myristoylated (Hantschel and Superti-Furga, 2004), and this engages the C-terminal lobe of the ABL1 catalytic domain, similar to the conformation of inactivated SRC (Hantschel and Superti-Furga, 2004; Xu et al., 1997). Forms of ABL1b that lack the myristoyl moiety have constitutively active tyrosine kinase activity. The BCR-ABL1 fusion replaces the N-terminal 'cap' of ABL1 with BCR, therefore removing this auto-inhibitory mechanism. BCR has three important domains that act as docking platforms for accessory proteins, including (1) a putative calcium-dependent lipid binding site, (2) the central pleckstrin homology domain (PHD), and (3) a key tyrosine residue (Ren, 2005). Tyrosine 177 is important because it forms a docking site for multiple accessory proteins including GRB2, GRB10, 14-3-3 and ABL1 (Ren, 2005). However, it also has two other notable domains (1) a serine/threonine kinase domain and (2) a coiled-coil oligomerization domain (Ren, 2005). Mutations of the coiled-coil domain, that disrupt

oligomerization, will significantly impair the kinase activity and leukaemogenic properties of the BCR-ABL1 fusion protein (Smith et al., 2003).

The leukaemogenic properties of BCR-ABL1 are facilitated through the signalling pathways that are induced (figure 1.11). The BCR-ABL1 fusion protein is a hub that can dock specific accessory proteins and through constitutively active kinase activity it can drive oncogenic pathways. Phosphorylation of tyrosine 177 of the BCR module is essential for BCR-ABL1-mediated leukaemogenesis (Zhang et al., 2001). Phosphorylation of this site facilitates the recruitment of GRB2, which binds a guanine-nucleotide exchange factor (GEF) for RAS called Son of sevenless (SOS) (Ren, 2005). This complex leads to RAS activation and induction of its downstream targets. Also recruited to this complex is the scaffold adapter GRB2-associated binding protein 2 (GAB2) (Ren, 2005), which is phosphorylated by the ABL1 moiety of BCR-ABL1 (Sattler et al., 2002). BCR-ABL1-induced phosphorylation of GAB2 causes constitutive activation of the phosphatidylinositol 3 kinase (PI3K)/AKT pathway (Skorski et al., 1995). The importance of GAB2 was demonstrated using GAB2-/- mice, as expression of BCR-ABL1 in these animals is unable to transform primary myeloid cells (Sattler et al., 2002).

Although the RAS and PI3K/AKT pathways are key oncogenic pathways, BCR-ABL1 has many other targets as well. BCR-ABL1 can also phosphorylate SRC family kinases HCK, LYN and FGR. Phosphorylated HCK activates STAT5 (Ren, 2005; Ilaria and Van Etten, 1996), which up-regulates the cell-cycle regulator cyclin D1 increasing G1 to S phase progression (Nosaka et al., 1999). BCR-ABL1 activates the RAC GTPases that include downstream targets such as, CrKL, ERK, JNK and p38 (Thomas et al., 2007).

Imatinib is a specific inhibitor of BCR-ABL1, v-ABL, and cellular ABL1 (Buchdunger et al., 1996). It was shown to be an effective inhibitor of growth of BCR-ABL1 positive

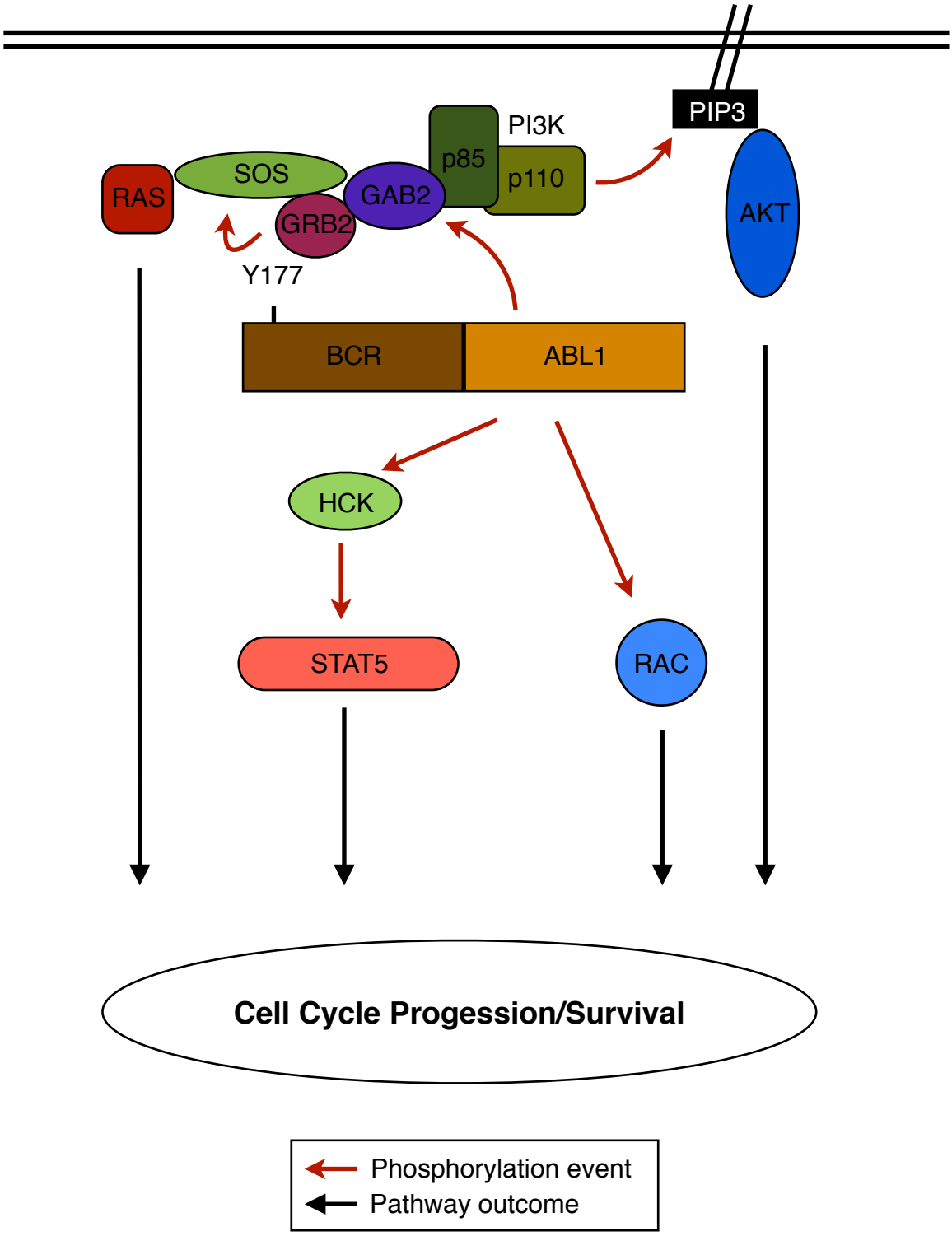


Figure 1.11 - Schematic diagram of BCR-ABL1 signalling in CML.

The diagram illustrates how the BCR-ABL1 fusion protein is able to increase proliferation and survival of leukaemic blasts. BCR-ABL1 can initiate signal transduction through multiple pathways, including activation of RAS and AKT, two factors that control critical proliferation and survival pathways. STAT5 and RAC pathways also have a significant role in the CML phenotype.

cells (Druker et al., 1996). Imatinib is now widely used to treat CML and next generation derivatives, Dasatinib and Nilotinib, are also in use in the clinic. However, these drugs are not cures, mutations of the ABL kinase pocket can lead to Imatinib resistance (Volpe et al., 2009), and Imatinib does not eradicate leukaemic stem cells (Chen et al., 2010).

1.6.3 K562 Cells.

The K562 cell line is derived from a patient with CML in blast crisis (Lozzio and Lozzio, 1975). K562 cells are positive for the philadelphia chromosome and encode the p210 BCR-ABL protein. The majority of cells are undifferentiated blasts, but a very small proportion will spontaneously differentiate down the myeloid linages to erythrocytes, megakaryocytes, granulocytes and monocytes. They can also be induced to differentiate down the erythroid and megakaryocytic lineages (Osti et al., 1997; Huang et al., 2004; Bütler et al., 1990). K562 cells are a useful cell line to study PRH activity because they express easily detectable levels of endogenous PRH protein and mRNA as well as high *Vegf* and *Vegfr1* expression. Therefore, K562 cells are ideal for manipulation of PRH expression levels.

1.7 Aims.

PRH is a known transcriptional repressor, however, few PRH target genes have been characterized in adult haematopoietic cells. The *Vegf*, *Vegfr1* and *Vegfr2* genes are potentially regulated by PRH, although whether this regulation occurs in haematopoietic cells and whether they are directly regulated by PRH is unknown. In this thesis the transcriptional regulation of the *Vegf*, *Vegfr1* and *Vegfr2* genes will be investigated. The specific protein-protein interactions and the functional characteristics of PRH that are required for transcriptional regulation will also be evaluated. PRH has diverse roles in the regulation of cell differentiation, cell survival and cell growth that are determined by its cellular environment. The second aim of

this thesis is to investigate the effect of PRH on cell growth and cell survival. Manipulation of endogenous PRH levels and introduction of exogenous PRH will be used to define the role of PRH in haematopoietic cells. PRH was recently shown to be phosphorylated by CK2 *in vitro*. The final aim of this thesis is to determine whether this post-translational modification occurs *in vivo* and what the functional significance of this modification is for PRH induced transcriptional repression and cell survival.

Materials and Methods

2 Materials and Methods

2.1 Materials.

2.1.1 Chemicals.

Table 1 - Chemicals and Reagents.

Chemical	Information
Acrylamide	Geneflow A2-0074 30% Acrylamide (37.5:1)
Agarose	Sigma A9539
Ammonium Persulphate	Sigma A3678
Ampicillin	Sigma A9518
Antibodies	Anti-Mouse IgG HRP(Santa Cruz sc-2005) Anti-Rabbit IgG HRP (Santa Cruz sc-2313) Anti-Goat IgG HRP (Santa Cruz sc-2020) Anti-Lamin A/C (H-110) (Santa Cruz sc-20681) Rabbit Polyclonal Anti-VEGF (VG76e) Mouse Monoclonal Anti-Phospho PRH (YKN5) Rabbit Polyclonal Anti-PRH Mouse Polyclonal

Chemical	Information
Antibodies	Anti-Myc tag (9E10) (New England Biolabs - Cell Signalling Technology #2276) Mouse Monoclonal Anti-PanTLE (C-19) (Santa Cruz sc-13373) Goat Polyclonal Anti-HA (12CA5) (Roche 11583816001) Mouse Monoclonal Anti-FLAG (Sigma F7425) Rabbit affinity-purified
Bacteriological Agar	Sigma A5306
Bactotryptone	Sigma
Bovine Serum Albumin (BSA)	First Link (UK) Ltd. Cat. 40-00-450
Bradford Reagent	Bio-Rad 500-001
Bromophenol Blue	Sigma B8026
Calcium Chloride	Sigma C1016
Complete EDTA-free (protease inhibitor cocktail tablets)	Roche 11 873 580 001
Dimethyl Sulfoxide (DMSO)	Sigma D8418
Dithiothreitol (DTT)	Park Scientific Ltd
DNA gel loading buffer 6X	
DNA markers	Fermentas GeneRuler™ 1Kb DNA Ladder (SM0311) GeneRuler™ 100bp DNA Ladder (SM0241)
Dynabeads® Protein A	Invitrogen 100-01D

Chemical	Information
Ethanol	Fisher Scientific
Ethidium Bromide	Fluka 46066
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma E3889
Ethylenediaminetetraacetic acid (EDTA)	Sigma E5134
Fetal Bovine Serum (FBS)	Sigma F7524
Formaldehyde (36.5%)	Sigma F8775
Glycerol	Fisher Scientific
Glycine	Sigma 241261
Glycogen	Calbiochem
HEPES	Sigma H3375
Igepal CA-630 (NP-40)	Sigma I8896
Magnesium Chloride	Sigma M8266
Methanol	Fisher Scientific
MG132 (Proteasome inhibitor)	Calbiochem 474790
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma T9281
Penicillin/Streptomycin	Sigma P4333
Phenol	Sigma P4557
Phenylmethylsulphonyl Fluoride (PMSF)	Sigma P7626
Polyoxyethylene Sorbitan Monolaurate (Tween 20)	Sigma P1379
Ponceau S solution	Sigma P7170

Chemical	Information
Potassium Chloride	Sigma P9541
Potassium phosphate (KH_2PO_4)	Sigma P9791
Protein Marker	Fermentas PageRuler™ Plus Prestained Protein Ladder (SM1811)
Random Hexamer Primers	Fermentas SO142
RPMI 1640 medium	Sigma R8758
Sodium Azide	Sigma S8032
Sodium chloride	Sigma S9888
Sodium deoxycholate	Sigma D6750
Sodium dodecyl sulfate	Sigma L4509
Sodium fluoride	Sigma S7920
Sodium orthovanadate (Na_3VO_4)	Sigma S6508
Sodium pyrophosphate	Sigma P8010
Sodium β-glycero phosphate	
Tetracycline	Sigma T3258
Thiazolyl blue formazan [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan] (MTT)	Sigma M2003
Triton X-100	Sigma T8787
Trizma Base	Sigma T4661
Yeast Extract	Sigma Y1625
β-mercaptoethanol	Sigma M6250

2.1.2 Media.

Table 2 - Media.

Media	Information
Luria-Bertani Medium (LB)	1% w/v Bactotryptone 0.5% w/v Yeast Extract 1% w/v NaCl
Luria-Bertani Agar (LB agar)	1% w/v Bactotryptone 0.5% w/v Yeast Extract 1% w/v NaCl 1.5% w/v Bacto Agar

2.1.3 Solutions.

2.1.3.1 Commonly Used Solutions.

Table 3 - Commonly Used Solutions.

Solution	Information
1X TAE buffer	40mM Tris-Acetate 1mM EDTA
TE buffer	10mM Tris-Cl (pH 8.0) 1mM EDTA (pH 8.0)
1X PBS	137mM NaCl 2.7mM KCl 10mM Na_2HPO_4 2mM KH_2PO_4 Adjusted to pH 7.4

2.1.3.2 Solutions Used in Cell Culture Protocols.

Table 4 - Chromatin Immunoprecipitation (ChIP) Solutions.

Solution	Information
ChIP Lysis Buffer	50mM Tris-Cl (pH 8.0) 10mM EDTA 1% SDS 1mM PMSF Protease Inhibitor Cocktail
ChIP RIPA Buffer	10mM Tris-Cl (pH 8.0) 140mM NaCl 1% v/v Triton X-100 1mM EDTA 0.5mM EGTA 0.1% w/v SDS 0.1% Sodium deoxycholate
Elution Buffer	20mM Tris-Cl (pH 7.5) 50mM NaCl 5mM EDTA
Elution Buffer + 1% SDS	20mM Tris-Cl (pH 7.5) 50mM NaCl 5mM EDTA 1% SDS

2.1.3.3 Solutions Used in Western Blot Protocols.

Table 5 - Whole Cell Protein Extraction Solutions.

Solution	Information
RIPA buffer	50mM Tris-Cl (pH 7.5) 150mM NaCl 1% v/v NP-40 0.5% w/v Sodium Deoxycholate 0.1% w/v SDS 5mM EDTA Protease Inhibitor Cocktail
1X SDS Loading Buffer	50mM Tris-Cl (pH 6.8) 100mM DTT 2% SDS 0.1% Bromophenol Blue 10% Glycerol

Table 6 - Post-Nuclear and Nuclear Fractionation of Protein Extracts Solutions.

Solution	Information
Buffer A	20mM Tris-Cl (pH7.5) 5mM $MgCl_2$ 1mM DTT Protease Inhibitor Cocktail

Solution	Information
Buffer B	50mM Tris-Cl (pH 7.5) 500mM NaCl 1% v/v NP-40 0.1% w/v SDS Protease Inhibitor Cocktail

Table 7 - Immunoprecipitation Solutions.

Solution	Information
15X Phosphatase Inhibitor Cocktail	50mM NaF 1mM Na_3VO_4 10mM Na β-glycero phosphate 1mM EDTA 5mM Na pyrophosphate
Lysis Buffer	50mM Tris-Cl (pH 8.0) 100mM NaCl 20mM NaF 10mM KH_2PO_4 1% v/v Triton X-100 100μM DTT 10% Glycerol 1X Phosphatase Inhibitors Protease Inhibitor Cocktail

Solution	Information
Binding Buffer	50mM Tris-Cl (pH 8.0) 100mM KCl 100μM EDTA 0.2% NP-40 1mg/ml BSA 2mM DTT 2.5% Glycerol 1X Phosphatase Inhibitors Protease Inhibitor Cocktail
Wash Buffer	100mM Tris-Cl (pH 8.0) 200mM NaCl 0.5% NP-40 2mM DTT Protease Inhibitor Cocktail

Table 8 - SDS Polyacrylamide Gel Solutions.

Solution	Information
1X Stacking Buffer	125mM Tris-Cl (pH 6.8) 0.1% w/v SDS
1X Resolving Buffer	375mM Tris-Cl (pH 8.8) 0.1% w/v SDS

Solution	Information
SDS polyacrylamide resolving gel	1X Resolving Buffer (See above) Acrylamide (% as required for molecular weight of proteins being resolved) 0.1% Ammonium Persulfate TEMED
SDS polyacrylamide stacking gel	1X Stacking Buffer (See above) 4.5% Acrylamide 0.1% Ammonium Persulfate TEMED

Table 9 - SDS Polyacrylamide Gel Electrophoresis Solutions.

Solution	Information
1X Running Buffer	2.5mM Tris-Cl 19.2mM Glycine 0.01% w/v SDS

Table 10 - Transfer of Proteins from SDS-PAG to Immobilon-P Membrane Solutions.

Solution	Information
1X Transfer Buffer	10mM Tris-Cl 100mM Glycine 0.005% w/v SDS 20% Methanol

Table 11 - Antibody Detection Solutions.

Solution	Information
PBS-Tween	1X PBS 0.05% Tween 20
Primary blocking solution	1X PBS 3% w/v Bovine Serum Albumin (BSA) 3mM Sodium Azide

2.1.3.4 Solutions Used in Flow Cytometric Protocols.

Table 12 - Apoptosis Assay Solutions.

Solution	Information
1X Annexin V Binding Buffer	10mM Hepes/NaOH (pH 7.4) 140mM NaCl 2.5mM $CaCl_2$

2.2 DNA Protocols.

2.2.1 DNA Electrophoresis.

1% agarose gel was made by mixing 1g of agarose with 100 ml of 1X TAE (see 2.1.3.1). This mixture was heated for 3 minutes in a microwave until the agarose had dissolved. To the gel tray 5μl of ethidium bromide (10mg/ml) was added upon which the gel solution was poured to allow thorough mixing. Combs were placed in the gel to produce the wells. After the gel had set the gel tray was placed into the electrophoresis tank (Fisher) and the gel was submerged in 1X TAE. DNA samples in loading buffer (diluted with sample to 1X loading buffer) were loaded alongside a marker, GeneRuler 100bp DNA Ladder (Fermentas SM0243) or GeneRuler 1kb DNA Ladder (Fermentas SM0313). The gel was run at 80-100V (constant voltage) for

45-60 minutes or until there was sufficient separation of the DNA fragments as determined using a UV trans-illuminator.

2.2.2 DNA Extraction

DNA extraction was performed using a Wizard SV gel purification kit (Promega Cat. A9281) according to the manufacturer's instructions.

2.2.3 Polymerase Chain Reaction (PCR)

10ng of plasmid DNA or 100ng of cDNA template was used with 2mM $MgCl_2$, 0.4mM dNTPs, 1X RedTaq Polymerase buffer (Sigma D4308), 10 units RedTaq Polymerase (Sigma D4308), and 10μM forward and reverse primers in a polymerase chain reaction in a final volume of 50μl.

Reactions were performed according to the manufacturer's instructions with a hot start for 5 minutes at 95ºC, followed by 30-35 cycles of 1 minute at 95ºC, 1 minute at 55-62ºC (primer dependent), and 1 minute at 72ºC. Samples were separated by agarose gel electrophoresis (see 2.2.1) in 1X DNA loading buffer and visualized under UV light.

2.2.4 Phenol: Chloroform: Isoamylalcohol (PCI) Extraction and DNA Precipitation.

DNA was made up to a volume of 400μl using autoclaved distilled H_2O (dH_2O). An equal volume of PCI (25:24:1) mixture, taken from the lower layer, was added to the DNA solution and vortexed for 1 minute. This was then centrifuged for 5 minutes at 13,000rpm (Eppendorf 5424). 350μl of the upper (aqueous) phase was carefully removed and transferred to a separate eppendorf. A further 50μl of dH_2O was added to the lower (organic) phase. This was vortexed and centrifuged as described previously, and 50μl of the upper phase was added to the first extraction.

10% v/v 3M sodium acetate (pH 5.2) was added to the extracted DNA with $2^1/_2$ volumes of 100% ethanol and 2μl glycogen (10mg/ml) as a DNA carrier. The sample

was vortexed, incubated at -20ºC for 1hour and then centrifuged at 13,000 rpm (Eppendorf 5424) for 10 minutes at 4ºC. The supernatant was removed and the pellet washed twice with 1ml ice cold 70% ethanol. The DNA pellet was then air-dried for 10 minutes and resuspended in the required volume of TE buffer.

2.2.5 Restriction Digestion

5μg of plasmid DNA was digested in a final volume of 20μl, in a reaction containing 1X buffer (enzyme specific) with 10units of enzyme for 1-2 hours at 37ºC (dependent on enzyme efficiency in used buffer). Reactions were separated on an agarose gel electrophoresis (see 2.2.1) in 1X DNA loading buffer. The gel was exposed to UV light to confirm digestion.

2.2.6 Phosphatase Reaction

Linearized vector DNA reactions were made up to a final volume of 40μl in 1X Calf Intestinal Alkaline Phosphatase (CIAP) buffer with 10 units of CAP enzyme (New England Biolabs M0290S) and incubated at 37ºC for 1 hour. Reactions were run using agarose gel electrophoresis (see 2.2.1) in 1X DNA loading buffer. The DNA fragment was identified under UV light, excised from the gel and transferred to a pre-weighed eppendorf for DNA extraction (see 2.2.2).

2.2.7 Ligation

Purified DNA insert was used 10 times more than the purified DNA vector in 1X T4 DNA ligase buffer with 10 units of T4 DNA ligase (Fermentas EL0014) in a final volume of 20μl. For sticky end ligations the reaction was incubated at room temperature overnight. For blunt end ligations the reaction was incubated on ice left at room temperature overnight.

2.3 Bacterial Protocols

2.3.1 Preparation of competent *Escherichia coli (E. coli)* XL-1 blue cells.

E. coli XL-1 blue cells were innoculated into 5ml LB broth and incubated at 37ºC overnight whilst shaking at 200rpm. 0.5ml overnight culture was innoculated into 50ml LB broth and incubated at 37ºC until the culture reached an optical density of 0.5 at 650nm. The culture was incubated on ice for 10 minutes and centrifuged at 4000rpm (Eppendorf 5810R) for 5 minutes at 4ºC. The pellet was resuspended in 25ml 0.1M $CaCl_2$ and incubated on ice for 20 minutes. This was centrifuged at 4000rpm (Eppendorf 5810R) for 5 minutes at 4ºC and the pellet resuspended in 2ml 0.1M $CaCl_2$ for 2 hours. 1.4ml 100% Glycerol was added to the cell suspension and then 100μl aliquots were either used for transformation (see 2.3.2) or stored at -80ºC.

2.3.2 Transformation of XL-1 blue cells

For plasmid transformation of competent XL-1 blue cells, 50ng of plasmid DNA was mixed with 80μl competent XL-1 blue cells (see 2.3.1) and incubated on ice for 20 minutes. The cells were heat shocked at 42.5ºC for 2 minutes and returned to ice for 2 minutes. To the cells, 1 ml of LB broth was added before incubating at 37ºC for 45 minutes. The cultures were centrifuged at 13,000rpm (Eppendorf 5424) for 15 seconds, 1ml of the LB broth supernatant was discarded. The cell pellet was resuspended in the remaining 80μl and then spread on an agar plate containing the appropriate antibiotic (ampicillin = 100mM, kanamycin = 50mM). Plates were incubated at 37ºC overnight.

2.3.3 Purification of Plasmid DNA

Plasmid DNA was prepared and purified using Qiagen mini (Qiagen 27104) or maxi (Qiagen 12163) prep kits according to the manufacturers instructions.

2.4 Cell Culture Protocols.

2.4.1 Cell culture

K562 cells were maintained in RPMI 1640 media (Sigma R8758) supplemented with 10% Fetal Bovine Serum (FBS)(Sigma F7524)(heated to 56ºC for 30 minutes to inactivate complement proteins), 100 units/ml Penicillin and 100μg/ml Streptomycin (Sigma P4333)(this will be referred to as RPMI media from this point, unless stated). Cells were grown at 37ºC in an atmosphere of 5% CO_2, 20% O_2 and 75% N_2. Cells were split once every two days or when the culture reached a density of 1 x10^6 cells/ml by centrifugation for 5 minutes at 1100 rpm (Eppendorf 5810R), washed in PBS and resuspended in the appropriate volume of fresh medium.

2.4.2 Transient Transfection

1x10^7 K562 cells were centrifuged at 1100 rpm (Eppendorf 5810R) for 5 minutes and washed in PBS. The cell pellet was resuspended in 200μl of RPMI media. 10μg expression plasmid (or as stated in the text) was added to a Genepulser 0.4 cm electroporation cuvette (Bio-Rad 165-2088). The cells were transferred into the 0.4cm electroporation cuvette onto the expression plasmid to mix. The cells were then electroporated using a Bio-Rad Genepulser at 250V, 975μF. Electroporated cells were transferred to 4ml of pre-warmed RPMI media in a 6 well plate and incubated for 24-48 hours at 37ºC in an atmosphere of 5% CO_2, 20% O_2 and 75% N_2.

2.4.3 ShRNA Transfection.

5x10^6 K562 cells were centrifuged at 1100 rpm (Eppendorf 5810R) for 5 minutes and washed in PBS. The cell pellet was resuspended in 100μl of solution V (Amaxa VCA-1003). 10μg of pRS EGFP (shRNA control) or 5μg pRS PRH49 and pRS PRH51 plasmids (or as stated in the text) were added to an Amaxa 0.4 cm nucleofector cuvette. The cells were transferred into the 0.4cm nucleofector cuvette onto the shRNA plasmid to mix. The cells were then transfected using an Amaxa

Nucleofector on program T-016. The cells were transferred to 4ml of pre-warmed media in a 6 well plate and incubated overnight at 37ºC. 24 hours post-transfection the cells were treated and kept in 5ml RPMI media containing 1μg/ml Puromycin (Sigma P8833). Cells were maintained at 37ºC in an atmosphere of 5% CO_2, 20% O_2 and 75% N_2.

2.4.4 Growth Analysis.

K562 cells were maintained after shRNA transfection under selection until at least a cell density of $3x10^5$ cells/ml in 10ml of RPMI media was attained. At this point all cell populations were split to a cell density of $3x10^5$ cells/ml in 10ml of RPMI media. The cells were counted daily using trypan blue exclusion. For this 10μl trypan blue (Fluka 93595) was mixed with 10μl of cells and then the number of clear cells were counted on a haemocytometer (blue cells were not counted). The cell populations were set back to $3x10^5$ daily or as required. All dilutions of the cells were recorded and the cumulative growth calculated using the equation (conc x vol x dilution factor = total number of cells).

An example of this calculation:

Day 1 - $3x10^5$ cells/ml in 10ml (total cells = $3x10^5$ x 10 x 1 = $3x10^6$)

Day 2 - $6x10^5$ cells/ml in 10ml (total cells = $6x10^5$ x 10 x 1 = $6x10^6$) Then split 1 in 2.

Day 3 - $6x10^5$ cells/ml in 10ml (total cells = $6x10^5$ x 10 x (1x2) = $1.2x10^7$) Then split 1 in 2.

Day 4 - $6x10^5$ cells/ml in 10ml (total cells = $6x10^5$ x 10 x (1x2x2) = $2.4x10^7$)

2.4.5 MTT assay

Cells were seeded in a 96-well plate at a density of ~$1x10^4$ cells/well in 180μl RPMI media. Cells were incubated with VEGF165 (50ng/ml), SU1498 (1μM or 2μM) (Boguslawski et al., 2003), anti-VEGF (VG76e) antibody (50μg/ml)(Fraser et al., 2000) or the equivalent volume of DMSO (Sigma D8418) in each case. Cells were

incubated for 72 hours and then 20μl MTT (5mg/ml, Sigma M2003) was added to each well and incubated for 3 hours. Cell plates were centrifuged at 4000rpm (Eppendorf 5810R) for 5 minutes and the MTT crystals were dissolved by pipetting in 200μl DMSO. After incubation at 37ºC for 5 minutes the optical density (OD) was read at 540nm and 650nm (background) using a Versamax (Molecular Devices) plate reader. The background OD was subtracted from the reading at 540nm. To calculate cell numbers, serial dilutions were made from a known number of cells, and a standard curve was produced for calculating cell numbers.

2.4.6 Chromatin Immunoprecipitation (ChIP).

Five transfections of $1x10^7$ K562 cells were transfected with 5μg pMUG1 Myc-PRH (or as described in the text)(see 2.4.2) per chromatin preparation. After 24 hours incubation at 37ºC, the transfected cells were transferred to a single T25 cell culture flask. 48 hours post-transfection cells were washed twice in ice cold PBS and resuspended in 500μl PBS. Formaldehyde was added to a final concentration of 1% and incubated at room temperature on a rotary wheel for 8 minutes. Glycine was added to a concentration of 0.125M to stop cross-linking reaction and centrifuged at 13,000rpm (Thermo Scientific Heraeus Fresco 21) for 10 minutes at 4ºC. Cells were washed twice in PBS. Cells were resuspended in lysis buffer (see 2.1.3.5) and split into fractions of $2.5x10^6$ cells per 130μl volume in an eppendorf. Lysates were sonicated in a Biorupter (Diagenode) for 10 minutes on medium power at 4ºC. Lysates were cleared by centrifugation at 13,000rpm (Thermo Scientific Heraeus Fresco 21) for 10 minutes at 4ºC. To check the efficiency of the sonication, 20μl of the chromatin lysate was diluted 1 in 25 in lysis buffer and incubated with 120μg Proteinase K at 68ºC for 2 hours. A phenol chloroform isoamyl alcohol (PCI) extraction (see 2.2.4) was performed in the presence of 50μg glycogen. 2μg of DNA

was separated on a 1.5% agarose gel (see 2.2.1) to check DNA fragmentation was in the range of 200-600bp in length.

Dynabeads® Protein A magnetic beads (Invitrogen 100-01D) were collected by applying a magnet and then washed in 500μl ChIP RIPA buffer (see 2.1.3.5). The beads were resuspended in 100μl ChIP RIPA buffer per immunoprecipitation. 3μl of either anti-Myc antibody (or as stated in the text) or IgGs were added to 100μl of the protein A beads and incubated at 4ºC for 2 hours on a rotary wheel. The chromatin lysates prepared above were diluted to 250μg/ml in lysis buffer and 100μl (25μg) of chromatin lysate was added to the antibody:bead complex and incubated at 4ºC overnight on a rotary wheel. The beads were collected by applying a magnet. Eight washes were performed at room temperature, the first two washes were carried out in ChIP RIPA buffer, followed by two washes in ChIP RIPA buffer containing 500mM NaCl (see 2.1.3.5), two washes in ChIP RIPA buffer with 0.5% NP40 (see 2.1.3.5), and finally two washes in TE buffer. After the final wash beads were resuspended in 150μl of elution buffer with 1% SDS (see 2.1.3.5). The beads suspension and 40μg Proteinase K was incubated at 68ºC for 2 hours. Beads were collected by applying a magnet. The supernatant was retained in a separate eppendorf (bound fraction). Beads were resuspended in a further 150μl elution buffer with 1% SDS. Beads were collected as before and the supernatant was added to the bound fraction.200μl elution buffer (see 2.1.3.5) was added to the bound fraction and a PCI extraction and DNA precipitation (see 2.2.4) was performed using 50μg glycogen. Pellets were resuspended in 50μl TE buffer and the concentration of DNA was measured. DNA obtained was analysed by qPCR (see 2.6.3).

2.5 Western Blot Protocols.

2.5.1 Whole Cell Protein Extraction.

Whole cell extracts were made from $1x10^7$ K562 cells. The cell pellet was centrifuged

at 1100rpm (Eppendorf 5810R) for 5 minutes and then washed in PBS. The pellet was resuspended in 150μl RIPA buffer (see 2.1.3.3) and the cell suspension drawn up and down several times through a 3 X Monojet needle (1.1x50mm, 19Gx2"). the cell suspension was then incubated on ice for 30 minutes, and then centrifuged at 13000rpm (Thermo Scientific Heraeus Fresco 21) for 15 min at 4°C. The extracts were incubated at 100ºC for 2 minutes in 1X SDS-loading buffer (see 2.1.3.3) and separated by electrophoresis (see 2.5.5) or stored at -20ºC.

2.5.2 Post-nuclear and Nuclear Fractionation of Protein Extracts.

Nuclear and post-nuclear extracts were made as follows: $2x10^7$ K562 cells were centrifuged at 1100rpm (Eppendorf 5810R) and the pellet was resuspended in 150μl Buffer A (see 2.1.3.3) at 4°C for 10 minutes. 15μl NP40 (10%v/v) was added to the lysate and mixed by vortexing. The lysate was centrifuged at 13,000rpm (Thermo Scientific Heraeus Fresco 21) at 4ºC for 1 minute and the supernatant removed and stored at -80°C in 1X SDS-loading buffer (see 2.1.3.3) as the soluble post-nuclear fraction. The nuclei in the pellet were resuspended in 150μl Buffer B (see 2.1.3.3) for 20 minutes at 4°C and then centrifuged at 13,000rpm (Thermo Scientific Heraeus Fresco 21) for 1 minute at 4ºC. The supernatant was removed and stored at -80°C in 1X SDS-loading buffer as the soluble nuclear extract.

2.5.3 Immuno-precipitation (IP).

Whole cell extracts from $2x10^7$ K562 cells were resuspended in 200μl lysis buffer (see 2.1.3.4), incubated at 4ºC for 30 minutes and centrifuged at 13,000rpm (Thermo Scientific Heraeus Fresco 21) for 10 minutes at 4ºC. The supernatant was transferred to a new eppendorf and binding buffer (see 2.1.3.4) was added at a 1:1 ratio.The supernatant was incubated with the primary antibody at 4ºC for 2 hours on a rotary wheel. Protein G sepharose beads (30μl per IP)(Sigma P3296) were equilibrated with binding buffer and added to the IP. This was incubated for 4 hours at 4ºC on a rotary

wheel. The immuno complexes were centrifuged at 13,000rpm (Eppendorf 5424) for 30 seconds. The pellet was washed in 300μl wash buffer (see 2.1.3.4) three times. The precipitate was then resuspended in 30μl of 4X SDS-Loading buffer (see 2.1.3.3) and heated to 95^{o}C for 2 minutes.

2.5.4 Preparation of SDS polyacrylamide gel.

The front and back plates were assembled into the casting brace according to the manufacturer's instructions (Bio-Rad) and an SDS polyacrylamide resolving gel was prepared (see 2.1.3.3) and poured between the gel plates. After the resolving layer had set, an SDS polyacrylamide stacking gel was prepared (see 2.1.3.3) and poured onto the resolving layer. The well comb was placed into the stacking layer.

2.5.5 Separation of proteins by molecular weight.

The SDS polyacrylamide gel was placed into the separation tank as described in the manufacturer's instructions (Bio-Rad Mini-PROTEAN Tetra Electrophoresis System). Protein samples (as prepared in 2.5.1 and 2.5.2) were loaded onto the gel along with PageRuler Plus Prestained Protein Ladder (Fermentas SM1811). The SDS-polyacrylamide gel was then run at 140V (constant voltage) for 80 minutes or until the bromophenol blue dye front had reached the bottom of the gel.

2.5.6 Transfer of proteins from SDS-polyacrylamide gel to Immobilon-P membrane.

Immobilon-P membrane (Millipore IPVH00010) was soaked in methanol for 35 seconds to hydrate, then washed in distilled H_2O for 1-2 minutes and equilibrated in 1X Transfer Buffer for at least 5 minutes. The gel transfer apparatus (Bio-Rad Mini Trans-Blot Cell System) and 3MM Whatman filter paper (Whatman 3030917)was also soaked in 1X Transfer Buffer. After protein separation the gel was removed from the gel plates and the stacking gel was removed. One piece of 3MM Whatman filter paper was placed onto the cathode side of the transfer casing. Onto this the gel was

placed, followed by Immobilon-P membrane, and then followed by another piece of 3MM Whatman filter paper. A plastic tube was carefully rolled over the stack of layer to remove air bubbles. The transfer casing was locked and assembled into the transfer apparatus, and then filled with 1X Transfer Buffer. The apparatus was run at 270mA for 2 hours and 30 minutes.

2.5.7 Antibody detection of proteins.

After transfer the membrane was transfered into 1X PBS-T and rinsed twice, successful protein transfer was detected by staining the membrane with 40ml Ponceau S stain for 1-2 minutes. The membrane was washed in 1X PBS-T to remove Ponceau S stain and then incubated in a 1X PBS-T solution containing 10% (w/v) milk on a rotating platform at room temperature for 18 hours.

The membrane was washed 5 times for 5 minutes in 1X PBS-T, before a 1 hour incubation in primary blocking solution (see 2.1.3.3) with the required primary antibody (see table 8). A further five 5 minute washes in 1X PBS-T were performed, prior to a 1 hour incubation in 1X PBS-T solution containing 10% (w/v) milk and the required secondary antibody. This was followed with a further five washes as described above.

ECL Western Blotting detection solutions 1 and 2 (GE Healthcare RPN2106) were mixed 1:1 and placed on Saran wrap. Excess PBS-T was removed from the membrane before soaking in the mixed ECL detection solution for 1 minute. Excess detection solution was removed from the membrane, prior to wrapping in Saran wrap and exposing to ECL hyperfilm (GE Healthcare 28-9068-37) for different exposure times. These were then developed to reveal the chemiluminescent signal.

Table 13 - Antibodies.

Antibody	Species	Working Dilution	Information
Anti-Lamin A/C (H-110)	Rabbit Polyclonal	1 in 10000	Santa Cruz sc-20681
Anti-Tubulin	Mouse Monoclonal	1 in 10000	NeoMarkers MS-581-P1
Anti-VEGF (VG76e)	Mouse Monoclonal	1 in 500	Prof. R. Bicknell (University of Birmingham)
Anti-PhosphoPRH (YKN5)	Rabbit Polyclonal	1 in 2500	Dr. P-S. Jayaraman (University of Birmingham)
Anti-PRH	Mouse Polyclonal	1 in 2000	Dr. P-S. Jayaraman (University of Birmingham)
Anti-PanTLE (C-19)	Goat Polyclonal	1 in 2000	Santa Cruz sc-13373
Anti-HA (12CA5)	Mouse Monoclonal	1 in 5000	Roche 1 583 816 001

2.5.8 Densitometric Analysis.

Densitometric analysis was performed using the open source ImageJ software according to the programs documentation. Briefly, Western blot film was scanned and opened using ImageJ. Rectangle boxes were drawn to identify the lanes of the gel and a lane plot analysis was performed. The area under the plots were calculated and quantified. Proteins of interest were compared to loading controls. Untreated or

control samples were set to 100%. For half-life calculations the percentages were calculated and averaged for three independent experiments. These averages were plotted, the equation for the line of best fit was then used to calculate the half-life of the protein.

2.6 Real Time Quantitative PCR (qPCR) Protocols.

2.6.1 mRNA Extraction.

Total RNA was extracted from $2x10^6$ K562 cells. Cells were centrifuged at 1100rpm (Eppendorf 5810R) and the cell pellet was used with the RNeasy mini kit (Qiagen 74104), according to the manufacturer's instructions. The DNase1 digestion step was performed using the DNase set (Qiagen 79254).

2.6.2 cDNA Production.

0.5-1μg RNA was reverse-transcribed to single strand cDNA using random primers (Fermentas SO142), 200U of Superscript III reverse transcriptase (Invitrogen 18080044) and 200U of Ribolock RNase Inhibitor (Fermentas EO0381) in a final volume of 20μl. The reactions were incubated at 25ºC for 10 minutes, 42ºC for 90 minutes followed by a 72ºC step for 30 minutes.

2.6.3 qPCR.

Real time PCR reactions were performed using the Rotor-gene detection system (rotor-gene 6) on an RG-3000 machine. Oligonucleotide primers for Gapdh, Prh, Vegf, Vegfr1 and Vegfr2 (see table 9) were used. A 15μl reaction mixture, containing 0.2μM primers, 1X SensiMix SYBR Green (Quantace QT650-05) and 100-400ng of cDNA, was amplified using the following thermal cycler parameters: denaturation at 95ºC for 10 minutes prior to 40-50 cycles of 95ºC for 15 seconds denaturation, 55-62ºC for 20 seconds (annealing primer dependent) and 72ºC for 5 seconds extension. A form of the ΔΔCT method was used to calculate the mRNA levels (Pfaffl, 2001).

Table 14 - Primers.

Gene	Direction	Sequence	Annealing Temperature
Gapdh	FWD	5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3'	55ºC for 19 seconds
Gapdh	REV	5'-TCC TTG GAG GCC ATG TGG GCC AT-3'	55ºC for 19 seconds
Prh	FWD	5'-CAC CCG ACG CCC TTT TAC AT-3'	60ºC for 20 seconds
Prh	REV	5'-GAA GGC TGG ATG GAT CGG C-3'	60ºC for 20 seconds
Vegf	FWD	5'-ATC AGC GCA GCT ACT GCC ATC C-3'	62ºC for 20 seconds
Vegf	REV	5'-TCT CCT ATG TGC TGG CCT TGG TG-3'	62ºC for 20 seconds
Vegfr1	FWD	5'-TGG CCA TCA CTA AGG AGC ACT CC-3'	60ºC for 20 seconds
Vegfr1	REV	5'GGA ACT GCT GAT GGC CAC TGT G-3'	60ºC for 20 seconds
Vegfr2	FWD	5'-TTA GTG ACC AAC ATG GAG TCG TG-3'	60ºC for 20 seconds
Vegfr2	REV	5'-TAG TAA AGC CCT TCT TGC TGT CC-3'	60ºC for 20 seconds

2.7 Flow Cytometric Protocols.

2.7.1 Cell Cycle Profile.

For cell cycle analysis 1x10^5 K562 cells were washed twice in PBS and resuspended

in RPMI media. Ipegal (1% final) and propidium iodide (P.I.; 50μg/ml final) were added immedately before running. Data was collected using a FACScalibur apparatus (Becton Dickinson) and results were analysed with Becton Dickinson Cell Quest software. For each sample at least 10,000 events were collected.

2.7.2 Apoptosis Assay.

For Annexin V analysis ~1x10^5 K562 cells were washed twice in PBS and resuspended in 1x Binding buffer (10mM Hepes/NaOH (pH 7.4), 0.14M NaCl, 2.5mM $CaCl_2$). 5μl Annexin V APC (Becton Dickinson, 550474) and propidium iodide (P.I. ; 5μg/ml final) was incubated for 30 minutes at room temperature. Data was collected as above. For EGFP transfected cells a EGFP positive (FL1) gate was applied. FL3 was used for propidium iodide and FL4 was used for Annexin V APC.

2.7.3 Carboxy-Fluorescein diacetate Succinimidyl Ester (CFSE) Assay.

1x10^5 K562 cells were incubated at 37ºC for 15 mins in 10μM CellTrace™ CFSE (Invitrogen C34554) in PBS. Cells were centrifuged and the dye removed. Cells were resuspended in RPMI medium, plated out and incubated for 30 minutes at 37ºC. Cells were washed, resuspended in RPMI medium and cultured for 5 days. Samples were taken on day 1 and 5. Data was collected and analyzed as above.

2.8 Plasmids.

pMUG1 Myc-PRH.

This plasmid encodes a fusion protein consisting of amino acids 7-270 of human PRH fused to an N-terminal Myc 9E10 epitope. As previously described (Bess et al., 2003; Swingler et al., 2004).

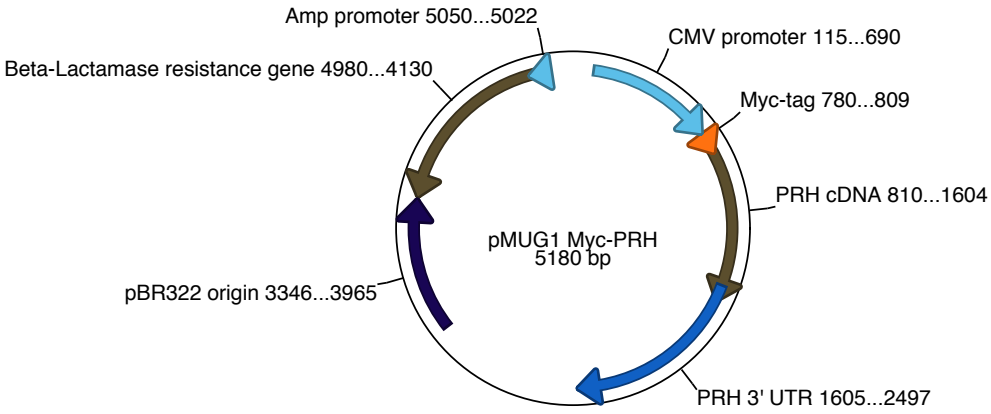


Figure 2.1 - Schematic diagram of pMUG1 Myc-PRH.

The diagram illustrates the plasmid features including the beta-lactamase resistance gene (for ampicillin resistance), the CMV promoter and the Myc-tag and PRH coding region.

pMUG1 Myc-PRH LL23,24AA.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutated to replace an alanine at residues 23 and 24 instead of a leucine. As previously described (Desjobert et al., 2009).

pMUG1 Myc-PRH F32E.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutagenised to replace a glutamic acid at residue 32 instead of a phenylalanine. As previously described (Swingler et al., 2004).

pMUG1 Myc-PRH N187A.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutated to replace an alanine at residue 187 instead of an asparagine. As previously described (Desjobert et al., 2009).

pMUG1 Myc-PRH RR188,189AA.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutated to replace alanine residues at 188 and 189 instead of arginine residues. As previously described (Desjobert et al., 2009).

pMUG1 Myc-PRH SS163,177EE.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutagenised to replace glutamic acid residues at 163 and 177 instead of serine residues. As previously described (Soufi et al., 2009).

pMUG1 Myc-PRH SS163,177CC.

This plasmid was created using a QuikChange® kit (Stratagene) according to the manufacturer's protocol. pMUG1 Myc-PRH was mutated to replace cysteine residues at 163 and 177 instead of serine residues. Mutagenesis was performed by Hannah Williams (University of Bristol) and given as a gift.

pCMV2 Flag-TLE.

The pCMV2 Flag-TLE plasmid expresses Flag-tagged TLE1 and has been previously described (Swingler et al., 2004).

pRc/CMV-CK2.

The plasmids pRc/CMV-CK2α-HA, pRc/CMV-HA-CK2β and pRc/CMV-CK2α-K68M-HA (Soufi et al., 2009) express HA-tagged CK α and β subunits and a kinase-dead CK2α mutant respectively and were a kind gift from Professor David Litchfield (University of Ontario).

pEGFP-C1.

The plasmid pEGFP expresses an enhanced green fluorescence protein. The plasmid is commercially available from Clontech.

pEGFP-PRH.

The pEGFP-PRH plasmid was created by inserting the PRH cDNA from a pBlueScript clone into the EcoRI and KpnI sites of pEGFP-C1. As previously described (Desjobert et al., 2009).

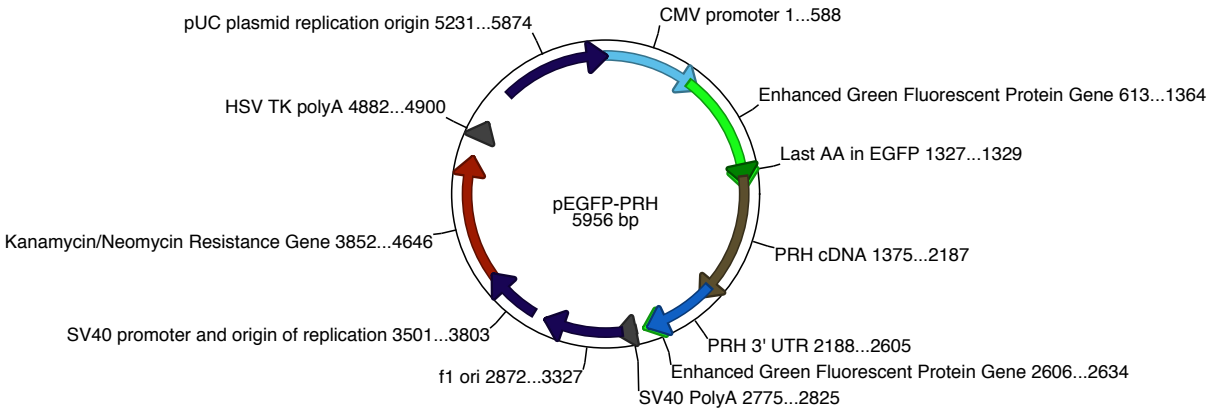


Figure 2.2 - Schematic diagram of pEGFP-PRH.

The diagram illustrates the plasmid features including the kanamycin/neomycin resistance gene, the CMV promoter and the EGFP gene fused to the PRH coding region.

pBJ Flt-1.

The pBJ Flt-1 plasmid is a mammalian expression plasmid that encodes for VEGFR1 (Flt1) and is a kind gift from D. Bates.

pBJ KDR.

The pBJ KDR plasmid is a mammalian expression plasmid that encodes for VEGFR2 (KDR) and is a kind gift from D. Bates.

pcDNA3 VEGF165.

The pcDNA3 VEGF165 plasmid is a mammalian expression plasmid that encodes for VEGF165 and is a kind gift from D. Bates.

pRS (shRNA).

The pRS shRNA plasmid constructs were obtained from ORIGENE (Rockville, MD). The PRH 49 shRNA plasmid contains the sequence, 5'- CGC GGA CGG TGA ACG ACT ACA CGC ACG CC -3', to target the N-terminal region of the PRH mRNA with a 7bp loop followed by the complement of the above sequence. For the PRH 51 shRNA plasmid contains the sequence 5'- CCA GGA AGA CCT TGA ATC AGA GAT TTC AG -3' to target the C-terminal region of the PRH mRNA. The EGFP shRNA plasmid contains the sequence 5'- CAC AAG CTG GAG TAC AAC TAC AAC AGC CA -3' to target EGFP with no human homology (Origene).

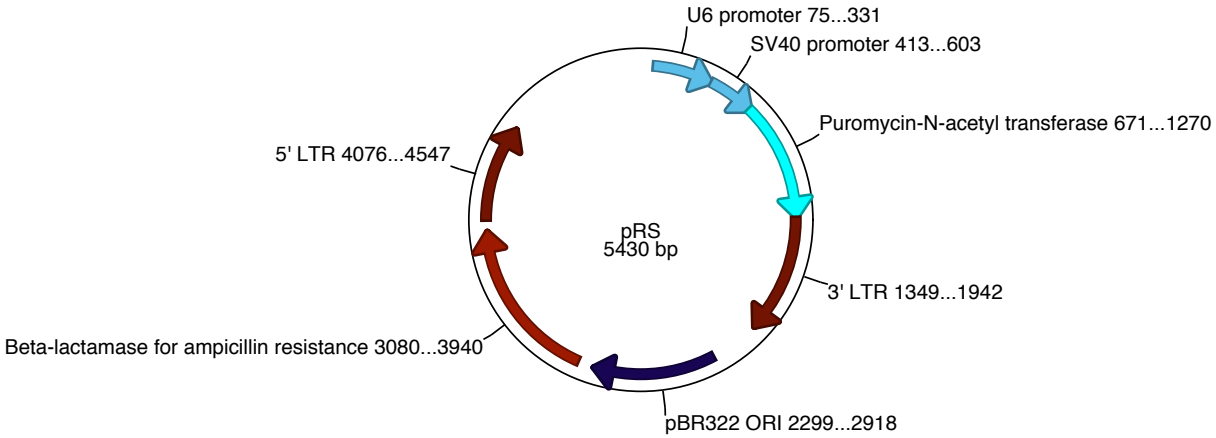


Figure 2.3 - Schematic diagram of pRS plasmid.

The diagram illustrates the plasmid features including the beta-lactamase resistance gene (for ampicillin resistance), the puromycin-N-acetyl transferase gene (for puromycin resistance) and the U6 promoter (NB/ the shRNA sequence follows the U6 promoter).

Transcriptional regulation of VSP gene expression by PRH and TLE

3 Transcriptional regulation of VSP gene expression by PRH and TLE.

3.1 Introduction

Gene regulation by PRH in haematopoietic progenitor cells is currently poorly understood. Although microarray data has revealed potential PRH target genes in murine embryonic stem (ES) cells (Guo et al., 2003), the mechanism through which PRH is regulating these genes is unknown. This chapter will investigate the regulation of genes in the VEGF signalling pathway (VSP), specifically the *Vegf*, *Vegfr1* and *Vegfr2* genes, by PRH. Over-expressed wild-type PRH will be compared to mutants of PRH to characterize the properties of PRH that are required for transcriptional regulation of these genes. Selective knock-down of PRH using targeted shRNAs will also be used to validate the targets. Finally, the role of the TLE-PRH interaction will be evaluated.

3.2 PRH regulation of the *Vegf, Vegfr1* and *Vegfr2* genes.

It has been shown previously that PRH over-expression down regulates *Vegfr1* and *Vegfr2* expression in HUVECs (Nakagawa et al., 2003). It is unknown whether this regulation occurs within haematopoietic progenitor cells, a context where PRH is normally highly expressed. It has also been shown that *Vegf* expression is up-regulated in PRH -/- ES cells (Guo et al., 2003) and in -/- mice embryos (Hallaq et al., 2004), but this regulation has not been investigated in any detail. To investigate the regulation of all three genes (*Vegf*, *Vegfr1* and *Vegfr2)* by PRH, K562 haematopoietic cells were used. Transcriptional regulation by PRH has been investigated in this

K562 cells previously (Swingler et al., 2004). Moreover K562 cells are known to express both VEGF and *Vegfr1* mRNA (Dias et al., 2000). K562 cells are therefore a useful cell line for investigating the regulation of the VSP genes by PRH. In this chapter I am going to use PRH over-expression and PRH knockdown to investigate the regulation of VSP gene expression by PRH in K562 cells.

3.2.1 PRH over-expression.

To determine the amount of over-expression achieved by transient transfection and whether the amount of exogenous PRH expressed is near to physiological levels, an expression plasmid encoding the cDNA for PRH (pMUG1 Myc-PRH) was transiently transfected in increasing amounts into K562 cells by electroporation as previously described (Bess et al., 2003). The expression plasmid contains a fusion gene consisting of a Myc-tag (9E10 epitope) joined to the N-terminus of human PRH cDNA encoding amino acids 7-270. In mammalian cells this fusion protein can be expressed and it will repress transcription in a similar manner to the wild-type PRH (Swingler et al., 2004). Cells were harvested after 48 hours, whole cell protein extracts were then prepared and analyzed by Western blotting (see section 2.5 of the Materials and Methods). Proteins were separated by a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG). After transfer to Immobilon P membrane, proteins were detected using specific antibodies. Western blot analysis was performed using a PRH antibody (mouse) which can detect both endogenous PRH (figure 3.1 - top panel, lower band) and exogenous Myc-PRH (figure 3.1 - top panel, upper band). A Lamin A/C antibody was used as a protein loading control (figure 3.1 - bottom panel). Figure 3.1 shows that the exogenous PRH is clearly detectable when 5μg of pMUG1 Myc-PRH is transfected into K562 cells (top panel, lane 5). There is a modest increase in the level of exogenous PRH present when 10μg pMUG1 Myc-PRH plasmid is used and the over-expressed Myc-tagged PRH appears to be expressed

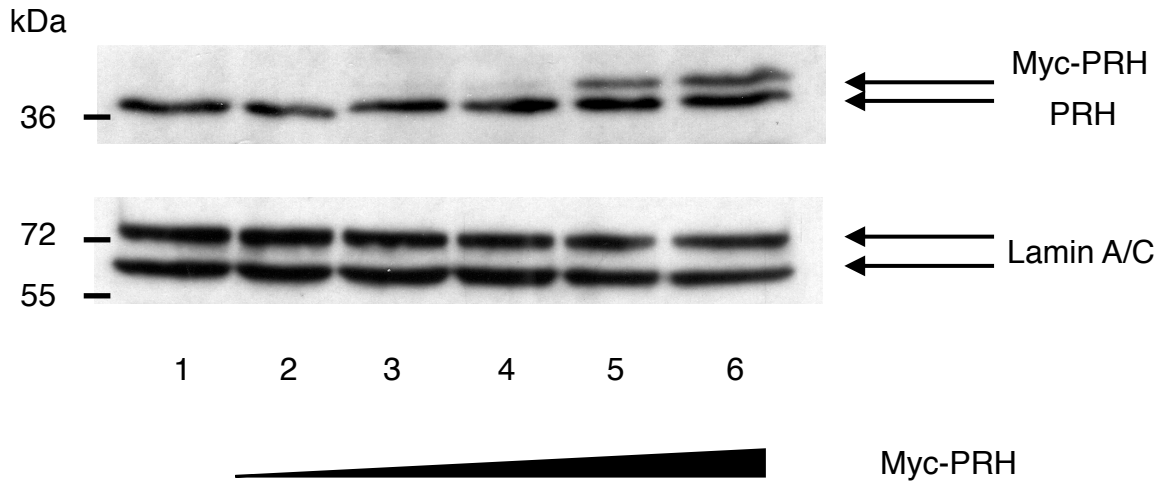


Figure 3.1 - Myc-PRH expression in K562 cells.

Western blot of whole-cell extracts from K562 cells transiently transfected with increasing amounts (0, 0.5, 1, 2, 5, 10μg) of the pMUG1 Myc-PRH plasmid expressing a Myc-tagged PRH protein. Protein extracts were made 48 hours post-transfection. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon P PVDF membrane. Proteins were stained with a mouse anti-PRH antibody (top panel), and a Lamin A/C antibody (bottom panel). Data representative of two independent experiments.

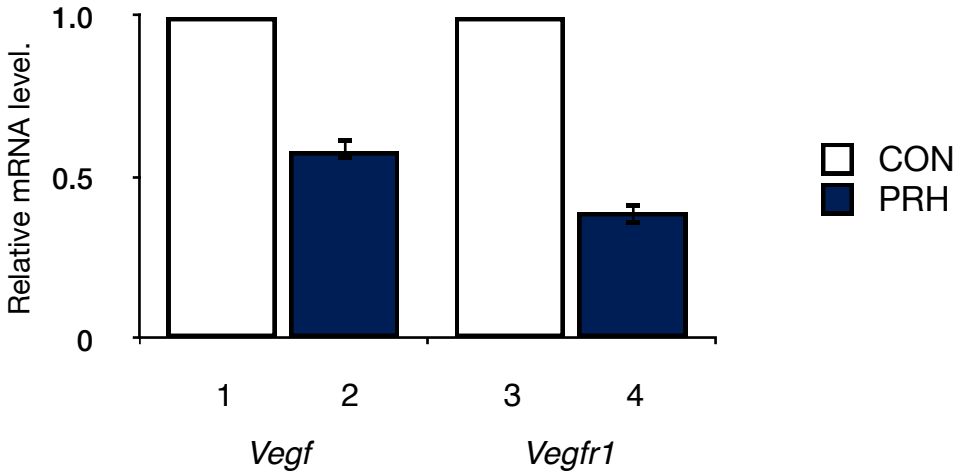
at roughly equivalent levels to endogenous PRH (figure 3.1 - top panel, lane 6). Since 10μg of PRH results in expression levels that are within the physiological range, subsequently we used 10μg pMUG1 Myc-PRH plasmid for all over-expression experiments.

To examine the regulation of *Vegf* and *Vegfr1* expression by over-expressed PRH, K562 cells were transfected with pMUG1 Myc-PRH or pMUG1 (CON) and 48 hours post-transfection the cells were harvested. RNA was extracted, as described in section 2.6.1 of the Materials and Methods chapter. 0.5μg of the extracted RNA was reverse transcribed to produce cDNA (see section 2.6.2 for details). The amount of cDNA for each gene was then analyzed using quantitative PCR (qPCR) with gene specific primers for *Vegf*, *Vegfr1* and *Gapdh* (internal control gene) (see table 9 in section 2.6.3 for details). The relative expression of *Vegf* and *Vegfr1* were analyzed using the ΔΔCt method (as modified by Pfaffl (Pfaffl, 2001)). Figure 3.2 [A] shows

that *Vegf* mRNA expression is repressed (lane 2 compared to lane 1) to approximately 60% of its endogenous level by over-expression of PRH. Similarly, *Vegfr1* mRNA expression is repressed to approximately 40% of its endogenous level by over-expression of PRH (figure 3.2 [A] - lane 4 compared to lane 3). The effect of PRH over-expression on *Vegfr2* expression could not be quantified because although expression for *Vegfr2* was detectable in untreated cells, the signal for *Vegfr2* was undetectable in the PRH over-expressed samples. It can be concluded that PRH over expression results in down regulation the *Vegf*, *Vegfr1*, and *Vegfr2* genes in K562 cells.

To determine whether regulation of VSP mRNA expression by PRH results in reduced protein synthesis for each gene, whole cell protein extracts from cells over-expressing Myc-PRH were made for Western blot analysis. Proteins were separated and transferred to Immobilon P membrane for immuno-detection as described above. Western blot analysis was performed using the VEGF (VG76e) antibody (Fraser et al., 2000) to detect VEGF protein levels (figure 3.2 [B] - top panel), and a Lamin A/C antibody was used as a loading control (figure 3.2 [B] - bottom panel). VEGF protein levels are decreased to 27% of the unrepressed state in cells over-expressing PRH compared to control cells (figure 3.2 [B] - top panel, lanes 2 and 1, respectively). This suggests that repression of *Vegf* mRNA by PRH is reflected in the level of VEGF protein expressed in the cell. The effect of PRH over-expression on VEGFR1 protein levels could not be determined with the antibody available (data not shown). The VEGFR2 antibodies available were also unable to detect VEGFR2 protein in K562 cells (data not shown). The lack of a signal for VEGFR1 and VEGFR2 in untransfected cells prevented further investigation of VEGFR1 and VEGFR2 protein levels.

[A]



[B]

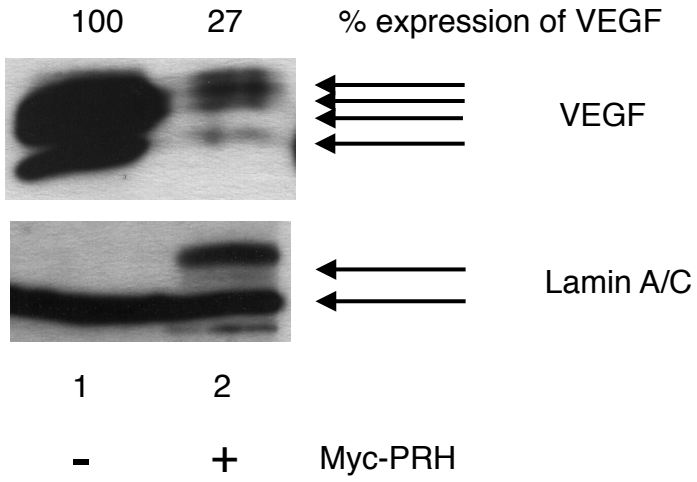


Figure 3.2 - The effect of exogenous PRH on *Vegf, Vegfr1* mRNA and VEGF protein levels.

[A] *Vegf* and *Vegfr1* mRNA levels in K562 cells 48 h after transfection with 10*μ*g pMUG1 (lanes 1 and 3, CON) or pMUG1 Myc-PRH (lanes 2 and 4, Myc-PRH). RNA was extracted and 0.5*μ*g of extracted RNA was reverse transcribed to cDNA. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using gene specific primers and comparing to qPCR reactions for *Gapdh*. Values are means and standard deviations (SD) (n = 5). [B] Western blot of whole-cell extract from K562 cells transfected with empty pMUG1 (lane 1) or pMUG1 MycPRH (lane 2). Protein extracts were made 48 hours post-transfection. Proteins were separated by SDS-PAGE and transferred to an Immobilon P PVDF membrane. Proteins were stained with VEGF (VG76e) antibody (top panel) or Lamin A/C antibody (bottom panel). Data representative of two independent experiments.

3.2.2 PRH knockdown.

To determine whether the *Vegf*, *Vegfr1* and *Vegfr2* genes are regulated by the endogenous PRH protein in K562 cells, PRH was knocked down using *Prh* specific shRNAs. Cells transfected with the shRNA plasmids activate the RNA interference pathway to knock down the expression of *Prh* mRNA in a sequence specific manner (Paddison et al., 2002; Rao et al., 2009). PRH shRNA 1 targets the *Prh* mRNA sequence in the first exon within the coding sequence, between bases 374 and 402. PRH shRNA 2 targets the *Prh* mRNA sequence within the coding sequence of the fourth exon, between bases 789 and 817 (figure 3.3). K562 cells were transfected with a single PRH shRNA plasmid (PRH shRNA 1 or PRH shRNA 2), a combination

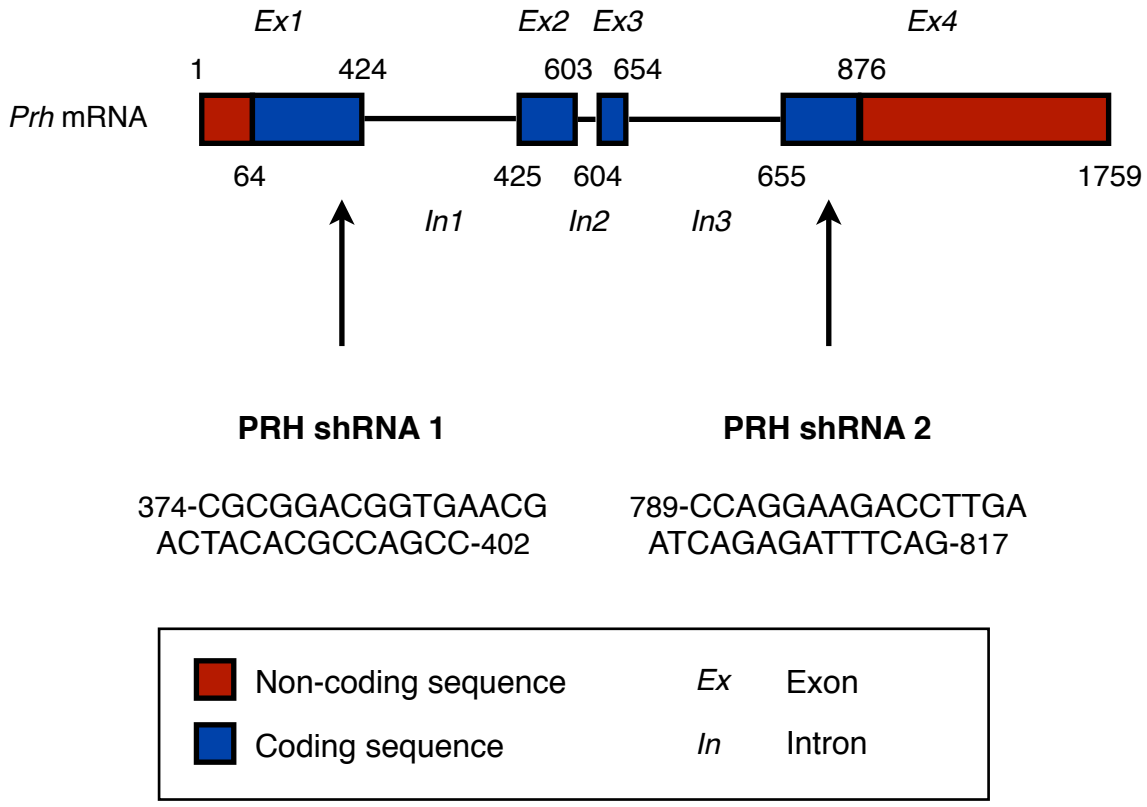


Figure 3.3 - Schematic diagram of the target of PRH shRNAs 1 and 2.

Schematic diagram of PRH shRNA targeting of the *Prh* mRNA sequence. PRH shRNA 1 targets the *Prh* mRNA in the first exon within the coding sequence. PRH shRNA 2 targets the *Prh* mRNA in the fourth exon within the coding sequence.

of these PRH targeted shRNA plasmids (PRH shRNA 1+2), or a GFP targeted shRNA plasmid that was used as a control. 24 hours post-transfection the transfected cells were selected using 1μg/ml puromycin. After 10 days of puromycin selection the knockdown cells were harvested for RNA and protein extraction.

To determine the level of PRH protein in PRH knockdown cells, whole cell protein extracts were extracted and analyzed by Western blot as previously described. Western blot analysis was performed using a rabbit anti-PRH antibody (figure 3.4 [A] - top panel) and an anti-Lamin A/C antibody was used as a loading control (figure 3.4 [A] - bottom panel). Figure 3.4 [A] shows that PRH protein is reduced to 35% and 55% of endogenous levels for the PRH shRNA 1 and PRH shRNA 2 plasmids respectively (figure 3.4 [A] - top panel, lanes 2 and 3). Combination of PRH shRNA plasmids 1 and 2 further reduces the level of PRH expression to 7% of the control level (figure 3.4 [A] - top panel, lane 4). The combination of PRH shRNA plasmids produced the most significant knockdown and therefore this combination of shRNA plasmids was used for all further PRH knockdown experiments. Knockdown of PRH protein levels were further confirmed by Western blot analysis using the mouse anti-PRH antibody (figure 3.4 [B] - top panel). In the PRH shRNA sample (lane 1) of the top panel of figure 3.4 [B] no band can be clearly identified for the mouse anti-PRH antibody, however for the control sample (lane 2), a strong band is observed for the mouse anti-PRH antibody. Thus two different PRH antibodies both show a dramatic reduction in PRH protein levels in PRH knockdown cells.

RNA was extracted from PRH knockdown and control cells as previously described. Reverse transcription of the extracted RNA produced cDNA, which was analyzed by qPCR using gene specific primers for *Prh* and *Gapdh* (internal control). A significant decrease in the level of *Prh* mRNA was detected in the PRH shRNA knockdown cells compared to the control shRNA plasmid cells (figure 3.4 [C] - column 2 compared to

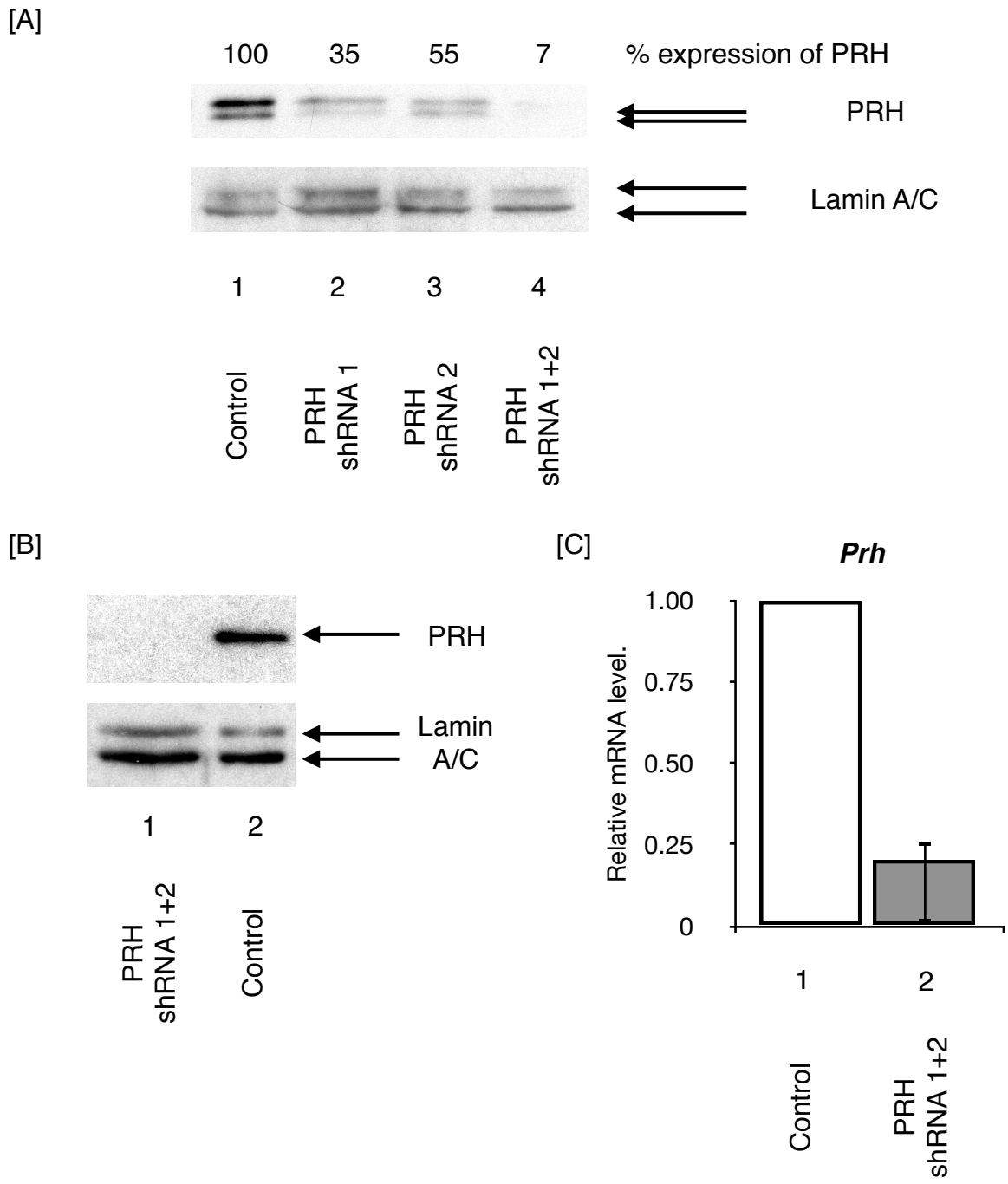


Figure 3.4 - Knock-down of PRH protein and *prh* mRNA by shRNA transfection.

[A] Western blot of whole-cell extracts from K562 cells co-transfected with shRNA plasmids control shRNA (lane 1), PRH shRNA 1 (lane 2), PRH shRNA 2 (lane 3), and PRH shRNA (1+2) (lane 4). Protein extracts were made 48 hours post-transfection. Proteins were separated by SDS-PAGE and transferred to an Immobilon P PVDF membrane. Proteins were stained with rabbit anti-PRH antibody (top panel) and anti-Lamin A/C antibody (bottom panel). [B] As for [A] except extracts from PRH shRNA 1+2 (lane 1) and control shRNA (lane 2), using mouse anti-PRH antibody (top panel) and anti-Lamin A/C antibody (bottom panel). Data representative of three independent experiments. [C] *Prh* mRNA levels after PRH shRNA 1+2 co-transfection. RNA was extracted and 0.5μg of extracted RNA was reverse transcribed to cDNA. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using gene specific primers and comparing to qPCR reactions for *Gapdh*. White bar represent control shRNA cells (lane 1) and grey bar PRH shRNA 1+2 cells (lane 2). Values are means and SD (n = 5).

column 1), confirming the knockdown of *Prh* mRNA as well as PRH protein levels.

To investigate the regulation of the *Vegf, Vegfr1* and *Vegfr2* by endogenous PRH, protein and RNA was extracted from PRH knockdown cells and control cells for analysis. Protein was extracted and analyzed by Western blot as described above. Western blot analysis was performed using an anti-VEGF (VG76e) antibody (Fraser et al., 2000) (figure 3.5 [A] - top panel) to detect VEGF protein expression, a rabbit anti-PRH antibody (figure 3.5 [A] - middle panel) and an anti-Lamin A/C antibody (figure 3.5 [A] - bottom panel) was used as a loading control. In each of the samples where PRH is knocked down (figure 3.5 [A] - middle panel, lanes 2-4), VEGF protein expression is strongly elevated (figure 3.5 [A] - top panel, lanes 2-4) compared to the control cells (figure 3.5 [A] - top panel, lane 1). This suggests that loss of PRH expression de-represses VEGF protein expression.

RNA was extracted from PRH knockdown and control cells as previously described. Reverse transcription of the extracted RNA produced cDNA, which was analyzed by qPCR using gene specific primers for *Prh, Vegf, Vegfr1, Vegfr2* and *Gapdh*. *Prh* mRNA expression in PRH knockdown cells was approximately 20% of the *Prh* mRNA expression levels in control cells (figure 3.5 [B] - lane 2 compared to lane 1), confirming knockdown of *Prh* mRNA. In PRH knockdown cells *Vegf* mRNA expression is elevated compared to control cells (figure 3.5 [B] - lanes 4 and 3) with over four times the expression of *Vegf* mRNA. Similarly, *Vegfr1* mRNA expression in PRH knockdown cells is approximately three and a half times greater than the *Vegfr1* mRNA expression in the control cells (figure 3.5 [B] - lanes 6 and 5). Notably, *Vegfr2* mRNA expression is also elevated over two fold in PRH knockdown cells compared to control cells (figure 3.5 [B] - lanes 8 and 7). These results show that endogenous PRH represses endogenous *Vegf, Vegfr1 and Vegfr2* expression in K562 cells.

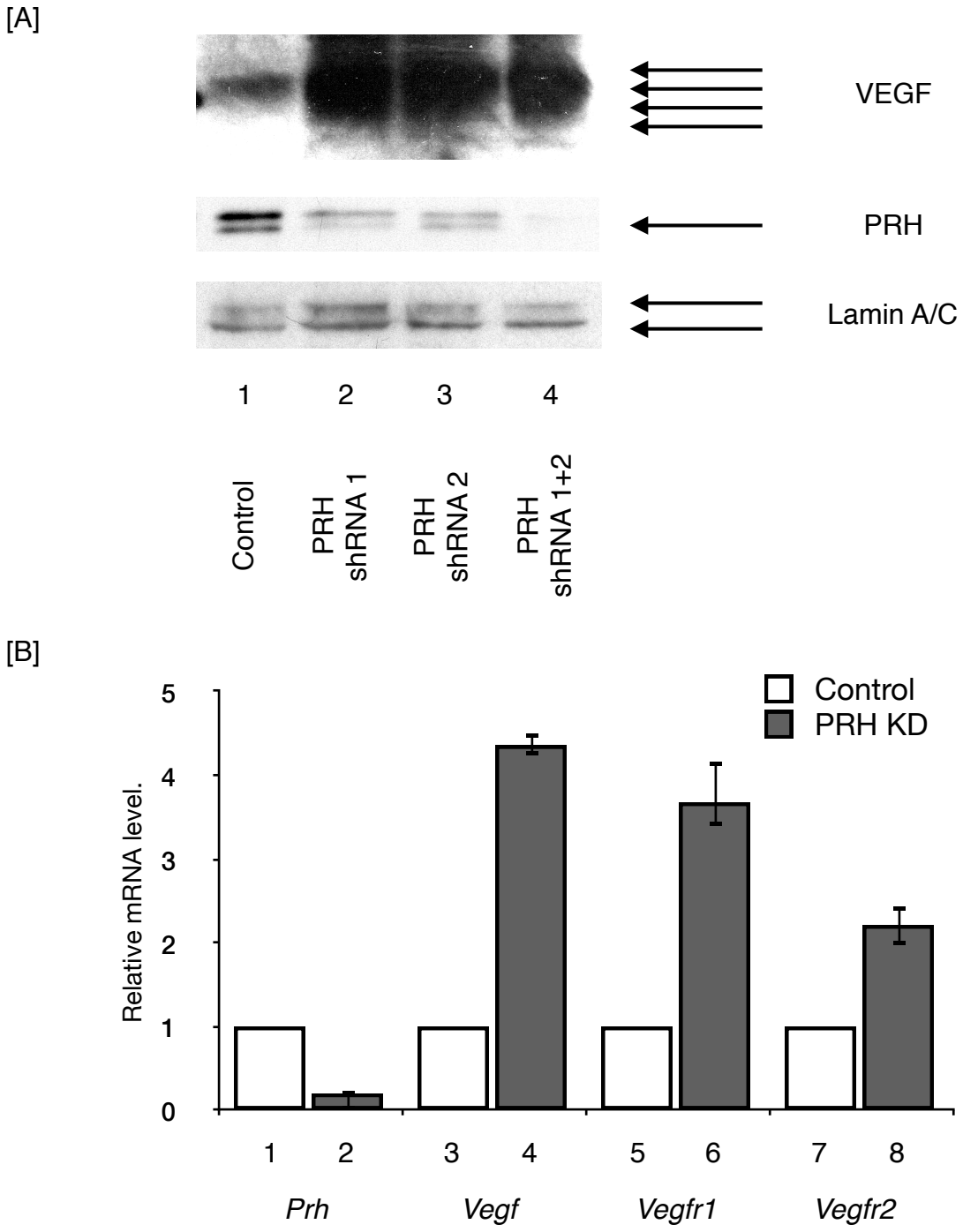


Figure 3.5 - The effect of PRH shRNA knock-down on VSP gene expression.

[A] Western blot of whole-cell extracts from K562 cells co-transfected with shRNA plasmids control shRNA (lane 1), PRH shRNA 1 (lane 2), PRH shRNA 2 (lane 3), and PRH shRNA (1+2) (lane 4). Protein extracts were made 48 hours post-transfection. Proteins were separated by SDS-PAGE and transferred to an Immobilon P PVDF membrane. Proteins were stained with anti-VEGF (VG76e) antibody (top panel), rabbit anti-PRH antibody (middle panel) and anti-Lamin A/C antibody (bottom panel). Data representative of two independent experiments. [B] *Prh, Vegf, Vegfr1* and *Vegfr2* mRNA levels after PRH shRNA 1+2 co-transfection (PRH KD). mRNA levels were determined as in figure 3.2. White bars represent control shRNA cells and grey bars PRH knockdown cells. Values are means and SD (n = 5).

3.3 PRH-TLE interaction is required for *Vegf*, *Vegfr1* regulation.

PRH can regulate gene expression through multiple mechanisms (Soufi and Jayaraman, 2008), for example PRH binds via its homeodomain and recruits co-repressor proteins such as TLE1 to bring about direct transcriptional repression (Swingler et al., 2004). PRH can also act post-transcriptionally by interacting with eIF4E (Topisirovic et al., 2003a), as outlined in the introduction.

To distinguish between these direct and indirect regulatory mechanisms, PRH mutants in which specific protein interactions or DNA binding are disrupted were used and the regulation of *Vegf* and *Vegfr1* genes by these mutated proteins was investigated. PRH requires the ability to bind to DNA through the homeodomain for direct transcriptional repression (Guiral et al., 2001). Using electrophoretic mobility shift assays (EMSA), DNA binding has been shown to be disrupted by a mutation within the homeodomain at residue 187 which alters an asparagine to an alanine (figure 3.6 - PRH N187A) (Guiral et al., 2001; Desjobert et al., 2009). Interaction between PRH and eIF4E can lead to post-transcriptional regulation of mRNA transport by PRH. A double mutation at residues 23 and 24 from leucine residues to alanine residues has been reported to abrogate this interaction (figure 3.6 - PRH LL23,24AA) (Topisirovic et al., 2003a). PRH interacts with the co-repressor Groucho/ TLE through a conserved Eh-1 motif located within the proline-rich N-terminal domain of PRH (Swingler et al., 2004). This interaction has been shown to be important for transcriptional repression (Swingler et al., 2004). Mutation of the Eh-1 motif in PRH from phenylalanine at residue 32 to a glutamic acid residue (figure 3.6 - PRH F32E) has previously been shown to abolish binding of TLE to PRH in pull-down and co-IP assays (Swingler et al., 2004).

K562 cells were transfected with 10μg pMUG1 Myc-PRH, or pMUG1 Myc-PRH F32E, pMUG1 Myc-PRH LL23,24AA, pMUG1 Myc-PRH N187A or pMUG1 as a

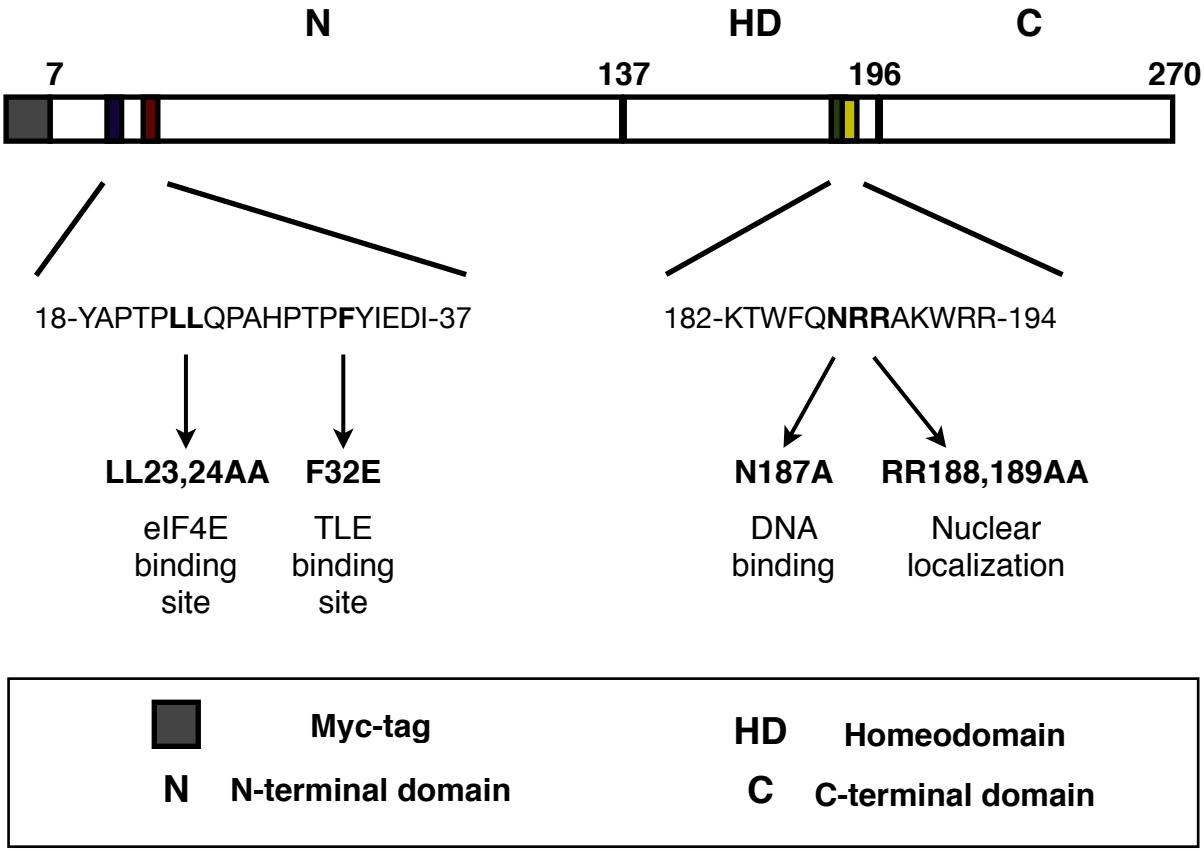


Figure 3.6 - Schematic diagram of Myc-PRH point mutations.

Schematic diagram of the PRH protein in pMUG1 Myc-PRH and targeted mutations in the protein. The LL23,24AA mutation is defective in eIF4E interaction. The F32E mutation reduces PRH binding to TLE. PRH N187A mutant is deficient in DNA binding. The PRH RR188,189AA mutant does not localize to the nucleus.

control. After 48 hours the cells were harvested and protein was extracted. Protein expression was analyzed by Western blot, as previously described, using an anti-Myc (9E10) antibody (figure 3.7 [A] - top panel) to detect exogenous Myc-PRH and an anti-Lamin A/C antibody was used as a loading control (figure 3.7 [A] - bottom panel). Each of the PRH mutant proteins are expressed at approximately equal levels (figure 3.7 [A] - top panel, lanes 3-5 respectively) compared to the wild-type Myc-PRH (figure 3.7 [A] - top panel, lane 2). Therefore, any difference observed in gene expression is a consequence of the specific protein mutation and not through variations in protein expression levels.

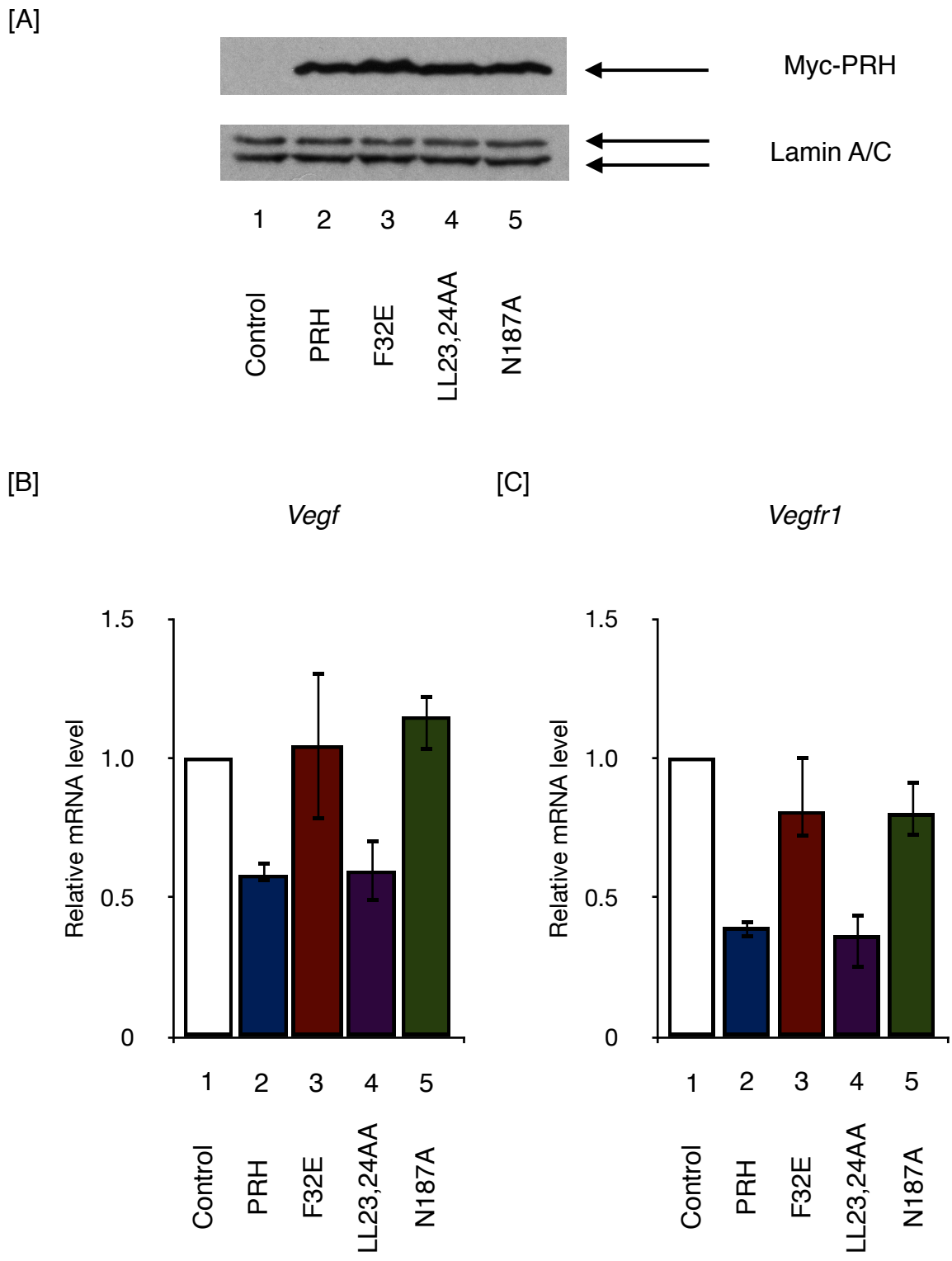


Figure 3.7 - Characteristics of PRH required for *Vegf* and *Vegfr1* gene regulation.

[A] Western blot of Myc-PRH expression in K562 cells 48 h after transfection with 10 μg pMUG1 (lane 1, control) or pMUG1 vectors expressing PRH (PRH {lane 2}, PRH F32E {lane 3}, PRH LL23,24AA {lane 4}, or PRH N187A {lane 5}). Whole-cell extracts were run on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) as described for figure 3.2 and stained with Myc and Lamin A/C antibodies. Data representative of five independent experiments.[B] *Vegf* mRNA levels in K562 cells 48 hours after transfection with the vectors described for panel [A]. mRNA levels were determined as for figure 3.2. Values are means and SD (n = 3). [C] As for panel [B] except that relative levels of expression of *Vegfr1* are shown. Values are means and SD (n = 3).

RNA was extracted from cells transfected with empty expression vector (pMUG1), or expression vectors for PRH, or each of the mutants, 48 hours post-transfection. The mRNA was reverse transcribed to produce cDNA which was analyzed as described above by qPCR. As previously observed (figure 3.2 [A]) the wild-type Myc-PRH represses *Vegf* mRNA expression when compared to the control cells (figure 3.7 [B] - lanes 2 and 1). The PRH F32E (figure 3.7 [B] - lane 3) and PRH N187A (figure 3.7 [B] - lane 5) mutants both have little or no effect on mRNA expression of *Vegf* compared to the control cells (figure 3.7 [B] - lane 1). In contrast the PRH LL23,24AA (figure 3.7 [B] - lane 4) mutant represses *Vegf* mRNA expression as efficiently as the wild-type Myc-PRH (figure 3.7 [B] - lane 2) when compared to control cells (figure 3.7 [B] - lane 1).

The effect of these PRH mutations was also investigated for *Vegfr1* mRNA expression, as described for the regulation of *Vegf* mRNA expression above. Myc-PRH represses *Vegfr1* mRNA expression to approximately 40% of its unrepressed state (figure 3.7 [C] - lane 2 compared to lane 1) as previously observed (figure 3.2 [A]). However, the PRH F32E (figure 3.7 [C] - lane 3) and PRH N187A (figure 3.7 [C] - lane 5) mutants have a substantially reduced capacity to repress *Vegfr1* mRNA expression, only repressing *Vegfr1* mRNA expression to approximately 80% of the unrepressed state (figure 3.7 [C] - lane 1). The LL23,24AA (figure 3.7 [C] - lane 4) mutation has no effect on the ability of PRH to repress *Vegfr1* mRNA expression, repressing mRNA expression as well as the wild-type Myc-PRH (figure 3.7 [C] - lane 2) when compared to control cells (figure 3.7 [C] - lane 1). It can be concluded that both DNA binding and interaction with TLE are required for the repression of the *Vegf* and *Vegfr1* genes. However, PRH interaction with eIF4E would appear to have no role in the regulation of *Vegf* and *Vegfr1* mRNA expression. This data suggests that PRH regulates these genes by a direct transcriptional mechanism.

To confirm the role of TLE in the regulation of the *Vegf* and *Vegfr1* genes, co-expression of PRH and TLE-1 was carried out in control and PRH knockdown cells. PRH knockdown and control cells were transfected with pMUG1 alone, or pMUG1 Myc-PRH alone, or pCMV2 Flag-TLE-1 alone, or with pMUG1 Myc-PRH and pCMV2 Flag-TLE-1. 48 hours post-transfection the cells were harvested and RNA was extracted. The extracted RNA was reverse transcribed and analyzed by qPCR as detailed above. Figure 3.8 [A] shows that in control cells when PRH is exogenously expressed repression of *Vegf* is observed (lane 2) when compared to control cells (lane 1). In control cells when TLE-1 is exogenously expressed, repression of the *Vegf* mRNA expression is also observed (figure 3.8 [A] - lane 3) when compared to the control (figure 3.8 [A] - lane 1). However, when both PRH and TLE-1 are co-expressed, further repression of *Vegf* is observed (figure 3.8 [A] - lane 4) compared to the control (figure 3.8 [A] - lane 1), or indeed compared to PRH or TLE-1 alone (figure 3.8 [A] - lane 2 and 3 respectively). In PRH knockdown cells as expected *Vegf* levels are de-repressed compared to control cells (figure 3.8 [A] - lane 5). Re-introduction of exogenous PRH results in repression of *Vegf* mRNA expression compared to control PRH knockdown cells (figure 3.8 [A] - lanes 6 and 5). In contrast with over-expression of TLE-1 in control cells, over-expression of TLE-1 in PRH knockdown cells has no significant effect on *Vegf* mRNA expression compared to control PRH knockdown cells (figure 3.8 [A] - lanes 7 and 5). Strikingly, increased repression of *Vegf* mRNA expression is observed when TLE-1 and PRH are co-expressed in PRH knockdown cells compared to expression of PRH alone in PRH knockdown cells (figure 3.8 [A] - lanes 8 and 6). This demonstrates that the presence of PRH is required for the repression of the *Vegf* gene by TLE-1. It is likely that in control cells, the repression observed when TLE-1 is over-expressed alone is through binding to endogenous PRH bound at the *Vegf* gene promoter.

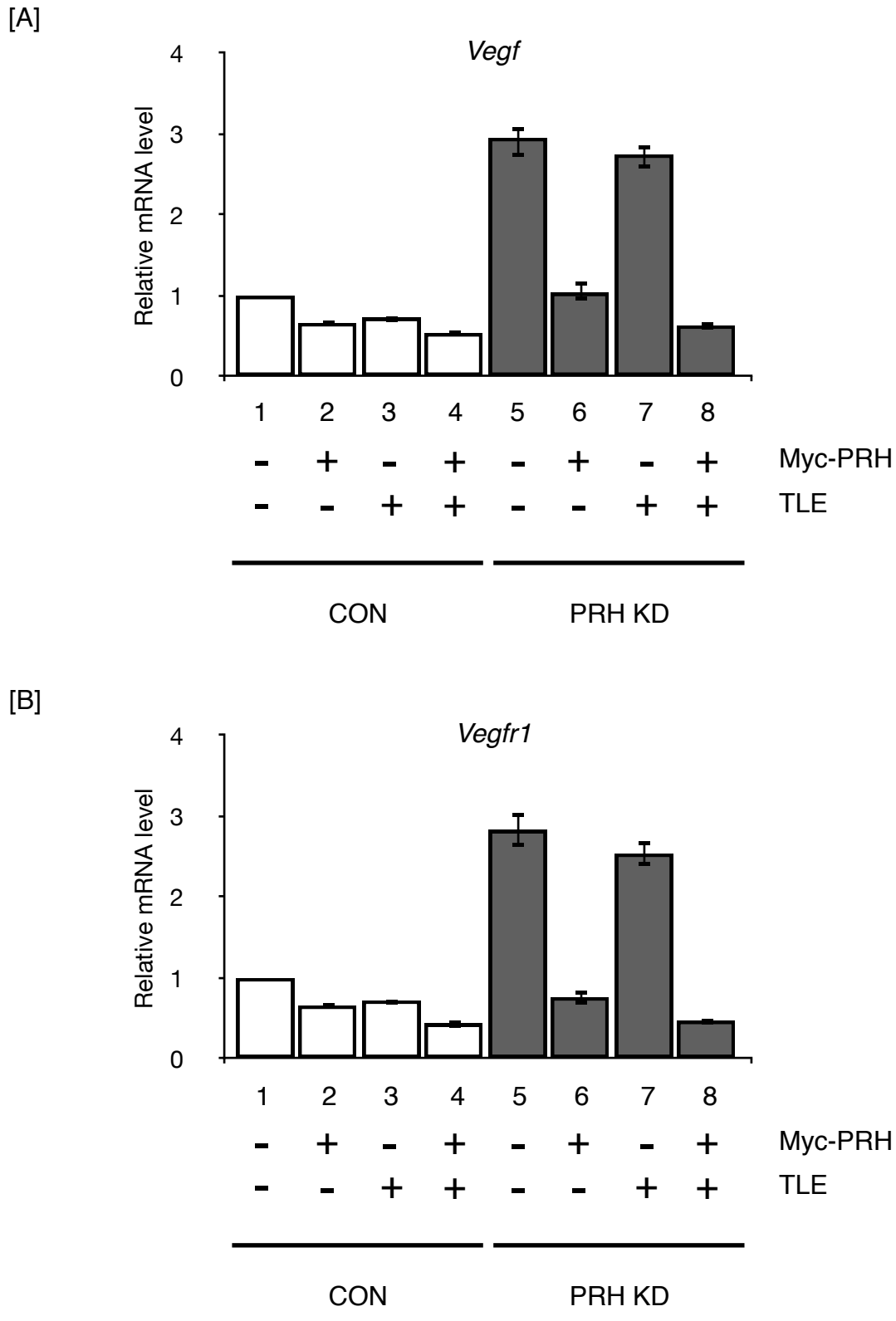


Figure 3.8 - PRH and TLE co-repression of *Vegf* and *Vegfr1* gene expression.

[A] *Vegf* mRNA levels in K562 shRNA control cells (CON) and PRH shRNA cells (PRH KD) 48 hours after transfection with pMUG1-MycPRH (5 μg), pCMV2-TLE1(2.5 μg), or both plasmids were determined as for figure 3.2. Values are means and SD (n = 3). [B] As for panel [A] except that relative levels of expression of *Vegfr1* are shown. Values are means and SD (n = 3).

The role of TLE-1 in *Vegfr1* mRNA expression was also investigated in the same experiment. As observed for *Vegf* mRNA expression exogenous expression of PRH in control cells results in repression of *Vegfr1* mRNA compared to the control (figure 3.8 [B] - lanes 2 and 1). In control cells when TLE-1 is exogenously expressed, *Vegfr1* mRNA expression is also repressed compared to the control (figure 3.8 [B] - lanes 3 and 1). Co-expression of both PRH and TLE-1 in control cells results in further repression of *Vegfr1* mRNA expression (figure 3.8 [B] - lane 4) compared to the control (figure 3.8 [B] - lane 1), TLE-1 only cells (figure 3.8 [B] - lane 3), or PRH only cells (figure 3.8 [B] - lane 2). Again in PRH knockdown cells *Vegfr1* mRNA expression is elevated. Re-introduction of exogenous PRH into PRH knockdown cells results in repression of *Vegfr1* mRNA expression compared to control PRH knockdown cells (figure 3.8 [B] - lanes 6 and 5). However, unlike over-expression of TLE-1 in control cells, over-expression of TLE-1 in PRH knockdown cells has no significant effect on *Vegfr1* mRNA expression (figure 3.8 [B] - lane 7) compared to control PRH knockdown cells (figure 3.8 [B] - lane 5). As seen for *Vegf* expression, increased repression of *Vegfr1* mRNA expression is observed when TLE-1 and PRH are co-expressed in PRH knockdown cells (figure 3.8 [B] - lane 8) compared to expression of PRH alone (figure 3.8 [B] - lane 6). It can be concluded that PRH is required for the repression of the *Vegfr1* gene by TLE-1. It is likely that in control cells TLE-1 expression alone represses *Vegfr1* mRNA expression by interacting with endogenous PRH bound at the *Vegfr1* gene promoter. These data confirm the results obtained with the PRH mutants suggesting that recruitment of TLE-1 is essential for transcriptional repression by PRH at the *Vegf* and *Vegfr1* promoters.

3.4 PRH is directly associated with the promoters of the VSP genes.

The data above suggests that PRH binds to the *Vegf* and *Vegfr1* promoters to repress gene transcription and that TLE-1 is also required for this transcriptional

regulation. To further investigate whether PRH directly regulates the *Vegf* and *Vegfr1* genes through promoter association, chromatin immuno-precipitation (ChIP) experiments were performed to determine the regions of PRH binding. ChIP is a technique that cross-links proteins to the regions of chromatin they are bound to in the cell, the cells are then lysed and sonicated to fragment the chromatin into fragments smaller than approximately 800bp. Immuno-precipitation of the protein of interest (for example PRH) is performed using a protein specific antibody conjugated to a bead. The cross-links are then reversed and the DNA fragments bound to the protein of interest are purified. This DNA can be used to determine enrichment of specific promoter regions by qPCR.

To perform ChIP experiments the optimum sonication conditions to produce chromatin fragments from K562 cells was necessary. For this $2.5x10^6$ or $5x10^6$ K562 cells were formaldehyde cross-linked before sonication. Cells were either sonicated on medium power for 5 minutes or 10 minutes at each concentration. The cross-links were then reversed and the DNA was purified. 2μg of purified DNA was run on a 1.5% agarose gel and visualized under UV light. For $2.5x10^6$ cells sonicated for 5 minutes on medium power, DNA fragments of between 300 and 600 base pairs in length are observed, with an average fragment size of 450bp (figure 3.9 - lane 1). Using double the number of cells, $5x10^6$ cells, and sonicating for 5 minutes on medium power, an increase in fragment sizes to between 250 and 900 base pairs long is observed, compared to half the number of cells under the same conditions (figure 3.9 - lanes 3 and 1). Sonication of $2.5x10^6$ cells on medium power for 10 minutes gave fragments of between 250 and 600 base pairs in length, with an average base pair fragment of 425 base pairs (figure 3.9 - lane 2). Yet sonication of $5x10^6$ cells for 10 minutes decreases the fragment size to between 200 and approximately 600 base pairs in size (figure 3.9 - lane 4), and this gives an average

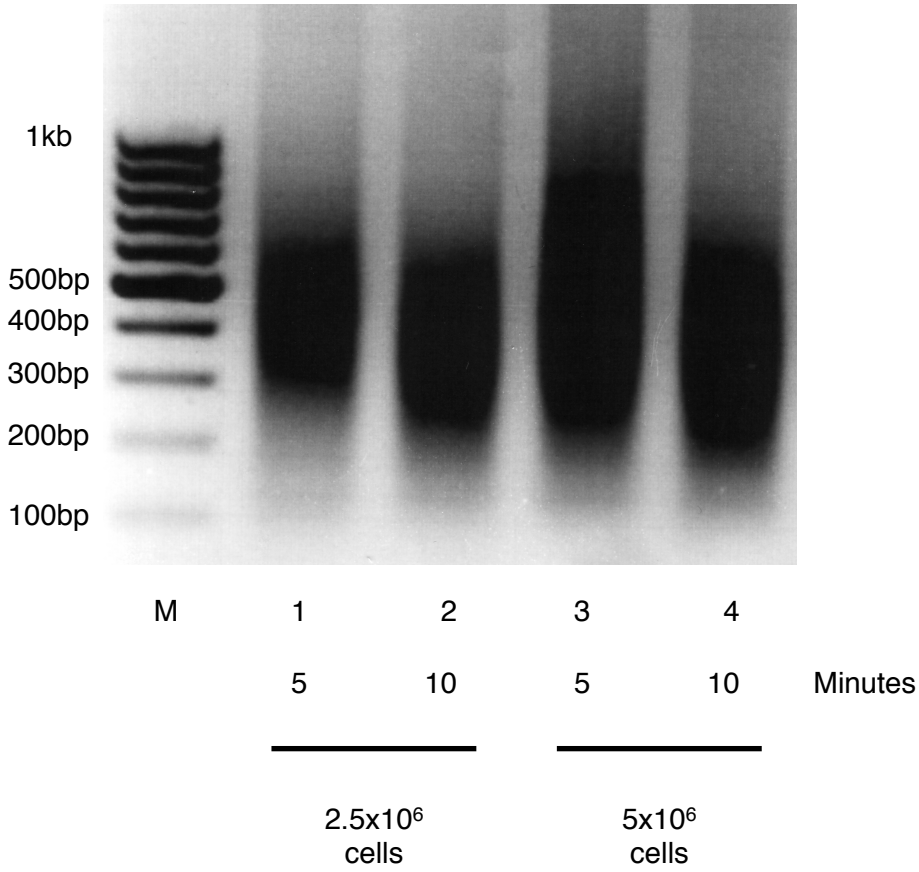


Figure 3.9 - Optimization of chromatin fragments used for ChIP experiments.

$2.5x10^6$ (lanes 1 and 2) or $5x10^6$ (lanes 3 and 4) un-transfected K562 cells were cross-linked with formaldehyde and sonicated for either 5 (lanes 1 and 3) or 10 (lanes 2 and 4) minutes on medium power. The chromatin fragments were purified and separated on a 1.5% agarose gel.

fragment size of 400 base pairs in length. Therefore for all ChIP assays, $5x10^6$ K562 cells were sonicated on medium power for 10 minutes because this condition produces an optimal average fragment size and allows processing of a large number of cells.

Previous work has shown that PRH can either regulate gene transcription through association with single binding sites or it can bind to multiple binding sites within a gene promoter. For example, PRH regulates promoters that contain a TATA box sequence and no other SELEX-defined PRH binding sites (Guiral et al., 2001). PRH

can also bind to multiple adjacent binding sites such as those within the *Goosecoid* promoter and induce transcriptional repression (Williams et al., 2008). *In vitro* DNase 1 protection experiments have shown that PRH protects a large region of DNA at least 200bp in length suggesting that PRH either spreads along DNA or wraps DNA around it (Williams et al., 2008). Therefore, to determine whether PRH binds across a small region of DNA or at multiple regions across the *Vegfr1* promoter, several regions across the promoter were assayed for PRH association.

To investigate where PRH binds to the *Vegfr1* promoter, 5μg of pMUG1 Myc-PRH was transfected into K562 cells. 24 hours after transfection formaldehyde was added to the cells to cross-link DNA bound proteins to chromatin. The cells were then sonicated to shear the chromatin into DNA fragments of 200-600bp in length. Chromatin was immuno-precipitated using an anti-myc or anti-IgG antibody, as a control. Protein-DNA cross-links were then reversed and the immuno-precipitated chromatin was purified. To determine the relative amount of DNA binding by PRH across the *Vegfr1* promoter, qPCR was performed using primers specific for regions within the *Vegfr1* promoter and the amount of input DNA was compared to the amount of immuno-precipitated DNA. This was then compared to the amount of binding by PRH observed at the control *Gapdh* promoter, which gives the fold enrichment of Myc-PRH DNA binding. Figure 3.10 shows the relative position of PCR primer pairs that were used to analyze PRH DNA binding at the *Vegfr1* gene promoter, these primers cover a region of over 9 kilobases from -7500 to +1700 of the promoter in relation to the transcription start site (TSS). Enrichment of Myc-PRH immuno-precipitated regions of DNA is observed across the *Vegfr1* promoter as black bars, white bars represent the input DNA and grey bars represent the IgG immuno-precipitation control (figure 3.10). For regions bound by Myc-PRH the black bar will be greater than the white bar (input) and the grey bar (IgG). For regions not bound by

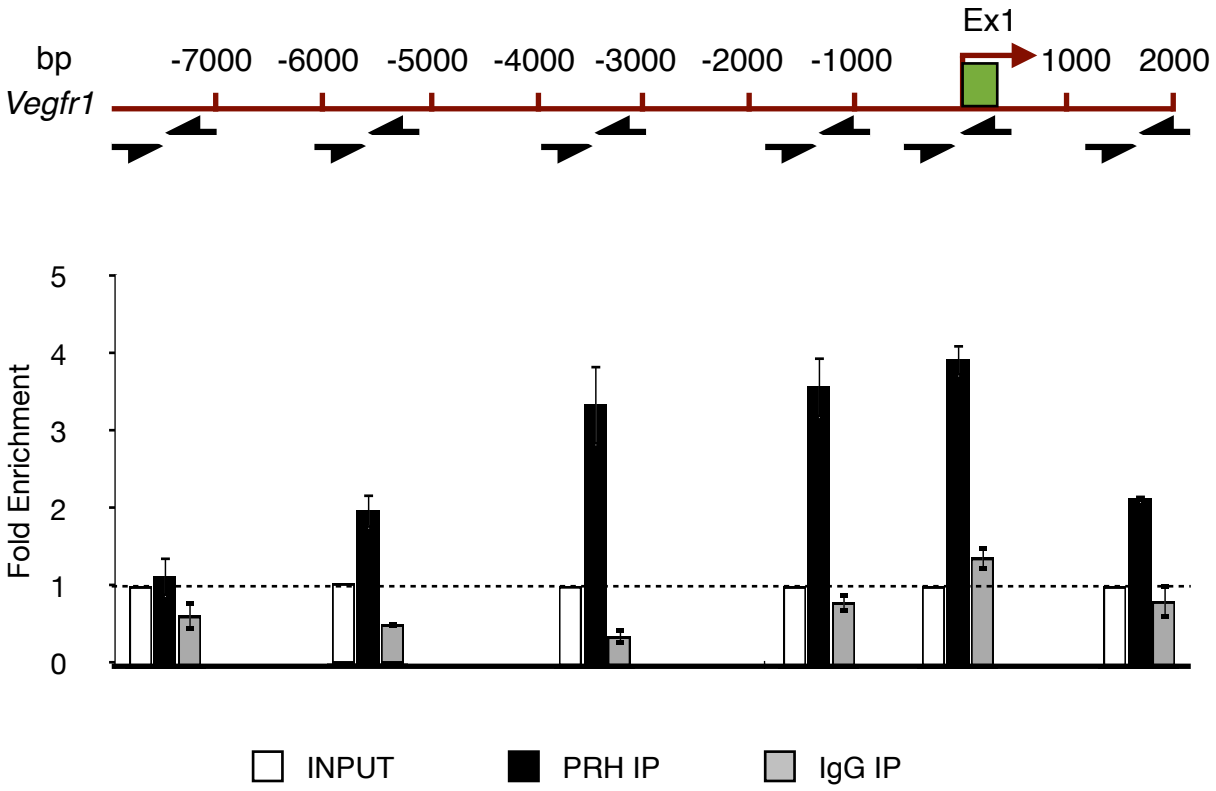


Figure 3.10 - PRH binds to multiple regions of the *Vegfr1* promoter.

Chromatin Immuno-precipitation of Myc-PRH from K562 cells transfected with 5 μg pMUG1 Myc-PRH. PRH enrichment levels (black bars) were compared to input DNA (white bars) and IgG enrichment levels (grey bars), which were determined by using purified DNA from the immuno-precipitation in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the *Gapdh* promoter. Dotted line represents the threshold of enrichment compared to the input DNA. Values are taken from a single immuno-precipitation and SD of 3 qPCR reactions. Data representative of 3 sets of chromatin.

Myc-PRH the black bar will be approximately equal or less than either the white bar or the grey bar. Myc-PRH does not bind to the region -7500 bp upstream of the *Vegfr1* TSS (figure 3.10) compared to the input DNA. However, Myc-PRH is found to be weakly associated with the *Vegfr1* promoter -5600 bp upstream of the TSS and 1700 bp downstream of the TSS (figure 3.10). Strong association of Myc-PRH with the *Vegfr1* promoter is observed at -3500 bp and -1200 bp upstream of the TSS, in addition to the region covering the *Vegfr1* TSS (figure 3.10). Therefore, PRH is

associated with the promoter of *Vegfr1* at multiple regions across approximately 7500 bp of promoter DNA.

To investigate whether mutation of the TLE interaction site in the N-terminal domain of PRH or a mutation in the DNA binding domain of PRH affects the binding of PRH to the *Vegfr1* promoter, 5*μ*g of pMUG1 vector expressing Myc-PRH, or Myc-PRH F32E, or Myc-PRH N187A was transfected into K562 cells. 24 hours after transfection chromatin was prepared as described above. Chromatin was then immuno-precipitated using an anti-Myc antibody or an anti-IgG as a control. Relative enrichment was analyzed across the *Vegfr1* promoter as described above. The binding of Myc-PRH F32E (figure 3.11 [B]) across the *Vegfr1* promoter gave a similar trend as observed for the wild-type Myc-PRH (figure 3.11 [A]). There was no binding observed for Myc-PRH F32E at the -7500bp region upstream from the *Vegfr1* TSS or at the +1700bp region downstream of the TSS (figure 3.11 [B]). However, Myc-PRH F32E binds to the -5600, -3500 and -1200bp regions upstream of the TSS and the region over the TSS (figure 3.11 [B]). The strongest binding observed for Myc-PRH F32E is at the proximal promoter regions, -1200bp upstream and the region covering the TSS (figure 3.11 [B]). The Myc-PRH N187A, DNA binding deficient mutant of PRH, does not immuno-precipitate any of these 6 regions across the *Vegfr1* promoter (figure 3.11 [C]). This suggests that mutation of the TLE interaction domain has no effect on the ability of PRH to bind to these region of the *Vegfr1* promoter, although mutation of the DNA binding domain of PRH does prevent association of PRH with the *Vegfr1* promoter.

To determine whether PRH and TLE both associate at the *Vegfr1* promoter, 5*μ*g of pMUG1 Myc-PRH, or 5*μ*g pCMV Flag-TLE-1, or 5*μ*g of each of these plasmids was transfected into K562 cells. 24 hours after transfection chromatin was prepared as described above. Chromatin was then immuno-precipitated using an anti-Myc

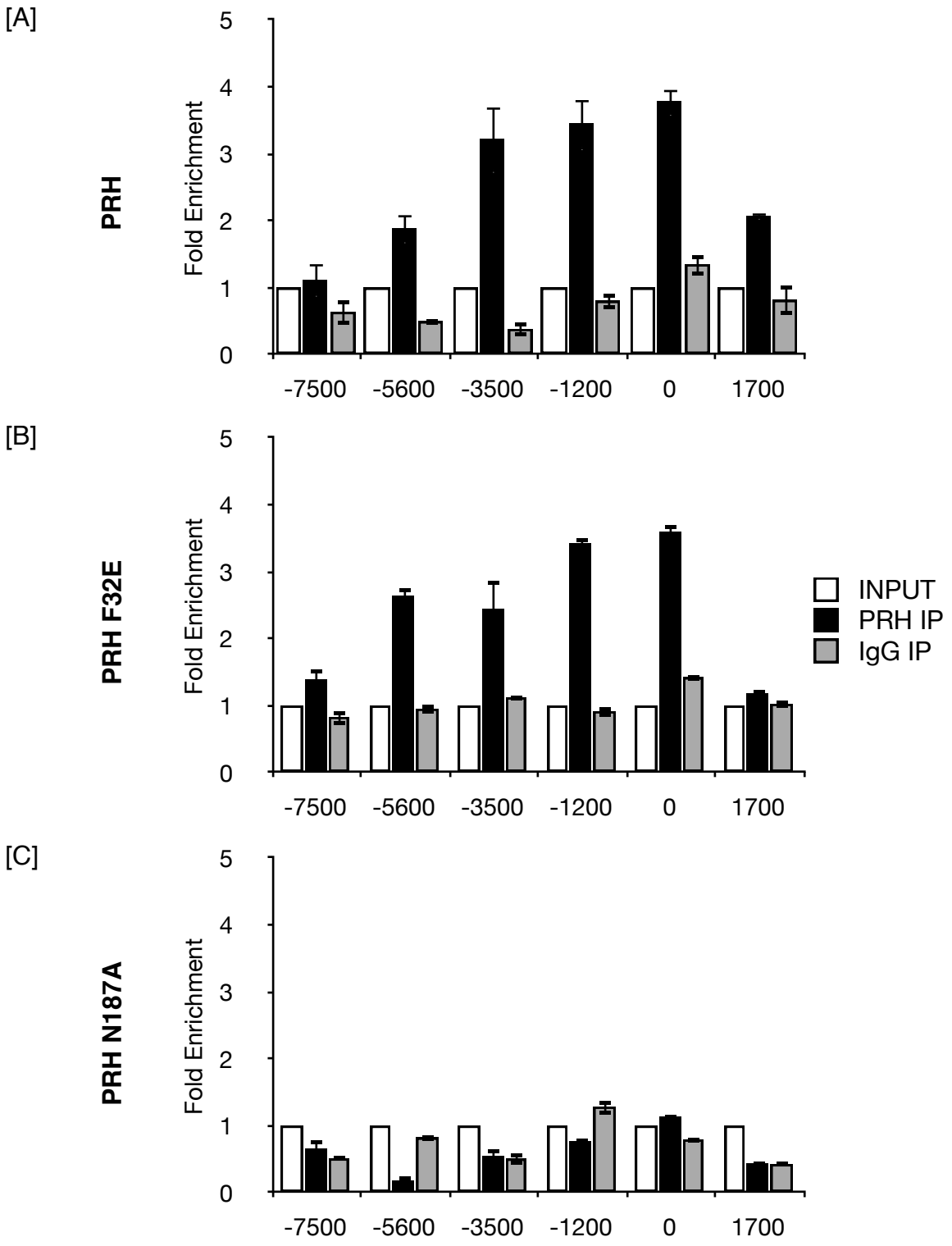


Figure 3.11 - PRH binding to the *Vegfr1* promoter requires a functional homeodomain but not interaction with TLE.

[A] Chromatin Immuno-precipitation of Myc-PRH from K562 cells transfected with 5 μg pMUG1 Myc-PRH. PRH enrichment levels (black bars) were compared to input DNA (white bars) and IgG enrichment levels (grey bars), which were determined by using purified DNA from the immuno-precipitation in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the Gapdh promoter. Values are taken from a single representative immuno-precipitation and SD of 3 qPCR reactions. Data representative of 3 sets of chromatin. [B] As for panel A except cells were transfected with 5μg pMUG1 Myc-PRH F32E. [C] As for panel A except cells were transfected with 5μg pMUG1 Myc-PRH N187A.

antibody, or an anti-Flag antibody or an anti-IgG as a control. Relative enrichment was analyzed across the *Vegfr1* promoter as described above. For the transfections involving Flag-TLE-1, immuno-precipitation of Flag-TLE-1 is represented by a dark red bar. For regions of the chromatin bound by Flag-TLE-1, the dark red bars will be greater than the white bar (input) and the grey bar (IgG). In cells expressing Flag-TLE-1 alone, Flag-TLE-1 is not strongly associated with the *Vegfr1* promoter (figure 3.12 [B]). However, Flag-TLE-1 does associate with the -1200bp upstream region of the *Vegfr1* promoter (figure 3.12 [B]). Yet, there is little association across the rest of the promoter regions that were assayed. Significantly, in cells expressing Myc-PRH and Flag-TLE-1, not only is binding of Myc-PRH observed across the *Vegfr1* promoter but Flag-TLE-1 is also associated strongly at the upstream regions, -5600, -3500, -1200bp from the TSS, and at the TSS (figure 3.12 [C]). Although no binding is observed for the -7500bp upstream region or the +1700bp downstream region where Myc-PRH is not strongly associated (figure 3.12 [C]). This suggests that PRH and TLE-1 bind to the *Vegfr1* promoter but TLE is not strongly associated with the promoter in the absence of PRH.

To further investigate the recruitment of TLE to the *Vegfr1* promoter, chromatin was prepared from K562 cells expressing Myc-PRH and Flag-TLE-1, or Myc-PRH F32E and Flag-TLE-1, or Myc-PRH N187A and Flag-TLE. Chromatin was then immuno-precipitated using an anti-Myc antibody, or an anti-Flag antibody or an anti-IgG as a control. Relative enrichment was analyzed across the *Vegfr1* promoter as described above. As described above for figure 3.12, in cells expressing Myc-PRH and Flag-TLE-1, both proteins immuno-precipitate the same regions of the *Vegfr1* promoter (figure 3.13 [A]). In contrast, in cells expressing Myc-PRH F32E and Flag-TLE-1, only Myc-PRH F32E is found associated with the *Vegfr1* promoter and no association is observed for Flag-TLE-1 (figure 3.13 [B]). Myc-PRH F32E is strongly associated to

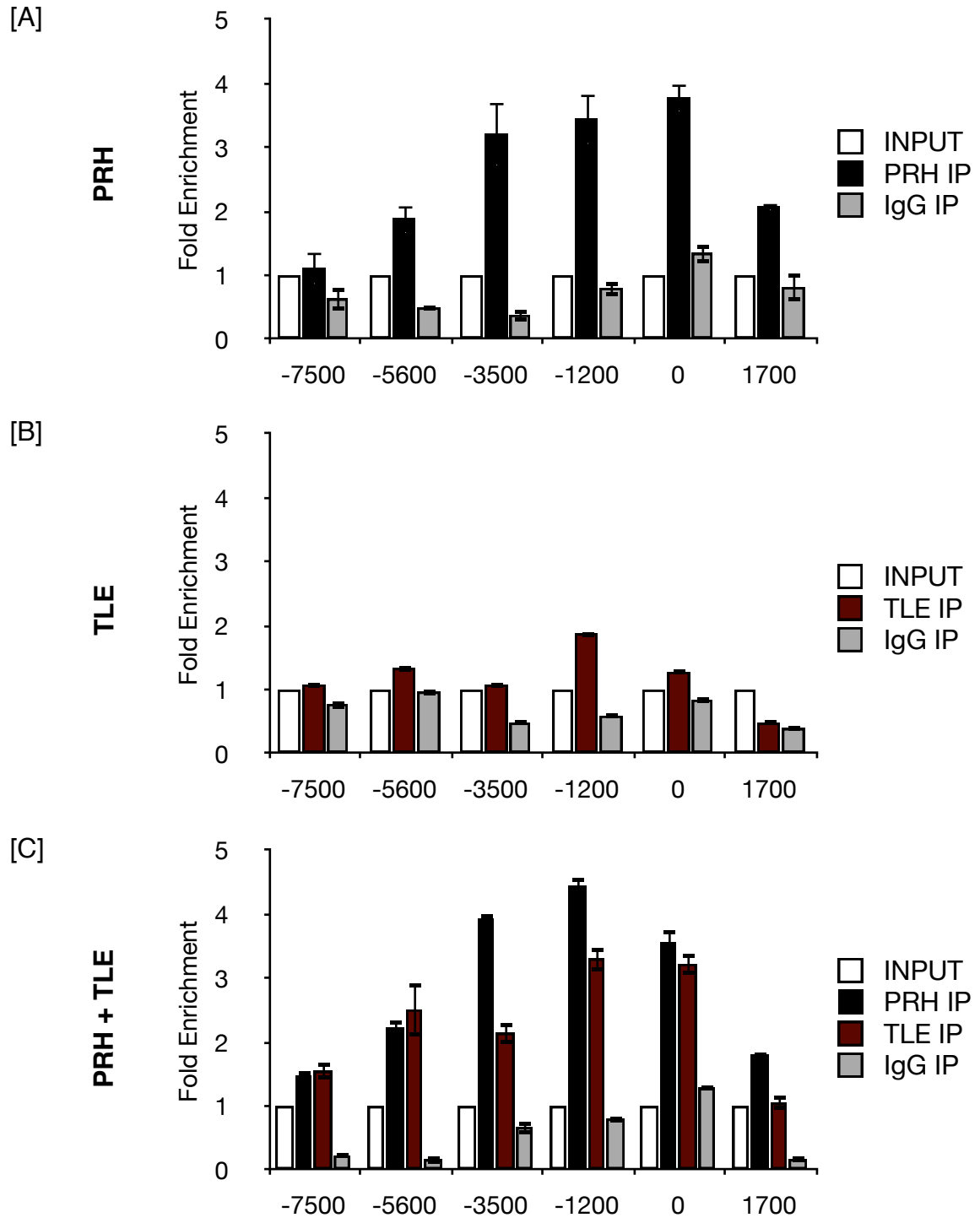


Figure 3.12 - TLE associates with the *Vegfr1* promoter in the presence of PRH.

[A] Chromatin Immuno-precipitation of Myc-PRH or Flag-TLE from K562 cells transfected with 5 μg pMUG1 Myc-PRH. PRH enrichment levels (black bars) or TLE enrichment levels (dark red bars) were compared to input DNA (white bars) and IgG enrichment levels (grey bars), which were determined by using purified DNA from the immuno-precipitation in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the Gapdh promoter. Values are taken from a single representative immuno-precipitation and SD of 3 qPCR reactions. As shown in 3.11. [B] As for panel A except cells were transfected with 5μg pCMV Flag-TLE. [C] As for panel A except cells were transfected with 5μg pMUG1 Myc-PRH and 5μg pCMV Flag-TLE. Data representative of 3 sets of chromatin.

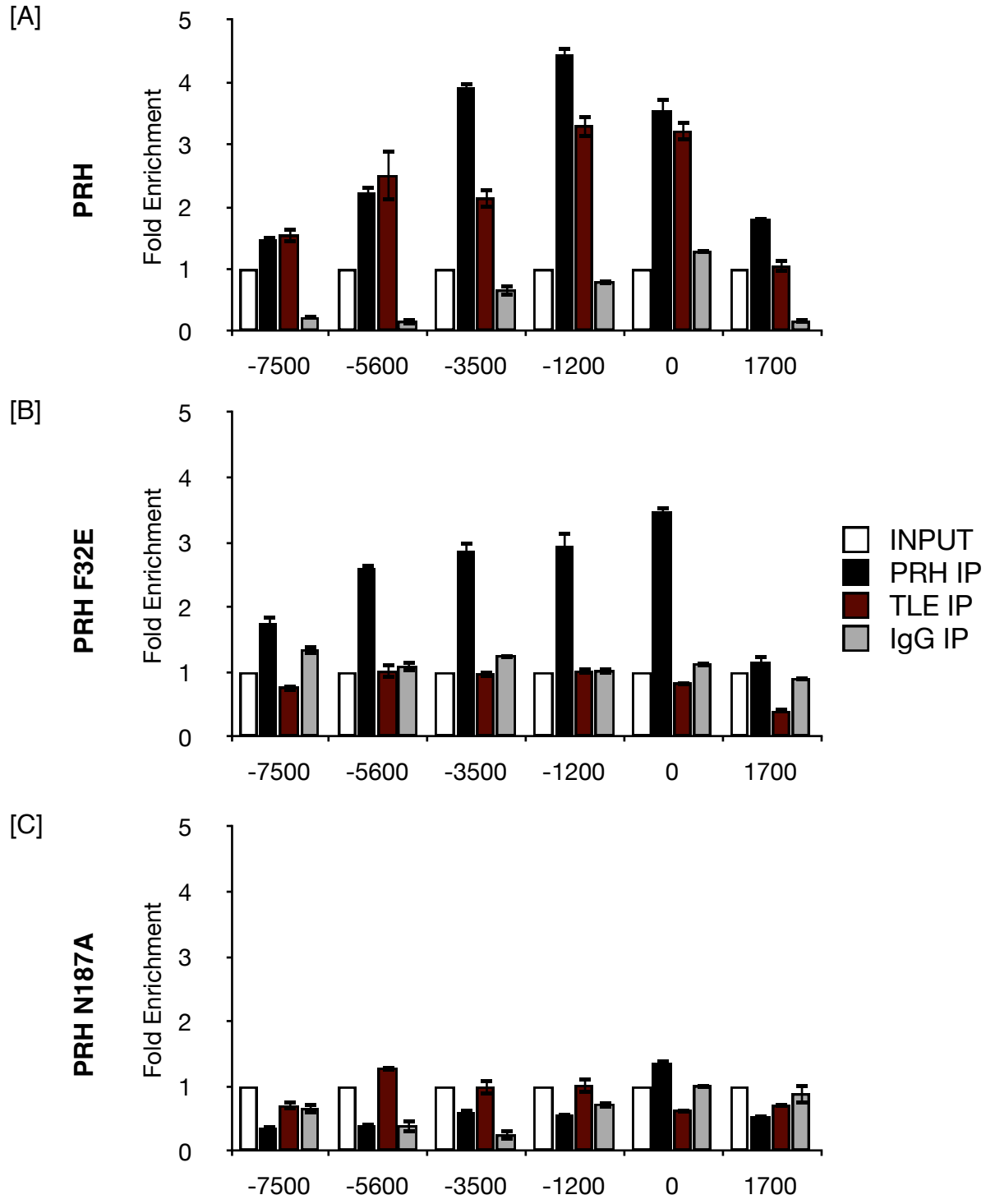


Figure 3.13 - TLE is not recruited to the *Vegfr1* promoter by PRH mutants that do not interact with TLE or bind to DNA.

[A] Chromatin Immuno-precipitation of Myc-PRH or Flag-TLE from K562 cells transfected with 5 μg pMUG1 Myc-PRH and 5μg pCMV Flag-TLE. PRH enrichment levels (black bars) or TLE enrichment levels (dark red bars) were compared to input DNA (white bars) and IgG enrichment levels (grey bars), which were determined by using purified DNA from the immuno-precipitation in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the Gapdh promoter. Values are taken from a single representative immuno-precipitation and SD of 3 qPCR reactions. [B] As for panel A except cells were transfected with 5μg pMUG1 Myc-PRH F32E. [C] As for panel A except cells were transfected with 5μg pMUG1 Myc-PRH N187A. Data representative of 3 sets of chromatin.

the upstream regions -5600, -3500 and -1200bp from the TSS, as well as at the TSS, similar to previous observations in cells expressing Myc-PRH F32E alone (figure 3.11 [B]). In cells expressing both Myc-PRH N187A and Flag-TLE-1, neither protein is associated at any of the regions assayed across the *Vegfr1* promoter (figure 3.13 [C]). These data confirm that the interaction between PRH and TLE-1 within the N-terminal domain of PRH is vital for the recruitment of TLE-1 to the *Vegfr1* gene.

To determine whether PRH binds at the promoter region of the *Vegf* gene, the same chromatin, as used above, from cells expressing Myc-PRH, or Myc-PRH F32E, or Myc-PRH N187A was analyzed as described above, at a single site at the *Vegf* gene promoter. Figure 3.14 [A] shows that in cells expressing Myc-PRH, PRH is bound to the *Vegf* gene promoter -4500bp from the TSS. In cells expressing Myc-PRH F32E, PRH F32E protein is also found associated with this *Vegf* promoter region (figure 3.14 [A]). However, in cells expressing Myc-PRH N187A, no PRH N187A is found associated with the -4.5 kb region upstream of the *Vegf* promoter (figure 3.14 [A]). This demonstrates that PRH directly binds to the *Vegf* promoter and that the interaction with TLE is not required for DNA binding.

To further establish whether TLE is associated at the *Vegf* gene where PRH binds, chromatin from cells expressing Myc-PRH, or Myc-PRH F32E, or Myc-PRH N187A with Flag-TLE-1, as used above, was analyzed as described above, at a single site at the *Vegf* gene promoter. In cells expressing TLE-1 alone no association of TLE is observed at the *Vegf* gene promoter (data not shown). In cells expressing Myc-PRH and Flag-TLE-1, PRH and TLE-1 are bound at -4500bp upstream from the TSS in the *Vegf* gene promoter (figure 3.14 [B]). Yet, in cells expressing Myc-PRH F32E and Flag-TLE-1, only PRH F32E protein is also found associated with this *Vegf* promoter region and no association of TLE-1 is observed (figure 3.14 [B]). However, in cells expressing Myc-PRH N187A and Flag-TLE-1, neither PRH N187A or TLE-1 are

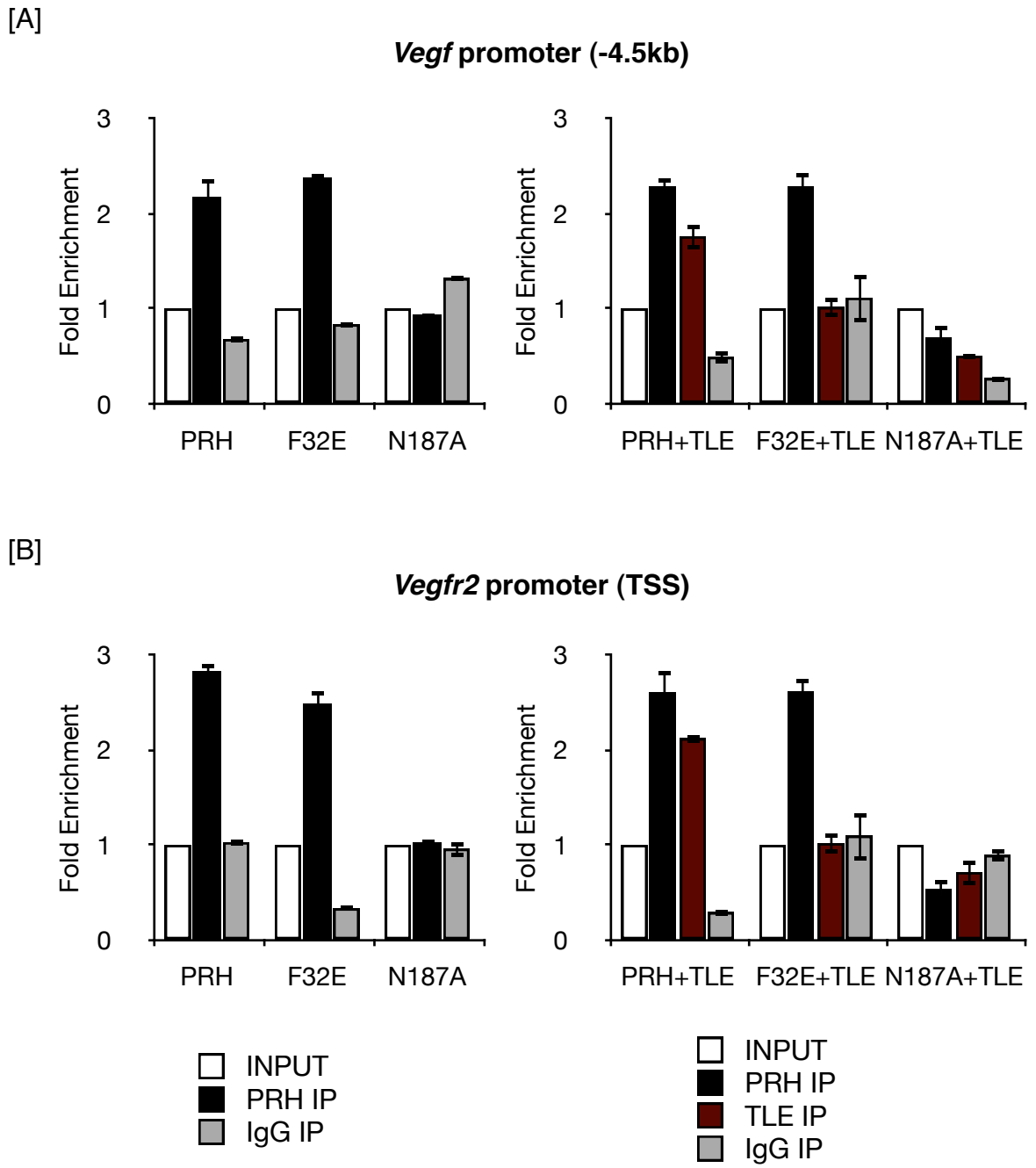


Figure 3.14 - PRH binds to the *Vegf* and *Vegfr2* promoters and recruits TLE.

[A] Chromatin Immuno-precipitation of Myc-PRH from K562 cells transfected with 5 μg pMUG1 plasmid expressing Myc-PRH or Myc-PRH F32E or Myc-PRH N187A. PRH enrichment levels (black bars) were compared to input DNA (white bars) and IgG enrichment levels (grey bars), which were determined by using purified DNA from the immuno-precipitation in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the Gapdh promoter. Values are taken from a single representative immunoprecipitation and SD of 3 qPCR reactions. [B] Chromatin Immuno-precipitation of Myc-PRH or Flag-TLE from K562 cells transfected with 5 μg pMUG1 plasmid expressing Myc-PRH or Myc-PRH F32E or Myc-PRH N187A and 5μg pCMV Flag-TLE. Enrichment levels were determined as for panel A, TLE enrichment is represented by dark red bars. [C] As for panel A except using *Vegfr2* promoter specific primers. [D] As for panel B except using *Vegfr2* promoter specific primers.

found associated with the -4500bp upstream region of the *Vegf* promoter. This suggests that PRH directly binds to the *Vegf* promoter and recruits TLE-1 to the promoter.

To determine whether PRH also binds to the *Vegfr2* promoter, the same chromatin from cells expressing Myc-PRH, or Myc-PRH F32E, or Myc-PRH N187A, as above, was analyzed as described above, at a single site at the TSS of the *Vegfr2* gene. PRH is bound to the *Vegfr2* gene promoter at the TSS, in cells that express Myc-PRH (figure 3.14 [C]). PRH F32E protein also binds to the TSS of the *Vegfr2* gene, in cells that express Myc-PRH F32E (figure 3.14 [C]). Although PRH N187A is not found to associate with the TSS of the *Vegfr2* promoter, in cells expressing Myc-PRH N187A (figure 3.14 [C]). This data suggests that PRH directly binds to the *Vegfr2* promoter and that the PRH F32E mutant is able to bind DNA.

To further establish whether TLE can associate with PRH at the *Vegfr2* gene, chromatin from cells expressing Myc-PRH, or Myc-PRH F32E, or Myc-PRH N187A with Flag-TLE-1, as used above, was analyzed as described above, at a single site at the TSS of the *Vegfr2* gene. Cells that express TLE-1 alone have no association of TLE-1 at the *Vegfr2* gene TSS (data not shown). In cells expressing Myc-PRH and Flag-TLE-1, PRH and TLE-1 are associated with the TSS region in the *Vegfr2* gene (figure 3.14 [D]). Yet, in cells expressing Myc-PRH F32E and Flag-TLE-1, the PRH F32E protein associates with this region of the *Vegfr2* gene but no association of TLE-1 is observed (figure 3.14 [D]). However, in cells expressing Myc-PRH N187A and Flag-TLE-1, neither PRH N187A or TLE-1 associate with the *Vegfr2* gene TSS (figure 3.14 [D]). This suggests that PRH directly binds to the *Vegfr2* gene proximal promoter and recruits TLE-1 to the promoter.

3.5 PRH induces nuclear retention of TLE.

TLE exists in multiple phosphorylation states (Buscarlet et al., 2009; Nuthall et al., 2004; Nuthall et al., 2002b; Nuthall et al., 2002a; Eberhard et al., 2000; Husain et al., 1996). Within the haematopoietic compartment TLE can partner with several DNA-binding proteins including Hes-1 (Fisher et al., 1996), Runx-1 (Aronson et al., 1997) and Pax5 (Linderson et al., 2004), as well as PRH (Swingler et al., 2004). It has been shown that several of these proteins have a role in regulating TLE phosphorylation and modulating its activity (Nuthall et al., 2002a; Eberhard et al., 2000). For example expression of Hes1 can induce CK2-dependent hyper-phosphorylation of TLE, which can be detected by retarded mobility of TLE proteins on a SDS-PAG (Nuthall et al., 2002a). Western blot detection of TLE proteins show that there are 2 bands, the lower band corresponds to hypo-phosphorylated TLE and the upper band corresponds to hyper-phosphorylated TLE (figure 3.15). This phosphorylation increases the nuclear retention of TLE proteins (Nuthall et al., 2002a).

To determine whether over-expressed PRH can alter the phosphorylation status and nuclear retention of TLE, pMUG1 or pMUG1 Myc-PRH was transfected into K562 cells. After 48 hours the cells were harvested, and nuclear and post-nuclear extracts were isolated. Post-nuclear extracts contain all cytoplasmic proteins and some loosely associated nuclear proteins. The nuclear extracts contain tightly associated nuclear proteins. Equal amounts of protein were loaded and run on a SDS-PAG. The SDS-PAG was transferred to Immobilon P membrane and Western blotted with an antibody that recognizes multiple endogenous TLE proteins (anti-panTLE antibody) (figure 3.15 - top panel). The blot was stripped of antibody and re-probed with an anti-Myc-tag (9E10) antibody (figure 3.15 - upper middle panel) for exogenous Myc-PRH, or anti-Lamin A/C antibody (figure 3.15 - lower middle panel) or anti-Tubulin antibody (figure 3.15 - bottom panel). Lamin A/C and Tubulin antibodies were used

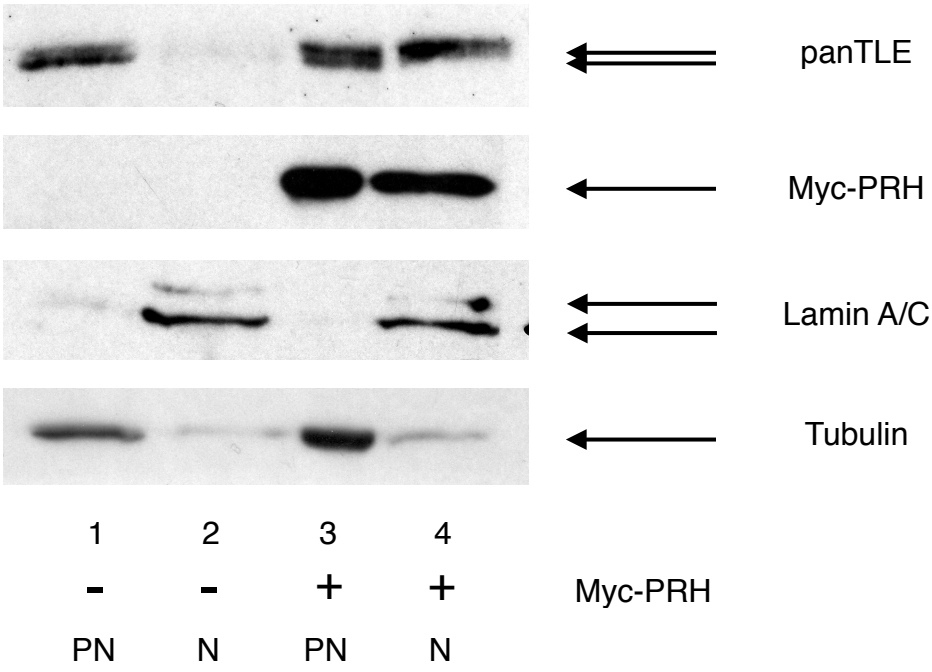


Figure 3.15 - PRH increases TLE nuclear retention.

Untransfected K562 cells and cells transfected with pMUG1 Myc–PRH were fractionated into post-nuclear (PN) and nuclear (N) extracts. The proteins were then separated by SDS-PAGE and western blotted for endogenous TLE using a pan-TLE antibody (top panel), Myc–PRH using an anti-Myc antibody (upper middle panel), Lamin A/C (lower middle panel) and Tubulin (bottom panel). Data representative of three independent experiments.

as fractionation and loading controls. In un-transfected K562 cells endogenous TLE proteins are primarily localized to the post-nuclear compartment (figure 3.15 - top panel, lane 1) and the relative intensity of the two proteins detected by the panTLE antibody suggests that the majority of TLE is hypo-phosphorylated (figure 3.15 - top panel, lane 1, lower band). However, in Myc-PRH transfected cells, TLE becomes equally distributed between the nuclear and post-nuclear compartments (figure 3.15 - top panel, lanes 3 and 4). Furthermore, the relative intensity of the two bands detected suggests that the majority of TLE is now hyper-phoshorylated in both the nuclear and post-nuclear compartments (figure 3.15 - top panel, lanes 3 and 4, upper

band). Myc-PRH is present in both the nuclear and post-nuclear fractions (figure 3.15 - upper middle panel, lanes 3 and 4). These results suggests that PRH expression increases nuclear retention of TLE proteins, as well as influencing the hyper-phosphorylation of TLE proteins.

To investigate whether the interaction of PRH and TLE is required for nuclear retention of TLE by PRH, the PRH F32E mutant was expressed in K562 cells. Extracts were prepared and run as previously described. The Western blot was stained with antibodies to panTLE (figure 3.16 - top panel) and re-probed with an anti-Myc-tag (9E10) antibody (figure 3.16 - upper middle panel), or anti-Lamin A/C antibody (figure 3.16 - lower middle panel) or anti-Tubulin antibody (figure 3.16 - bottom panel). Again Lamin A/C and Tubulin antibodies were used as fractionation and loading controls. As previously observed over-expression of exogenous wild-type PRH increases nuclear retention of endogenous TLE (figure 3.16 - top panel, lane 5) compared to control cells (figure 3.16 - top panel, lane 4). However, over-expression of PRH F32E is unable to increase the nuclear retention of endogenous TLE (figure 3.16 - top panel, lane 6). PRH F32E also has no effect on the phosphorylation status of endogenous TLE (figure 3.16 - top panel, lanes 3 and 6) compared to control cells (figure 3.16 - top panel, lanes 1 and 4). Since it has been shown that this mutation abolishes the interaction of PRH and TLE we conclude that interaction between PRH and TLE is required for the nuclear retention and hyper-phosphorylation of endogenous TLE.

PRH nuclear localization is reported to be mediated by two arginine residues (188 and 189) in the PRH homeodomain (figure 3.6), within the DNA recognition helix (Topisirovic et al., 2003a). In K562 cells this mutation does not influence PRH localization (Desjobert et al., 2009) but it does disrupt DNA binding (Unpublished observations, Jayaraman et al.). To determine whether DNA binding by PRH is

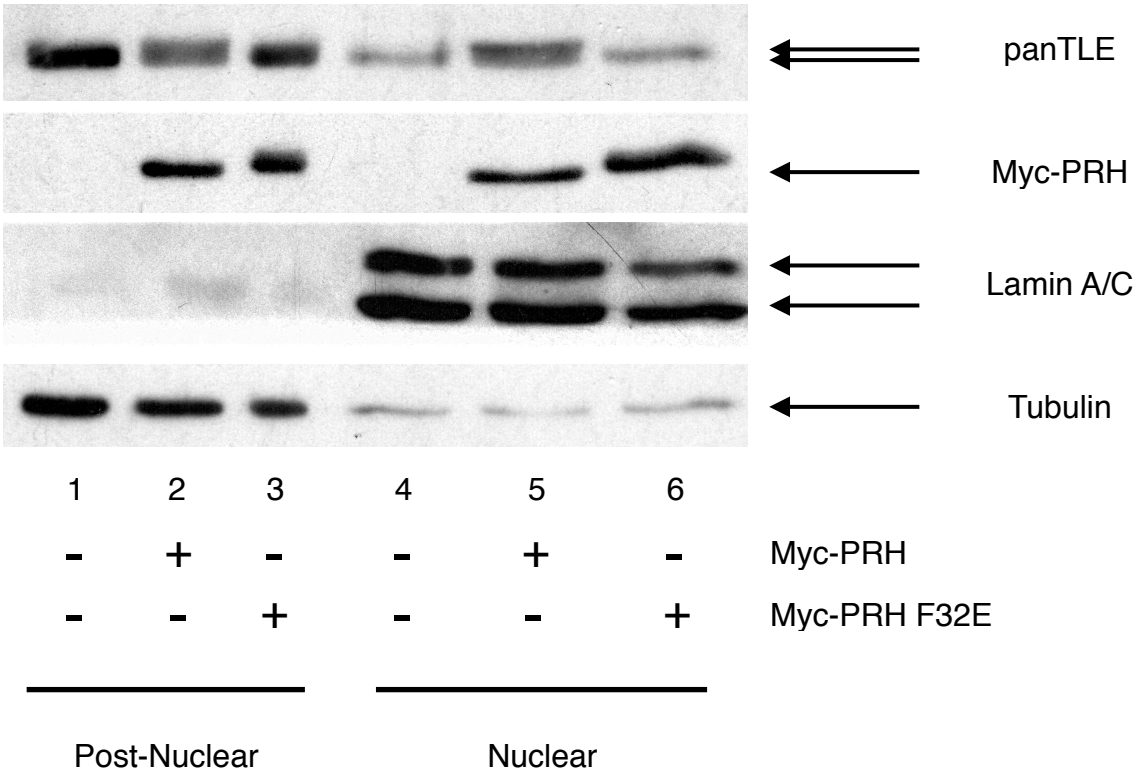


Figure 3.16 - PRH requires the TLE binding motif for increased nuclear localization of TLE.

Un-transfected K562 cells and cells transfected with pMUG1 containing Myc–PRH and Myc–PRH F32E were fractionated into post-nuclear and nuclear extracts. Proteins were separated by SDS-PAGE and transferred to an Immobilon P PVDF membrane. Proteins were stained with panTLE (top panel), Myc (upper middle panel), Lamin A/C (lower middle panel) and Tubulin (bottom panel). Data representative of three independent experiments.

required for nuclear retention of endogenous TLE, PRH N187A or PRH RR188,189AA were expressed in K562 cells. Extracts were prepared and run as previously described. The Western blot was as described above. Lamin A/C (figure 3.17 - lower middle panel) and Tubulin (figure 3.17 - bottom panel) antibodies were used as fractionation and loading controls. The PRH N187A mutant was unable to hyper-phosphorylate or retain TLE to the nuclear fraction (figure 3.17 - top panel, lane 7), in contrast to the wild-type PRH (figure 3.17 - top panel, lane 6). Similarly, PRH RR188,189AA was unable to hyper-phosphorylate or retain endogenous TLE in

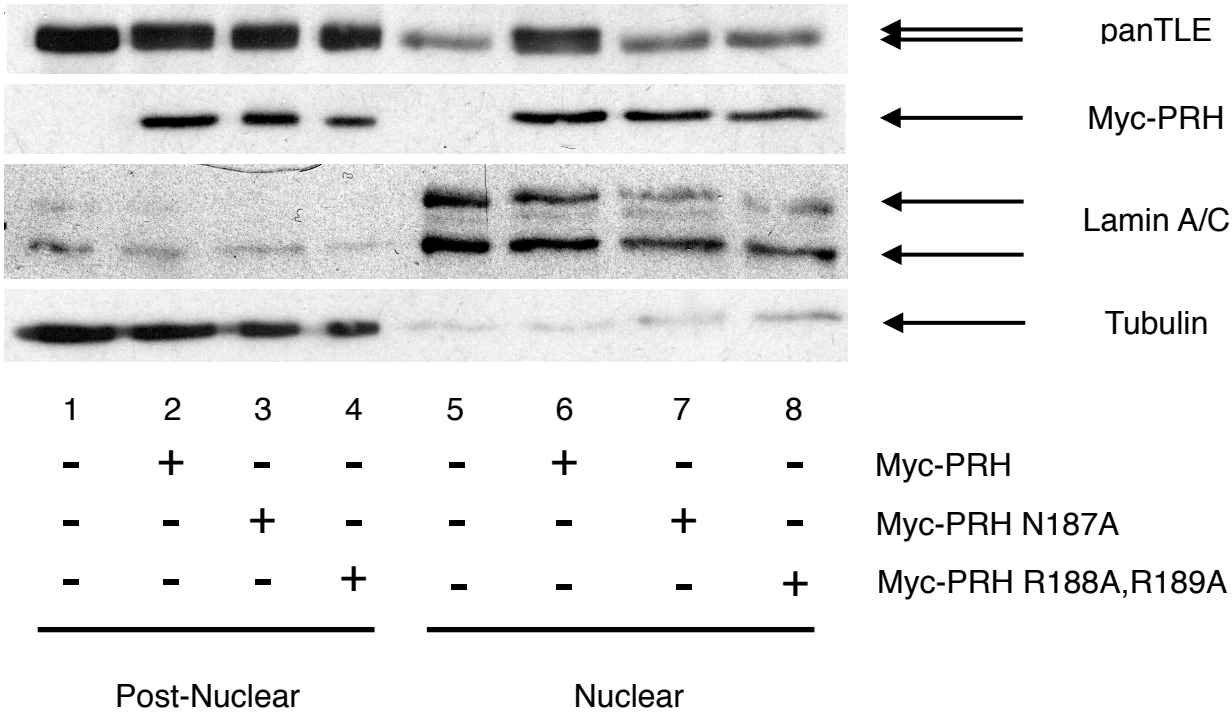


Figure 3.17 - PRH DNA binding capacity is required for increased nuclear localization of TLE.

Untransfected K562 cells and cells transfected with pMUG1 containing Myc–PRH or Myc–PRH N187A or Myc-PRH RR188,189AA were fractionated into post-nuclear and nuclear extracts. Proteins were separated by SDS-PAGE and transferred to an Immobilon P PVDF membrane. Proteins were stained with panTLE (top panel), Myc (upper middle panel), Lamin A/C (lower middle panel) and Tubulin (bottom panel). Data representative of three independent experiments.

the nuclear fraction (figure 3.17 - top panel, lane 8). Since both the PRH N187A and PRH RR188,189AA mutants are found in the tightly associated nuclear compartment and are defective in DNA binding, it can be concluded that DNA binding by PRH is essential for both the nuclear retention and hyper-phosphorylation of endogenous TLE.

3.6 Discussion

Considering the importance of the VSP genes in normal physiological processes such as angiogenesis and their contribution to malignancy, it is surprising that so little is known about direct negative regulators of these genes. This chapter demonstrates

a direct role for PRH and its co-repressor TLE, as negative regulators of *Vegf, Vegfr1* and *Vegfr2.* Transcriptional repression of the *Vegf* and *Vegfr1* genes is observed when PRH is over-expressed, and knock-down of PRH de-represses *Vegf, Vegfr1* and *Vegfr2* expression. Mutations to the DNA binding domain or mutation of the TLE interaction motif of PRH prevent transcriptional repression. PRH is known to interact with TLE via the Eh-1 motif in the N-terminal domain of PRH, and although this interaction has been shown to be significant for the transcriptional repression of an artificial promoter (Swingler et al., 2004), it was unknown whether the interaction was functional at an endogenous PRH target gene. However, this chapter shows that PRH and TLE co-operate to repress both *Vegf* and *Vegfr1* gene expression, and PRH and TLE will also bind together at the promoters of these genes. Importantly, TLE is not recruited to these promoter regions by the PRH F32E mutant, which does not interact with TLE, but does bind DNA.

Over-expression of TLE-1 alone represses *Vegf* and *Vegfr1* gene transcription. However, TLE-1 repression of *Vegf* and *Vegfr1* is PRH dependent because no repression is observed when TLE-1 is expressed in PRH knock-down cells. This suggests that TLE maybe a limiting factor for further repression of *Vegf* and *Vegfr1*. To support this hypothesis over-expression of TLE-1 alone is weakly associated with the *Vegfr1* promoter, suggesting that the exogenous TLE is binding to the endogenous PRH bound at the *Vegfr1* promoter. Interestingly, TLE-1 association is not observed when Myc-PRH N187A is co-expressed with TLE-1. Immuno-fluorescence staining of TLE-1 and PRH N187A, has shown that PRH N187A tightly binds TLE-1 and they congregate in large foci in the nucleus but not the chromatin compartment (Desjobert et al., 2009). Furthermore, the PRH N187A protein acts as a trans-dominant negative against wild-type PRH by diminishing available endogenous TLE (Desjobert et al., 2009). Therefore, it is likely that over-expression of PRH

N187A sequesters TLE-1 in these foci. Also, TLE-1 is not associated with the *Vegfr1* promoter DNA when co-expressed with PRH F32E. Although, since PRH F32E binds to this region it may be that PRH F32E dilutes the amount of endogenous PRH that can bind TLE-1 at the *Vegfr1* promoter.

Over-expression of exogenous PRH induces nuclear retention of endogenous TLE proteins and this requires PRH DNA binding ability, suggesting that the function of PRH and probably the localization of PRH is important for the regulation of TLE. Interestingly, the ability of PRH to localize to the nucleus is not affected by mutating the DNA binding domain of PRH at residue 187 and so PRH localization to the nucleus alone is not sufficient for nuclear retention of TLE. It is most likely that DNA binding by PRH and the association of other proteins, recruited during DNA binding, are vital for TLE nuclear retention. In ROS17/2.8 cells, exogenous expression of Hes-1 can induce hyper-phosphorylation and induce the nuclear retention of TLE (Nuthall et al., 2002a). This hyper-phosphorylation of TLE is CK2 dependent. Inhibition of CK2 in cells expressing Hes-1 reduces TLE nuclear retention and this CK2 hyper-phosphorylated form of TLE is associated with chromatin. Exogenous expression of PRH also induces hyper-phosphorylation of TLE (Desjobert et al., 2009). Therefore it is likely that the post-translational modification of TLE is required for nuclear retention, and this occurs when TLE is recruited to the DNA by transcription factors, including PRH.

Out of the three genes, regulation of the *Vegfr1* gene is the least characterized. As described in the introduction (see 1.4.2.1 and figure 1.7), *Vegfr1* is positively regulated by HIF-2α and Ets-1 in response to hypoxia or EGF/FGF signalling. Interestingly, the binding sites for these proteins are within the first 100 bp upstream of the TSS, with a second HRE around 1 kb upstream of the TSS. ChIP experiments show that PRH bound strongly around the TSS and to a region -1.2 kb upstream from

the TSS of the *Vegfr1* gene. Therefore, it is likely that PRH induced transcriptional repression of *Vegfr1* could be acting through blocking the binding of transcriptional activators. Previous work that showed that PRH only weakly interacts with the region about the TSS of the *Vegfr1* gene and more strongly at the -1.2kb upstream region (Noy et al., 2010). This work also showed that when using *Vegfr1* luciferase reporter constructs the activity of the promoter was elevated if the -700 to -1200 region was removed, suggesting endogenous PRH is associated with this region. However, the *Vegfr1* promoter reporter constructs containing promoter fragments from -700 to +155 or -200 to +155 were strongly repressed by exogenous PRH. Strong association of endogenous PRH to the -1.2 kb upstream region of the *Vegfr1* promoter would explain why *Vegfr1* expression is elevated in PRH knock-down cells and further support ChIP results that demonstrate association of TLE with this region.

It is now widely accepted that large multimeric complexes of proteins are required to activate transcription, through the formation of the mediator and the RNAP II complexes (see 1.1 for details). Large multimeric complexes are also formed for transcriptional repression. The region -1 kb upstream of the TSS of the *Vegfr1* gene contains a cluster of six ATTA or TAAT elements within 500 bp, and the central four core homeodomain binding sequences are clustered within 200 bp. The *Goosecoid* promoter is the only other promoter, that PRH represses, that has been analyzed in detail, and PRH binds to a region within this promoter that contains 5 ATTA sites within a 200 bp region (Williams et al., 2008). It is tempting to suggest that PRH regulates this region of the *Vegfr1* promoter as an oligomer that wraps the promoter DNA around an octameric PRH core, in a similar fashion to that proposed for the *Goosecoid* gene. Using the approximate molecular weight of a PRH octamer, Williams et al calculated the length of DNA that could wrap around a PRH octamer and found it to be one and a half times the length of DNA a nucleosome wraps

around itself (Williams et al., 2008). Therefore a PRH octamer could wrap approximately 200 bp fragments of DNA around its circumference (Williams et al., 2008). Thus suggesting that PRH could preferentially bind to clusters of ATTA DNA sequences, as found in the *Vegfr1* promoter. Interestingly, there are other ATTA clusters in the *Vegfr1* promoter including one around -5.6 kb upstream of the TSS, a region PRH also binds.

TLE co-represses *Vegfr1* expression, and is capable of forming oligomeric structures that are critical for TLE induced transcriptional repression (Chen et al., 1998). TLE recruits further factors such as HDACs that can alter and compact the DNA (Chen et al., 1999). Consequently, interaction of PRH and TLE would multiply the depletion attraction forces and could contribute to the spreading of TLE (Martinez and Arnosti, 2008) or possibly facilitating the cooperative binding of PRH to less favourable DNA binding sequences. Therefore, PRH repression of the *Vegfr1* gene is likely to be one of three potential models for repression (figure 3.18). PRH could binds to single PRH binding sites in the promoter and recruit co-repressors to repress transcription. Repression would be realized through inhibiting the binding of other transcription factors that activate gene expression. In this model PRH would inhibit the accessibility of transcription factor binding sites through recruitment of TLE and HDACs that increase chromatin compaction. In the second model, PRH could bind to clustered core ATTA sites as an oligomer, thus condensing local regions of chromatin by wrapping DNA (Soufi and Jayaraman, 2008). In addition PRH could recruit co-repressors and HDACs to further condense this local structure, again inhibiting transcription factor binding sites but over a larger area. This could result in looping of DNA, as PRH oligomers bound at distant promoter regions (1-2 kb away from each other), which contributes to condense the higher order chromatin structure. Importantly, there would still be accessible promoter DNA within the looped regions.

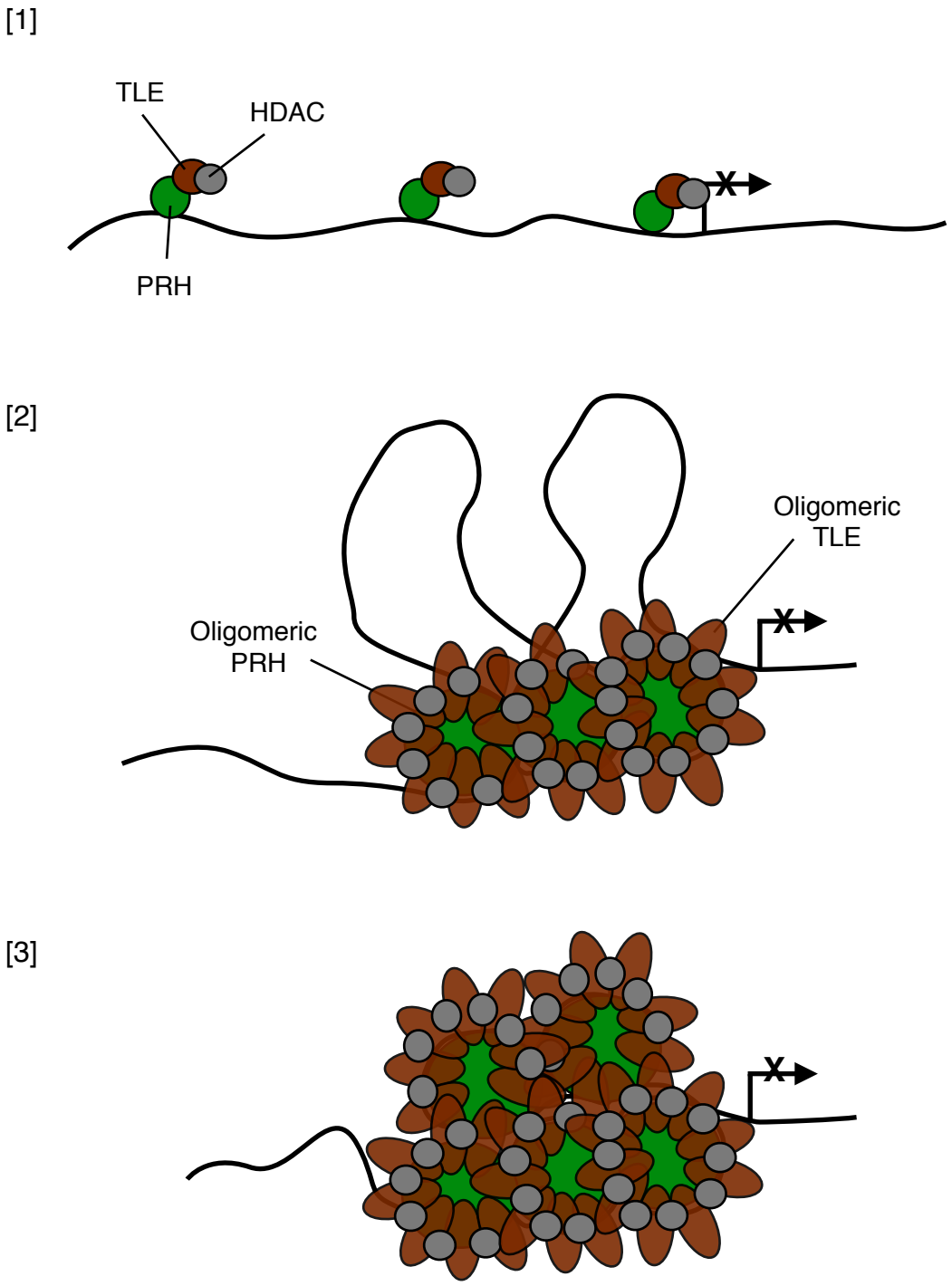


Figure 3.18 - Proposed models for PRH and TLE transcriptional repression.

The diagram illustrates three models for PRH and TLE gene repression. [1] PRH binds to single sites within the promoter of the target gene, PRH recruits co-repressors and inhibits binding of other transcription factors where PRH is bound. [2] PRH binds as an oligomeric complex to the promoter and recruits co-repressors which act together to condense chromatin around the binding sites. However, there are loops of DNA that are still accessible to other transcription factors but large areas surrounding the PRH binding site are inaccessible. [3] PRH binds as an oligomeric complex to the promoter and recruits co-repressors which act together to condense chromatin and spread condensing large areas of DNA even in the absence of specific PRH binding sites.

In the third model, PRH binds to multiple binding sites as an oligomer and wraps the DNA around its octameric core. Oligomerization of PRH octamers condenses chromatin structure and facilitates cooperative binding to low affinity PRH binding sites, allowing PRH to spread along the chromatin fiber. PRH also recruits co-repressors, which increases the compaction further condensing chromatin structure, so the entire promoter is virtually inaccessible for transcription factor binding.

VEGF and the VEGF receptors, VEGFR1 and VEGFR2, are essential survival factors for haematopoietic stem cells (Gerber et al., 2002), and control proliferation and survival in endothelial cells (Hoeben et al., 2004). Intriguingly, as shown in this chapter, PRH transcriptionally represses all three genes coordinately. Therefore, manipulation of PRH activity will directly influence the VSP and its downstream targets, potentially controlling cell survival or proliferation. The implications of this regulation will not be discussed here as the following chapter will investigate the importance of PRH regulation of the VSP genes on cell growth and survival.

VEGF:VEGF receptor axis controls cell survival

4 VEGF:VEGF receptor axis controls cell survival.

4.1 Introduction

The role of PRH on the regulation of cell growth, differentiation and survival has predominantly been investigated during development. PRH controls the differentiation and proliferation of blast colony-forming cells (BL-CFC) produced from embryonic stem cell-derived embryoid bodies, BL-CFC have similar characteristics to the haemangioblast present in the early embryo (Kubo et al., 2005; Guo et al., 2003). Yet the influence of PRH on the adult system is less well characterized and the role of PRH on the proliferation of the myeloid haematopoietic compartment is not well understood. This chapter will characterize the role of PRH in growth and survival of human haematopoietic cells of myeloid lineage through perturbing the expression level of PRH. In addition the contribution of VEGF signalling to growth will be assessed using drugs to manipulate VEGF signalling and by over-expressing components of the VSP.

4.2 PRH inhibits cell growth

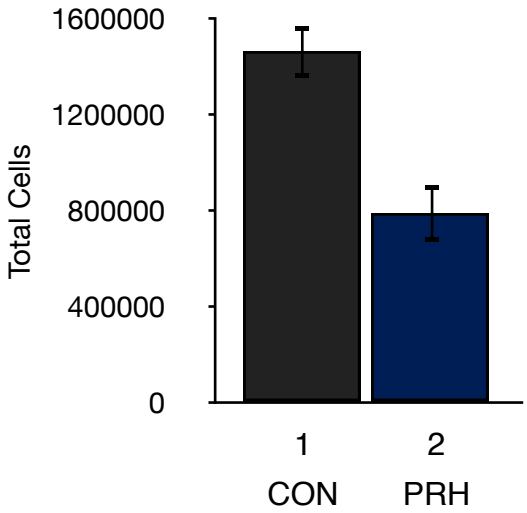
To investigate the effect of PRH expression on cell growth, K562 cells were transfected with 5μg pMUG1 Myc-PRH or 5μg pMUG1 (as a control). Cells were counted, using trypan blue exclusion (as described in section 2.4.4 of the Materials and Methods chapter), at 24, 48 and 72 hours post-transfection. After 72 hours there is a significant decrease in cell growth for the PRH over-expressed cells (figure 4.1 [A] - lane 2) compared to the control cells (figure 4.1 [A] - lane 1). This data shows that PRH is decreasing the growth of K562 cells.

To further investigate the effect of PRH on cell growth, K562 cells were transfected with pEGFP in addition to pMUG1 Myc-PRH or pMUG1 (control). The expression of EGFP was observed using fluorescence assisted cell sorting (FACS) at 24, 48 and 72 hours post transfection. The number of EGFP expressing cells at 24 hours was set as 100% for both populations of cells. Both control and PRH expressing cells had equivalent transfection efficiencies. Figure 4.1 [B] shows that cells expressing PRH have a strong reduction in EGFP expression over 72 hours and express 50% less EGFP by 72 hours post-transfection. However, control cells maintain 90% expression of EGFP over 72 hours after transfection (figure 4.1 [B]). This data indicates that PRH expression may be altering cell proliferation or survival.

To determine whether PRH inhibits cell cycle checkpoint progression, EGFP and PRH expressing cells or EGFP control cells were permeabilized and stained with propidium iodide, 24 hours post-transfection, and analyzed using FACS. Propidium iodide stains the DNA and can be used to analyze the proportion of cells in each phase of the cell cycle. The amount of DNA in a cell doubles during S phase, therefore the amount of DNA in the cell indicates which phase of the cell cycle the cell is in. In FACS cells are first identified by their morphology, using forward scatter (FSC) and side scatter (SSC) parameters, and the DNA content of cells is then analyzed by the intensity of propidium iodide staining (FL3). Figure 4.2 shows a representative set of data for cells expressing PRH and control cells. There is little difference in the distribution of cells throughout the cell cycle in PRH transfected cells compared to control cells. Thus PRH does not appear to be influencing cell cycle progression.

To determine whether PRH alters cell survival by increasing the amount of apoptosis, the PRH transfected and control cells were co-stained with an Annexin V APC antibody and propidium iodide, 24 hours post-transfection and analyzed using FACS.

[A]



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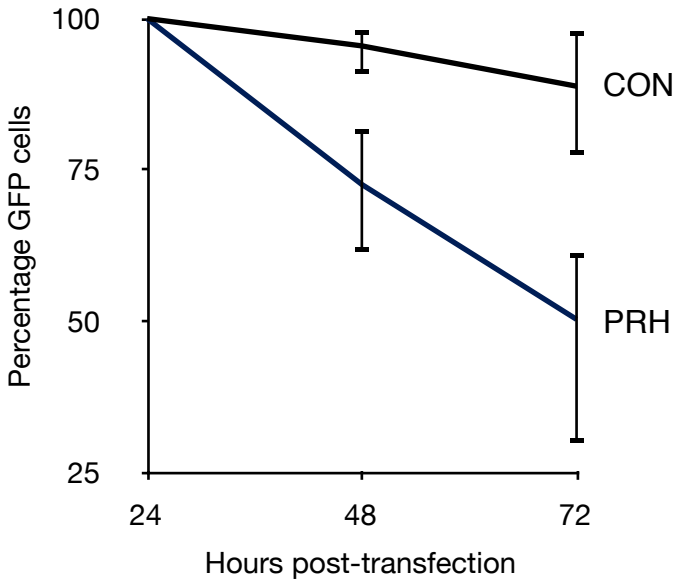
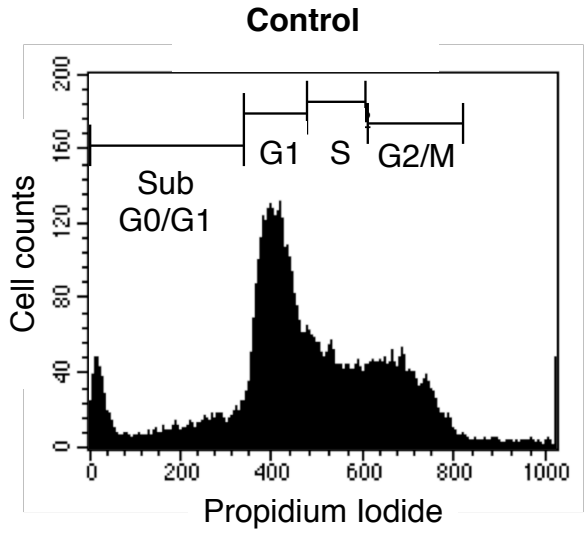
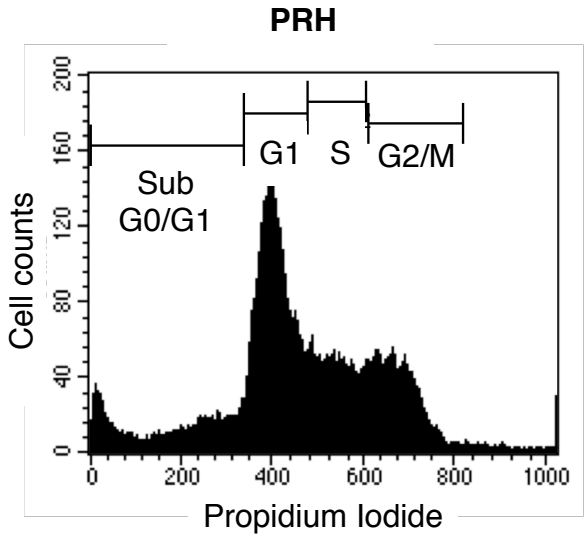


Figure 4.1 - The effect of PRH on cell number.

[A] Cells transfected with pMUG1 (lane 1) or pMUG1-Myc-PRH (lane 2). Total number of cells, counted using trypan blue exclusion, 72 hours after transfection. Values are means and standard deviation (n=3). [B] Cells transfected with pEGFP alone or pEGFP and pMUG1-Myc-PRH. The percentage of EGFP-expressing cells 24 h post-transfection was set as 100%, and the change from this was tracked over 72 h. Values are means and standard deviation (n = 3).



Sub G0/G1	13.1%
G1	44.0%
S	20.1%
G2/M	21.0%



Sub G0/G1	14.1%
G1	44.7%
S	20.2%
G2/M	19.3%

Figure 4.2 - Cell cycle for K562 cells transfected with PRH.

Cells transfected with pEGFP and pMUG1 or pMUG1 Myc-PRH. 24 h post-transfection cells were treated with Igepal and propidium iodide and analyzed by flow cytometry. Propidium iodide staining determines the phase of the cell cycle. One representative histogram of 3 is shown.

In this case the cells were identified on their morphology (FSC vs SSC) and by expression of EGFP (FL1). EGFP positive cells were sorted into cells that stain with propidium iodide alone (FL3), Annexin V APC alone (FL4), or stain with both. Early during apoptosis phosphatidylserine is translocated from the inner plasma membrane to the outer plasma membrane of the cell. The Annexin V protein has a strong affinity for phosphatidylserine, therefore cells in early apoptosis display Annexin V on the surface of the cell. This allows the use of Annexin V as a cell membrane marker for apoptotic cell death. Whereas cells that are dying necrotically, stain with propidium iodide and do not display Annexin V on their cell surface. This produces four distinct cell populations; live cells (propidium iodide and Annexin V APC negative cells), cells undergoing necrosis (propidium iodide positive, Annexin V APC negative cells), cells in early apoptosis (propidium iodide negative, Annexin V APC positive cells), and cells in late apoptosis (propidium iodide positive, Annexin V APC positive cells). Figure 4.3 shows a representative set of data for cells expressing PRH (lower panel) and control cells (upper panel). The PRH expressing cells have a significant increase in early and late apoptotic cells, 18.2% and 17.8% respectively (figure 4.3 - lower panel), compared to the control cells, 14.7% and 2.1% respectively (figure 4.3 - upper panel). There is a reduction in the number of live cells for the PRH expressing cells (figure 4.3 - lower panel) compared to the control cells(figure 4.3 - upper panel). There is little difference in the level of necrosis between PRH expressing (figure 4.3 - lower panel) and control cells (figure 4.3 - upper panel). It can be concluded that elevated PRH expression in cells increases cell apoptosis rather than reducing cell proliferation.

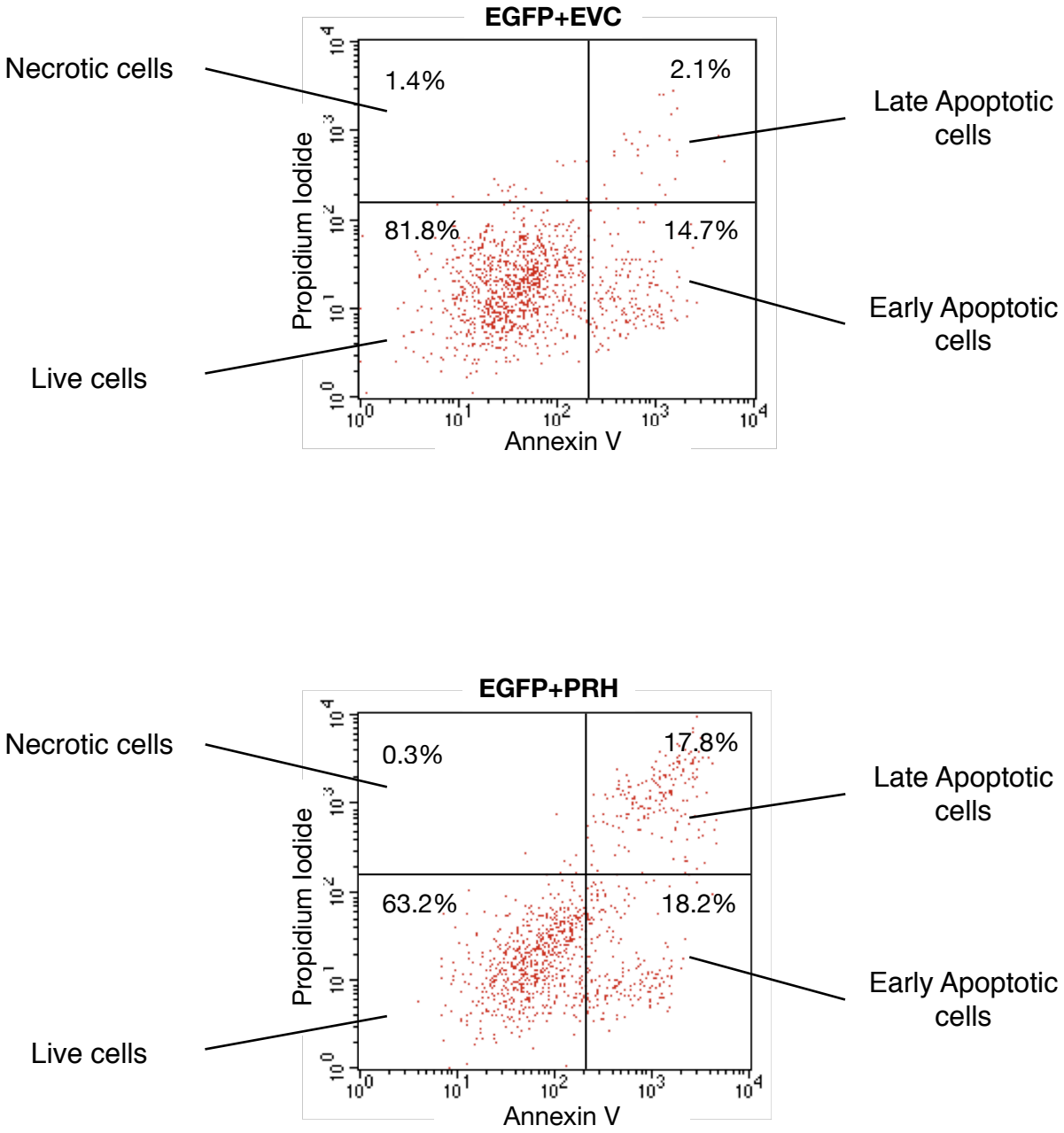


Figure 4.3 - The effect of PRH on apoptosis.

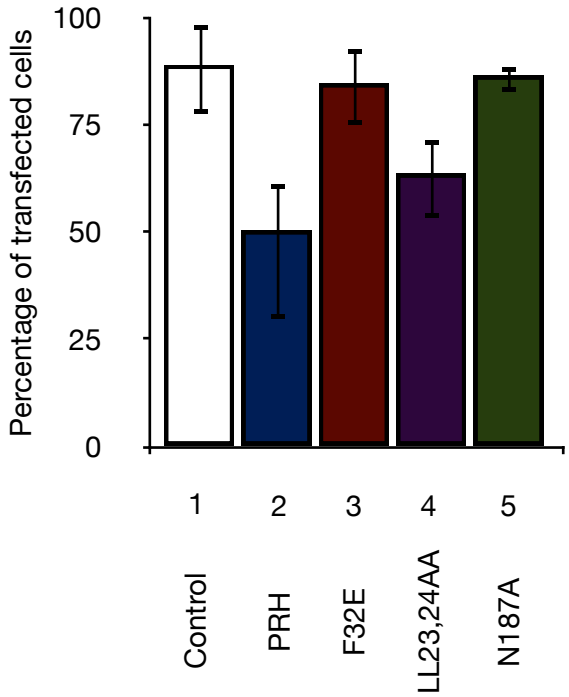
EGFP and empty vector (upper panel) or EGFP and Myc-PRH (lower panel) expressing cells were dual stained with propidium iodide (PI)/Annexin V APC antibody (AV) and analyzed by flow cytometry. The dot plot shows the percentages of live cells (PI- AV-), necrotic cells (PI+), early apoptotic cells (AV+), and late apoptotic cells (AV+ PI+) after gating for EGFP positive cells. One representative dot plot of 3 is shown.

4.3 DNA binding and interaction with TLE are required for PRH dependent for cell apoptosis.

To determine whether transcriptional regulation by PRH has a role in regulating cell growth, cells co-expressing EGFP and PRH or EGFP with either PRH F32E or PRH LL23,24AA or PRH N187A or empty vector were analyzed using FACS at 24, 48 and 72 hour time points. In each case the percentage of EGFP expression at 24 hours was calculated and set as 100%. All transfections had equivalent transfection efficiencies. The number of cells expressing EGFP at 48 and 72 hours was calculated relative to the 24 hour time point. Figure 4.4 [A] compares the percentage of cells expressing EGFP at 72 hours in the absence or prescence of PRH. As previously shown in figure 4.1, the percentage of cells expressing EGFP in the PRH transfected cell population (figure 4.4 [A] - lane 2) is approximately half that observed for the control cells (figure 4.4 [A] - lane 1). In contrast expression of PRH N187A (figure 4.4 [A] - lane 5) has no effect on the percentage of cells expressing EGFP. Similarly expression of PRH F32E (figure 4.4 [A] - lane 3) also has no effect on the percentage of EGFP positive cells. Interestingly, expression of PRH LL23,24AA (figure 4.4 [A] - lane 4) results in 40% fewer EGFP positive cells than the control cells (figure 4.4 [A] - lane 1). These data suggest that the interaction of PRH with TLE and with DNA are important requirements for the regulation of cell growth. They also suggest that the interaction with eIF4E has a relatively minor role in the regulation of cell growth in this case.

To determine whether transcriptional regulation by PRH plays a role in inducing apoptosis, EGFP and PRH transfected or control EGFP cells were stained with propidium iodide and Annexin V APC antibody 24 hours after transfection and analyzed by FACS. As before transfected cells were identified by morphology and expression of EGFP. EGFP positive cells were isolated and examined for apoptosis

[A]



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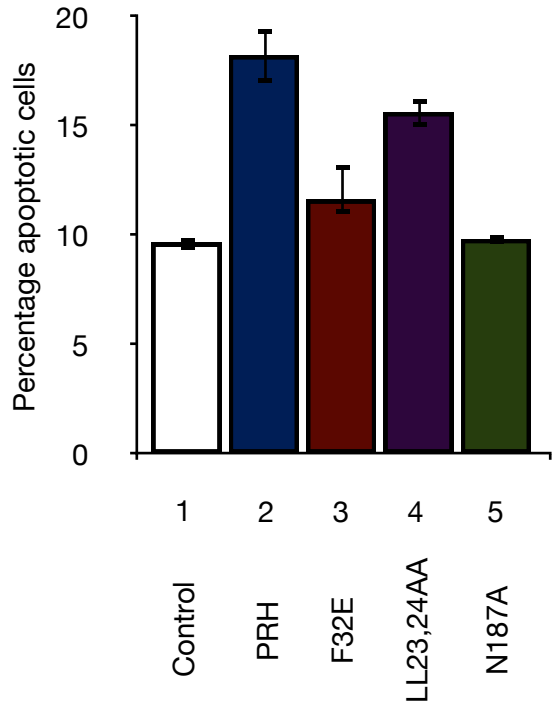


Figure 4.4 - Characteristics of PRH required to induce apoptosis.

[A] Percentage of EGFP positive cells in co-transfection experiments. K562 cells were transfected with pEGFP and either pMUG1 (Control) or pMUG1 vectors expressing PRH (PRH), PRH F32E (F32E), PRH LL23,24AA (LL23,24AA), or PRH N187A (N187A), and the percentage of EGFP positive cells was measured 72 hours post-transfection. Values are means and SD (n = 3). [B] K562 cells were transfected as for [A] and dual stained with PI/AV antibody 24 hours post-transfection for analysis by flow cytometry. Total apoptosis shown (n = 3).

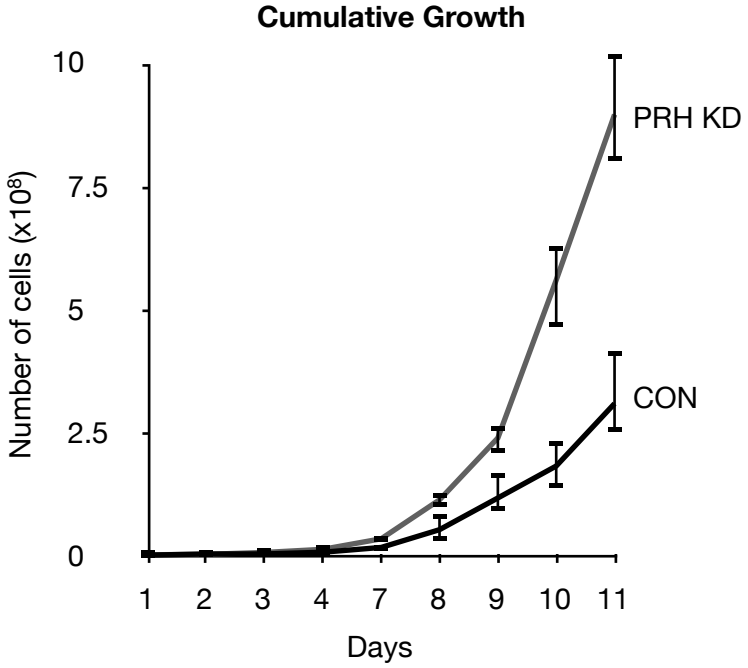
or necrotic cell death by staining with propidium iodide and Annexin V APC as described above. As observed previously cells expressing PRH (figure 4.4 [B] - lane 2) have approximately twice as many apoptotic cells (early and late) compared to the control cells (figure 4.4 [B] - lane 1). In contrast cells expressing PRH N187A (figure 4.4 [B] - lane 1), show no change in the total number of apoptotic cells compared to the control cells (figure 4.4 [B] - lane 5). A small increase in cells undergoing apoptosis is observed for cells expressing PRH F32E (figure 4.4 [B] - lane 3) compared to control cells (figure 4.4 [B] - lane 1), however, there is substantially less apoptosis than the wild-type PRH cells (figure 4.4 [B] - lane 2). Cells expressing PRH LL23,24AA (figure 4.4 [B] - lane 4) have more cells in apoptosis than the control cells (figure 4.4 [B] - lane 1), although still less than the wild-type PRH cells (figure 4.4 [B] - lane 2). These results show that mutations that block DNA binding by PRH and perturb the recruitment of the PRH co-repressor dramatically reduces the pro-apoptotic effects of PRH. Whereas mutations that block post-translational regulation by PRH have a relatively minor effect on the pro-apoptotic function of PRH. This further supports the hypothesis PRH induces apoptosis through transcriptional repression of genes that require the PRH-TLE interaction for repression.

4.4 Knockdown of PRH increases cell survival

Expression of VEGF and its receptors are associated with or contribute to several haematopoietic malignancies (Dias et al., 2000; Dias et al., 2002). Since both VEGF ligand and its receptors are up-regulated in the PRH knockdown cells, it is expected that the PRH knockdown cells will either proliferate faster or have a survival advantage compared to the control cells.

To investigate the role of endogenous PRH on cell growth and survival, PRH was knocked down using *Prh* specific shRNAs. $5x10^6$ cells were transfected with a control shRNA or PRH shRNA 1+2 (as described in Chapter 3). Transfected cells were

[A]



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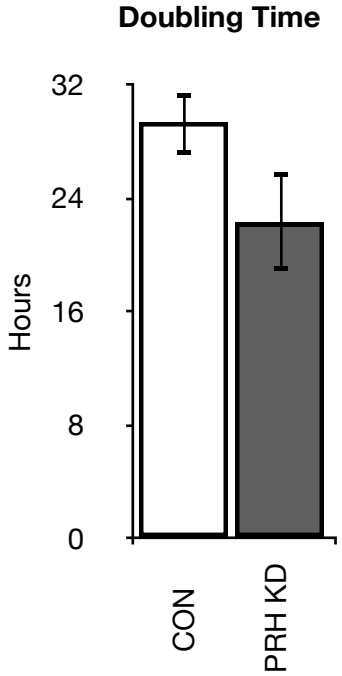


Figure 4.5 - Knock-down of PRH increases cellular growth.

[A] Cumulative growth curves for K562 cells transfected with control shRNA (CON) or PRH shRNA 1+2 (PRH KD). Cells were selected with puromycin 24 hours post-transfection. After 7 days, 3 x10^6 cells of each cell type were plated out and counted over 11 days. Values are means and SD (n = 3). [B] Graph of the doubling time for K562 cells transfected with SVC shRNA or PRH shRNA (1+2) (grey bar). Values are means and SD (n = 3).

selected using 1μg/ml puromycin, 24 hours post-transfection. After 7 days of puromycin selection the cells were diluted to $3x10^5$ cells/ml and counted every 24 hours. The cells were diluted to $3x10^5$ cells/ml every day or 2 days as required. The cumulative growth was then calculated as described in section 2.4.4 of the Materials and Methods. As shown in figure 4.5 [A] PRH knockdown (PRH KD) cells grow substantially faster than control (CON) cells over 11 days, with approximately 2-3 times the cumulative number of cells by day 11. From these data the mean doubling time for each of the cell populations was calculated. Figure 4.5 [B] shows that the doubling time for the PRH knockdown cells is approximately 5 hours less than the control cells.

To further investigate this growth difference between PRH knockdown cells and control cells, the cell cycle profile was analyzed for each population. The populations of cells were permeablized using 1% Igepal and the DNA was stained with 50μg/ml propidium iodide before analyzing by FACS. Viable cells were gated according to their morphology (FSC vs SSC) and were analyzed by the intensity of propidium iodide stain (FL3), which reflects the stage the cells are at in the cell cycle. The PRH knockdown cells and control cells have the same proportion of cells in G1 and S phase (figure 4.6), 43% and 17.5% respectively. However, in the PRH knockdown population of cells there is a small but significantly ($p=0.003$) increase of cells in the G2/M phase with an average of 28.5% compared to 23.5% for the control cells population (figure 4.6). There is also a slight but significant ($p=0.008$) decrease of cells in the Sub G0/G1 phase for the PRH knockdown population (6% of the total cells) compared to the control population (9.5% of the total cells)(figure 4.6). Results were taken from three independent PRH knockdown experiments. A reduction in the sub G0/G1 phase of the cell cycle indicates that there are less cells that are undergoing apoptosis. The increase of cells in the G2/M phase of the cell cycle could

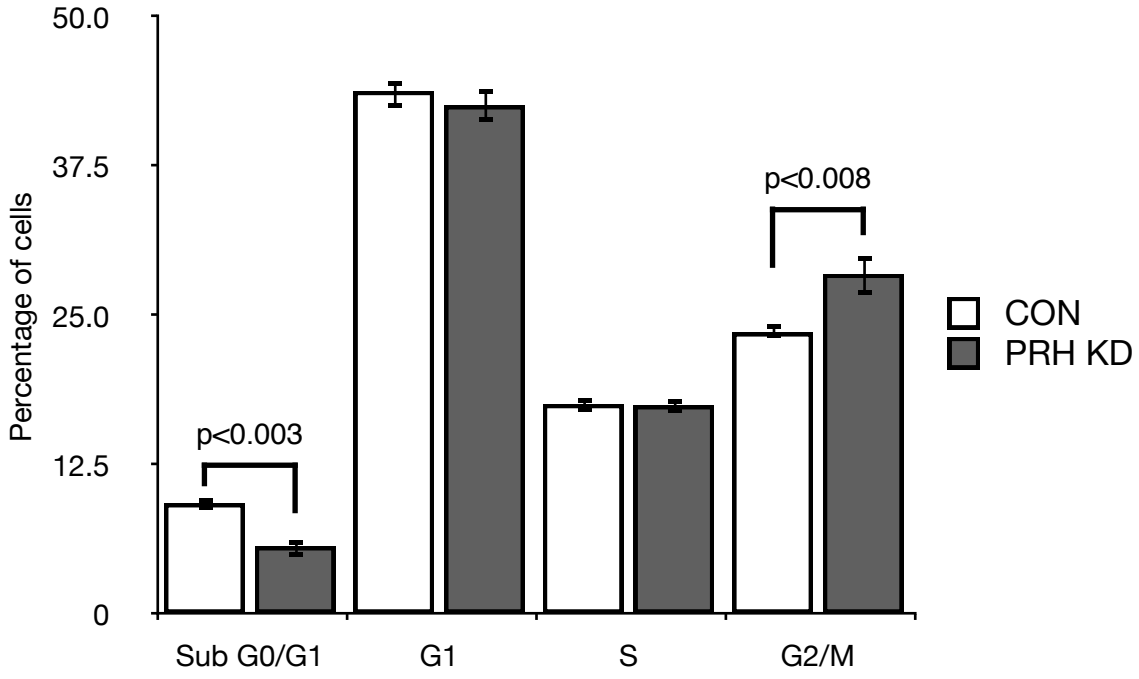


Figure 4.6 - Cell cycle profile for PRH knock-down cells.

Percentage distribution of cells in each stage of the cell cycle. Propidium Iodide staining of K562 cells transfected with control shRNA (CON) or PRH shRNA 1+2 (PRH KD) is shown (n = 3).

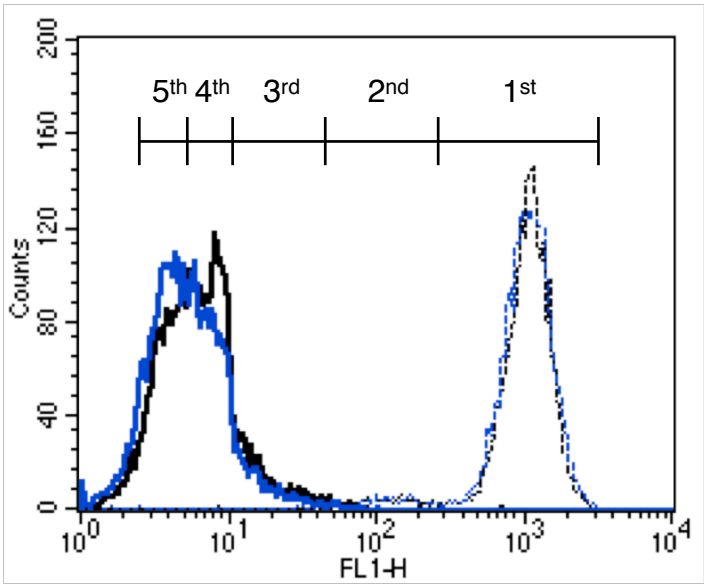
suggest that PRH plays a role in the G2 cell cycle check point. As there is no change in the G1 and S phases of the cell cycle PRH does not appear to have any effect on DNA replication.

To investigate whether the PRH knockdown cells cycle through the cell cycle faster than the control cells, PRH knockdown and control cells were treated with CFSE. CFSE is an inter-membrane fluorescence collating agent; the intensity of the fluorescence emitted is proportional to the cell number and reduces through each cell division. Therefore, if the PRH knockdown cells have an increased rate of cycling through the cell cycle there will be a distinct decrease in fluorescence in the PRH

knockdown population compared to the control cells. PRH knockdown and control cells were established as previously described above. After treatment with CFSE the cells were grown for a further 5 days. On days 1 and 5 a sample of the cell population was analyzed by FACS. On day 1 both the control cells (figure 4.7 [A] - dashed black line) and PRH knockdown cells (figure 4.7 [A] - dashed blue line) had more than 98% of the population in a single peak for the 1st generation of cells stained with CFSE. By day 5 the population of cells were predominantly spread out between the 3rd, 4th and 5th daughter cell populations (figure 4.7 [A] - black and blue solid lines). The PRH knockdown cells contain approximately 10% more cells in the 5th daughter cell population than the control cells (figure 4.7 [B]). This suggests that there is little difference in the rate of cycling through the cell cycle. Thus the increased growth of PRH knockdown cells appears to be through a decrease in apoptosis.

To confirm that apoptosis is reduced in PRH knockdown cells, PRH knockdown and control cells were stained with propidium iodide and Annexin V APC antibody and analyzed by FACS. Viable cells were sorted for staining of propidium iodide and Annexin V APC. Figure 4.8 shows a representative set of data from three independent experiments for PRH knockdown cells (lower panel) and control cells (upper panel). The PRH knockdown cells have a reduction in early and late apoptotic cells, 14.1% and 1.4% respectively (figure 4.8 - lower panel), compared to the control cells, 17.5% and 3% respectively (figure 4.8 - upper panel). There is also an increase in the number of live cells in the PRH knockdown population (figure 4.8 - lower panel) compared to the control population (figure 4.8 - upper panel). There is little difference in the level of necrosis between PRH knockdown cells (figure 4.8 - lower panel) and control cells (figure 4.8 - upper panel). This 25% reduction of total apoptosis in PRH knockdown cells very likely contributes considerably to the growth of these cells. These data confirm that PRH controls cell growth by influencing apoptosis.

[A]



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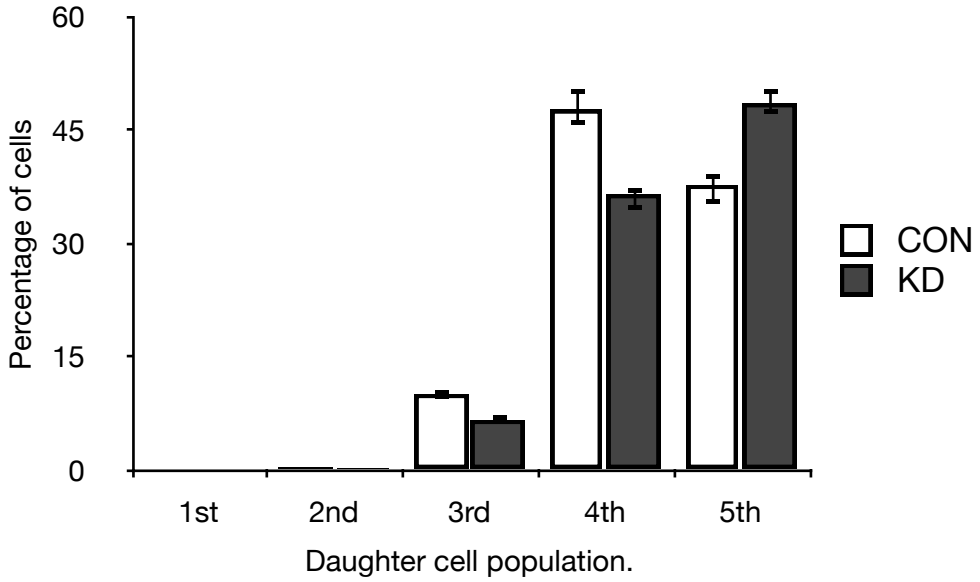


Figure 4.7 - The rate of proliferation of PRH knock-down cells.

[A] CFSE staining of control shRNA (CON) or PRH shRNA 1+2 (PRH KD) at day 1 (right hand peak) and day 5 (left hand peak). Representative histogram of 3 independent experiments. [B] Graph of the percentage of K562 cells transfected with SVC shRNA (white bars) or PRH shRNA (1+2) (grey bar) in each of the daughter cell populations. Values are means and SD (n = 3).

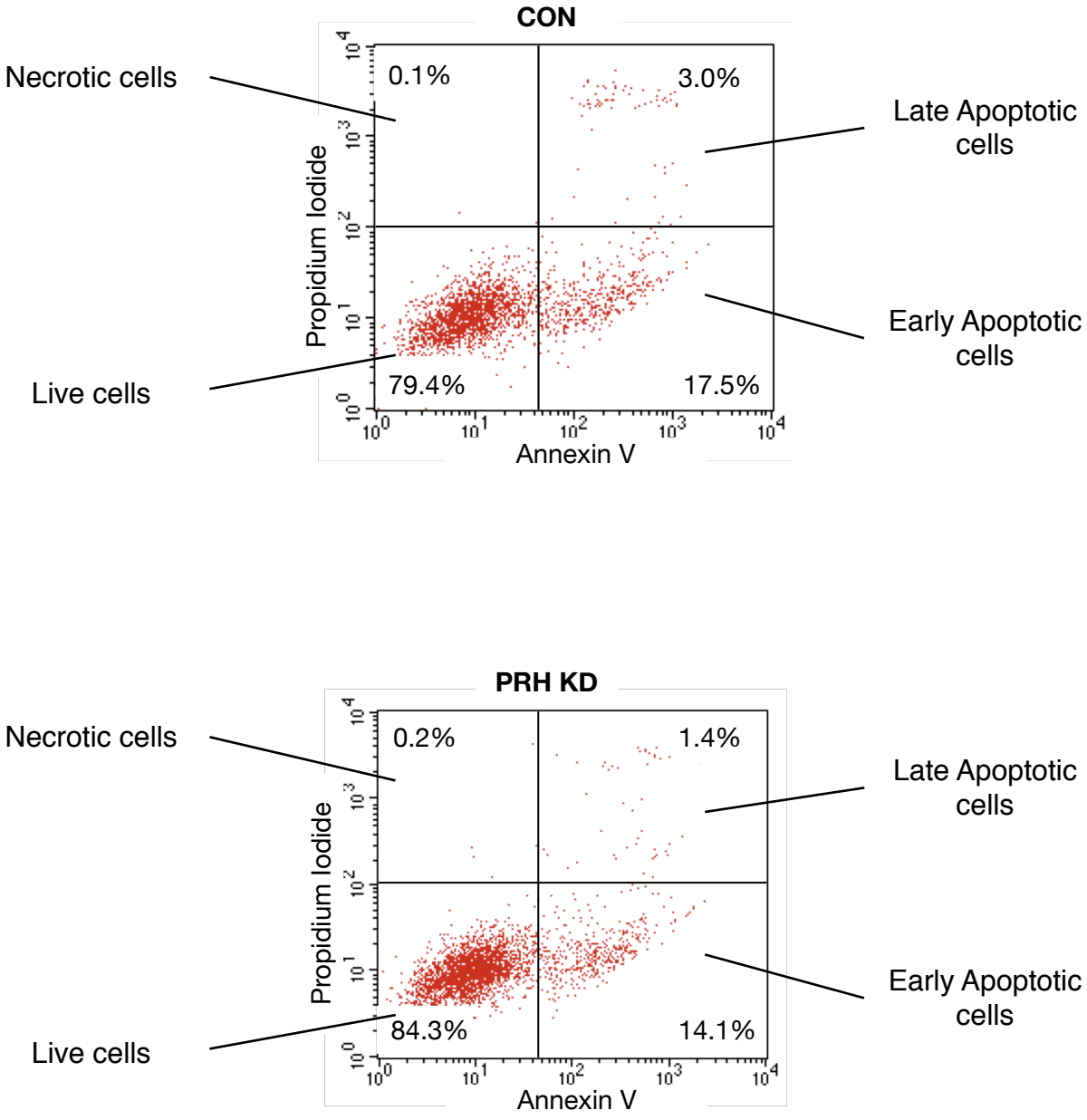


Figure 4.8 - The level of PRH knock-down cells undergoing apoptosis.

PRH knockdown (lower panel) and control cells (upper panel) were dual stained with propidium iodide (PI)/Annexin V APC antibody (AV) and analyzed by flow cytometry. The dot plot shows the percentages of live cells (PI- AV-), necrotic cells (PI+), early apoptotic cells (AV+), and late apoptotic cells (AV+ PI+). One representative dot plot of 3 is shown.

4.5 Cell survival induced by VEGF signalling is dependent on PRH levels

VEGF paracrine signalling is essential for proliferation and survival of endothelial progenitors (Zachary, 2003). Autocrine VEGF signalling through its receptors is known to be an essential mechanism for cell survival in haematopoietic progenitors (Gerber et al., 2002; Katoh et al., 1995; Katoh et al., 1998; Dias et al., 2002), and more recently has also been shown to be involved in regulating proliferation and survival in endothelial cells (Bates et al., 2003; Gee et al., 2005).

To investigate the role of VEGF signalling in haematopoietic progenitor cell survival, all three VSP genes, VEGF, VEGFR1 and VEGFR2 were co-expressed in K562 cells either with EGFP or exogenous EGFP-PRH. As control samples, cells expressing EGFP alone or EGFP-PRH alone were used. Cells were then assessed for cell death using Annexin V staining and propidium iodide as outlined above. As before viable transfected cells were identified according to their morphology and expression of EGFP. EGFP positive cells were then examined for propidium iodide and Annexin V APC staining. In cells expressing VEGF, VEGFR1, and VEGFR2 (figure 4.9 [A] - lane 2) there is a significant reduction ($p=0.05$) in the level of total apoptosis (early and late apoptosis) compared to the control cells (figure 4.9 [A] - lane 1). As previously observed, cells expressing PRH alone (figure 4.9 [A] - lane 3) are more apoptotic than control cells (figure 4.9 [A] - lane 1). However, cells expressing PRH together with VEGF, VEGFR1 and VEGFR2 (figure 4.9 [A] - lane 4) have significantly ($p=0.005$) reduced apoptosis in comparison to cells expressing PRH alone (figure 4.9 [A] - lane 3). This suggests that increased expression of genes in the VEGF signalling pathway is sufficient to induce survival signalling and block apoptosis even in the presence of PRH.

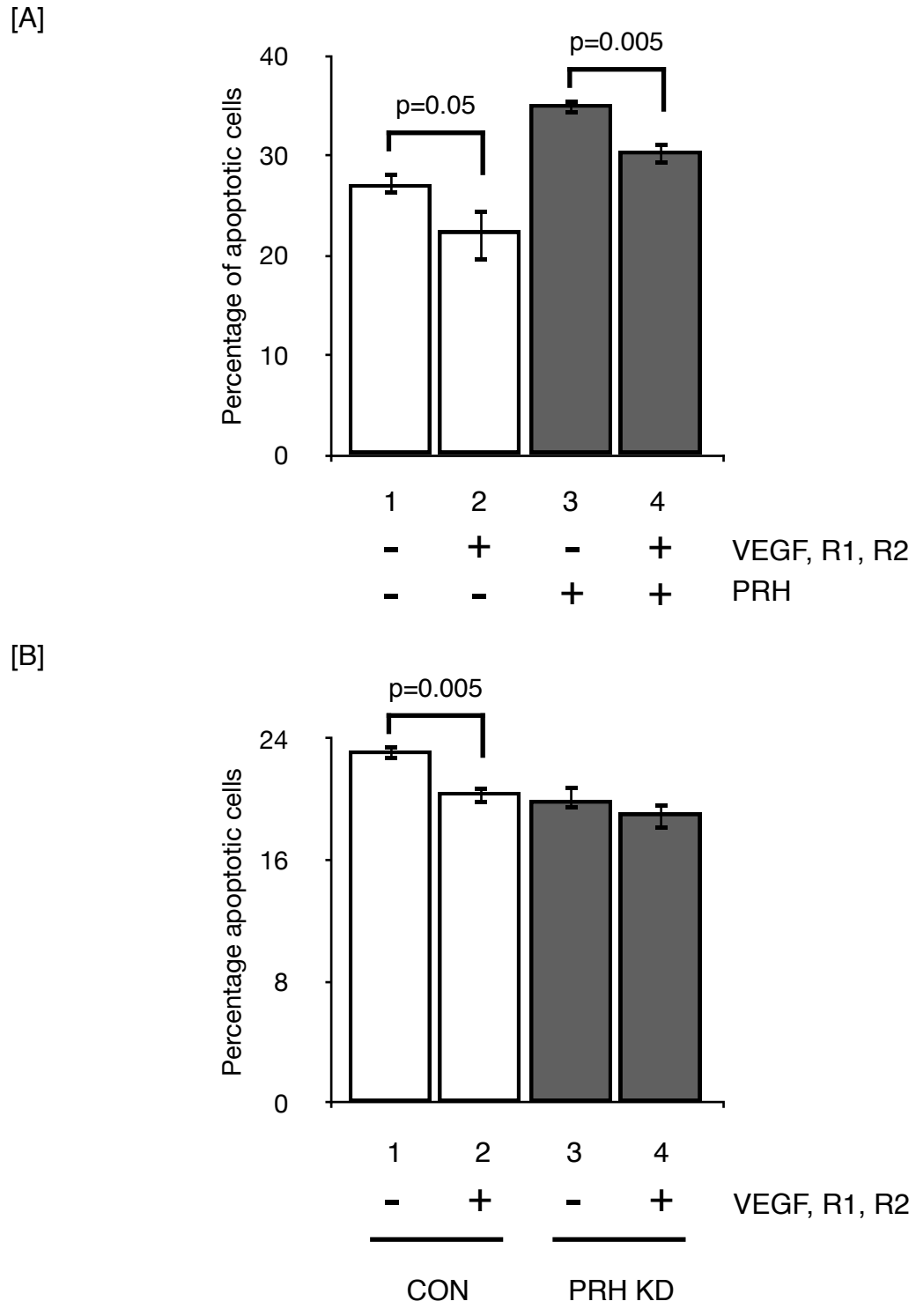


Figure 4.9 - Contribution of VSP to PRH induced apoptosis.

[A] K562 cells transfected with 3μg pEGFP (white bars) or pEGFP-PRH (grey bars), with or without 3 μg of each of the vectors expressing VEGF, VEGFR-1, and VEGFR-2 (bars 2, and 4). 24 hours post-transfection the cells were dual stained with PI/AV antibody for analysis by flow cytometry. The graph shows the means and SD (n = 3).[B] K562 cells transfected with SVC shRNA (CON - white bars) or PRH shRNA 1+2 (PRH KD - grey bars) were retransfected with 3 μg of each of the vectors expressing VEGF, VEGFR-1, and VEGFR-2 (bars 2, and 4). Cells were analyzed as in panel [A]. The graph shows the means and SD (n = 3).

To determine whether increased expression of VEGF, VEGFR1 and VEGFR2 are important for cell survival in the absence of PRH; VEGF, VEGFR1 and VEGFR2 were expressed in PRH knockdown and control cells. Propidium iodide and Annexin V APC staining was performed and cell death was analyzed by FACS as previously described. Control cells expressing VEGF, VEGFR1 and VEGFR2 (figure 4.9 [B] - lane 2) have a significant (p=0.005) decrease in total cell apoptosis compared to control cells alone (figure 4.9 [B] - lane 1). PRH knockdown cells (figure 4.9 [B] - lane 3) have reduced apoptosis compared to the control cells (figure 4.9 [B] - lane 1). However, PRH knockdown cells co-expressing VEGF, VEGFR1 and VEGFR2 (figure 4.9 [B] - lane 4) show no change in the level of apoptosis compared to the PRH knockdown cells alone (figure 4.9 [B] - lane 3). Thus, VEGF and its receptors are only able to counteract apoptosis caused by PRH in cells that express PRH. This strongly suggests that down-regulation of VSP gene expression and VEGF signalling mediates PRH induced apoptosis in K562 cells.

To confirm that VEGF signalling in the PRH knockdown cells is important for cell survival, PRH knockdown and control cells were treated with exogenous VEGF165 protein or an equivalent volume of DMSO. Cells were seeded into 96 well plates and treated with 50 ng/ml VEGF165 protein or DMSO and grown for 72 hours. Cell numbers were measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay for viable cells that measures the activity of mitochondrial reductase enzymes which reduce MTT to give a purple formazan dye. Reactions were performed in quadruplicate and readings were taken at 0 hours to ensure equal loading of cells. Data shown is the mean and standard error from a set of four from a single experiment and is representative of five independent experiments. As expected, the PRH knockdown population (figure 4.10 [A] - lane 3) have significantly more cells (p=0.01) than the control cell population (figure 4.10 [A] -

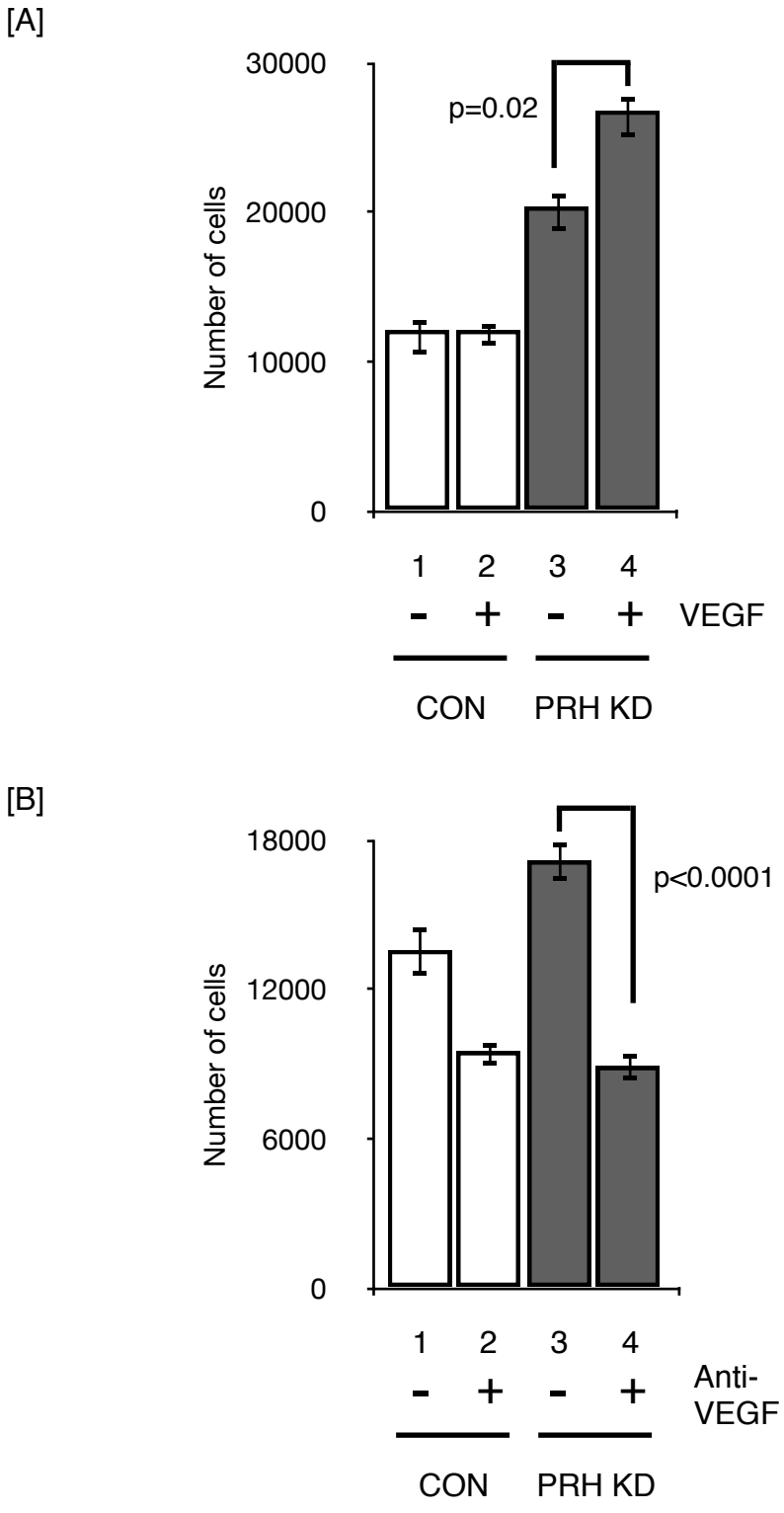


Figure 4.10 - Role of VEGF signalling in PRH knock-down cell growth.

[A] K562 cells were transfected with SVC shRNA (CON - white bars) or PRH shRNA 1+2 (PRH KD - grey bars) and then incubated with 50 ng/ml VEGF (bars 2 and 4) or an equal volume of DMSO (bars 1 and 3) for 72 hours. An MTT assay was then used to calculate cell numbers. Values are means and SD (n = 4). [B] As for panel A except that cells were incubated with 50 μg/ml anti-VEGF antibody. Values are means and SD (n = 4). Data representative of five independent experiments.

lane 1). Untreated control cells (figure 4.10 [A] - lane 1), and control cells treated with VEGF165 (figure 4.10 [A] - lane 2) have an equal number of cells. However, the PRH knockdown cells treated with VEGF165 (figure 4.10 [A] - lane 4) have significantly more cells ($p=0.02$) than the PRH knockdown untreated population (figure 4.10 [A] - lane 3). This demonstrates that VEGF can stimulate cell growth or survival better in the PRH knockdown cells than the control cells and suggests that there is increased VEGF signalling in the PRH knockdown cells.

To further investigate the importance of VEGF signalling in the PRH knockdown cells, PRH knockdown and control cells were established as previously described, and treated with 50μg/ml of a VEGF specific antibody or an equivalent volume of DMSO. Cell numbers were calculated using an MTT assay after 72 hours of treatment. Data shown is the mean and standard error from a single experiment performed in quadruplicate and is representative of five independent experiments. As observed in figure 4.10 [A] there are more PRH knockdown cells (lane 3) than control cells (lane 1). Treating PRH knockdown cells with an anti-VEGF antibody (figure 4.10 [B] - lane 4) reduces cell growth significantly ($p<0.0001$) compared to untreated PRH knockdown cells (figure 4.10 [B] - lane 3) and negates the growth advantage of the PRH knockdown cells relative to control cells (figure 4.10 [B] - lane 2). Clearly PRH knockdown cells respond more dramatically to VEGF antibodies than control cells suggesting that de-repression of *Vegf* in PRH knockdown cells contributes to cell survival.

To investigate the role of the VEGF receptors in PRH knockdown cell survival, PRH knockdown and control cells were treated with either DMSO or 2mM Sutent (SU11248); a receptor tyrosine kinase inhibitor which inhibits VEGFR2 signalling (Sun et al., 2003) and potentially VEGFR1 signalling (Barbarroja et al., 2009), PDGFR (Sun et al., 2003; Abrams et al., 2003), KIT(Abrams et al., 2003), and FLT3

signalling (Deeks and Keating, 2006). Cell numbers were calculated using an MTT assay after 72 hours of treatment. Data shown is the mean and standard error from a single experiment performed in quadruplicate and is representative of three independent experiments. PRH knockdown cells treated with Sutent (figure 4.11 [A] - lane 4) have significantly (p=0.01) reduced cell growth compared to untreated PRH knockdown cells (figure 4.11 [A] - lane 3), whereas Sutent has no effect on control cells (figure 4.11 [A] - lane 1 and 2). Treating PRH knockdown cells with Sutent (figure 4.11 [A] - lane 4) also reduces the growth advantage of the PRH knockdown cells compared to the control cells (figure 4.11 [A] - lane 2). This evidence suggests that signalling through tyrosine kinase receptors, including the VEGF receptors is important for cell survival and that de-repression of *Vegfr1* and *Vegfr2* in PRH knockdown cells contributes to the observed increase in cell growth.

To distinguish between the effect of VEGF receptor signalling and the effect of other receptor tyrosine kinases, control and PRH knockdown cells were also treated with 2mM Sugen (SU1498), a receptor tyrosine kinase inhibitor, which specifically inhibits VEGFR2 receptor signalling (Strawn et al., 1996), or an equivalent volume of DMSO. As described above the cell numbers were calculated using an MTT assay after 72 hours of treatment. Data shown is the mean and standard error from a single experiment performed in quadruplicate and is representative of three independent experiments. PRH knockdown cells treated with Sugen (figure 4.11 [B] - lane 4) have significantly (p=0.0001) reduced cell growth compared to untreated PRH knockdown cells (figure 4.11 [B] - lane 3). Treating PRH knockdown cells with Sugen (figure 4.11 [B] - lane 4) also removes the growth advantage of PRH knockdown cells compared to the control cells (figure 4.11 [B] - lane 2). This suggests that VEGF signalling through VEGFR2 is important for cell survival and that de-repression of *Vegfr2* in PRH knockdown cells is responsible for increased cell growth.

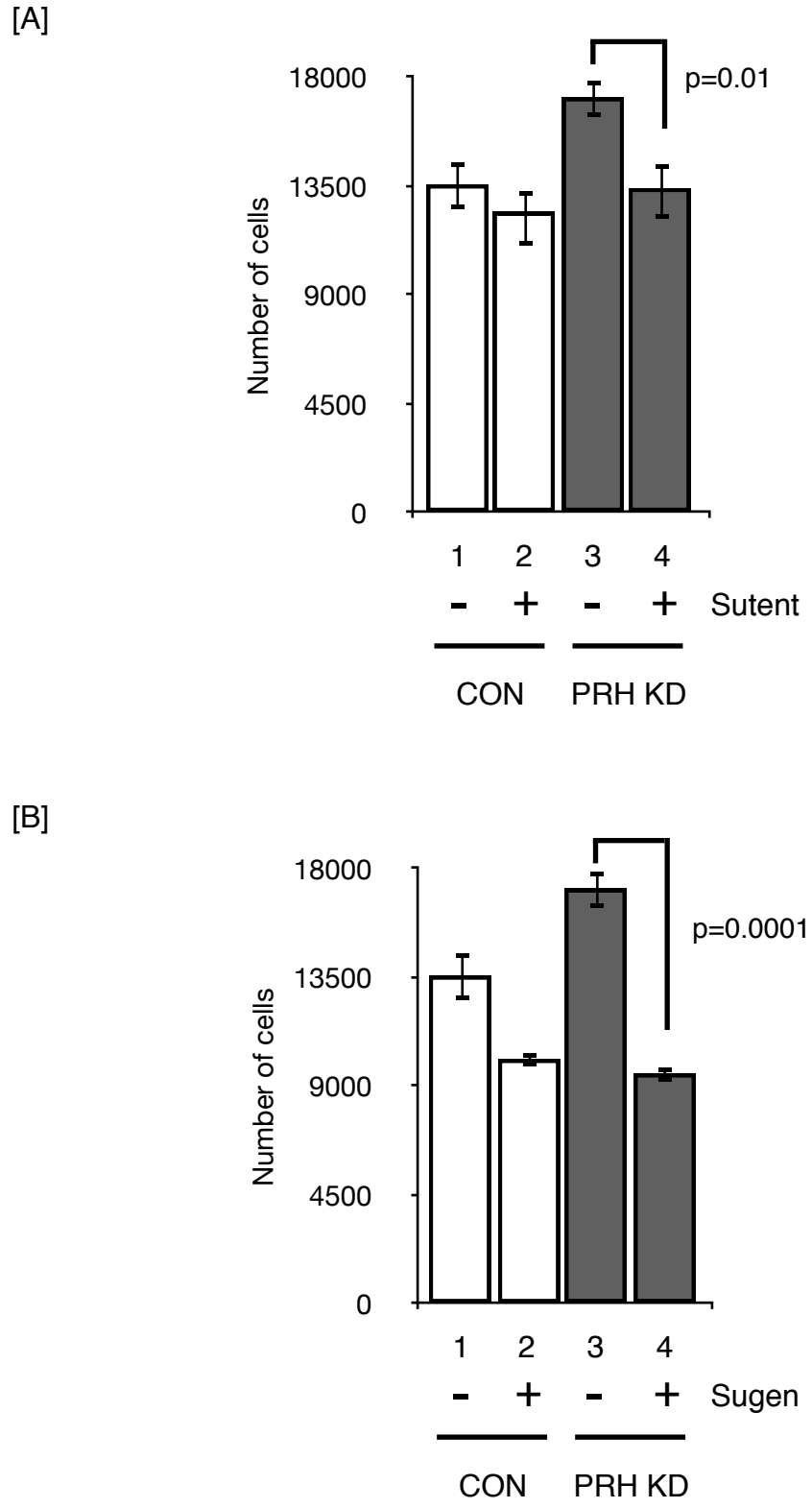


Figure 4.11 - The role of VEGF receptor signalling in PRH knock-down cell growth.

[A] K562 cells were transfected with SVC shRNA (CON - white bars) or PRH shRNA 1+2 (PRH KD - grey bars) and then incubated with 2 mM SU11248 (bars 2 and 4) or an equal volume of DMSO (bars 1 and 3) for 72 hours. An MTT assay was then used to calculate cell numbers. Values are means and SD (n = 4). [B] As for panel A except that cells were incubated with 2 mM SU1498 (bars 2 and 4). Values are means and SD (n = 4). Data representative of three independent experiments.

4.6 Discussion

As demonstrated in this chapter PRH is a regulator of cell survival. Increased survival is observed in PRH knock-down cells, and over-expression of PRH induces apoptosis. PRH regulation of cell survival has not previously been described. However, inhibition of cell proliferation by PRH has been described in the myeloid compartment (Jayaraman et al., 2000; Topisirovic et al., 2003b). In U937 cells, a myeloid cell line displaying monocytic characteristics, PRH induces G1 cell cycle arrest by inhibiting eIF4E mRNA recruitment of cyclin D1 to the ribosome (Topisirovic et al., 2003a). PRH has also been shown to inhibit cell transformation by E26 Myb-Ets expressing viruses infected into multi-potential progenitors (MEPs) (Jayaraman et al., 2000). Therefore, an important question is why does PRH induce apoptosis in K562 cells, yet PRH inhibits cell proliferation in other myeloid-derived cell lines and model systems. It is likely that the role of PRH is dependent on its cellular environment. K562 cells contain the Philadelphia chromosome and the resulting BCR-ABL fusion protein activates a variety of signalling pathways that activate cell cycle controlling proteins (Kurzrock et al., 2003). For example, activation of the Ras pathway, leads to MEK/ERK activation, resulting in increased cyclin D1 expression, CDK2-cyclin E activation and rapid G1 phase progression (Keenan et al., 2001; Lents et al., 2002; Phillips-Mason et al., 2000). Over-expression of a mutant of PRH (PRH LL23,24AA), which disrupts the interaction with eIF4E, has little effect on cell growth and apoptosis compared to wild-type PRH in K562 cells. This suggests that translational repression of cyclin D1 mRNA transport by PRH, may only have a small effect in K562 cells because of the constitutive activation of the Ras pathway. PRH knock-down cells proliferate slightly faster than control cells but this effect is not as significant as the regulation of survival in these cells. It is possible that this slight increase in proliferation is because a decrease in PRH results in increased eIF4E

mRNA transport of cyclinD1. The key characteristics of PRH in K562 cells is the ability of PRH to bind DNA and interact with the co-repressor TLE. These features significantly reduce PRH induced apoptosis. Therefore transcriptional regulation by PRH is critical for PRH regulation of K562 cell survival.

This chapter illustrates the role of VEGF signalling in the control of myeloid cell survival. Increased cell survival through VEGF signalling was shown here using over-expression of VEGF, VEGFR1 and VEGFR2 in K562 cells. This supports previously published data demonstrating VEGF autocrine signalling induces survival of haematopoietic stem cells (Gerber et al., 2002). Also, work carried out by Ruan et al, showed that VEGF expression in K562 cells is vital for cell growth in culture and solid tumour formation in nude mice (Ruan et al., 2004). In addition PRH antagonizes VEGF survival signalling through transcriptional regulation. This was demonstrated in cells treated with a VEGF neutralizing antibody or VEGF receptor inhibitors, where PRH knockdown cells were more sensitive to depletion of VEGF or inhibition of VEGF receptor signalling than control cells. In addition, over-expression of VEGF and its receptors from PRH-independent promoters antagonizes the effects of PRH over-expression. One explanation of these results is that PRH knockdown cells are reliant on the VSP for their increased survival. In support of this idea is data showing that PRH knockdown cells have enhanced survival when grown in the presence of exogenous VEGF, whereas control cells are unresponsive to exogenous VEGF.

In this chapter over-expression of both VEGFR1 and VEGFR2 were found to increase survival of K562 cells. The role of VEGFR1 induced signalling versus VEGFR2 induced signalling is controversial in endothelial cells, where it has been suggested that VEGFR1 may only act as a decoy receptor (Hiratsuka et al., 1998). However, in the haematopoietic compartment VEGFR1 signalling is vital for macrophage migration (Hiratsuka et al., 1998), as well as monocyte migration

(Barleon et al., 1996). In addition both VEGFR1 and VEGFR2 are able to promote cell survival through autocrine signalling in haematopoietic stem cells (Gerber et al., 2002). A recent study treated AML patient samples and AML cell lines with a VEGF receptor inhibitor, with specificity for both VEGFR1 and VEGFR2, and observed increased apoptosis (Barbarroja et al., 2009), similar to evidence shown in this chapter. Also, Barbarroja et al show that combined VEGFR inhibitor and chemotherapy has a better therapeutic efficacy than chemotherapy alone (Barbarroja et al., 2009), demonstrating the importance of VEGF signalling for haematopoietic cell survival. In this chapter we used a low specificity small molecule receptor tyrosine kinase inhibitor, SU11248, and a VEGFR2 specific inhibitor, SU1498, which reduced PRH knock-down cell growth significantly. This data suggests that VEGFR2 signalling is important for K562 cell survival. However, it is important to note SU1498 was selected for specificity against VEGFR2 and checked for PDGF specificity, but its activity against VEGFR1 has not been determined (Strawn et al., 1996). Therefore, it cannot be ruled out that VEGFR1 may also be inhibited by this compound. Both VEGFR1 and VEGFR2 are thought to activate several similar pathways including the Akt survival pathway, therefore it is likely there are redundancies between these receptors and they both contribute to PRH knock-down cell survival signalling.

As a transcriptional regulator of VSP genes, which control cell survival, it is tempting to suggest a role for PRH in myeloid-derived leukaemic disease. It is known that dysregulation of PRH activity, such as aberrantly low levels of PRH or mis-localization of PRH, has been observed in a subset of human AML and CML patient samples (Topisirovic et al., 2003b). Although a correlation between increased VEGF-induced survival signalling and PRH activity in primary AML or CML samples has not been investigated, there is a body of previous work that has validated the

significance of VEGF survival signalling in malignant haematopoietic cells. For example, it is known that VEGF and the VEGF receptors, VEGFR1 and VEGFR2, are up-regulated in many haematopoietic malignancies (Dias et al., 2002). Leukaemia samples and leukaemic myeloid-derived cell-lines express VEGF and functional VEGFR2, where autocrine signalling stimulates cell survival and proliferation (Dias et al., 2000). In addition, VEGF has been shown to promote cell survival by protecting haematopoietic cells from apoptosis induced by gamma-ray irradiation and chemotherapeutic drugs, etoposide and doxorubicin (Katoh et al., 1995; Katoh et al., 1998). Therefore, it is possible that CML and AML patients that have perturbed PRH activity are likely to have a higher tolerance of chemotherapeutic agents and increased survival signalling. Furthermore, in HL60 leukaemic cells, VEGF was found to up-regulate Hsp90 and induce Bcl-2 expression, leading to apoptosis inhibition (Dias et al., 2002). It has also been reported that MCL1, a BCL2 family member, is up-regulated after VEGF treatment, with reduced caspase 3 activity (Katoh et al., 1998). This suggests that VEGF is a key factor for haematopoietic stem cell survival through control of the Bcl-2 family genes, although it is likely multiple pathways including activation of the Akt pathway (as discussed in the introduction). There is strong evidence that supports a role for VEGF signalling in haematopoietic malignancies and deregulation of PRH activity could be a key step in the regulation of these VSP genes.

The role of PRH in regulating cell growth within the haematopoietic compartment appears to be complex. In this chapter PRH is shown to be a regulator of myeloid cell survival through transcriptional regulation of the VSP genes. However, PRH can also regulate cell proliferation within the myeloid compartment, as I have previously described above (Topisirovic et al., 2003b; Topisirovic et al., 2003a)(Jayaraman et al., 2000). This suggests that PRH has a general role in myeloid cells as a tumour

suppressor. In contrast, in the lymphoid compartment, PRH can act as an oncogene. For example, expression of PRH under the control of a T-cell specific promoter results in increased proliferation of T-cells (Mack et al., 2002) and PRH was found to be up-regulated in some B-cell leukaemias (Hansen and Justice, 1999). Also, over-expression of PRH is sufficient for self-renewal of thymocytes and accumulation of subsequent mutations that allow for leukaemic transformation (McCormack et al., 2010). These observations suggest a myeloid-lymphoid divide for PRH control of cell growth and survival, suggesting that tissue specific factors are important in controlling the PRH induced response.

Role of CK2 phosphorylation of PRH

5 Role of CK2 phosphorylation of PRH.

5.1 Introduction.

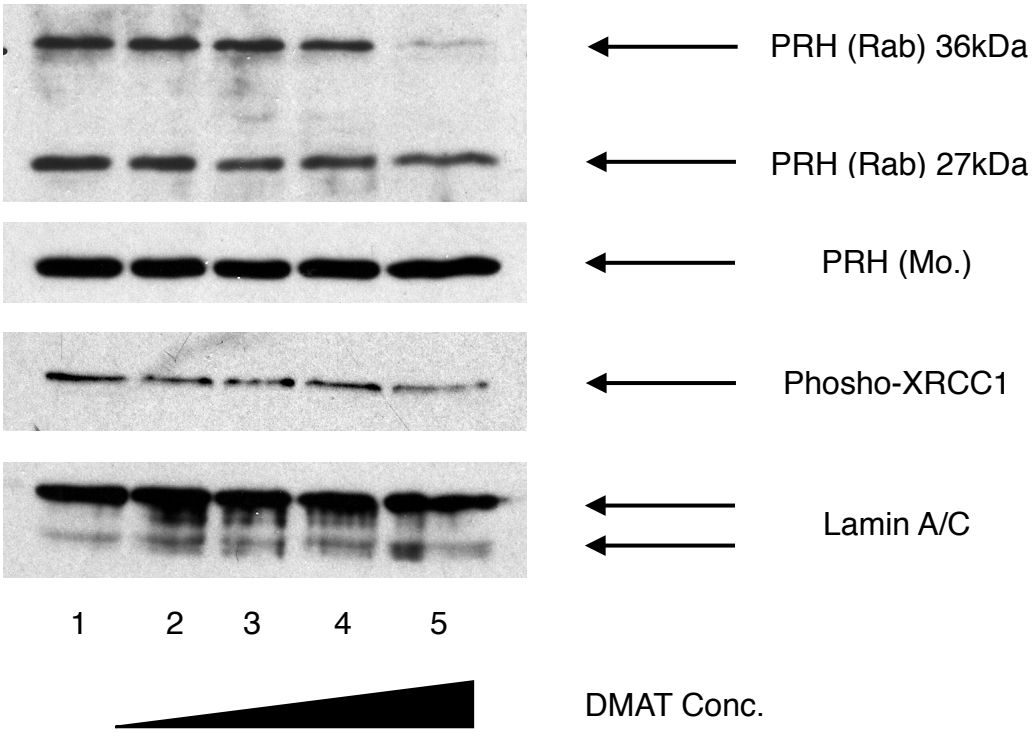
Protein kinase CK2 (CK2) is a ubiquitously expressed protein kinase that is activated in response to stress signals (Sayed et al., 2000). There are multiple targets of CK2 phosphorylation, including components of the Akt signalling pathway and regulators of apoptosis. CK2 is therefore defined as a pro-survival factor (Ahmed et al., 2002). Unsurprisingly, CK2 is commonly activated in malignant diseases of most cell types, including AML and CML (Kim et al., 2007; Mishra et al., 2007). *In vitro* studies identified PRH as a binding partner of CK2β. It was further established that CK2 phosphorylates the serine residues 163 and 177 of the PRH homeodomain, which inhibits DNA binding *in vitro* (Soufi et al., 2009). However, the significance of CK2 phosphorylation of PRH in cells is unknown. Evidence that CK2 phosphorylates PRH in vivo and the effect of phosphorylation on PRH transcriptional activity and stability will be presented in this chapter. The clinical significance of CK2 activity and regulation of PRH by CK2 will also be discussed.

5.2 Phosphorylation of PRH *in vivo*.

It has been demonstrated previously by Soufi et al that antibodies generated in mouse and rabbit preferentially recognize the unphosphorylated and phosphorylated conformations of PRH, respectively (see figure 2B (Soufi et al., 2009)). These antibodies were utilized to investigate whether endogenous PRH is phosphorylated by CK2 *in vivo*.

To determine whether PRH is phosphorylated in vivo by CK2, K562 cells were treated with increasing amounts of a specific CK2 inhibitor 2-Dimethylamino-4,5,6,7-

[A]



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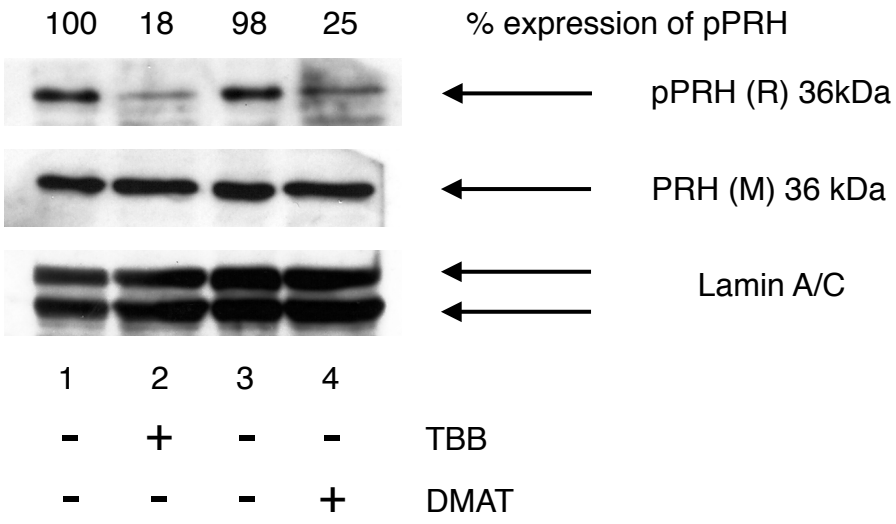


Figure 5.1 - *In vivo* phosphorylation of PRH by CK2.

[A] K562 cells were treated with 10μM, 20μM, 40μM, 80μM DMAT or the equivalent volume of DMSO. The proteins were then separated by SDS-PAGE and western blotted for endogenous phosphorylated PRH using a rabbit anti-PRH antibody (top panel), total endogenous PRH using a mouse anti-PRH antibody (upper middle panel), CK2 phosphorylated XRCC1 using an anti-phospho-XRCC1 antibody (lower middle panel) and Lamin A/C was used as a control (bottom panel). Data representative of two independent experiments. [B] K562 cells were treated with 80μM DMAT (lane 2) or 160μM TBB (lane 4) or the equivalent volume of DMSO (lanes 1 and 3). The proteins were then separated by SDS-PAGE and western blotted as for panel A. Data representative of two independent experiments.

tetrabromo-1H-benzimidazole (DMAT) (Pagano et al., 2004) for 24 hours. Whole cell protein extracts were made from treated and untreated cells. Equal amounts of protein were separated by SDS-PAGE, and the proteins were transferred onto an Immobilon P membrane. Western blot analysis was performed by staining the membrane with the rabbit anti-pPRH antibody (figure 5.1 [A] - top panel) for phosphorylated PRH (pPRH). The blot was stripped of antibody and re-probed with the mouse anti-PRH antibody (figure 5.1 [A] - upper middle panel) for unphosphorylated PRH (PRH), or an anti-pXRCC1 antibody (figure 5.1 [A] - lower middle panel). XRCC1 protein is known to be phosphorylated by CK2 and serves as a CK2 phosphorylation specific control. Blots were also probed with an anti-Lamin A/C antibody, as Lamin A/C is used as a loading control (figure 5.1 [A] - bottom panel). In untreated K562 cells, a strong pPRH band can be observed at 36kDa (figure 5.1 [A] - top panel, upper band, lane 1) and the strength of this band decreases with increasing DMAT concentration (figure 5.1 [A] - top panel, upper band, lanes 2-5). However, the level of unphosphorylated PRH protein is unchanged by DMAT treatment (figure 5.1 [A] - upper middle panel, lanes 1-5). The pPRH rabbit antibody also detects a band at 27kDa (figure 5.1 [A] - top panel, lower band, lane 1) and will be discussed later in section 5.7 of this chapter. To confirm that DMAT inhibits CK2 activity the membrane was re-probed with an antibody against CK2 phosphorylated XRCC1 (pXRCC1). Figure 5.1 [A] lower middle panel, lanes 1-5, show a gradual decrease in pXRCC1 staining with increased DMAT concentration.

To further confirm that PRH is phosphorylated by CK2, K562 cells were treated with 80μM DMAT or 150μM TBB, a second CK2 specific inhibitor, or the equivalent volume of DMSO. After 24 hours the cells were harvested and whole cell protein extracts were made. Western blot analysis was performed as above, using the rabbit anti-pPRH antibody (figure 5.1 [B] - top panel), or the mouse anti-PRH antibody

(figure 5.1 [B] - middle panel), or an anti-Lamin A/C antibody (figure 5.1 [B] - bottom panel). As previously observed, K562 cells treated with DMAT have reduced staining down to only 25% of the untreated levels of pPRH (figure 5.1 [B] - top panel, lane 4 compared to lane 3). K562 cells treated with an alternative CK2 inhibitor, TBB, also have reduced levels of pPRH (figure 5.1 [B] - top panel, lane 2), 18% expression compared to untreated K562 cells (figure 5.1 [B] - top panel, lane 3). The level of unphosphorylated PRH is unaffected by either treatment (figure 5.1 [B] middle panel). This shows that PRH is phosphorylated in K562 cells by CK2.

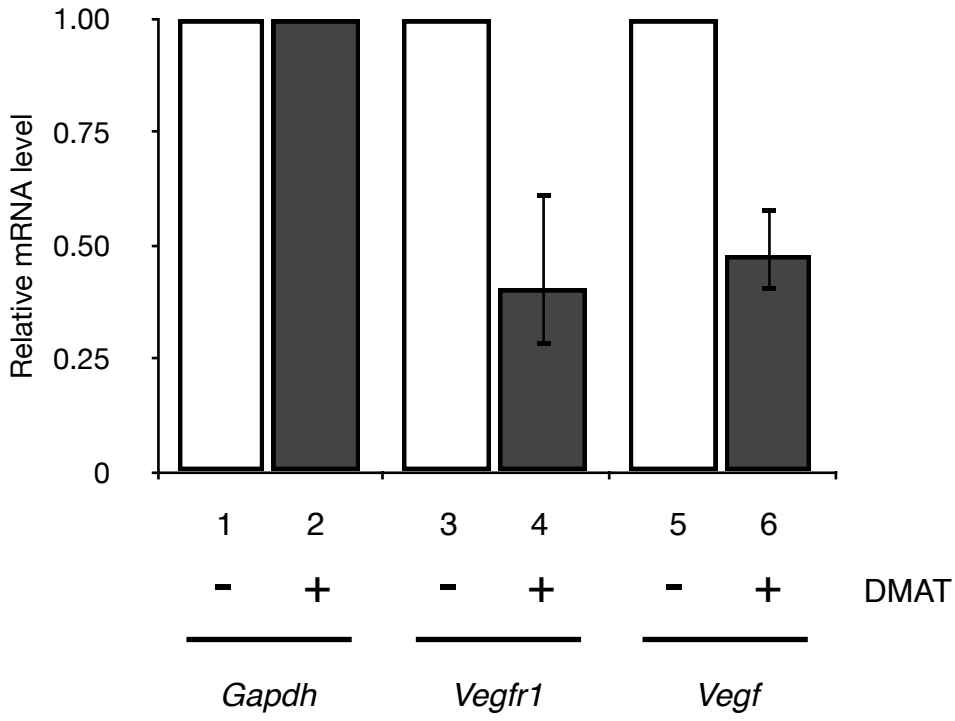


Figure 5.2 - Transcriptional repression of *Vegf* and *Vegfr1* expression by CK2 inhibition.

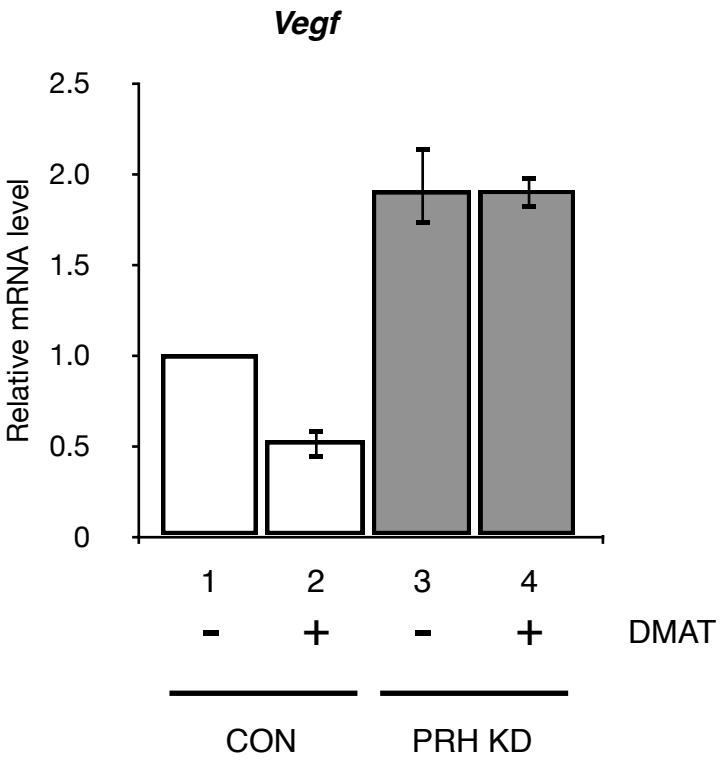
K562 cells were treated with 80μM DMAT or the equivalent volume of DMSO for 24 hours. RNA was extracted and reverse transcribed the cDNA. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using gene specific primers and comparing to qPCR reactions for *Gapdh*. Values are means and standard deviations (SD) (n = 3).

5.3 CK2 phosphorylation of PRH regulates gene expression of *Vegf and Vegfr1.*

To investigate the role of CK2 phosphorylation on the regulation of *Vegf* and *Vegfr1* gene expression, K562 cells were treated with 80μM DMAT or an equivalent volume of DMSO. After 24 hours of treatment the cells were harvested, RNA was extracted and reverse transcribed to cDNA. The amount of cDNA for each gene was then analyzed using qPCR with gene specific primers for *Vegf*, *Vegfr1* and *Gapdh*. *Gapdh* was used as an internal control gene, therefore all qPCR data was normalized against *Gapdh* mRNA expression (figure 5.2 - lanes 1 and 2). Relative to *Gapdh* levels, *Vegf* gene expression is down-regulated by approximately 50% in the DMAT treated cells (figure 5.2 - lane 6) compared to the untreated cells (figure 5.2 - lane 5). The expression of *Vegfr1* is also repressed by approximately 60% in the cells treated with DMAT (figure 5.2 - lane 4) compared to cells that have not been treated (figure 5.2 - lane 3). Evidently, CK2 activity plays a role in the regulation of *Vegf* and *Vegfr1* gene expression. CK2 activity could potentially increase the amount of unphosphorylated PRH which can bind DNA and repress the *Vegf* and *Vegfr1* genes.

To determine whether CK2 regulation of *Vegf* gene expression is dependent on endogenous PRH, PRH was knocked down using *Prh* specific shRNAs, as described previously. PRH knockdown and control cells were treated with 80μM DMAT or the equivalent volume of DMSO for 24 hours. mRNA levels were measured as outlined above to determine gene expression levels. In cells treated with DMAT, the level of *Vegf* expression is repressed by approximately 50% (figure 5.3 [A] - lane 2) when compared to untreated cells (figure 5.3 [A] - lane 1). When PRH protein levels are knocked down by specific shRNAs (figure 5.3 [A] - lane 3) *Vegf* expression is elevated compared to the control cells (figure 5.3 [A] - lane 1), as previously described. However, in the PRH knockdown cells, treatment with DMAT (figure 5.3

[A]



[B]

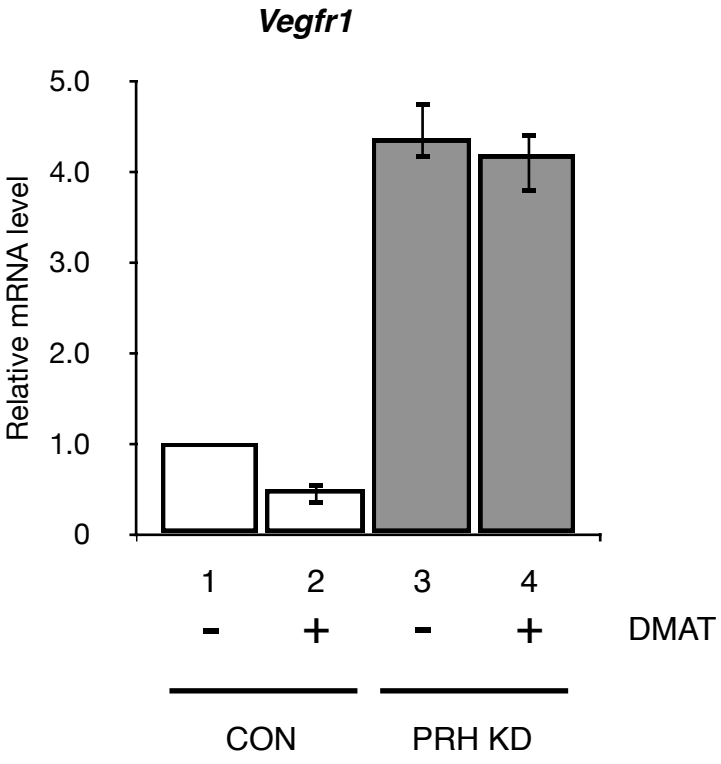


Figure 5.3 - Repression of *Vegf, Vegfr1* by CK2 inhibitors requires PRH expression.

[A] *Vegf* mRNA levels in K562 shRNA control cells (CON) and PRH shRNA cells (PRH KD) 24 hours after treatment with 80μM DMAT or the equivalent volume of DMSO. RNA was extracted and 0.5μg of extracted RNA was reverse transcribed to cDNA. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using gene specific primers and comparing to qPCR reactions for *Gapdh*. Values are means and standard deviations (SD) (n = 3). [B] *Vegfr1* mRNA levels from the same cells as in panel A.

[A] - lane 4) has no effect on the level of *Vegf* gene expression compared to untreated PRH knockdown cells (figure 5.3 [A] - lane 3). This demonstrates that PRH is necessary for CK2 induced regulation of *Vegf* gene expression.

Analysis of *Vegfr1* expression levels in knockdown and control cells treated with DMAT shows that in control cells *Vegfr1* expression is reduced to approximately 40% (figure 5.3 [B] - lane 2) of its unrepressed state (figure 5.3 [B] - lane 1). ShRNA knockdown of PRH (figure 5.3 [B] - lane 3) results in elevated expression of *Vegfr1* compared to the control cells (figure 5.3 [B] - lane 1), as previously described in Chapter 3. However, in PRH knockdown cells that are treated with DMAT (figure 5.3 [B] - lane 4) there is no effect on the level of *Vegfr1* gene expression compared to untreated PRH knockdown cells (figure 5.3 [B] - lane 3). Again this demonstrates that PRH is necessary for CK2 induced regulation of *Vegfr1* gene expression.

To determine how CK2 phosphorylation of PRH regulates PRH function as a transcriptional repressor protein, mutations were made at serines 163 and 177. To mimic phosphorylation of these residues these two serines were mutated to glutamic acid residues (figure 5.4). In addition, these serines were also mutated to cysteines (figure 5.4), to prevent phosphorylation and to imitate unphosphorylated serine residues. Mutagenesis was performed by Hannah Williams and the plasmids were gifted to me for these studies.

To further investigate the role of CK2 regulation of *Vegf* gene expression through PRH; PRH, a PRH mutant that cannot be phosphorylated by CK2 (PRH S163C,S177C), and the PRH phospho-mimic (PRH S163E,S177E) were expressed in K562 cells alone or with CK2 α and β. 48 hours post-transfection, RNA was extracted and reverse transcribed to produce cDNA which was analyzed as described above by qPCR. As previously observed, when PRH is expressed, *Vegf* gene expression is repressed (figure 5.5 [A] - lane 2) when compared to the

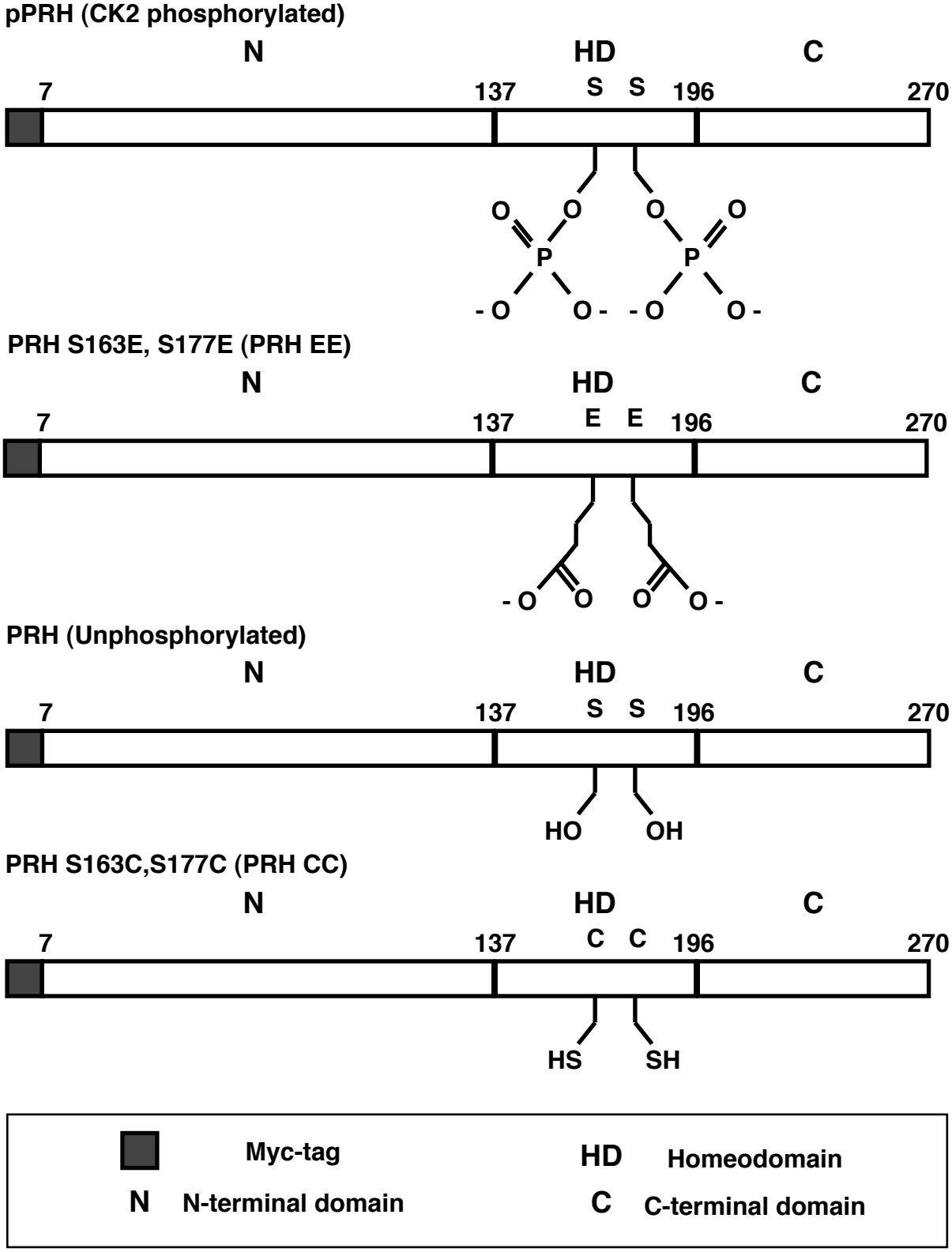


Figure 5.4 - Schematic diagram of Myc-PRH CK2 phosphorylation site point mutations.

Schematic diagram of the PRH protein in pMUG1 Myc-PRH and targeted mutations in the protein. The PRH S163E,S177E mutant mimics CK2 phosphorylated PRH. The PRH S163C,S177C mutant is unable to be phosphorylated.

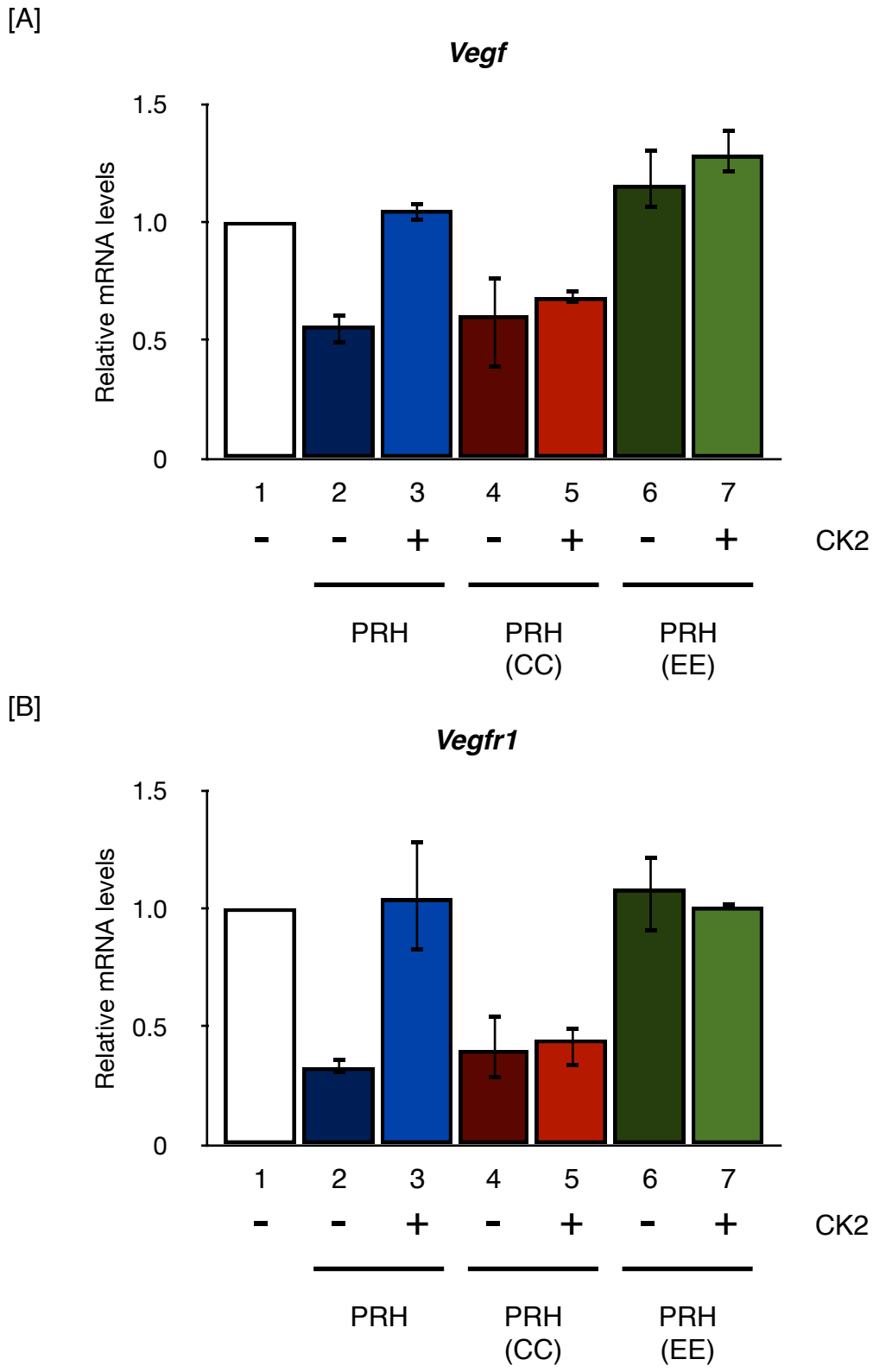


Figure 5.5 - Regulation of *Vegf, Vegfr1* expression by CK2 phosphorylation mutants of PRH.

[A]*Vegf* mRNA levels in K562 cells 48 h after transfection with 5 μg pMUG1 (lane 1) or pMUG1 vectors expressing PRH (PRH [lane 2], PRH S163C,S177C [lane 3], or PRH S163E,S177E [lane 4]) alone or with 3μg pRc/CMV-CK2α-HA, and 5μg pRc/CMV-HA-CK2β. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using gene specific primers and comparing to qPCR reactions for *Gapdh*. Values are means and SD (n = 3). [B] *Vegfr1* mRNA levels in K562 cells 48 hours after transfection with the vectors described for panel [A]. Values are means and SD (n = 3).

expression of *Vegf* in control cells (figure 5.5 [A] - lane 1). However, when PRH and CK2 are co-expressed PRH no longer represses *Vegf* expression (figure 5.5 [A] - lane 3). *Vegf* gene expression is also reduced in cells expressing PRH S163C,S177C (figure 5.5 [A] - lane 4) to a similar level as observed in cells expressing PRH (figure 5.5 [A] - lane 2). Yet, when PRH S163C,S177C and CK2 are co-expressed (figure 5.5 [A] - lane 5) there is no significant difference in repression of *Vegf* expression when compared to PRH S163,S177C alone (figure 5.5 [A] - lane 4). Additionally, cells expressing PRH S163E,S177E alone (figure 5.5 [A] - lane 6) or cells expressing PRH S163E,S177E and CK2 (figure 5.5 [A] - lane 7) have unchanged *Vegf* expression compared to control cells (figure 5.5 [A] - lane 1). This suggests that the serine residues 163 and 177 within the homeodomain are vital for CK2 dependent antagonism of PRH repression at the *Vegf* gene.

Analysis of *Vegfr1* gene expression in the same experiment shows that *Vegfr1* is repressed when PRH is expressed, (figure 5.5 [B] - lane 2) when compared to the expression of *Vegfr1* in control cells (figure 5.5 [B] - lane 1). However, when PRH and CK2 are co-expressed, repression of *Vegfr1* expression is no longer observed (figure 5.5 [B] - lane 3) compared to the control cells (figure 5.5 [B] - lane 1). Cells expressing PRH S163C,S177C (figure 5.5 [B] - lane 4) also have repressed *Vegfr1* gene expression compared to the control (figure 5.5 [B] - lane 4). Yet, cells expressing PRH S163C,S177C and CK2 (figure 5.5 [B] - lane 5) have no significant difference in *Vegfr1* expression compared to PRH S163,S177C alone (figure 5.5 [B] - lane 4). Furthermore, expression of PRH S163E,S177E alone (figure 5.5 [B] - lane 6) or PRH S163E,S177E and CK2 (figure 5.5 [B] - lane 7) has no effect on expression (figure 5.5 [B] - lane 1). These data demonstrate that CK2 antagonizes PRH repression of *Vegfr1* expression through the phosphorylation of serine residues 163 and 177 within the homeodomain of PRH.

5.4 CK2 phosphorylation of PRH regulates DNA binding of the *Vegf and Vegfr1* promoters.

To investigate the whether CK2 phosphorylation inhibits DNA binding of PRH *in vivo*, chromatin immuno-precipitation assays were carried out with cells expressing; PRH, PRH S163C,S177C, or PRH S163E,S177E alone or co-expressing CK2. K562 cells were transfected with pMUG1 vectors expressing Myc-PRH (PRH, PRH S163C,S177C, or PRH S163E,S177E) alone or with CK2α and CK2β expression vectors. ChIP was performed as outlined in Chapter 3. PRH binds to the *Vegfr1* promoter, as described in section 3.4, at multiple sites across a 10kb region. As previously observed, in cells expressing PRH alone enrichment of the upstream -3000 and -1000 regions (figure 5.6 [A] - black bars at -3000 and -1000) and the region covering the ATG (figure 5.6 [A] - black bar at 0) are observed compared to the input (total pool of DNA fragments)(figure 5.6 [A] - white bars at -3000, -1000, and 0). In addition there is moderate enrichment of the upstream -5000 and downstream +1700 regions (figure 5.6 [A] - black bars at -5000 and +1700) when compared to the input DNA (figure 5.6 [A] - white bars at -5000 and +1700). No enrichment is observed at the distal upstream promoter region of *Vegfr1,* -7500bp from the ATG, in cells containing PRH alone (figure 5.6 [A] - black bar compared to white bar at -7500). However, in cells expressing PRH and CK2, no significant enrichment is observed across the entire 7.5kb regulatory region for *Vegfr1* (figure 5.6 [B]). For cells containing PRH S163C,S177C (figure 5.6 [C]), a similar pattern of enrichment is observed as for wild-type PRH (figure 5.6 [A]). Substantial enrichment is observed for regions of *Vegfr1* at distances of -3000, -1000 and 0bp from the ATG (figure 5.6 [C] - black bars compared to white bars). There is also some enrichment observed for the -5000 and +1700 regions of the *Vegfr1* promoter, but there is no enrichment at the -7500bp upstream region (figure 5.6 [C] - black bars compared to white bars), as

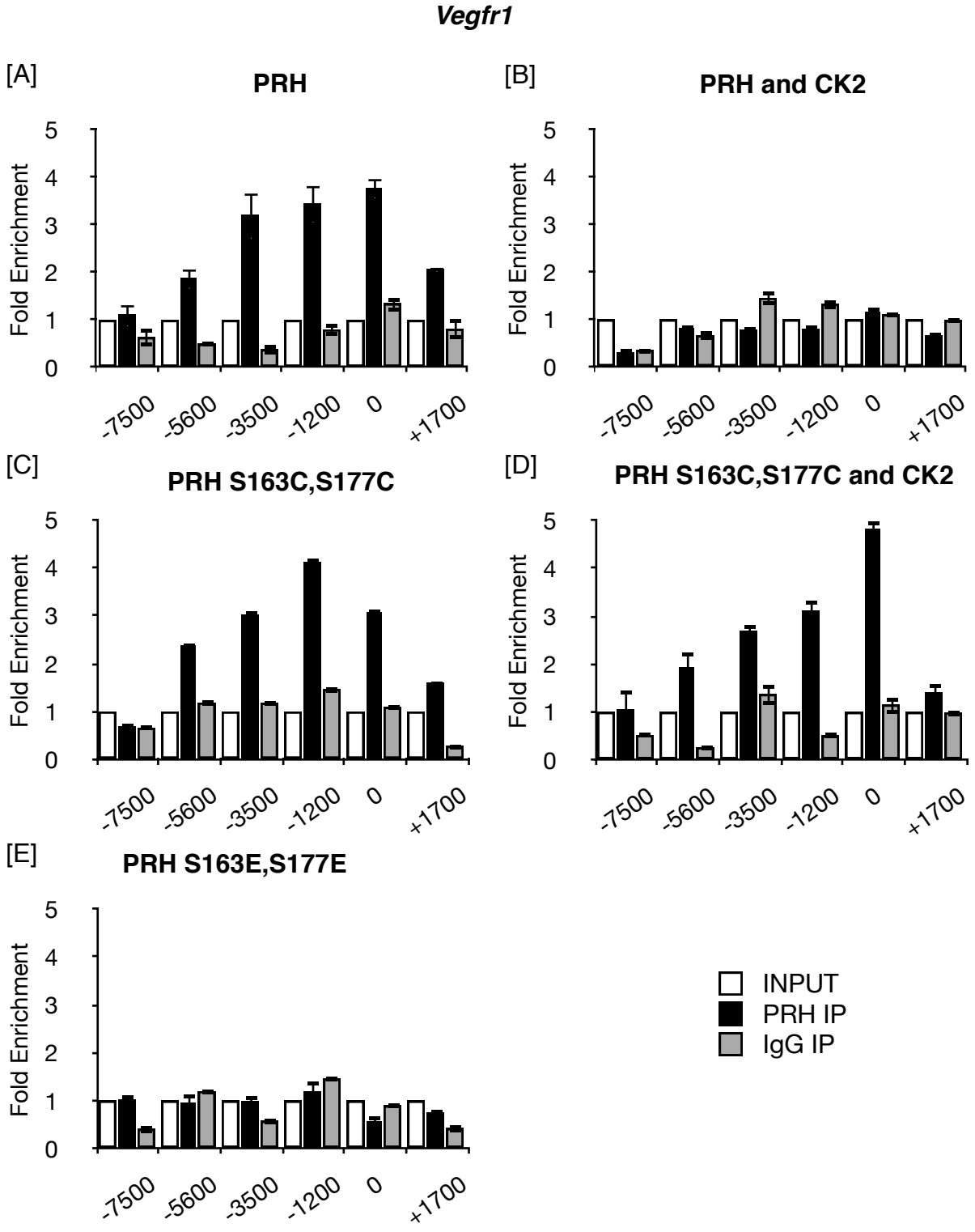


Figure 5.6 - CK2 phosphorylation controls PRH binding to the *Vegfr1* promoter.

[A] Chromatin Immuno-precipitation of Myc-PRH from K562 cells transfected with 5 μg pMUG1 Myc-PRH. Enrichment levels were determined by using the purified DNA in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the *Gapdh* promoter. Values are taken from a single immuno-precipitation and SD of 3 qPCR reactions. [B] As for panel A with cells transfected with 5μg pMUG1 Myc-PRH, 3μg pRc/CMV-CK2α-HA, and 5μg pRc/CMV-HA-CK2β. [C] As for panel A with cells transfected with 5μg pMUG1 Myc-PRH S163C,S177C. [D] As for panel A with cells transfected with 5μg pMUG1 Myc-PRH S163C,S177C, 3μg pRc/CMV-CK2α-HA, and 5μg pRc/CMV-HA-CK2β. [E] As for panel A with cells transfected with 5μg pMUG1 Myc-PRH S163E,S177E.

observed for PRH (figure 5.6 [A]). Expression of CK2 along with PRH S163C,S177C (figure 5.6 [D]) also results in binding across the promoter as observed for PRH S163C,S177C alone (figure 5.6 [C]). PRH S163C,S177C is bound to the -5000, -3000, -1000, and 0 bp upstream from the ATG site of *Vegfr1* in the presence of CK2, although there is no binding at the -7500 and +1700 regions of the promoters. No binding of PRH S163E,S177E is observed across the *Vegfr1* promoter (figure 5.6 [E]). This data was collected from one set of chromatin, a further repeat that only analyzed the -1000bp region upstream of the TSS was performed and confirmed the observation that CK2 activity disrupts PRH binding but does not affect PRH S163C,S177C DNA binding activity (figure 5.7 [A]). PRH DNA binding to the *Vegf* gene promoter was also analyzed from these chromatin samples. Enrichment of the -5.5kb region of the *Vegf* gene promoter is observed in immuno-precipitations of PRH from cells expressing PRH alone (figure 5.7 [B] - lanes 1-3). However, in cells expressing PRH and CK2 no enrichment is observed (figure 5.7 [B] - lanes 4-6). As observed at the *Vegfr1* gene promoter, PRH S163C,S177C binds to the *Vegf* gene promoter in cells expressing PRH S163C,S177C alone (figure 5.7 [B] - lanes 7-9) or in cells expressing PRH S163C,S177C and CK2 (figure 5.7 [B] - lanes 10-12). The PRH S163E,S177E does not associate with the *Vegf* gene promoter (figure 5.7 [B] - lanes 13-15). These data show that CK2 phosphorylation of PRH can inhibit DNA binding *in vivo* and that the serine residues 163 and 177 within the homeodomain are the targets of this CK2 phosphorylation.

5.5 CK2 phosphorylation of PRH regulates cell growth and apoptosis.

To investigate the role of CK2 phosphorylation of PRH in controlling growth potential, K562 cells were transfected with empty vector (pMUG1) or PRH (pMUG1 Myc-PRH) alone or PRH with the CK2 subunits α and β, or PRH with the β subunit and a

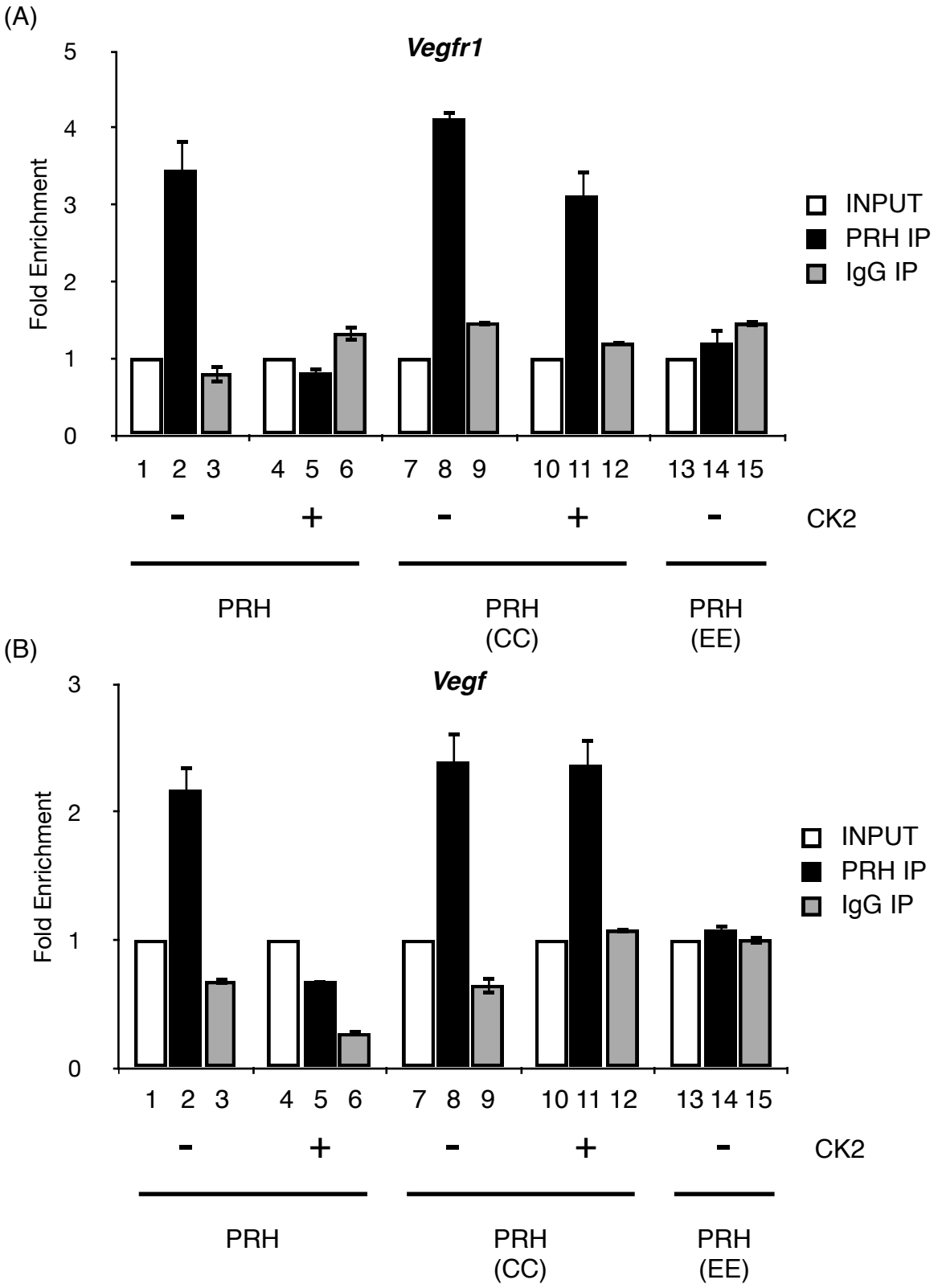


Figure 5.7 - CK2 phosphorylation of PRH inhibits DNA binding at *Vegfr1* and *Vegf*.

CK2 phosphorylation controls PRH binding to the [A] *Vegfr1* promoter and [B] *Vegf* promoter. Chromatin Immuno-precipitation of Myc-PRH from K562 cells transfected with 5 *μ*g pMUG1 vector containing (Myc-PRH, Myc-PRH S163C,S177C, Myc-PRH S163E,S177E) with or without 3*μ*g pRc/CMV-CK2α-HA, and 5*μ*g pRc/CMV-HA-CK2β. Enrichment levels were determined by using the purified DNA in a qPCR reaction using promoter specific primers and comparing to qPCR reactions for the *Gapdh* promoter. Values are taken from a single immuno-precipitation and SD of 3 qPCR reactions. Data representative of 3 sets of extracted chromatin.

mutated α subunit of CK2, or PRH with the β subunit of CK2 only. 24 hours after transfection viable cells were counted using trypan blue exclusion staining and cells were diluted to equal starting concentrations. After 72 hours the cells were re-counted. In the cells expressing PRH alone (figure 5.8 - lane 2) a reduction in the total number of cells after 72 hours was observed compared to the control cells (figure 5.8 - lane 1). However, the growth of cells expressing both subunits of CK2, as well as PRH (figure 5.8 - lane 3) is unaffected compared to the control cells (figure 5.8 - lane 1). Yet, for cells expressing PRH with the β subunit alone (figure 5.8 - lane 5) or the β subunit and a mutated CK2α subunit (figure 5.8 - lane 4), reduced growth

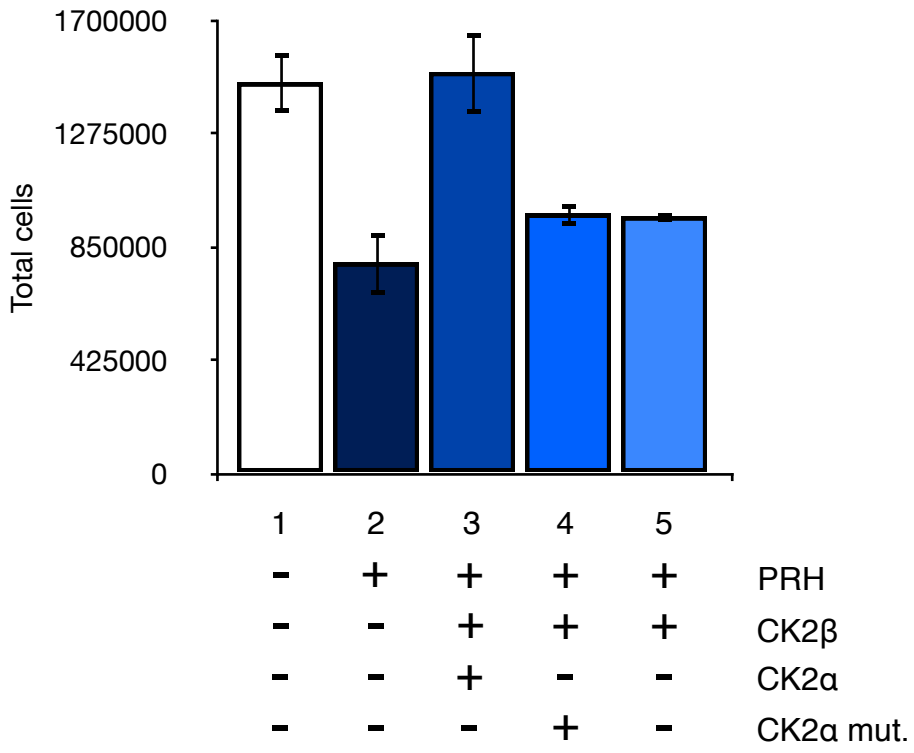


Figure 5.8 - CK2 antagonizes PRH induced cell growth.

CK2 antagonises PRH repression of growth. K562 cells were transfected with 5*μ*g pMUG1 Myc-PRH alone, or with 3*μ*g pRc/CMV-CK2α-HA and 5*μ*g pRc/CMV-HA-CK2β, or with 3*μ*g pRc/CMV-CK2α-K68M-HA and 5*μ*g pRc/CMV-HA-CK2β, or with 5*μ*g pRc/CMV-HA-CK2β. K562 cells transfected with 5*μ*g pMUG1 was used as a control. Populations were set to equal numbers of cells and counted after 72 hours by trypan blue exclusion. Values are means and SD (n=3).

is still observed in comparison to the growth of control cells (figure 5.8 - lane 1). This suggests that phosphorylation of PRH by CK2 antagonizes growth inhibition by PRH.

To determine whether phosphorylation within the PRH homeodomain is essential for the CK2 dependent antagonism of growth inhibition by PRH, K562 cells expressing GFP or GFP with PRH or PRH S163C,S177C or PRH S163E,S177E with or without expression of CK2 α and β were used. 24 hours post-transfection viable cells were counted in the presence of trypan blue dye as described above. Cells expressing PRH alone (figure 5.9 [A] - lane 2) have a reduction in the total number of cells after 72 hours compared to the control cells (figure 5.9 [A] - lane 1), as previously observed. However, for cells expressing PRH and both subunits of CK2 (figure 5.9 [A] - lane 3) no change in the growth of these cells is observed when compared to the control cells (figure 5.9 [A] - lane 1). Expression of PRH S163C,S177C in K562 cells (figure 5.9 [A] - lane 4) results in a reduction in the total number of cells after 72 hours, similar to that seen with PRH expressing cells (figure 5.9 [A] - lane 2). In contrast to wild type PRH and CK2, the expression of CK2 has no effect on PRH S163C,S177C (figure 5.9 [A] - lane 5). The growth of cells expressing PRH S163E,S177E (figure 5.9 [A] - lane 6) is similar to control cells (figure 5.9 [A] - lane 1) and co-expression of PRH S163E,S177E and CK2 (figure 5.9 [A] - lane 7) has no effect on the growth of the cells. These results suggest that the CK2 phosphorylation sites within the homeodomain of PRH are vital for CK2 dependent antagonism of PRH activity in relation to cell growth.

Chapter 4 showed that PRH inhibits growth by increasing apoptosis. To determine whether CK2 antagonises PRH induced apoptosis, K562 cells were transfected as described above and stained with PI and Annexin V APC 24 hours post-transfection and analyzed using flow cytometry. Transfected cells were identified through expression of EGFP. EGFP positive cells were examined by staining with PI and

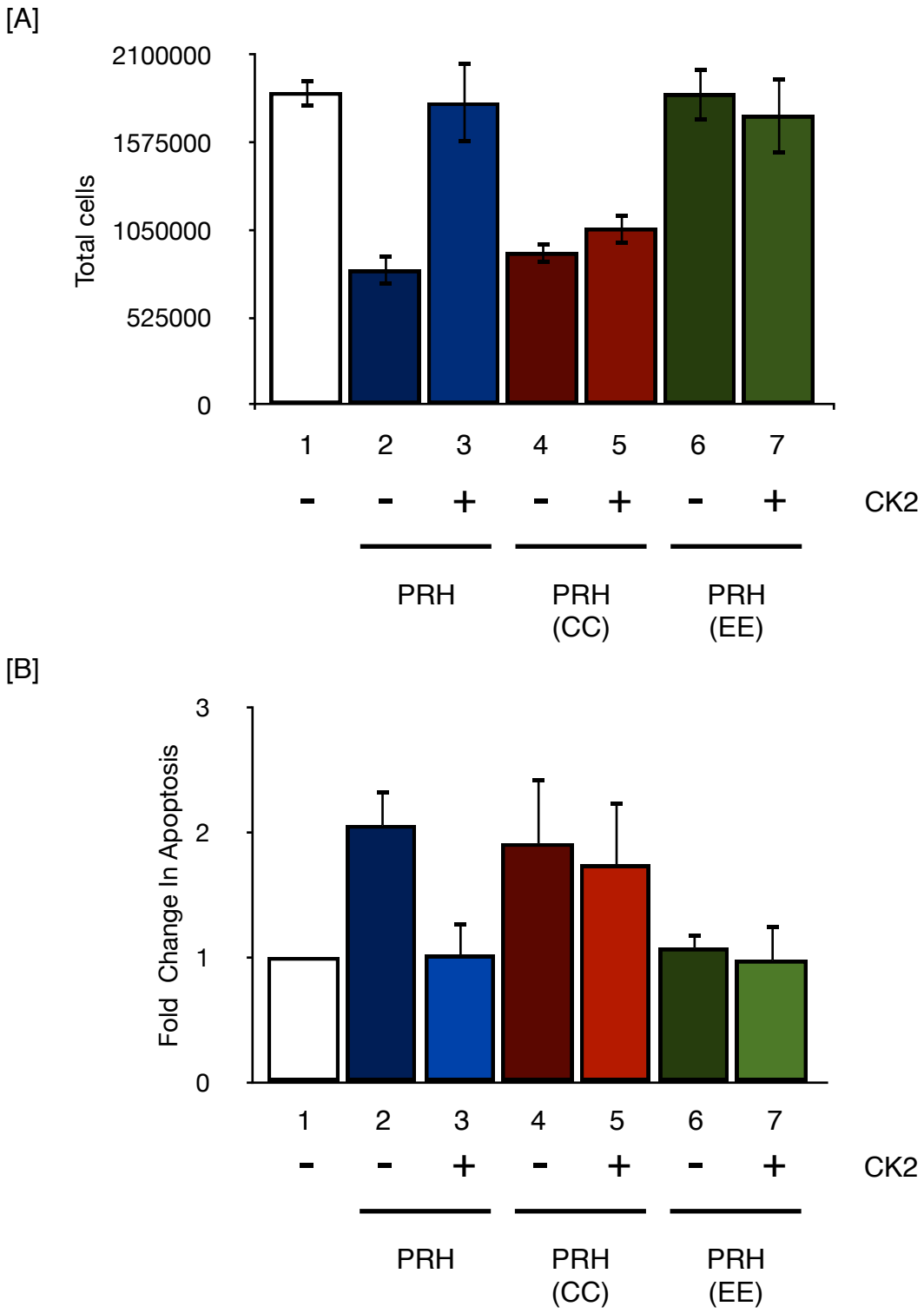


Figure 5.9 - The effect of CK2 on PRH induced cell apoptosis.

CK2 antagonizes PRH repression of growth and induction of apoptosis. [A] K562 cells were transfected with 2*μ*g pEGFP and 5*μ*g pMUG1 or 2*μ*g pEGFP and 5*μ*g pMUG1 vector containing (Myc-PRH, Myc-PRH S163C,S177C, Myc-PRH S163E,S177E) with or without 3*μ*g pRc/CMV-CK2α-HA and 5*μ*g pRc/CMV-HA-CK2β. Populations were set to equal numbers of cells and counted after 72 hours by trypan blue exclusion. Values are means and SD (n=3). [B] Cells as described for panel A were stained with propidium iodide and an anti-Annexin V APC antibody after 24 hours. Fold change in total apoptosis compared to the EGFP control. Values are means and SD (n=3).

Annexin V APC. As observed in previous experiments, cells expressing PRH (figure 5.9 [B] - lane 2) have approximately double the total number of cells in apoptosis (early and late) compared to the control cells (figure 5.9 [B] - lane 1). However, cells co-expressing PRH and CK2 (figure 5.9 [B] - lane 3) have an equal number of cells undergoing apoptosis compared to the control cells (figure 5.9 [B] - lane 1). In cells expressing PRH S163C,S177C (figure 5.9 [B] - lane 4) the total number of cells undergoing apoptosis is increased in comparison to the control cells (figure 5.9 [B] - lane 1) to a similar level as cells expressing the wild-type PRH (figure 5.9 [B] - lane 2). Co-expression of PRH S163C,S177C and CK2 in K562 cells (figure 5.9 [B] - lane 4) is still elevated when compared to the control cells (figure 5.9 [B] - lane 5). Expression of PRH S163E,S177E alone (figure 5.9 [B] - lane 6) or with CK2 (figure 5.9 [B] - lane 7) has no effect on cell apoptosis compared to the control cells (figure 5.9 [B] - lane 1). Since CK2 cannot block PRH induced apoptosis when PRH is mutated at serines 163 and 177 it can be concluded that the PRH induced apoptosis observed in K562 cells is antagonized by CK2 phosphorylation at these positions within the homeodomain of PRH.

5.6 BCR-ABL1 increases CK2 phosphorylation of PRH.

K562 cells are derived from a patient diagnosed with CML, and they contain the Philadelphia chromosome that encodes for the p210 BCR-ABL1 fusion protein, which drives proliferation (Lozzio and Lozzio, 1975). Imatinib is a small molecule inhibitor of ABL1 that is used to treat CML patients who have the Philadelphia chromosome. Imatinib binds to the ATP pocket of the ABL1 moiety and stabilizes the inactive form (O'hare et al., 2007). Dasatinib is a second generation BCR-ABL1 inhibitor, which binds only to the active conformation of the ATP binding pocket of ABL1 (O'hare et al., 2007). Imatinib and Dasatinib are not structurally related molecules. Inhibition of p190 BCR-ABL1 in lymphoid cells using Imatinib results in inhibition of CK2 activity

and this is proposed to occur through the direct interaction of BCR-ABL1 and the CK2α subunit (Mishra et al., 2003). Since direct inhibition of CK2 with DMAT increases transcriptional repression of VSP genes when PRH is present, I wanted to determine whether inhibition of CK2 activity indirectly through inhibition of BCR-ABL1 activity would have the same effect. K562 cells were therefore treated with the BCR-ABL1 inhibitors, Imatinib or Dasatinib, or an equivalent volume of DMSO for 8 hours. Whole cell protein extracts were made, equal amounts of protein were separated on a SDS-PAG, and the proteins were transferred onto an Immobilon P membrane. Western blot analysis was performed by staining the membrane with a rabbit anti-PRH antibody (figure 5.10 - top panel) for phosphorylated PRH. The blot was stripped of antibody and re-probed with a mouse anti-PRH antibody (figure 5.10 - upper middle panel) for unphosphorylated PRH, or an anti-pXRCC1 antibody (figure 5.10 - lower middle panel), or an anti-Lamin A/C antibody which was used as a loading control (figure 5.10 - bottom panel). In DMSO treated K562 cells, a strong band can be observed at 36kDa (figure 5.10 - top panel, lane 1), which correlates to full-length pPRH. The pPRH band is markedly reduced in K562 cells treated with 100μM Imatinib (figure 5.10 - top panel, lane 2). In addition, K562 cells treated with the more potent BCR-ABL1 inhibitor Dasatinib (30μM) also show reduced pPRH levels (figure 5.10 - top panel, lane 4) compared to DMSO treated K562 cells (figure 5.10 - top panel, lane 3). However, the level of unphosphorylated PRH protein is unchanged by either treatment (figure 5.10 - middle panel, lanes 1-4). In K562 cells treated with either Imatinib or Dasatinib, antibody staining for CK2 phosphorylated XRCC1, an unrelated CK2 target, was also substantially reduced (figure 5.10 - lower middle panel, lanes 2 and 4) indicating that CK2 activity is reduced in cells treated with BCR-ABL1 inhibitors. These data show that inhibition of BCR-ABL1 activity

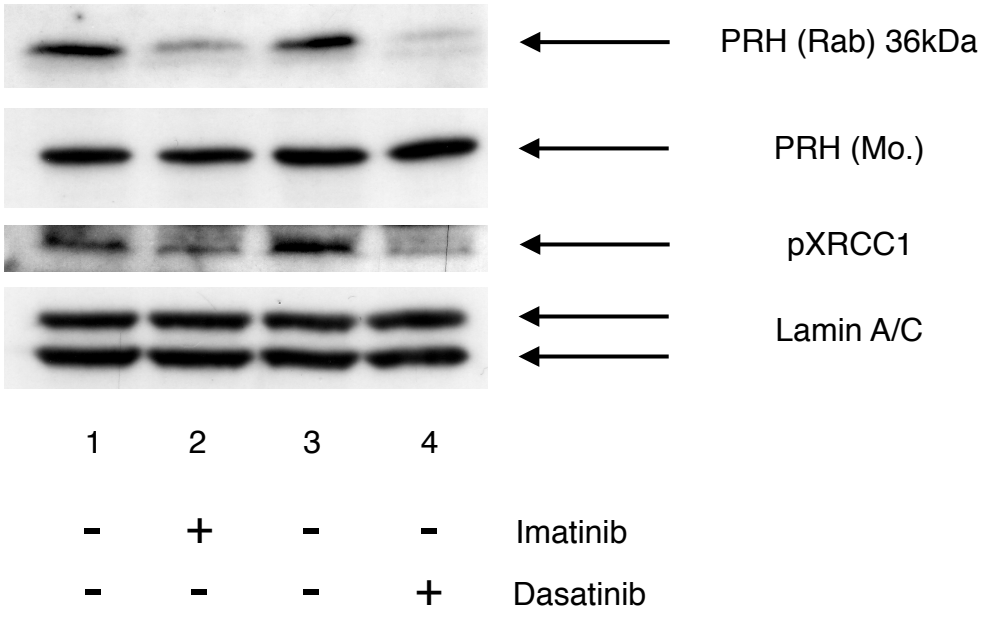


Figure 5.10 - Inhibition of BCR-ABL1 decreases CK2 phosphorylation of PRH.

K562 cells were treated with 100μM Imatinib (lane 2) or 30μM Dasatinib (lane 4) or the equivalent volume of DMSO (lanes 1 and 3). Proteins were then separated by SDS-PAGE and western blotted for endogenous phosphorylated PRH using a rabbit anti-PRH antibody (top panel), unphosphorylated PRH using a mouse anti-PRH antibody (upper middle panel), pXRCC1 (lower middle panel) and Lamin A/C was used as a control (bottom panel). Data representative of three independent experiments.

results in decreased phosphorylation of the unrelated CK2 target proteins PRH and XRCC1.

To determine whether inhibition of BCR-ABL1 activity affects the regulation of VSP genes, RNA was extracted from PRH KD and control cells that were treated as described above. Expression of the *Vegf* gene was analyzed using qPCR as described previously. In control cells treated with Imatinib the level of *Vegf* expression was reduced approximately 35% when compared to the level of *Vegf* expression for cells treated with DMSO (figure 5.11 [A] - lane 2 and 1). For control cells treated with Dasatinib the level of *Vegf* expression was more robustly repressed to almost 50% compared to the levels observed for K562 cells treated with DMSO (figure 5.11 [A] -

lane 3 and 1). However, in PRH KD cells treated with Imatinib *Vegf* expression was unchanged compared to the DMSO treated PRH KD cells (figure 5.11 [A] - lane 5 and 4). The level of expression of *Vegf* in PRH KD cells treated with Dasatinib was slightly reduced compared to PRH KD cells treated with DMSO (figure 5.11 [A] - lane 6 and 4), however, this decrease is not significant. This demonstrates that inhibition of the p210 BCR-ABL1 fusion protein activity decreases the expression of the PRH target gene, *Vegf*.

To investigate the regulation of *Vegfr1* gene expression by p210 BCR-ABL1 activity, qPCR analysis was performed as above except using *Vegfr1* specific primers. The level of *Vegfr1* expression was reduced to almost 50% in control cells treated with Imatinib when compared to the level of *Vegf* expression for cells treated with DMSO (figure 5.11 [B] - lane 2 and 1). In control cells treated with Dasatinib, *Vegfr1* expression was repressed to approximately 65% of the level observed for control cells treated with DMSO (figure 5.11 [B] - lane 3 and 1). Conversely, in PRH KD cells treated with Imatinib, the level of *Vegfr1* expression is unchanged from PRH KD cells treated with DMSO (figure 5.11 [B] - lane 5 and 4). Furthermore, the level of *Vegfr1* expression is unaffected by Dasatinib treatment of PRH KD cells when compared with PRH KD cells treated with DMSO (figure 5.11 [B] - lane 6 and 4). This shows that the expression level of two PRH target genes, *Vegf* and *Vegfr1*, are both controlled through the activity of the p210 BCR-ABL1 fusion protein.

To determine whether PRH plays a role in Dasatinib induced cell death of BCR-ABL1 positive cells, PRH KD and control cells were treated with 30μM Dasatinib for 16 hours. Cell numbers were determined using MTT assays, starting with equal numbers of cells as described previously. Data shown is the mean and standard error from a single experiment performed in quadruplicate and is representative of three independent experiments. Dasatinib treatment of control cells reduces cell number

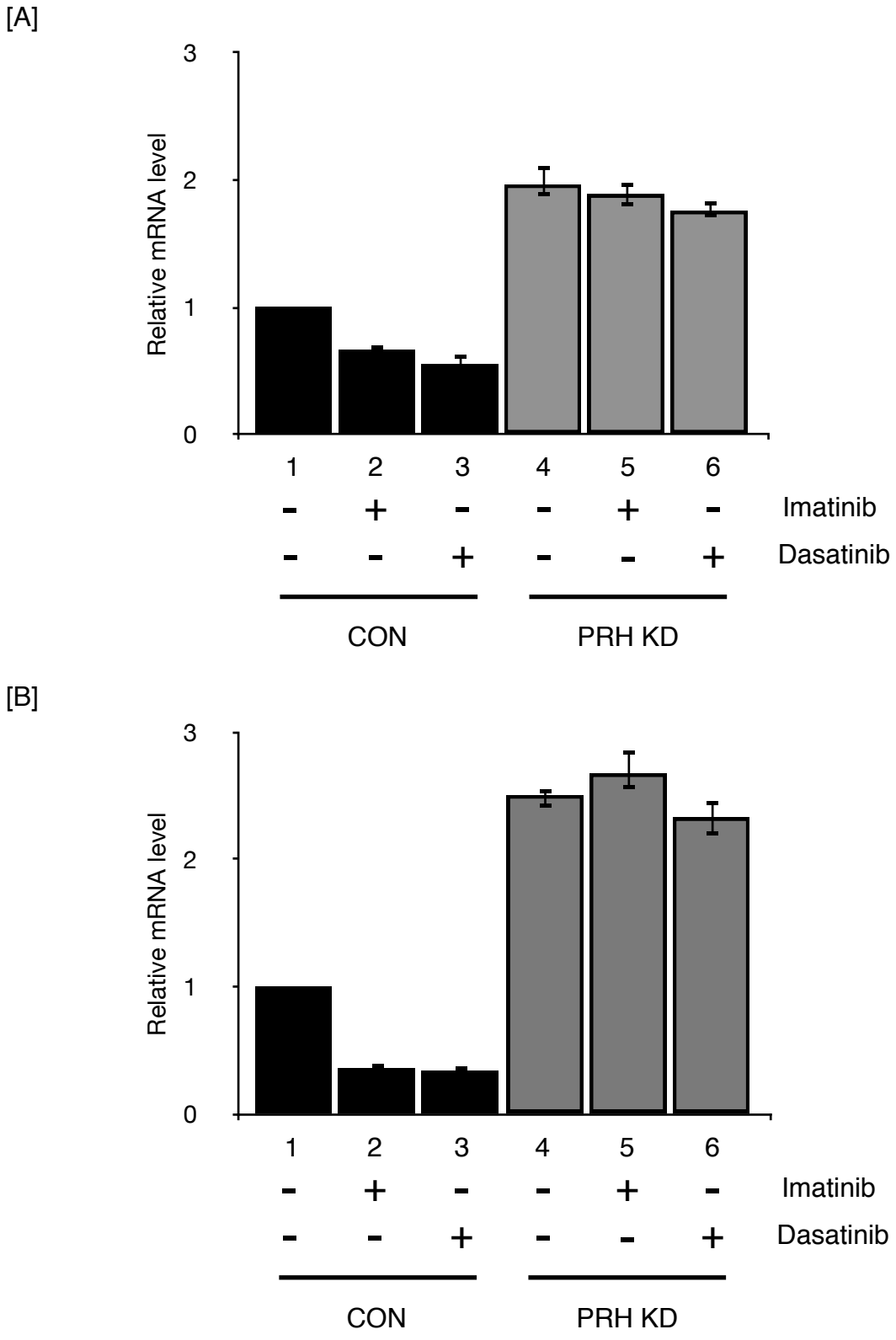


Figure 5.11 - Inhibition of BCR-ABL1 reduces *Vegf* and *Vegfr1* expression.

[A] PRH knockdown (PRH KD) and Control (CON) cells were treated with 100μM Imatinib or 30μM Dasatinib or the equivalent volume of DMSO for 24 hours. RNA was extracted and cDNA was reverse transcribed. mRNA levels were determined by using the synthesized cDNA in a qPCR reaction using *Vegf* specific primers and comparing to qPCR reactions for *Gapdh*. Values are means and standard deviations (SD) (n = 3). [B] As for panel A, except using *Vegfr1* specific primers.

after 16 hours of treatment when compared to control cells treated with DMSO (figure 5.12 - lanes 2 and 1). PRH KD cells treated with Dasatinib are also significantly reduced compared to PRH KD cells treated with DMSO (figure 5.12 - lanes 4 and 3) However, there is a significant increase in the number of PRH KD cells treated with Dasatinib compared to control cells treated with Dasatinib (p=0.001) (figure 5.12 - lanes 4 and 2). These data show that PRH plays a role in the cell death processes induced by the BCR-ABL1 inhibitor, Dasatinib.

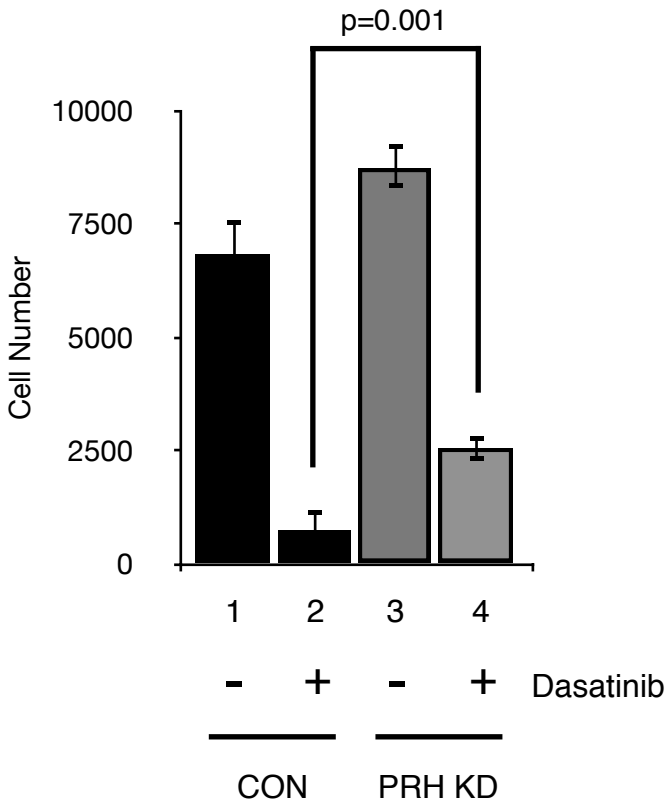


Figure 5.12 - Absence of PRH reduces BCR-ABL1 inhibitor potency in K562 cells.

Control and PRH KD cells were treated with 30μM Dasatinib (bars 2 and 4) or an equal volume of DMSO (bars 1 and 3) for 16 hours. An MTT assay was then used to calculate cell numbers. Values are means and SD (n = 4). Data representative of three independent experiments.

5.7 Phosphorylation of PRH reduces proteasome dependent protein stability and nuclear localization.

Previous studies have demonstrated a role for CK2 in the regulation of degradation for specific proteins. This regulation can be positive or negative depending on the target protein. For example, CK2 phosphorylates the tumour suppressor PML and specifically targets this protein for degradation by the proteasome (Scaglioni et al., 2006). However, CK2 phosphorylation of c-myc is vital for the proliferation of T-cell lymphomas and pharmacological inhibition or knock-down of CK2 reduces the stability of c-myc (Channavajhala and Seldin, 2002). Thus alteration of protein stability is a feature of some CK2 target proteins.

In K562 cell extract full length pPRH (36kDa) detected by rabbit pPRH antibody is always accompanied by a 27kDa product and this product is present even in DMAT treated cells, although there is a small reduction over 24 hours (figure 5.1 [A]). To determine whether the endogenous 27kDa PRH band observed in figure 5.1 [A] is a CK2 dependent cleavage product of full-length PRH, K562 cells or cells expressing PRH with or without co-expression of the α and β subunits of CK2 were harvested after 24 hours and whole cell protein extracts were made. Proteins were separated by SDS-PAG, transferred to a PVDF membrane and stained with an anti-Myc antibody. The membrane was stripped of antibody and re-stained with the rabbit anti-PRH antibody or an anti-Tubulin antibody, which was used as a loading control (figure 5.13 - lower panel). The Myc antibody detects a strong 36kDa band and a weak 27 kDa band for PRH from cells transfected with Myc-PRH alone (figure 5.13 - top panel, lane 2). In cells expressing Myc-PRH and CK2, the Myc antibody detects a stronger band at 27kDa (figure 5.13 - top panel, lane 3) compared to the 27kDa band observed in cells expressing Myc-PRH alone (figure 5.13 - top panel, lane 2). Re-probing of the membrane with the rabbit anti-PRH antibody confirms that a 36kDa

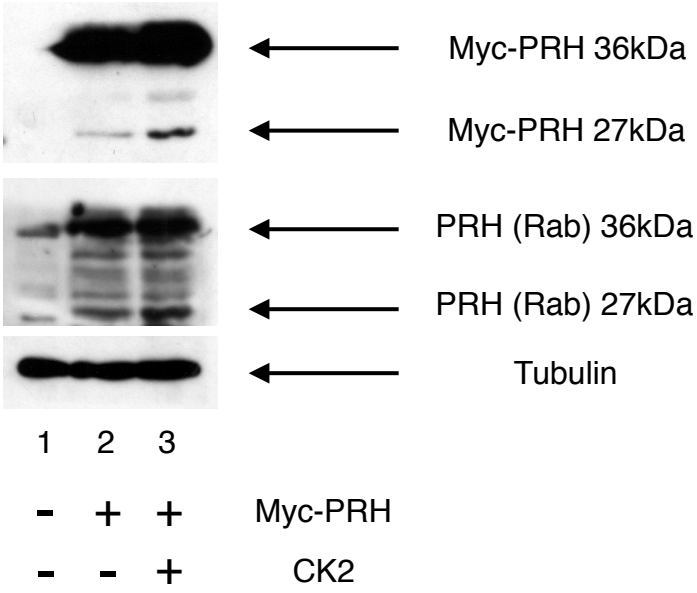


Figure 5.13 - CK2 phosphorylation increases cleavage of PRH.

Untransfected K562 cells (lane 1) or cells transfected with 5μg pMUG1 Myc-PRH (lanes 2) or cells transfected with 5μg pMUG1 Myc-PRH, 3μg pRc/CMV-CK2α-HA and 5μg pRc/CMV-HA-CK2β. The cells were harvested and whole cell protein was extracted. Proteins were separated on a SDS-PAG and transferred to a PVDF membrane that was stained with a anti-Myc (top panel), or a rabbit anti-PRH antibody (middle panel), or an anti-Tubulin antibody (lower panel). Data representative of two independent experiments.

and a 27kDa band are present in cells alone (figure 5.13 - middle panel, lane 1). In cells expressing Myc-PRH, the myc tag slightly retards the mobility of the 36kDa and 27kDa PRH proteins, therefore bands corresponding to the exogenous Myc-PRH protein are observed at 37kDa and 28kDa (figure 5.13 - middle panel, lane 2). In cells expressing Myc-PRH and CK2, the 28kDa PRH band (figure 5.13 - middle panel, lane 3) is stronger than in cells expressing Myc-PRH alone (figure 5.13 - middle panel, lane 2). However, the 36kDa PRH species is equally expressed in the presence or absence of CK2 (figure 5.13 - middle panel, lanes 2 and 3). This suggests that elevated CK2 expression increases the presence of the 27kDa PRH band. It can be concluded that the 27kDa PRH species is a C-terminal truncation of PRH because the Myc-tag is attached at the N-terminal domain (see figure 3.6).

Therefore, from this point forwards the 27kDa PRH species will be referred to as pPRHΔC.

To further investigate the presence of pPRHΔC, K562 cells were transfected with empty vector, PRH or the series of PRH mutants described in Chapter 3 (Myc-PRH LL23,24AA, Myc-PRH F32E, Myc-PRH N187A, Myc-PRH SS163,177EE, and Myc-PRH SS163,177CC). The cells were harvested after 24 hours and whole cell protein extracts were made. Proteins were separated by SDS-PAG, transferred to a PVDF membrane and stained with an anti-Myc antibody. The membrane was stripped of antibody and re-stained with anti-Tubulin antibody, which was used as a loading control (figure 5.14 [A] - lower panel). As described above, the Myc antibody detects a strong 36kDa band and a weak 27 kDa band for PRH from cells transfected with Myc-PRH alone (figure 5.14 [A] - top panel, lane 2). In cells expressing proteins with mutations that inhibit the interaction of PRH with eIF4E (PRH LL23,24AA) (figure 5.14 [A] - top panel, lane 3), or inhibit the interaction of PRH with TLE (PRH F32E) (figure 5.14 [A] - top panel, lane 4), the levels of expression of pPRHΔC are equivalent to the expression seen with wild-type Myc-PRH (figure 5.14 [A] - top panel, lane 2). However, the expression of pPRHΔC in cells expressing Myc-PRH N187A (figure 5.14 [A] - top panel, lane 5) appears slightly reduced compared to cells expressing wild-type Myc-PRH (figure 5.14 [A] - top panel, lane 2). Interestingly, higher levels of pPRHΔC are observed in cells expressing the Myc-PRH SS163,177EE mutant of PRH, which mimics CK2 phosphorylation of PRH (figure 5.14 [A] - top panel, lane 6), when compared to cells expressing the wild-type Myc-PRH protein (figure 5.14 [A] - top panel, lane 2). In addition, no pPRHΔC is observed in cells expressing the Myc-PRH SS163,177CC mutant of PRH, which inhibits CK2 phosphorylation of PRH (figure 5.14 [A] - top panel, lane 7). These data suggest that CK2 phosphorylation of PRH targets full-length PRH for cleavage and increases the

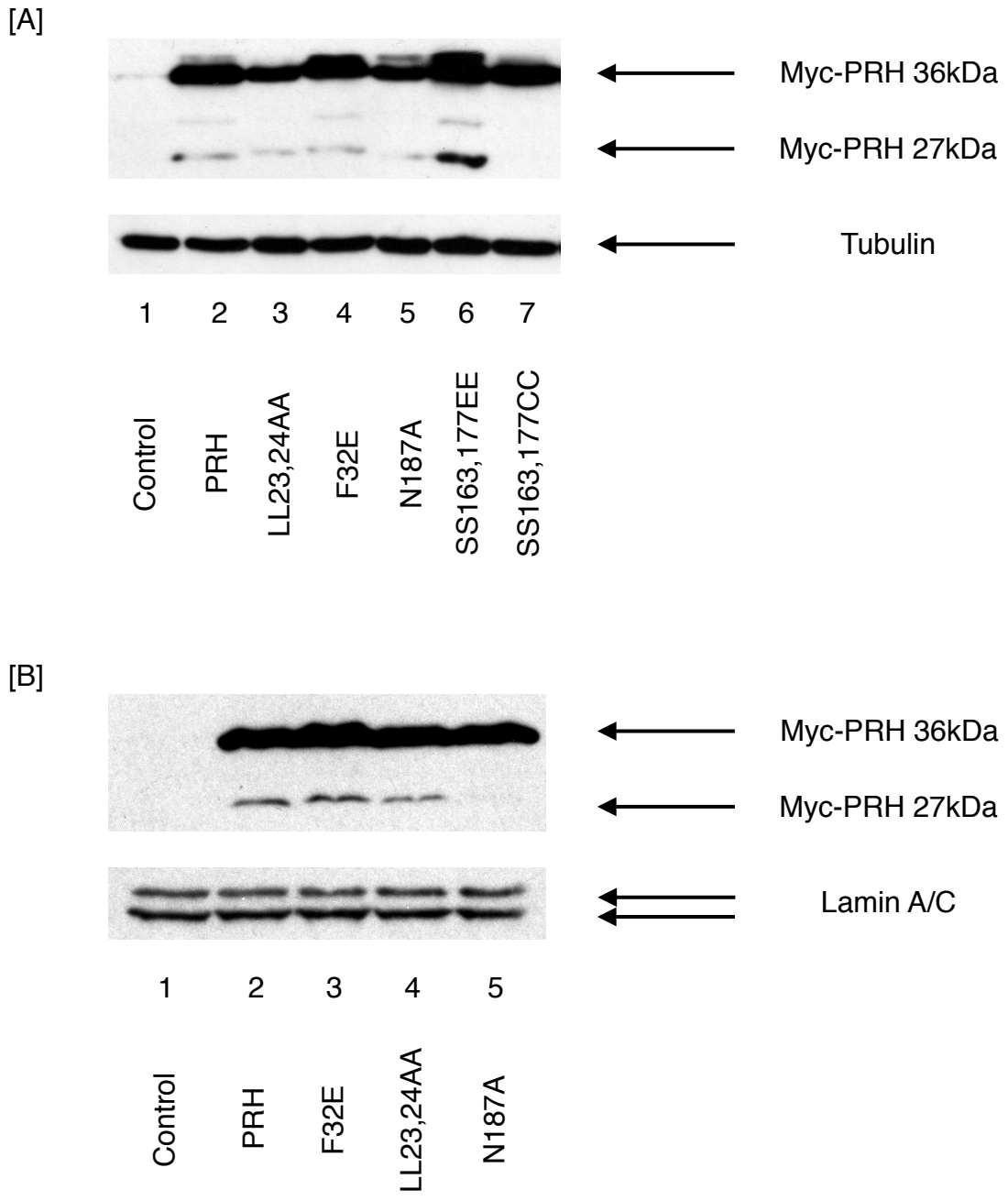


Figure 5.14 - CK2 phosphorylation increases cleavage of PRH.

[A] Western blot of Myc-PRH expression in K562 cells 48 h after transfection with 10 μg pMUG1 (lane 1, Control) or pMUG1 vectors expressing Myc-tagged PRH (PRH [lane 2], LL23,24AA [lane 3], F32E [lane 4], N187A [lane 5], SS163,177EE [lane 6], or SS163,177CC [lane 7]). Whole-cell extracts were separate by SDS-PAGE and transferred to a PVDF membrane that was stained with anti-Myc (top panel) and anti-Tubulin antibodies (lower panel). [B] As for [A] except extracts are from control cells (lane 1) or cells expressing PRH (lane 2), PRH F32E (lane 3), PRH LL23,24AA (lane 4), or PRH N187A (lane 5). A Lamin A/C antibody was used as a loading control. Data representative of three independent experiments.

production of pPRHΔC.

To further confirm there is a reduction in pPRHΔC production from the PRH N187A mutant, extract from cells expressing PRH, PRH F32E, PRH LL23,24AA, or PRH N187A were separated by SDS-PAGE and transferred to a PVDF membrane as described above for western blot analysis. The membrane was probed with an anti-Myc tag antibody (figure 5.14 [B] - top panel), to detect PRH. The membrane was then stripped and reprobed with an anti-Lamin A/C antibody, as a loading control (figure 5.14 [B] - lower panel). As described above, for cells expressing Myc-PRH, Myc-PRH F32E, or Myc-PRH LL23,24AA, a strong 36kDa band for PRH and a weak 27 kDa band for pPRHΔC is detected (figure 5.14 [B] - top panel, lanes 2-4). In contrast, for cells expressing Myc-PRH N187A, only a band at 36 kDa is observed (figure 5.14 [B] - top panel, lane 5). This confirms that DNA binding has a role in the production of pPRHΔC expression.

Anisomycin is a protein translation inhibitor that blocks the translation of mRNAs in the cell. With no new protein translation, the stability of a protein is entirely a consequence of its degradation rate and the half-life of a protein can be determined. To investigate the stability of endogenous full-length PRH and the pPRHΔC cleavage product, K562 cells were treated with 40μM anisomycin for 0, 4, 8 and 24 hours. The cells were harvested and whole cell protein extracts were made. Proteins were separated by SDS-PAG, transferred to a PVDF membrane and stained with a mouse anti-PRH antibody. The membrane was stripped of antibody and re-stained with a rabbit anti-PRH antibody or an anti-Lamin A/C antibody, which was used as a loading control (figure 5.15 - lower panel). Over 24 hours of treatment with anisomycin the level of un-phosphorylated PRH is unchanged (figure 5.15 - top panel). The rabbit anti-PRH antibody gives two specific PRH bands, one at 36 kDa for full-length PRH and another at 27 kDa that corresponds to endogenous pPRHΔC, as demonstrated

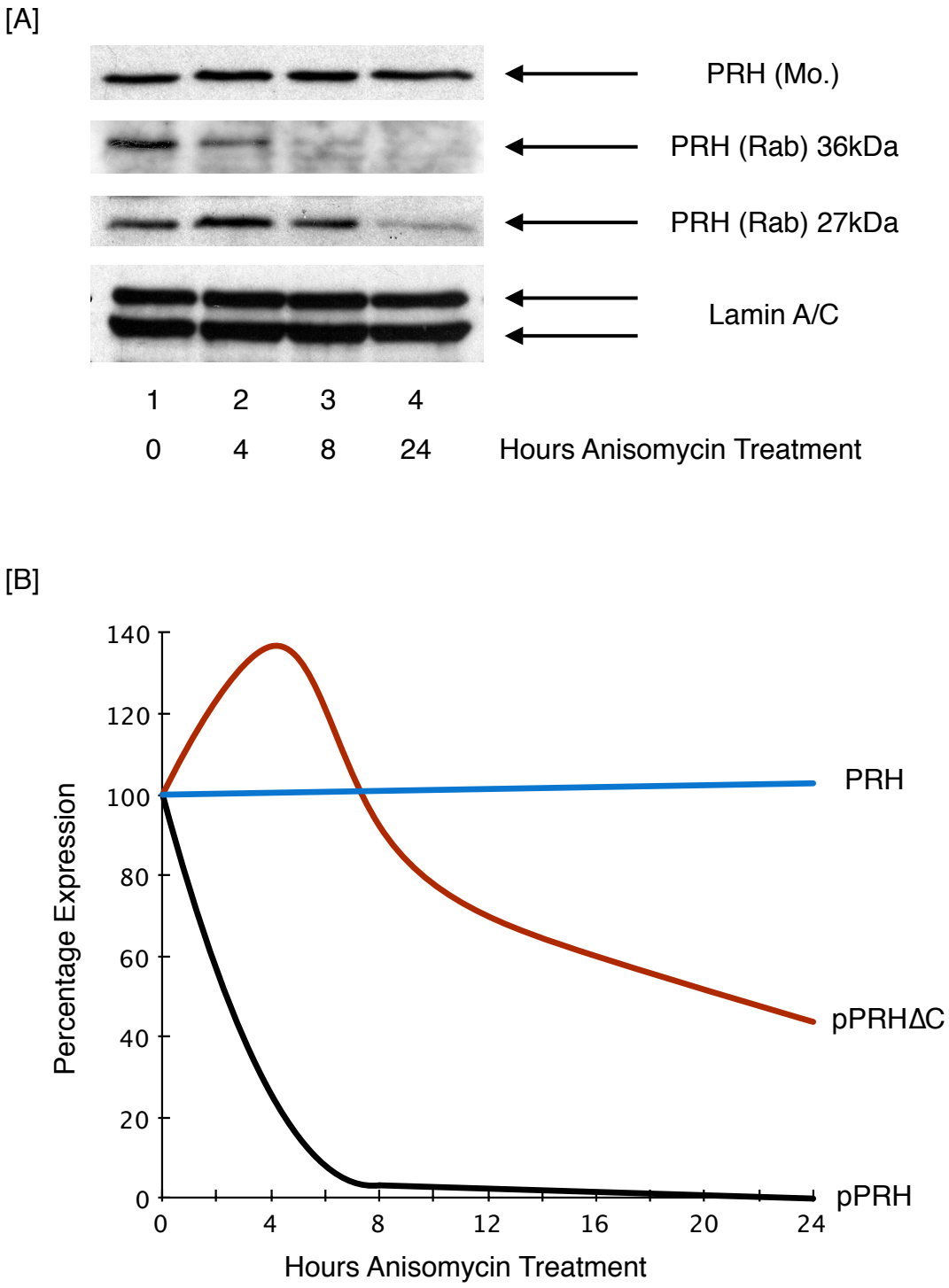


Figure 5.15 - CK2 phosphorylation reduces PRH stability.

[A] K562 cells were treated with 40μM anisomycin for 0, 4, 8, and 24 hours (lanes 1-4 respectively). The cells were harvested and whole cell protein was extracted. Proteins were separated on a SDS-PAG and transferred to a PVDF membrane that was stained with a mouse anti-PRH (top panel), or a rabbit anti-PRH antibody (middle panels), or an anti-Lamin A/C antibody (lower panel). Data representative of three independent experiments. [B] Graphical representation of the expression of PRH (blue line), pPRH (black line) and pPRHΔC (red line) over 24 hours treatment with 40μM anisomycin, determined by densitometric analysis of three independent Western blots.

in figure 5.1. The rabbit anti-PRH antibody preferentially binds to the phosphorylated form of PRH. However, the phosphorylated form of PRH (figure 5.15 - upper middle panel) is substantially less stable than the unphosphorylated form (figure 5.15 - top panel). Using densitometric analysis of three independent experiments, full-length phosphorylated PRH has a half-life of approximately 100 minutes (figure 5.15 [B] - black line). The half-life for the full-length unphosphorylated PRH could not be quantified because it is stable over the 24 hour time period assayed (figure 5.15 [B] - blue line). The half-life for the pPRHΔC protein also could not be quantified because it accumulates prior to degradation (figure 5.15 [B] - red line). However, pPRHΔC is more stable than full-length pPRH and accumulates around 4 hours of treatment, after which pPRHΔC is degraded (figure 5.15 - lower middle panel). These data suggest that phosphorylation of PRH by CK2 destabilizes the protein and targets it for degradation or cleavage into pPRHΔC prior to degradation.

Endogenous PRH is known to be present in the nucleus and the cytoplasm of cells. Biochemical fractionation of cells, as outlined in the Materials and Methods, results in two fractions: a post-nuclear fraction consisting of the cytoplasm and loosely held nuclear proteins, and a nuclear fraction consisting of tightly held nuclear proteins. To investigate the localization of PRH, full-length pPRH and pPRHΔC and their relative stabilities in the post-nuclear and nuclear compartments, K562 cells were treated with 40μM anisomycin for 0, 4, 8, 24 hours, harvested and fractionated into post-nuclear and tightly held nuclear protein fractions. Protein extracts were separated by SDS-PAGE and transferred onto a PVDF membrane for antibody staining. The membrane was probed with the mouse anti-PRH antibody. The membrane was then stripped of antibody and re-probed using the rabbit anti-PRH antibody, or an anti-Tubulin, or anti-Lamin A/C antibody, as fractionation and loading controls (figure 5.16 - lower panel and lower middle panel respectively). Unphosphorylated PRH is present in both the

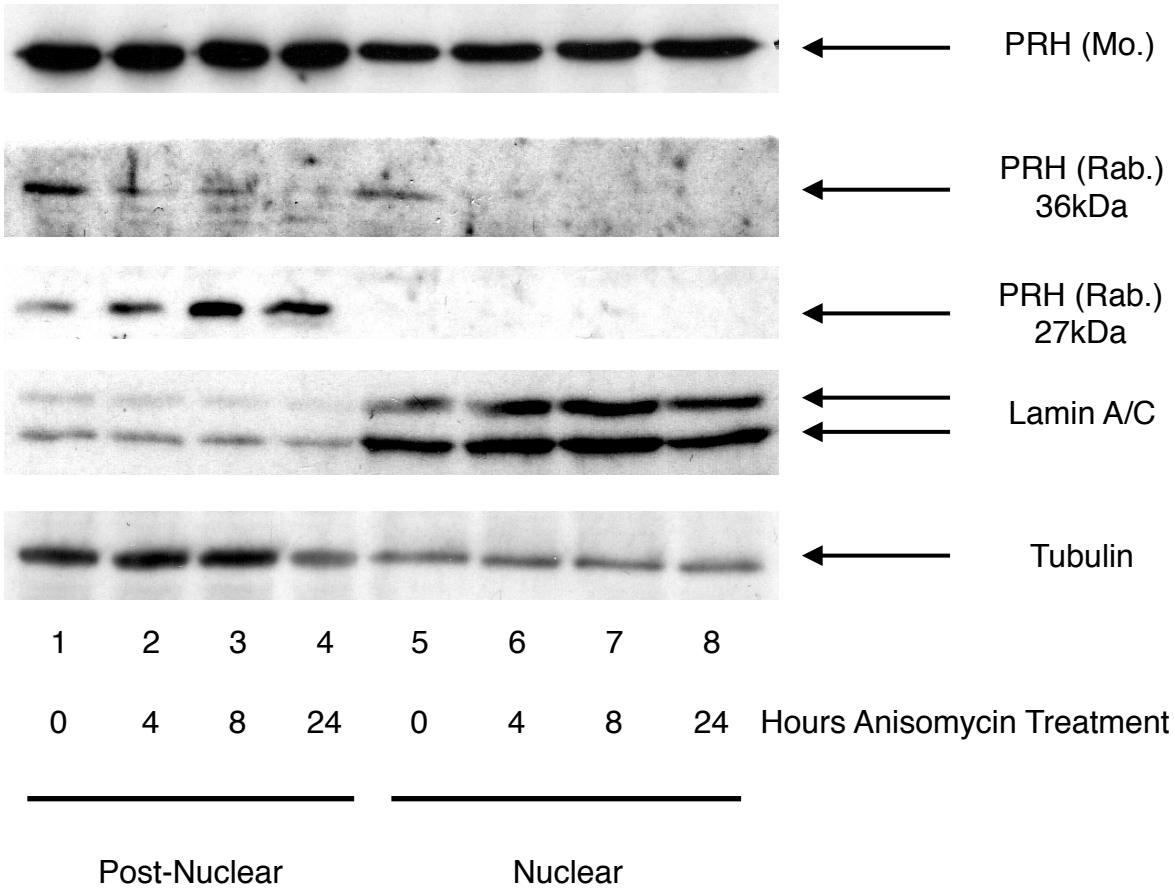


Figure 5.16 - Stability of endogenous PRH and CK2 phosphorylated PRH in nuclear and post-nuclear cellular fractions.

K562 cells were treated with 40μM anisomycin for 0, 4, 8, and 24 hours. The cells were harvested and fractionated into post-nuclear and nuclear protein extracts. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane that was stained with a mouse anti-PRH (top panel), or a rabbit anti-PRH antibody (upper and central middle panels), or an anti-Lamin A/C antibody (lower panel), or an anti-Tubulin (lower middle panel). Data representative of three independent experiments.

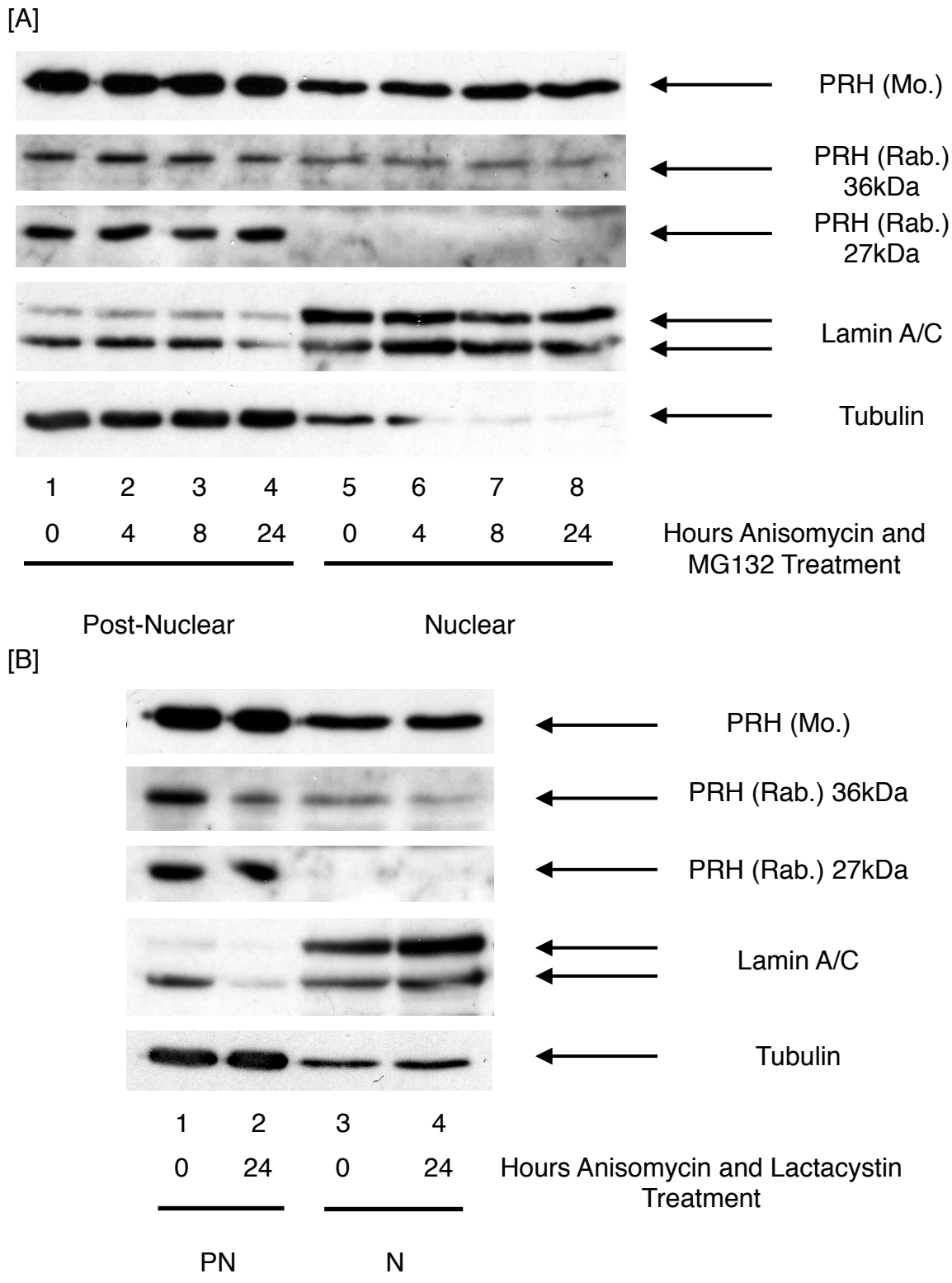


Figure 5.17 - Stability of endogenous PRH and CK2 phosphorylated PRH in nuclear and post-nuclear cellular fractions in the presence of proteasome inhibitors.

[A] K562 cells were treated with 40μM anisomycin and 10μM MG132 for 0, 4, 8, and 24 hours. The cells were harvested and fractionated into post-nuclear and nuclear protein extracts. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane that was stained with a mouse anti-PRH (top panel), or a rabbit anti-PRH antibody (upper and central middle panels), or an anti-Lamin A/C antibody (lower panel), or an anti-Tubulin (lower middle panel). Data representative of three independent experiments. [B] As for panel A except K562 cells were treated with 40μM anisomycin and 10μM lactacystin for 0 and 24 hours. Data representative of two independent experiments.

nuclear and post-nuclear fractions and it can be seen over 24 hours (figure 5.16 - top panel). In contrast although full-length pPRH is present in both the post-nuclear and nuclear fractions at the 0 hour time point, it is completely lost from both fractions after 24 hours (figure 5.16 - upper middle panel). Therefore just as observed previously in whole cell extracts (figure 5.15), pPRH protein (figure 5.16 - upper middle panel) has a shorter half-life than the unphosphorylated PRH (figure 5.16 - top panel). Interestingly, pPRH in the nuclear fraction has a shorter half-life than pPRH in the post-nuclear fraction (figure 5.16 - upper middle panel). After 4 hours of treatment with anisomycin the pPRH band in the nuclear fraction is no longer observed (figure 5.16 - upper middle panel, lanes 5-8), however the pPRH band persists in the post-nuclear fraction until 8 hours after treatment (figure 5.16 - upper middle band, lanes 1-4). Notably, pPRHΔC is located exclusively in the post-nuclear extract (figure 5.16 - central middle panel) and it accumulates until 8 hours after treatment (figure 5.16 - central middle panel, lanes 1-3), yet by 24 hours the band appears reduced compared to the 8 hour time point (figure 5.16 - central middle panel, lanes 4 and 3). One possible reason for these observations is that phosphorylation of PRH reduces its nuclear retention, thus the cleavage of PRH to produce pPRHΔC only occurs in the post-nuclear compartment. Alternatively pPRHΔC could be produced in the nucleus but is not retained in the nuclear compartment. In conclusion pPRH has a short half-life relative to PRH and both pPRH and pPRHΔC are not tightly retained in the nucleus.

PRH has been shown to interact with the HC8 subunit of the proteasome(Bess et al., 2003), however, the role of this interaction *in vivo* is unknown. CK2 phosphorylation of PML induces proteasome-dependent degradation of PML (Scaglioni et al., 2006). To investigate whether the proteasome is important for degradation of PRH protein, K562 cells were treated with anisomycin and the proteasome inhibitor, MG132, for 0,

4, 8 and 24 hours. MG132 is a specific small reversible proteasome inhibitor, which specifically inhibits the chymotrypsin-like activity of the 20S proteasome (Chen et al., 1995). The treated cells were harvested and fractionated into post-nuclear and nuclear protein fractions as before. Protein extracts were separated by SDS-PAGE and transferred onto a PVDF membrane for antibody staining. The membrane was stained with the mouse anti-PRH antibody and was then stripped of antibody and re-probed using the rabbit anti-pPRH antibody, or an anti-Tubulin, or anti-Lamin A/C antibody. It can be seen that the level of unphosphorylated PRH (figure 5.17 [A] - top panel) is not affected by anisomycin treatment over 24 hours in either the post-nuclear or the nuclear fractions (figure 5.16). However, in the presence of both

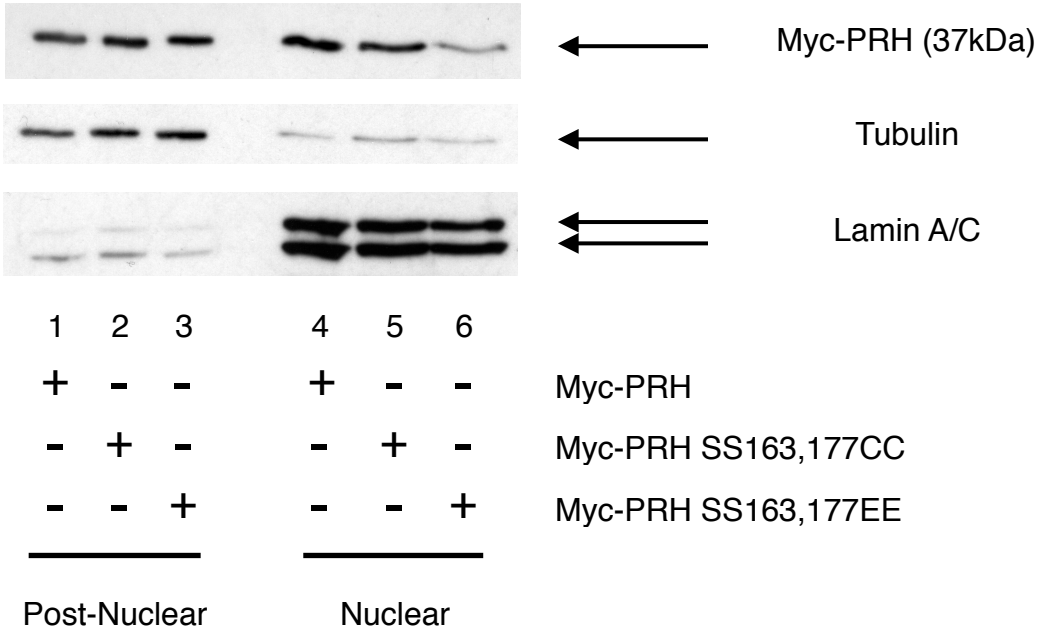


Figure 5.18 - CK2 phosphorylation mutants of PRH have reduced nuclear retention.

K562 cells transfected with pMUG1 containing Myc–PRH, Myc-PRH S163C,S177C and Myc–PRH S163E,S177E were fractionated into post-nuclear and nuclear extracts. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Proteins were stained with anti-Myc (top panel), anti-Tubulin (middle panel) and anti-Lamin A/C (bottom panel) antibodies. Data representative of two independent experiments.

anisomycin and MG132 pPRH is stabilized in both post-nuclear and nuclear fractions (figure 5.17 [A] - upper middle panel). Additionally, pPRHΔC present in the post-nuclear fraction only, is now stable over 24 hours (figure 5.17 [A] - central middle panel), and does not fluctuate over time compared to cells treated with anisomycin alone (figure 5.16). These results suggest that CK2 phosphorylation of PRH targets PRH for cleavage and degradation by the proteasome, yielding a 27kDa product that is not tightly held in the nucleus.

To confirm that the degradation is specific to the proteasome, K562 cells were treated with 40μM anisomycin and 10μM lactacystin for 0 and 24 hours. Lactacystin is an irreversible inhibitor of proteasome activity and is active against the trypsin-like and chymotrypsin-like proteolytic activity of the 20S proteasome (Jensen et al., 1995). The treated cells were then harvested and fractionated into post-nuclear and nuclear extracts. Protein extracts were run on a SDS-PAG before transferring the proteins to a PVDF membrane for antibody staining. The membrane was stained as described above. Unphosphorylated PRH (figure 5.17 [B] - top panel) is stable over 24 hours of treatment with anisomycin and lactacystin. pPRH is stabilized compared to cells treated with anisomycin alone (figure 5.16 - upper middle panel). In cells treated with anisomycin alone, pPRH is not present after 24 hours in either the post-nuclear or nuclear fraction (figure 5.16 - upper middle panel, lanes 4 and 8), however, when cells are treated with lactacystin and anisomycin, pPRH can be seen (figure 5.17 [B] - upper middle panel, lanes 2 and 4). The pPRHΔC protein is also stabilized in lactacystin and anisomycin treated cells (figure 5.17 [B] - central middle panel) compared to cells treated with anisomycin alone (figure 5.16 - central middle panel). This confirms that CK2 phosphorylation of PRH is a signal for proteasome cleavage of full-length PRH protein to a 27 kDa intermediate, pPRHΔC, before complete degradation.

To confirm that CK2 phosphorylation of PRH affects the nuclear retention of PRH, K562 cells were transfected with PRH, or PRH S163C,S177C, or PRH S163E,S177E. 24 hours after transfection the cells were harvested and fractionated into post-nuclear and nuclear extracts. Proteins were separated on a SDS-PAG and transferred to a PVDF membrane. The membrane was stained using an anti-Myc antibody to detect the expression of the transfected Myc-PRH proteins. Subsequently the membrane was stripped of antibody and stained with antibodies against Tubulin or Lamin A/C, which were used as loading and fractionation controls. PRH (figure 5.18 - top panel, lanes 1 and 4) and PRH S163C,S177C (figure 5.18 - top panel, lanes 2 and 5) can be detected in the post-nuclear and nuclear fractions. In contrast PRH S163E,S177E is predominantly observed within the post-nuclear fraction (figure 5.18 - top panel, lane 3) and is very weakly held in the nuclear fraction (figure 5.18 - top panel, lane 6) when compared to Myc-PRH or Myc-PRH S163C,S177C (figure 5.18 - top panel, lanes 4 and 5 respectively). It can be concluded that phosphorylation of PRH within the homeodomain by CK2 reduces the nuclear retention of the pPRH species.

5.8 Discussion.

Previous chapters have identified PRH as a key regulator of cell survival through the VSP in haematopoietic cells. *In vitro* experiments by Soufi et al demonstrated that DNA binding by PRH is blocked through CK2 phosphorylation of two serine residues within the homeodomain (Soufi et al., 2009). This work suggested that PRH function could be perturbed by the stress activated regulator of survival CK2. However, the significance of this phosphorylation in cells was not demonstrated. In this chapter PRH is shown to be phosphorylated by CK2 in cells. This phosphorylation blocks transcriptional repression of *Vegf* and *Vegfr1* by PRH. This finding supports previous data that shows phospho-mimic mutants of PRH are unable to repress an artificial

reporter construct (Soufi et al., 2009). I demonstrate here that CK2 phosphorylation of PRH inhibits DNA binding at the *Vegfr1* promoter and the *Vegf* promoter. CK2 inhibition of transcription factor DNA binding has been demonstrated for another group of homeodomain containing proteins, the Cut homeodomain proteins (Coqueret et al., 1998b). Cut homeodomain containing proteins are distinct from PRH and most other homeodomain containing proteins because they also contain three other DNA binding regions called Cut repeats. This work by Coqueret et al showed that CK2 could phosphorylate a key serine residue in each of these Cut repeats. Phosphorylation of these sites reduced transcriptional repression by mCut (Coqueret et al., 1998a). Cut homeodomain protein is a regulator of differentiation, cell growth and developmental processes (Nepveu, 2001). For example, Cut is a transcriptional repressor of c-Myc expression and therefore CK2 can promote growth through inactivation of Cut and this consequently leads to c-Myc activation (Dufort and Nepveu, 1994). CK2 appears to play a role in regulating many transcriptional repressors. In addition to blocking DNA binding by PRH and Cut, CK2 phosphorylation has been shown to alter the activity of the transcription factor, CTCF, from a transcriptional repressor into an activator of transcription (El-Kady and Klenova, 2005). CK2 phosphorylation of insect Hox proteins, of the UBX ortholog class, inhibits the repression of Distal-less (Dll) transcription, although this is thought to be mediated through sites C-terminal to its homeodomain (Taghli-Lamallem et al., 2008). Furthermore, there are multiple examples of CK2 phosphorylation of co-repressors. This includes the co-repressor and tumour suppressor PML, which is phosphorylated by CK2 and targeted for degradation by the proteasome (Scaglioni et al., 2006). Other targets include TLE (Nuthall et al., 2004) (which will be discussed further below) and SMRT (Zhou et al., 2001). However, these phosphorylation events appear to be linked to developmental processes rather than stress activated

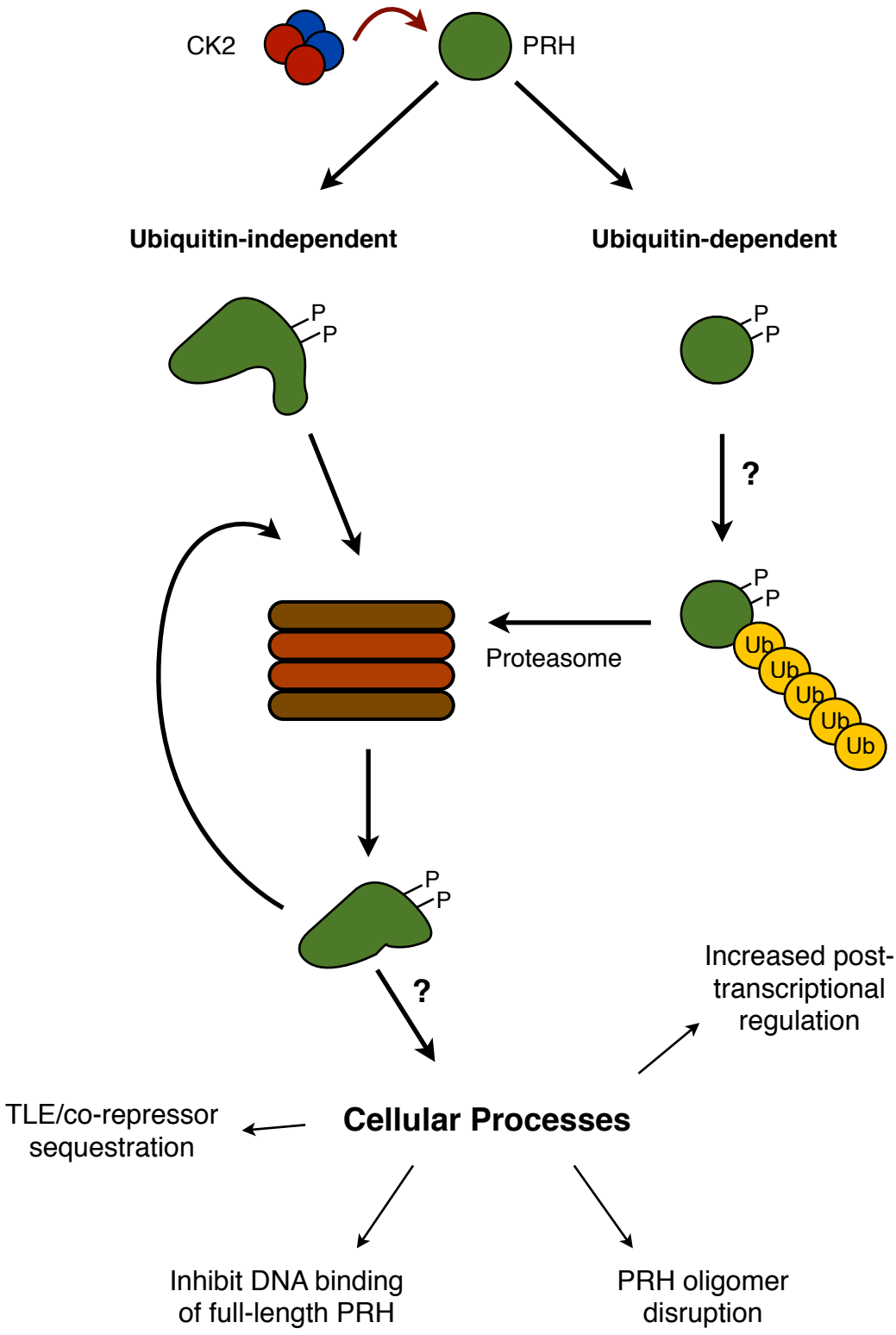


Figure 5.19 - Role of PRH truncation after CK2 phosphorylation.

Schematic diagram proposing a model for PRH degradation through ubiquitin-dependent or ubiquitin-independent pathways, and a role for PRH truncation by the proteasome in response to CK2 phosphorylation.

responses.

Another feature of proteins phosphorylated by CK2 is that the phosphorylation event can alter the stability or turn-over rate of the protein. The tumour suppressor protein PML is phosphorylated by CK2 at multiple sites with the major phosphorylation site serine 517 within the degron domain of PML (Scaglioni et al., 2006). There are also other phosphorylation sites, with phosphorylation of serine 517 priming phosphorylation of serine 514, as well as other minor CK2 phosphorylation sites within the downstream PEST domain (Scaglioni et al., 2006). Phosphorylation of serine 517 is critical for ubiquitin-mediated proteasomal degradation of PML (Scaglioni et al., 2006). Other proteins that have reduced stability after CK2 phosphorylation include, IκB (McElhinny et al., 1996), and SAG (He et al., 2007). In contrast, c-myc is a good example of how CK2 can reduce protein turnover and thereby increase protein stability (Channavajhala and Seldin, 2002). In the case of PRH, phosphorylation by CK2 appears to target PRH for proteasomal degradation producing a truncated cleavage product pPRHΔC, which appears to be more stable than the full length pPRH protein. At present it is unknown whether the proteasomal degradation of full-length PRH occurs through a ubiquitin dependent degradation pathway (figure 5.19). It is possible that the observed cleavage of PRH and subsequent degradation is independent of ubiquitin conjugation because it has previously been shown that PRH interacts with the HC8 subunit of the proteasome (Bess et al., 2003). This study by Bess et al demonstrated that despite the interaction there was little effect of the proteasome on the half-life of full length unphosphorylated PRH either *in vitro* or *in vivo*. However, these studies did not examine the effect of the proteasome on phosphorylated PRH. Interestingly, they did observe that partially degraded PRH is a better substrate for the 20S and 26S proteasomes than the full length PRH, and that degradation is mediated through a

ubiquitin-independent mechanism (Bess et al., 2003). As shown in this chapter, CK2 phosphorylated full length PRH is rapidly cleaved to form pPRHΔC, which retains the N-terminal domain and homeodomain of PRH. It is possible that CK2 phosphorylation of PRH may alter the conformation of PRH making it a better substrate for the proteasome or that phosphorylation may facilitate ubiquitylation and subsequent cleavage. Since the pPRHΔC product is relatively stable, compared to the full-length pPRH *in vivo*, it may very well have some role in other cellular processes in the cytoplasm. Although this product still contains the homeodomain it is very unlikely that it can bind DNA as it will be phosphorylated within the homeodomain. However, pPRHΔC could still bind to unphosphorylated PRH in the cytoplasm, as it retains the N-terminal domain that is necessary for PRH dimerization/oligomerization. Therefore, it is possible that pPRHΔC could alter the post-transcriptional regulation activity of PRH, because the eIF4E interaction site, also within the N-terminal domain, is retained. Alternatively, this pPRHΔC protein may disrupt PRH oligomer formation or it may sequester TLE, reducing TLE availability for full-length unphosphorylated PRH. Notably, pPRHΔC is not tightly held in the nuclear compartment, so it is unlikely to have direct transcriptional functions, although there could be indirect consequences on transcription through sequestration of TLE or other co-factors. Further experiments will be required to determine the role of this form of PRH.

Both PRH and PML are rapidly degraded upon CK2 phosphorylation (Scaglioni et al., 2006). PRH associates with PML and is a constituent of PNBs (Topisirovic et al., 2003a; Topcu et al., 1999). PNBs are nuclear matrix associated structures (Lallemand-Breitenbach and de Thé, 2010). PRH is also found at the nuclear matrix (P-S Jayaraman, unpublished observations). CK2 is a well established nuclear matrix factor that can re-localize to the nuclear matrix in response to growth factor stimulation (Guo et al., 1999; Faust and Montenarh, 2000). Therefore, CK2

phosphorylation of PRH and PML could be occurring within the nuclear matrix compartment. In support of this idea, proteasome and ubiquitin E3 ligases necessary for PML degradation have been observed to co-localize with PNBs (Lallemand-Breitenbach et al., 2008; Lallemand-Breitenbach et al., 2001). Similarly, PRH cleavage and production of pPRHΔC could also be occurring at PNBs in association with the nuclear matrix and may represent a mechanism for the removal of PRH from DNA and the nuclear matrix.

Not only does CK2 phosphorylation of proteins affect the transcriptional activity, DNA binding activity and stability of target proteins but it can also alter their sub-cellular localization. I have shown that the nuclear retention of the PRH protein is affected by CK2 phosphorylation. There are clear parallels between PRH and Ikaros in their function and regulation in general terms. For example, Ikaros is important for the regulation of haematopoiesis, with key roles in the differentiation of myeloid (Dumortier et al., 2003) and lymphoid (Wu et al., 1997) lineages, and is thought to have tumour suppressor functions (Winandy et al., 1995). Ikaros can also act as a transcriptional repressor or activator, like PRH. Work by Gurel et al demonstrated that Ikaros DNA binding capacity is negatively regulated by CK2 (Gurel et al., 2008). Furthermore, they showed that CK2 phosphorylation of Ikaros reduced its pericentromeric heterochromatin (PC-HC) localization (Gurel et al., 2008). This chapter shows that the sub-cellular localization of PRH is effected by CK2 phosphorylation in a similar way to Ikaros. Reduced nuclear retention of PRH is observed for the CK2 phospho-mimic mutant of PRH. Additionally, the endogenous full length pPRH protein is predominantly present in the post-nuclear fraction. There is a small amount of pPRH that is present in the nuclear fraction but it is lost from the nuclear fraction within four hours of treatment with anisomycin. Since the half-life of CK2 is very short it is likely that four hours after treatment CK2 activity has been lost

and there is no further phosphorylation of PRH by CK2 taking place in the cell (Lüscher and Litchfield, 1994). Therefore, the more rapid loss of pPRH from the nuclear compartment suggests that PRH is phosphorylated in the nuclear compartment. This phosphorylation then reduces nuclear retention and the pPRH remains only in the post-nuclear compartment. Thus, CK2 phosphorylation of PRH could be reducing PRH transcriptional repression activity through both reduction of DNA binding and through altered sub-cellular localization. Increased pPRH and its degradation product in the post-nuclear compartment could potentially enhance other

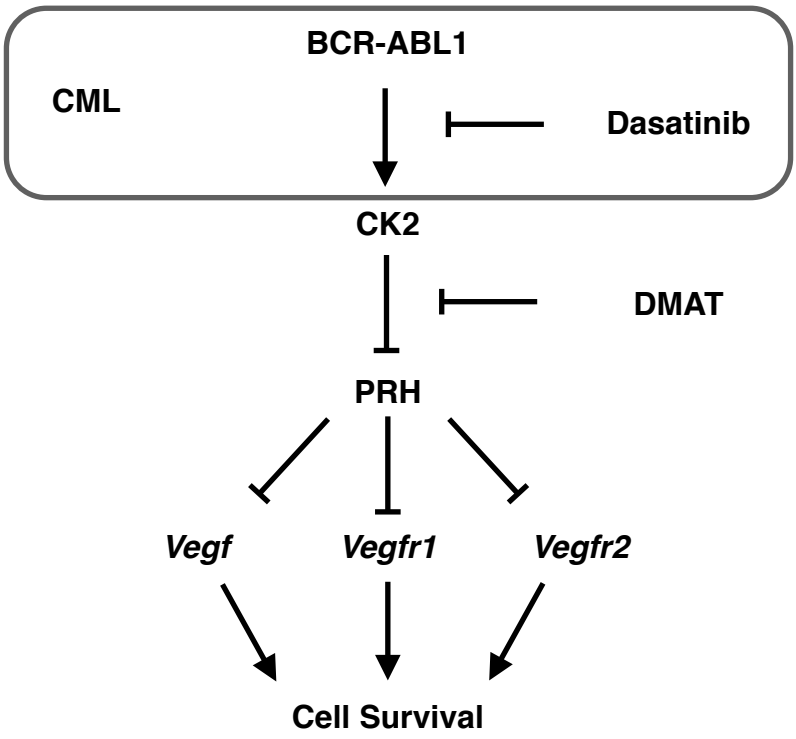


Figure 5.20 - PRH controls a component of BCR-ABL1 induced cell survival.

Schematic diagram proposing a model for how BCR-ABL1 and CK2 regulate VSP gene expression through PRH.

interactions or functions of PRH in the post-nuclear compartment. PRH and Ikaros are not the only examples of altered sub-cellular localization through CK2 phosphorylation. CK2 phosphorylation can either favour nuclear export, such as for S6 kinase 1 II (S6K1 II) (Panasyuk et al., 2006), or block nuclear export, such as for CDC34 (Block et al., 2001), demonstrating the diversity of outcome from CK2 phosphorylation.

CK2 is activated in response to stress signals such as osmotic shock or UV radiation. These signals activate p38 MAPK which can directly interact with CK2 and stimulates CK2 activities (Sayed et al., 2000). Stress-induced CK2 activation has been shown to be important for the induction of the NF-κB pathway. NF-κB is a pro-inflammatory transcription factor that is negatively regulated through an interaction with IκB. CK2 can phosphorylate the C-terminal domain of IκB and cause degradation of IκB, thus activating NF-κB nuclear translocation and transcriptional activity (Kato et al., 2003). PML phosphorylation and degradation (Scaglioni et al., 2006; Scaglioni et al., 2008), and p53 phosphorylation at serine 392 (Sayed et al., 2000) have also been observed in response to osmotic stress or UV radiation. Currently, it is unknown whether PRH responds to extracellular signals and although it has not been investigated in this thesis, PRH activity is very likely to also be regulated as a response to stress.

The role of CK2 in response to stress stimuli and the evolutionary conservation of the CK2 proteins suggests that they are fundamental for cell survival. Therefore, it is unsurprising that aberrant CK2 activity is a feature of many malignant diseases. One of the most obvious links to cancer is through the interaction of CK2 with the BCR-ABL1 fusion protein that is found in acute lymphoblastic leukaemia (ALL) and chronic myelogenous leukaemia (CML). The CK2α subunit was identified as a binding partner of the BCR moiety of the BCR-ABL1 oncogene (Mishra et al., 2003) and it is thought to be phosphorylated by the kinase activity of ABL1 (Hériché and Chambaz,

1998). Interestingly, in p190 BCR-ABL1 lymphoblastic leukaemia cells, monotherapy by a CK2 inhibitor (DMAT), has been shown to substantially reduce viability of these cells, suggesting that CK2 plays a critical role in BCR-ABL1 dependent transformation. However, although CK2 activity was shown to be increased in patients with CML in blast crisis (Phan-Dinh-Tuy et al., 1985), a role for CK2 in a CML model has not been clearly demonstrated. K562 cells are derived from a CML patient in blast crisis and contain the Philadelphia chromosome that encodes the p210 BCR-ABL1 fusion protein. In this chapter, phosphorylation of PRH by CK2 is significantly decreased when K562 cells are treated with either Imatinib or Dasatinib, two BCR-ABL1 inhibitors. Furthermore, target genes of PRH are repressed to a greater extent. Importantly however, increased repression of target genes is only observed in the presence of PRH. This evidence indicates that CK2 activity is a feature of BCR-ABL1 molecular biology and it likely highlights the significance of PRH regulation by CK2. Transcriptional repression of the VSP by PRH is a key process in the regulation of K562 cell survival. Therefore it can be concluded that dysregulation of PRH through CK2 and the consequent increased survival, through up-regulation of VSP genes, is a feature of p210 BCR-ABL1 CML.

Similar to DMAT treatment, Dasatinib also has no effect on repression in the absence of PRH. Dasatinib has a less potent effect on cell survival in the absence of PRH also. This suggests that there is a pathway from BCR-ABL1 inhibition by Dasatinib, to CK2 inhibition, increased PRH activity, observed as increased repression of multiple VSP genes and increased cell death. It can be inferred that increased CK2 activity as a consequence of BCR-ABL1 activities and consequent inactivation of PRH activity could be a contributor to BCR-ABL1 molecular biology (figure 5.20).

General Discussion

6 General Discussion.

6.1 General Discussion.

This thesis has demonstrated that PRH is a direct transcriptional repressor of three VSP genes, *Vegf*, *Vegfr1*, and *Vegfr2*. PRH directly binds to the promoter regions of these genes and recruits TLE proteins to bring about transcriptional repression. PRH expression can alter the sub-cellular localization of TLE and also induce phosphorylation of TLE (Desjobert et al., 2009). The role of PRH expression on cell growth and the induction of an apoptotic response has been investigated. Expression of the VSP genes that PRH regulates have been shown to be important for cell survival. Furthermore, post-translational modification of PRH has been demonstrated to be a key regulatory feature of PRH activity. Finally, a role for dysregulation of PRH function in CML, through altered CK2 phosphorylation, has been demonstrated.

As well as having a role in haematopoietic diseases, the direct regulation of the VSP genes by PRH could be important for embryonic development. As summarized in the introduction (see section 1.2.4) PRH expression is lost in the vascular system. However, PRH is required for the formation of the angioblast (Guo et al., 2003). It is likely that PRH expression must be lost in the haemangioblast for the angioblast to form due to direct repression of key endothelial lineage genes, such as *Vegfr2* and *Vegf*. This is supported by work from Nakagawa et al, who reported that PRH regulates a number of other endothelial genes although the mechanism of regulation for these genes was not investigated (Nakagawa et al., 2003). Therefore PRH is involved in lineage commitment of HSCs and angioblasts and this maybe partially through the direct regulation of the VSP genes.

Chapter 3 identified a role for TLE as a transcriptional repressor of *Vegf* and *Vegfr1* expression, through an interaction with PRH. TLE, like PRH, is phosphorylated by CK2. Nuthall et al demonstrated that the DNA-binding protein Hes-1, induced hyper-phosphorylation and increased the nuclear retention of TLE (Nuthall et al., 2002a) and that CK2 phosphorylation of serine residue 239 within the CcN domain of TLE was required. As demonstrated in chapter 3 PRH over-expression increases nuclear retention of endogenous TLE and is known to induce hyper-phosphorylation of TLE, although it is unknown whether this occurs in a CK2 dependent fashion. Interestingly, CK2 phosphorylation of TLE increases the transcriptional repression activity of TLE, whereas CK2 phosphorylation of PRH reduces transcriptional activity. Therefore, there appears to be conflicting outcomes of CK2 activity on transcription, where it both promotes and inhibits expression of PRH and TLE target genes. It is worth noting that in experiments performed by Nuthall et al, they used HEK293 cells, a cell line derived from human embryonic kidney cells, and not K562 cells, so there may be cell type differences or other kinases that can hyper-phosphorylate TLE. More likely however, is that this is a 'self-regulating' mechanism (figure 6.1). If CK2 activation is thought of as a survival response to stress, then there needs to be a point at which the cell can overcome this survival response and undergo apoptosis. In a steady state situation, there are low levels of TLE in the nucleus and some PRH bound at promoters of target genes repressing transcription. If a stress response is induced, then CK2 activity is increased and PRH activity is reduced, resulting in increased cell survival. However, if PRH expression becomes elevated and recruits TLE then CK2 may preferentially phosphorylate TLE instead of PRH, therefore increasing transcriptional repression. The outcome of this mechanism would be determined by the threshold of PRH. Up to a point, the level of expression of PRH would not be sufficient to recruit enough TLE to inhibit CK2 phosphorylation of PRH and increase

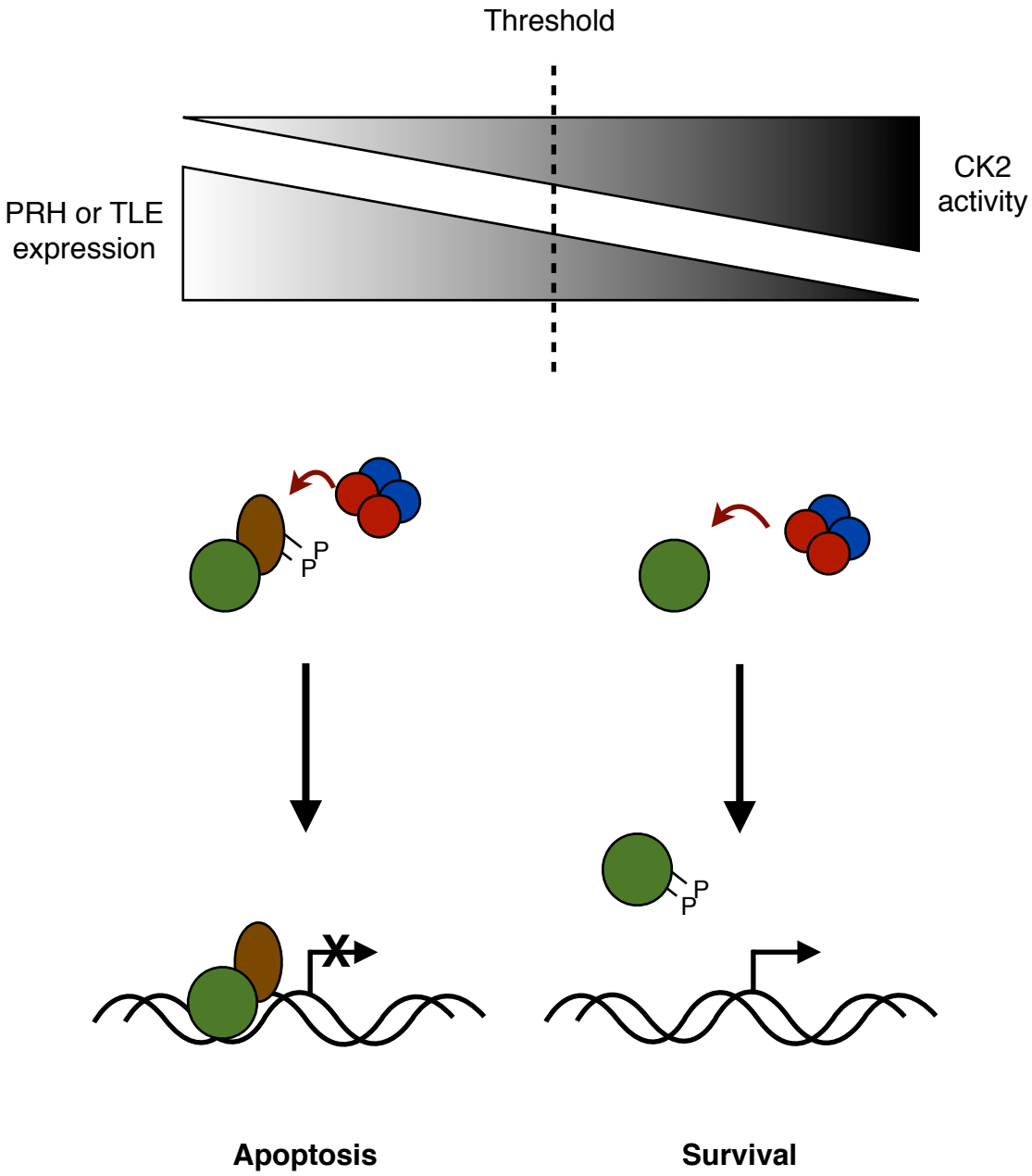


Figure 6.1 - Model for PRH versus TLE phosphorylation by CK2.

Schematic diagram showing how the levels of CK2 activity or the expression levels of PRH or TLE may favour PRH phosphorylation instead of TLE phosphorylation by CK2.

transcriptional repression, but once the threshold is surpassed the level of transcriptional repression would increase, promoting apoptosis (figure 6.1).

This thesis has demonstrated a role for PRH regulation in K562 CML cells. Figure 6.2 presents a model for the role of PRH within the regulation of this disease state and how it is manipulated to favour the progression of malignant phenotypes. This model shows that PRH, as an oligomer, recruits TLE and binds to regulatory sequences within the promoter regions of the VSP genes, *Vegf, Vegfr1*, and *Vegfr2*. It can be assumed that further proteins are also recruited to this repression complex, such as HDACs which are associated with TLE proteins. Transcriptional repression of the *Vegf*, *Vegfr1* and *Vegfr2* genes reduces the expression of the protein products of these genes. Subsequently there is a decrease in survival signalling through the VSP. In this system PRH acts as a regulator of cell survival through the VSP. However, CK2 is able to regulate PRH activity. Inappropriate expression or up-regulation of CK2 leads to direct restriction of PRH function through phosphorylation. CK2 inhibits DNA binding by PRH and induces proteolytic cleavage and degradation of PRH by the proteasome. Furthermore, CK2 restricts the nuclear localization of PRH. Consequently, this leads to increased expression of the VSP genes and enhanced survival signalling.

In addition to the implied role of CK2 in CML and ALL, CK2 activity has also been linked with a number of other haematopoietic diseases. AML is a heterogenous disease with no single dominant mutation that characterizes the disease. Recent work by Kim et al identified CK2 expression as a marker for a poor prognosis in AML patients (Kim et al., 2007). They found that CK2 expression correlated with a significant reduction in patient survival. Over-expression of CK2 also results in increased levels of key survival or anti-apoptotic genes (Kim et al., 2007). One of these genes is Bcl-2, a negative modulator of apoptosis, and interestingly, this gene

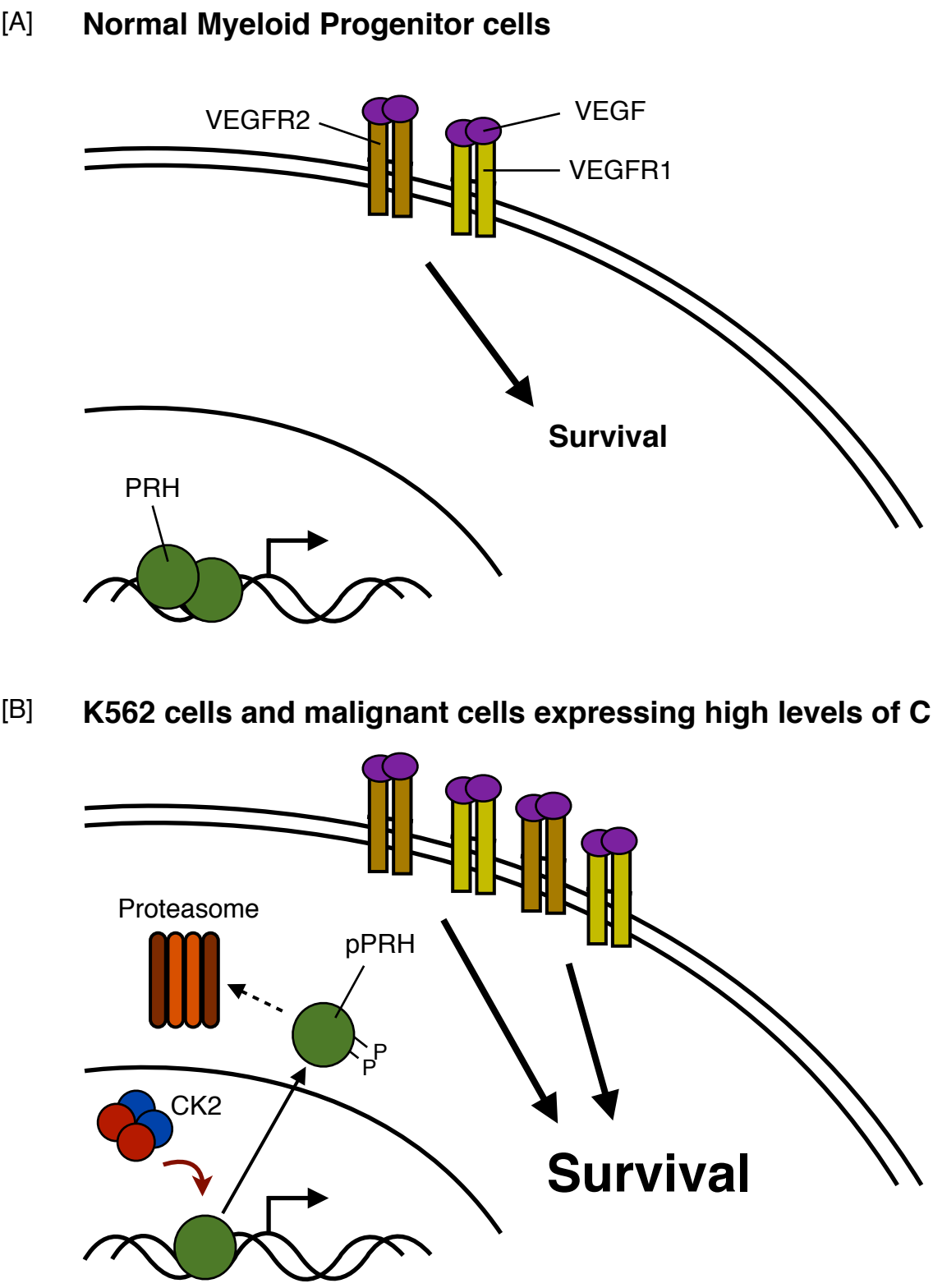


Figure 6.2 - Model of PRH dysregulation in K562 cells.

Schematic diagram showing how PRH dysregulation through elevated CK2 activity, in K562 cells (B), can drive cell survival through inhibition of PRH function and targeted degradation of PRH protein compared to a normal myeloid progenitor cell (A).

is also induced through VEGF signalling in haematopoietic cells (Dias et al., 2002), and is vital for VEGF survival signalling. Expression of PRH is reduced or PRH protein is mis-localized in a subset of AML patient samples, and CML patients in blast crisis (Topisirovic et al., 2003b). PRH regulates VEGF expression in haematopoietic cells. Interestingly, neo-vascularization of the bone marrow is an associated pathology of AML (Hussong et al., 2000; Padró et al., 2000; Matuszewski et al., 2007; Shih et al., 2009). It has been demonstrated that bone marrow samples taken from AML patients have significantly increased micro-vessel counts, whereas these decrease to control levels in patients undergoing chemotherapy or in patients that are in complete remission (Padró et al., 2000). In addition, it has been shown that the release of soluble mediators from AML cells stimulates increased proliferation of microvascular endothelial cells (Hatfield et al., 2009). VEGF is a potent endothelial cell mitogen and critical for angiogenesis (Leung et al., 1989; Ferrara et al., 1996). Reduced PRH expression results in elevated VEGF production. It is therefore possible that PRH levels in cells taken from AML patients may correlate with bone marrow neo-vascularization. It is worth noting that once CML has progressed to the blast crisis phase of the disease it behaves like an AML. For this reason K562 cells, derived from a patient CML in blast crisis, has also been described as a model of AML (Peinert et al., 2010; Tiziani et al., 2009). With the evidence demonstrated in this thesis, it is reasonable to hypothesize that PRH function could be altered in many more AML cases than the subsets previously analyzed. It is highly likely that PRH activity may also be perturbed in AML patients where the activity or expression of CK2 is high. High CK2 activity was found in over 30% of AML cases tested, this suggests that suppression of PRH function by CK2 may be a major contributor to AML. Since, PRH activity can also be affected when it is poorly expressed or mis-localized, this suggests that the role for PRH in AML may be significantly greater than

initially anticipated. Furthermore, the antibody against pPRH could potentially be used as a diagnostic tool, to identify AML patients with elevated CK2 activity and reduced transcriptional function of PRH.

In conclusion, PRH is a pleiotropic transcription factor, that can be dysregulated during normal haematopoiesis, resulting in aberrant survival signalling through the VSP. PRH is an interesting model of gene regulation within the haematopoietic compartment due to its oligomeric properties and relaxed binding specificity. However, further investigation is required to fully understand the mechanism through which PRH represses gene expression. One unanswered question is whether the relative position of binding sites compared to the TSS or the number of regions, within a promoter, bound by PRH determine high and low affinity gene targets of PRH. For example there are interesting differences in the regulation of the VSP genes by PRH. The *Vegfr1* gene is bound by PRH in the proximal promoter and at sites as far away as -5.6 kb upstream of the TSS. However, for the *Vegf* gene PRH binds in the distal promoter region, weak binding by PRH has been reported around -3.5 kb and -4.5 kb upstream of the TSS and promoter reporter assays further confirmed that the proximal promoter region of *Vegf* is not regulated by PRH (Noy et al., 2010). This could suggest differential cross-talk with other transcription factors bound to these promoters. For example, the distal regulation at *Vegf* may demonstrate weaker repression of some transcription factors, such as those that bind at the TSS compared to those that bind to upstream sites. Whereas PRH regulation of *Vegfr1* may be stronger for proximal bound transcription factors compared to distal ones. Additional investigation would be required to clarify this sort of cross-talk between different regulatory factors bound at a specific gene. It is also possible that the relative position that PRH binds compared to the TSS is irrelevant because of the three dimensional positioning of chromatin structure. For example, binding of PRH to

the *Vegf* promoter may induce looping so that these distal regions interact and induce a similar mechanism of regulation to genes where PRH binds to the proximal promoter, such as *Vegfr1* and *Vegfr2.* The use of chromatin conformation capture (3C) would be ideal for elucidating the mechanism of PRH induced transcriptional repression at *Vegf* and *Vegfr1* (Dekker et al., 2002). For 3C cells are formaldehyde cross-linked, thereby binding associated regions of chromatin together. This is followed by enzymatic digestion, leaving two DNA fragments bound by the formaldehyde cross-linker, and intramolecular ligation, so the two associated fragments are ligated together. Finally, PCR can be performed on the ligation product, with one primer annealing to each of the annealed fragments. If used with ChIP data, this technique could be utilized to determine whether PRH bound promoter regions are associated with the TSS of the *Vegf* and *Vegfr1* genes.

This thesis has also demonstrated the importance of post-translational regulation of PRH by CK2 and suggests that other post-translational modifications will also alter PRH functionality. Although the molecular basis of CK2 regulation of PRH has been investigated and discussed at length in this thesis there is still further work required to fully understand the significance of CK2 phosphorylation in disease. CK2 has a role in cancer for many tissues (Ruzzene and Pinna, 2010). Thus, CK2 phosphorylation of PRH may be relevant in other tissues where PRH is expressed. In the haematopoietic compartment, CK2 has also been observed to be highly up-regulated in AML patients with a poor survival prognosis (Kim et al., 2007). It is known that reduced PRH expression or activity is a feature of some AMLs (Topisirovic et al., 2003b; Jankovic et al., 2008). Therefore, AML patients with elevated CK2 activity may have reduced PRH activity without affecting PRH expression or the potential functionality of PRH. However, this work suggests that PRH is an important protein in

maintaining the homeostasis of myeloid progenitor cells, which may be relevant to a number of other cell types.

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