Protein-Protein Interactions Mediating Hypoxia Inducible Factor-1 Complex Function

by

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Abstract

The hypoxia-inducible factor 1 (HIF1) complex, comprised of a HIF1α and aryl hydrocarbon receptor translocator (ARNT/HIF1β) heterodimer, regulates the transcription of genes promoting tumour progression under hypoxia. Preliminary data suggests that the retinoblastoma protein (RB) attenuates HIF1-mediated transcription directly through interaction with the co-activator thyroid hormone receptor/retinoblastoma interacting protein 230 (TRIP230) to inhibit invasiveness of MCF7 human breast cancer cells. The objective of this study was to further characterize the invasive phenotype in MCF7 cells following loss of RB. Loss of RB resulted in a hypoxic-dependent increase in expression of HIF1 target genes involved in tumour progression. Protein-protein interaction analysis demonstrated that RB exists in complex with ARNT and TRIP230. Moreover, this complex dissociated in the presence of an ARNT-interacting peptide. Lastly, the C-terminal region of the TRIP230 RB-interaction domain has been identified as the minimal interaction domain. Altogether, these results further establish RB as an attenuator of HIF1-mediated transcription.

Keywords: hypoxia-inducible factor 1α, aryl hydrocarbon receptor nuclear translocator, retinoblastoma, thyroid hormone receptor/retinoblastoma interacting protein 230, tumour progression

To my Aunt Betty and Chris.

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List of Acronyms

Abl	Ableson tyrosineprotein
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
BCL	B-cell lymphoma
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BRG	Brm-related gene
BRM	Brahma
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
COOH	Carboxylic acid
CREB	cAMP response element-binding
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
E	Embryonic
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
EMT	Epithelial-mesenchymal transition
EPO	Erythropoietin
EPAS	Endothelial PAS domain protein
E2F	E2 family of transcription factors
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FIH	Factor-inhibiting HIF

G_0	Gap 0/resting phase
G_1	Gap 1/post-mitotic phase
G_2	Gap 2/pre-mitotic phase
GST	Glutathion-s-transferase
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-inducible factor
HRE	Hypoxia responsive element
ID	Inhibitor of DNA binding protein
IPAS	Inhibitory PAS domain protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
KOH	Potassium hydroxide
LNCaP	Lymph node carcinoma of the prostate
MAPK	Mitrogen-activated protein kinase
MCF7	Michigan cancer foundation 7
MDA-MB-231	M.D. Anderson-metastatic breast
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinases
MMTV	Mouse mammary tumour virus
mRNA	Messanger ribonucleic acid
mTOR	Mammalian target of rapamycin
MyoD	Myogenic differentiation antigen
NaCl	Sodium Chloride
Nfl	Neurofilament
p300	protein 300
p53	protein 53
PAS	Per-ARNT-SIM
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

Per	Period circadian protein
PDGF	Platelet derived growth factor
PHD	Prolyl hydroxylase
PP1	Protein phosphatase 1
PPI	Protein-protein interaction
PU1	Purine box factor 1
PVDF	Polyvinylidene fluoride
PyMT	Polyoma virus-middle T antigen
RACK1	Receptor of activated protein kinase 1
RB	Retinoblastoma
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RUNX2	Runt-related transcription factor
S	Synthesis phase
SDS	Sodium dodecyl sulfate
SIP1	Smad-interacting protein 1
SIM	Single-minded protein
SRC1	Steroid receptor co-activator 1
SWI/SNF	Switching defective/sucrose non-fermentable
SUV39H1	Suppressor of variegation 3-9 homologue 1
TAD	Transactivation domain
TAT	Trans-activator of transcription
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TR	Thyroid hormone receptor
TRIP15	Thyroid hormone interacting protein 15
TRIP230	Thyroid hormone receptor/retinoblastoma interacting protein 230
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
VHL	Von Hippel Lindau
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1. Introduction

The development of cancer is a multistep process involving dynamic changes within the cancer cell genome that activates oncogenes and/or silences tumour suppressor genes, which ultimately drive unregulated cell growth (Hanahan and Weinberg, 2011). Tumourigenesis was initially thought of as a cell autonomous process, where a tumour cell's ability to sustain hyperproliferation and promote invasion were acquired solely through genetic mutation within the tumour cell itself. There is a growing body of evidence demonstrating an additional level of complexity directing tumour cell behaviour that is influenced by the context of its external environment. On the one hand, an infiltration of immune cells to the immediate surroundings, or microenviroment, of the tumour can initiate a host immune response against tumour-specific antigens and suppress tumour progression (Tanaka *et al.*,1986). Conversely, as genetic mutations accumulate over time, tumour cells can adapt to evade the host response and create an environment that promotes tumour growth and invasion (O'Byrne and Dalgleish, 2001). As a result, it is necessary to understand how tumours interact with their external environment to gain further insight into the molecular mechanisms of tumour progression.

The tumour microenvironment promotes growth and invasion either directly, through the release of factors, or indirectly, through the inherently hypoxic tumour microenvironment. All solid tumours develop regions of hypoxia, where oxygen availability is limited due to the uncontrolled growth of tumour cells which exceeds that of the existing vasculature, coupled with an increase in oxygen consumption by tumour cells to support tumour growth (Tannock, 1972). In response, tumour cells release angiogenesis-stimulating factors to induce growth of new blood vessels that penetrate the tumour and sustain tumour growth by supplying oxygen and nutrients (Folkman *et al.*, 1989). In addition, hypoxia activates mechanisms for cell motility to promote invasion and metastasis of tumour cells. Without adequate vascularization, most tumours will not exceed a few cubic millimetres in volume and remain clinically silent

(Folkman, 1966). Targeting factors required for angiogenesis has long been an attractive target for anti-cancer therapies, but current therapies eventually become ineffective or worse, promote a more metastatic phenotype (Ebos *et al.*, 2009; Paez-McCaw *et al.*, 2009). Once metastasis occurs, the cancer becomes increasingly difficult to treat and the disease prognosis worsens. Thus, targeting hypoxic-responsive factors that promote invasion, rather than angiogenesis, may be more effective at combating tumour progression.

This study focuses on the activity of the hypoxia-inducible factor 1 (HIF1) transcription factor complex, the primary transcriptional regulator activated in response to hypoxia. HIF1 activity is typically up-regulated in several types of cancer in response to tumour hypoxia and regulates transcription of genes involved in both angiogenesis and metastasis (Zhong *et al.*, 1999). Of particular interest was the protein-protein interactions within the HIF1 complex, and how these interactions contribute to angiogenesis and invasiveness. By addressing the importance of these specific protein-protein interactions, this work will not only contribute to our basic understanding of HIF1 transcriptional regulation in response to hypoxia, but will also provide further insight into the role of hypoxia in tumour progression and help identify potential novel therapeutic targets.

1.1. Oxygen homeostasis

All organisms possess mechanisms for regulating oxygen homeostasis to maintain proper cellular oxygen concentrations and are essential for survival (Semenza, 1999). In mammals, development of the cardiovascular and respiratory systems ensures that oxygen delivery is maintained throughout the body to sustain energy production through oxidative metabolism. Oxygen is metabolized within the mitochondria to produce adenosine triphosphate (ATP), the main source of energy required for several cellular and systemic functions, including protein synthesis, protein degradation, sodium/potassium pumping, urea synthesis, and glucose metabolism (Wilson *et al.*, 1988; Bailey and Driedzic, 1996; Hellstrand *et al.*, 1984; Kashiwagura *et al.*, 1984). Normal physiological concentrations of oxygen, or normoxia, vary among adult cells, from 21% in the upper respiratory tract to 1% within the bone marrow (Simon,

2008). Too much oxygen within a cell can result in excess ATP production that leads to an accumulation of oxygen metabolism by-products, called reactive oxygen species, which can damage the cell and cause disease (Galanis, 2008). Conversely, when oxygen availability is limited, cells would not be able to produce enough ATP to maintain essential functions, which can lead to cell death and eventually tissue necrosis. Thus, maintaining appropriate physiological levels of oxygen throughout the body is essential.

1.1.1. Hypoxia

Hypoxia refers to an oxygen deficiency within cells, tissues or whole organisms. Hypoxic environments occur under normal physiological conditions in both the developing embryo and adult. Embryogenesis typically begins within a relatively low oxygen environment prior to development of the circulatory system (Mitchell and Yochim, 1968; Fischer and Bavister, 1993). Local hypoxic microenvironments within the embryo serve as a signal to stimulate and guide the growth of cells that will eventually give rise to various components of the body, including the nervous, cardiovascular and pulmonary systems, as well as the placenta and bone (Ruijtenbeek *et al.*, 2000; Tomita *et al.*, 2003; Adelman *et al.*, 2000; Wang *et al.*, 2007). In a similar manner, hypoxia serves as a stimulus for wound healing in adults. Disruption of vessels initiates an acute hypoxic response that helps initiate an inflammatory response and stimulates the growth of new tissue and surrounding blood vessels (Knighton *et al.*, 1981). More recently, hypoxic conditions have been identified within the microenvironment, or 'niches', of stem cells, and appears to influence their proliferation and differentiation (Grayson *et al.*, 2006; Santilli *et al.*, 2010).

Hypoxia develops in response to disease as well, such as anemia, hemorrhages and pneumonia (Weiskopf *et al.*, 1998; Jones *et al.*, 1968; Ferstenfeld *et al.*, 1975). Acute forms of hypoxia, for example during wound healing, can initiate an adaptive response that promotes healing. Chronic exposure to hypoxia that develop in response to disease, however, can initiate an inappropriate response that impairs processes necessary for healing, which includes excess production of reactive oxygen species, elevated blood pressure, and enhanced production of signalling factors due to the persistant recruitment of inflammatory cells. As a result, exposure to chronic hypoxia can have severe negative consequences, including heart failure within chronic lung

disease patients, and stroke in patients with obstructive sleep apnea (Yu *et al.*, 1999; Kline *et al.*, 2002; Brizel *et al.*, 1996; Lesske *et al.*, 1997). Longevity, in combination with poor lifestyle choices, have specifically put humans at risk for exposure to more chronic forms of hypoxia that can arise from tobacco-related lung diseases, artherosclerosis, cardiovascular disease and cancer.

Over time, exposure to hypoxia has favoured the establishment of an evolutionary conversed adaptive hypoxic response at the cellular level. When oxygen availability is too low, cells will adapt to survive by suppressing energy turnover and upregulating efficient ATP-producing pathways (Hochachka *et al.*, 1996). This adaptation occurs as a two step process: (1) the initiation of transcription of key hypoxia-sensitive transcription factors, which are then required for (2) the initiation of a second cycle of gene expression to regulate the synthesis of glycolytic enzymes to survive oxygen deprivation (Semenza *et al.*, 1994; Semenza and Wang, 1992). Research has identified the HIF1 complex as a key transcription factor within the adaptive hypoxic response that directly regulates the transcription of several genes, including those involved in cell proliferation, cell motility, angiogenesis and glucose metabolism. HIF1 activity is essential under conditions of hypoxia that arise during development and wound healing, however, research has now uncovered a role for HIF1 activity under conditions of hypoxia associated with disease, such as cancer. Nevertheless, the molecular mechanism behind the adaptive hypoxic response mediated by HIF1 has yet to be completely defined.

1.2. The bHLH/PAS family of proteins

The basic helix-loop-helix (bHLH)/Per-ARNT-SIM (PAS) proteins are a family of transcriptional factors whose activity is typically regulated through the detection of signals from the external environment (Kewley *et al.*, 2004). Members of this family are characterized by a helix-loop-helix domain adjacent to a PAS domain consisting of two degenerate repeats of amino acids, referred to as PAS A and PAS B. Members of this family are divided into two classes; class I factors, which detect and respond to specific signals, and class II factors, which act as general binding partners for class I factors. Members of the class I factors include the aryl hydrocarbon receptor (AhR), single-

minded proteins (SIM), and hypoxia-inducible factors (HIF), including HIF1 (Ema *et al.*, 1996; Wang *et al.*, 1995; Lindebro *et al.*, 1995). Upon stimulation, class I proteins heterodimerize with a member from class II, to form a transcriptionally active complex that recognize variant forms of the classic E-box core enhancer sequence. The most well characterized class II factor is the aryl hydrocarbon receptor nuclear translocator (ARNT), which is capable of forming a heterodimeric complex with AhR, SIM and HIF proteins (Hoffman *et al.*, 1991; Wang *et al*., 1995; Swanson *et al.*, 1995). Both the bHLH and PAS domains act as interfaces for dimerization, however, it is the bHLH domain that favours interaction with other PAS proteins, rather than forming a homodimer (Jiang *et al.*, 1996).

1.2.1. The HIF1 transcription factor complex

Higher metazoans, including humans, have three isoforms of hypoxia-inducible factors: HIF1α, HIF2α/Endothelial PAS domain protein (EPAS) 1 , and HIF3α/Inhibitory PAS domain protein (IPAS), encoded for by the *HIF1α*, *EPAS1*, and *HIF3α* genes, respectively (Semenza, 1999). The HIF1 transcription factor is a heterodimeric complex comprised of a HIF1α subunit and an ARNT subunit, also referred to as HIF1β (Wang *et al.*, 1995). Upon dimerization, HIF1 initiates transcription through hypoxia response elements (HRE), which are enhancer elements that contain the consensus core sequence RCGTG (Semenza *et al.*, 1996). HIF1 specifically interacts with the guanine residues, which lie within the major groove of deoxyribonucleic acid (DNA), and selectively induces gene expression at HREs upstream of promoters under conditions of hypoxia (Okina *et al.*, 1998). There are more than 70 direct target genes of HIF1, which encode for proteins involved in several cellular processes, including proliferation, survival, angiogenesis and glucose metabolism (Wenger *et al.*, 2005).

In response to hypoxia, HIF1 mediates an adaptive response that can be both cell autonomous and tissue restricted. Acute exposure to hypoxia throughout the entire body can occur during ascents into high altitude or in response to pneumonia, which can induce HIF1 activity to maximize oxygen uptake while minimize energy usage (Forster *et al.*, 1974; Tissot van Patot *et al.*, 2004; Tzouvelekis *et al.*, 2007). Hypoxia stimulates specialized cells within the kidney to produce erythropoietin (EPO), a glycoprotein hormone that stimulates the production of red blood cells and is a direct target of HIF1

transcription (Schuster *et al.*, 1987; Wang and Semenza, 1993). Transferrin and the transferrin receptor are up-regulated by HIF1 as well, to facilitate an increase in iron transport to the bone marrow, where the production of red blood cells occur (Tacchini *et al.*, 1999; Rolfs *et al.*, 1997). Cells must also undergo several changes in their metabolism to reduce energy consumption. Among these changes include an increase in pyruvate dehydrogenase kinase, which directs pyruvate away form the mitochondria to reduce the synthesis of ATP through oxidative metabolism (Kim *et al.*, 2006). A reduction in oxidative metabolism is then compensated for by an increase in glycolysis, a process that synthesizes ATP from glucose. HIF1 activates expression of several glycolytic enzymes, including glucose transporter proteins, glyceraldehyde-3-P-dehydrogenase, and lactate dehydrogenase (Graven *et al.*, 1999; Semenza and Wang, 1992; Iyer *et al.*, 1998).

Local environments of hypoxia also arise in cases where wounds sever blood vessels, restricting blood flow to a limited population of cells. HIF1 initiates synthesis of new blood vessels, or angiogenesis, by enhancing expression of vascular endothelial growth factor (VEGF), stromal-derived factor 1 and platelet-derived growth factor (PDGF) (Forsythe *et al.*, 1996; Hitchon *et al.*, 2002; Yoshida *et al.*, 2006). HIF1 also induces expression of the C-X-C chemokine receptor type 4 (CXCR4), the receptor for stromal-derived factor 1, and vascular endothelial growth factor receptor (VEGFR), the receptor for VEGF (Gerber *et al.*, 1997; Elvert *et al.*, 2003; Staller *et al.*, 2003). Both receptors are involved with the mobilization and recruitment of mesenchymal stem cells, bone marrow-derived angiogenic cells, and endothelial progenitor cells, all of which are required for remodelling of both vascular endothelial and smooth muscle cells.

1.2.2. HIF1α

HIF1α is the most extensively studied isoform of the hypoxia-inducible factors. HIF1α was first identified as a nuclear factor bound to the enhancer region of the *EPO* gene in response to hypoxia (Semenza and Wang, 1992). It is a 120-130 kD protein consisting of both an N-terminal and C-terminal nuclear localization signals and two transactivation domains, the N-TAD and the C-TAD, both of which serve as an interaction surface for co-activator proteins (Semenza, 1999). HIF1α also contains a central oxygen-dependent degradation domain, which overlaps with the N-TAD and

mediates the oxygen-dependent degradation of HIF1α. Expression is detected in mouse embryos as early as embryonic day (E) 8.5 and is an essential for development, as *HIF1α* -/- mice die at E9.5-10.5 (Iyer *et al.*,1998a, Ryan *et al.*, 1998). These mice demonstrate severe defects in blood vessel formation within the yolk sac, bronchial arches, cranium, somites and placenta.

Activity of HIF1α is largely dependent on oxygen availability and primarily occurs at the post-translational level (Figure 1). Under normoxia, HIF1α is constitutively expressed but is then rapidly degraded. Prolyl hydroxylases (PHDs) utilize oxygen as a substrate to hydroxylate HIF1α at two specific proline residues, P564 and P402, located within the oxygen degradation domain (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Hydroxylation of the oxygen degradation domain creates a docking site for Von Hippel Lindau (VHL) tumour suppressor protein binding, which then recruits elongins B and C, cullin 2, and RING-box protein 1 to form a functional E3-ubiquitin ligase complex (Iwai *et al.* 1999). VHL-E3 ubiquitin ligase complex ubiquitinates HIF1α, targeting it for proteosomal degradation by the 26S proteasome. Factor-inhibiting HIF (FIH) provides an additional level of post-translational control by hydroxylating a specific asparagine (N803) residue within the C-TAD domain of HIF1α, which blocks the binding of the co-activator CREB-binding protein (CBP)/protein 300 (p300) (Lando *et al.*, 2002; Lando *et al.*, 2002). Like PHDs, FIH activity is oxygen dependent. FIH has a higher affinity for oxygen compared to PHD and would thus require more severe hypoxia to become inactive (Koivunen *et al.*, 2004).

HIF1α can be targeted for proteosomal degradation through VHL-independent mechanisms of HIF1α proteosomal degradation. Receptor of activated protein C-kinase (RACK1) competes with heat-shock protein-90 for binding with HIF1α. Binding of RACK1 to HIF1α displaces heat-shock protein-90 and initiates recruitment of the E3-ubiquitin ligase complex (Liu *et al.*, 2007). Similar to the VHL-dependent pathway, HIF1α undergoes ubiquitination and is then targeted for proteosomal degradation. Glycogen synthase kinase-3 has also been reported to target HIF1α for degradation through ubiquitination in a prolyl-hydroxylation- and VHL-independent manner (Flugel *et al.*, 2007).

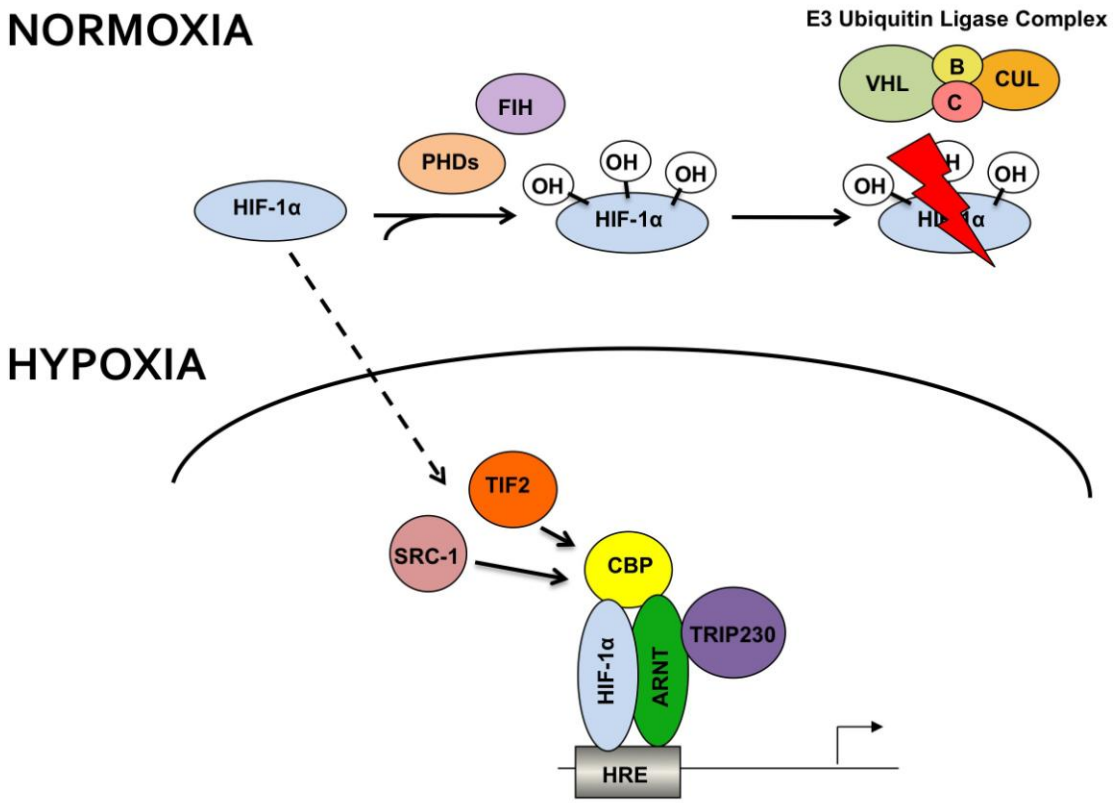

Figure 1. Regulation of the HIF1 transcription factor activity

Under normoxic conditions, the enzymes prolyl hydroxylase (PHD) and factor-inhibiting HIF (FIH), utilize oxygen as a substrate to hydroxylate HIF1α at two specific proline residues. These hydroxylated sites then serve as a docking site for the Von Hippel Lindau (VHL) E3 ubiquitin ligase complex, which then targets HIF1α for proteasomal degradation. Under hypoxic conditions, PHD and FIH can no longer hydroxylate HIF1α, allowing HIF1α protein to accumulate and translocate into the nucleus, where it will dimerize with ARNT and recruit co-activators to activate target genes through their hypoxia responsive elements (HRE).

Under hypoxia, oxygen is no longer available to serve as a substrate for both PHD and FIH activity. HIF1α can no longer undergo hydroxylation and results in an accumulation of HIF1α protein. HIF1α will then translocate into the nucleus, where it will dimerize with ARNT and recruit co-activators to activate transcription through the recognition of HREs in the regulatory regions of target genes. Expression of HIF1α is detected within 30 minutes of hypoxia with maximal expression occurring at 0.5% O_2 and half-maximal expression occurring between 1.5%-2% O_2 (Jiang *et al.*, 1996). HIF1 transcription is dependent on the recruitment of several co-activators that help stabilize the general transcription factors associated with ribonucleic acid (RNA) polymerase II. CBP/p300 is an essential co-activator that directly interacts with HIF1α and helps recruit other co-activators such as steroid receptor co-activator 1 (SRC1), or brahma (BRM)/Brm-related gene (BRG1) (Wang *et al.*, 2004; Arany *et al.*, 1996; Carrero *et al.*, 2000). SRC1 and transcription intermediary factor 2 are a part of the SRC1/p160 family of transcriptional co-activators that have histone acetyltransferase activity. These factors are recruited to the HIF1 complex in a hypoxic dependent manner to enhance transcription through acetylation of specific residues on histone tails, making the DNA more accessible to RNA polymerase II (Wang *et al.*, 2010).

Regulation of HIF1α activity can also occur through oxygen-independent mechanisms in a cell type-specific manner in response to growth factor stimulation. This allows cells to initiate HIF1 activity in response to other conditions of cellular stress, such nutrient deprivation. Both the mammalian target of rapamycin (mTOR) and mitrogen-activated protein kinase (MAPK) pathways activate HIF1α translation through the phosphorylation of translational repressors eukaryotic initiation factor (eIF) 4E-binding proteins (4E-BP1, 4E-BP2, and 4E-BP3). The mTOR signaling pathway is activated through stimulation by either growth factors, oncoproteins or cytokines, and results in the phosphorylation of 4E-BP proteins by extra-cellular-signal-regulated kinases (Magagnin *et al.*, 2008; Zhu *et al.*, 2009). Phosphorylation of 4E-BP decreases its affinity for eIF4E and enables complex formation with eIF4F. The eIF4F-complex is then allowed to interact with the 5' cap of HIF1α messanger RNA (mRNA) and initiate translation. Similarly, activation of the MAPK signal-integrating kinase through the MAPK pathway phosphorylates eIF4E, enabling eIF4F complex formation and initation of HIF1α mRNA translation (Kasuno *et al.*, 2004). Calcium availability has also been reported to inhibit

HIF1α degradation. An increase in calcium increases activity of calcimeurin A, which in turn dephosphorylates RACK1 and decreases RACK1 affinity for HIF1α (Liu *et al.*, 2007).

1.2.3. HIF2α and HIF3α

In addition to HIF1α, metazoan genomes encode for two other HIF1α isoforms, HIF2α and HIF3α. HIF1α and HIF2α are the most structurally and functionally similar in that they can both dimerize with ARNT and mediate transcription through HREs (Semenza and Wang 1999). Both contain an central oxygen degradation domain and the two transactivation domains, N-TAD and C-TAD. Several human tissues express both HIF1α and HIF2α, however, HIF2α is preferentially used over HIF1α in the endothelium, kidney, lung, heart and small intestine (Ema *et al.*, 1997; Tian *et al*, 1997; Wiesener *et al.*, 2003). Both share similar target genes, such as VEGF, but also have a distinct set of target genes and transcriptional co-activators. For example, HIF1α is responsible for the transcription of genes encoding enzymes for the glycolytic pathway, but oct4, cyclin D1 and transforming growth factor(TGF)-α are predominantly expressed under HIF2α (Hu *et al.*, 2003; Covello *et al.*, 2006; Raval *et al.*, 2005). The N-TAD serves as an interaction domain for HIF2α-specific co-activators and appears to contribute to target gene specificity of HIF2α, whereas the C-TAD interacts with several common co-activator proteins and promotes the expression of common target genes of both HIF1α and HIF2α (Hu *et al.*, 2007). Unlike HIF1α null mice, *HIF2α* -/- mice can survive until mid to late gestation, but eventually die due to severe defects within the pulmonary and cardiovascular systems.

The role of HIF3α is the least well characterized. Multiple isoforms of HIF3α exist, which all lack a C-TAD and therefore can not bind with the co-activator protein CBP/p300. Most contain an oxygen degradation domain but contain only one of the two conserved proline residues, which is targeted for ubiquitination in the presence of VHL and PHD in an oxygen-dependent manner (Maynard *et al.*, 2003; Heikkila *et al.*, 2011). HIF3α expression is even more limited compared to HIF1α and HIF2α, as it is only detected in the adult thymus, lung, brain, heart and kidney (Gu *et al.*, 1998). HIF3α variants are capable of interacting with both HIF1α and HIF2α, and appears to repress HIF1 transcription by preventing translocation of either HIF1α or HIF2α into the nucleus

(Heikkila *et al.*, 2011). The most well characterized splice variant of HIF3α, referred to as IPAS, is particularly unique in that it does not contain an oxygen degradation domain and functions in a oxygen-independent manner. Moreover, IPAS acts as a negative regulator of HIF1 transcription by interfering with HIF1 binding activity to HREs (Jang *et al.*, 2005; Maynard *et al.*, 2005). When expressed in VHL null clear-cell renal carcinoma cells, a decrease in HIF1 target gene expression is observed and suppresses tumour growth in a tumour xenograft mouse model, suggesting that HIF3α may have therapeutic potential.

1.2.4. HIF1β/ARNT

Similar to HIF1α, there are three paralogues of ARNT (ARNT/HIF1β, ARNT2/ARNT-like 2, and ARNT3/ARNT-like 3) (Takahata *et al.*, 1998; Hoffman *et al.*, 1991; Hirose *et al.*, 1996). ARNT was originally identified as a binding partner with AhR following treatment with the AhR ligand 2,3,7,8 tetracholorodibenzo-*p*-dioxin (Hoffman *et al.*, 1991). ARNT is now classified as a class II bHLH/PAS domain protein that serves as a general transcription factor binding partner for class I bHLH-PAS domain proteins, including AhR, HIF1α and SIM. ARNT is both constitutively and ubiquitously expressed, and remains within the nucleus through its N-terminal nuclear localization signal (Hirose *et al.*, 1996). *ANRT-/-* mice are not viable past E10.5 and show similar defects as *HIF1α* -/- mice, such as decreased vascularization of the placenta, yolk sac, bronchial arches, cranium, somites and placenta (Maltepe *et al.*, 1997; Kozak *et al.*, 1997). ARNT transcriptional activity is likely mediated through its C-TAD that serves as a platform for recruiting co-activator proteins and chromatin remodelling factors, including CBP/p300, SRC1/2, gyrA C-terminal domain-63, and coiled-coil co-activator-A (Whitelaw *et al.*, 1994; Beischlag *et al.*, 2002; Kobayashi *et al.*,1997).

While ARNT serves as a general binding partner for other bHLH/PAS proteins, ARNT is also capable of forming a homodimer that can mediate reporter gene transcription through a CACGTG E-box element. While the role of an ARNT-ARNT transcription factor is still unclear, DNA microanalysis has revealed several genes up-regulated in response to ARNT-ARNT, include those involved in cell growth, metabolism and apoptosis (Wang *et al.*, 2006). Furthermore, none of these genes were reported

targets of AhR-ARNT regulation, suggesting that these may be unique targets for ARNT homodimers.

1.3. Tumour metastasis

Metastasis refers to the physical displacement of tumour cells from the primary tumour site into the surrounding tissue and blood stream, towards a secondary site that facilitates growth of new tumours. According to the Canadian Cancer Society, approximately 177,800 new cases of cancer arise each year in Canada and one in every four persons diagnosed with cancer will die. Metastasis accounts for close to 90% of cancer-related deaths as these forms of cancer are particularly aggressive and develop rapid resistance to therapy (Chaffer and Weinberg, 2011).

Following analysis of over a century of cancer research, Douglas Hanahan and Robert Weinberg established the six hallmarks of cancers observed in tumour cells that are considered necessary for the acquisition and ultimately mestastasis of tumours (Hanahan and Weinberg, 2000).

1. Self-sufficient and sustained proliferation
2. Insensitivity towards anti-proliferation signals
3. Evade signals for apoptosis
4. Acquisition an infinite replication potential
5. Sustained angiogenesis
6. Acquisition of a more mobile phenotype

While subsequent research within more recent years has revealed an even greater level of complexity dictating a cancer cell phenotype, these six hallmarks still hold as an accurate foundation for the onset of cancer.

The primary hallmark of cancer is the ability to sustain cellular proliferation through the production of growth signals required for entry into the cell cycle. Normal cells respond to exogenous signals that allow for entry into the cell cycle. Cancer cells acquire the ability to produce their own growth signals, which eliminates the dependence on exogenous signals for proliferation. This is typically acquired through genetic

mutation, which up-regulates expression of growth signals or disrupt negative feedback loops of growth factor signal production.

Normal cells also rely on exogenous signals to exit from the cell cycle and terminate cell proliferation (Perona *et al.*, 2006). Cancer cells become insensitive to such signals by evading mechanisms of contact inhibition or becoming unresponsive to tumour suppressor protein activity. Tumour suppressors are a class of proteins responsible for inhibiting cell cycle progression, particularly in response to DNA damage or cellular stress (Curto *et al.*, 2007; Partanen *et al.*, 2009). Classical examples of tumour suppressor proteins include RB, which inhibits cell cycle progression in response to DNA damage, and the protein 53 (p53) protein, which responds to conditions of cellular stress or abnormal cellular conditions (Burkhart and Sage, 2008; Bieging and Attardi, 2012).

The third hallmark is the ability to evade signals of cell death. The process of programmed cell death, or apoptosis, can act as a natural defence against the development of cancer in response to excess DNA damage or cellular stress. For example, p53 can elicit apoptosis if cellular conditions become intolerable through the up-regulation of the pro-apoptotic factor bax (Selvakumaran *et al.*, 1994; Gao *et al*, 2001). In turn, bax activates additional pro-apoptotic factors through the release of cytochrome C, which coordinates the degradation of cellular components. When mutations accumulate and render proteins like p53 inactive, cancer cells acquire the ability to evade this process either through the up-regulation of apoptosis inhibitors or inactivation of proteins required for apoptosis.

Cell autonomous proliferation, insensitivity towards anti-proliferative signals and evading apoptosis signals are altogether not sufficient for tumour growth. Cancer cells must also overcome internal factors that limit replication potential. Healthy cells typically undergo a finite number of cell cycles before entering a state of senescence or apoptosis to prevent unhealthy cells from continuing replication or to limit growth of tissues and organs (Hayflick, 1997). Programs involved in regulating these processes work independently from those required to inhibit cell cycle progression and initiate apoptosis. Thus, cancer cells must manipulate such programs in order to develop the ability to undergo limitless replication, rendering them immortal. For example, telomerase is the

enzyme which protects against erosion of chromosome ends following each round of DNA replication and is essential maintaining cells in an immortalized state (Counter *et al.*, 1992). Cancer cells have significantly increased telomerase activity, enabling them to evade senescence and apoptosis, thus providing a means for limitless replication (Jong *et al.*, 1999; Sommerfeld *et al.*, 1996; Murakami *et al.*, 1997; Aue *et al.*,1998).

Induction of angiogenesis and acquisition of a more mobile phenotype are the final two hallmarks of cancer as defined by Hanahan and Weinberg. Similar to normal cells, tumour cells require nutrients and oxygen to sustain growth and proliferation. Thus, tumour cells acquire mechanisms to release signalling factors and attract an influx of immune cells to induce growth of new vasculature, or angiogenesis, to support tumour growth (Lorusso and Ruegg, 2008). While angiogenesis facilitates metastasis, cancer cells must become mobile in order to invade surrounding tissue and vasculature in order to metastasize. An increasing amount of evidence has demonstrated that this is primarly achieved through a process called epithelial-mesenchymal transition (EMT) (Thiery, 2002). EMT is a multistep process in which cells transition from a sendentary epithelial state towards a more mobile mesenchymal-like phenotype, in which cell surface molecules and the actin cytoskeleton are re-arranged to favour loosely adherent interactions with the extra-cellular matrix. Both angiogenesis and EMT are particularly important for tumour progression, and will be discussed in more detail.

1.3.1. Angiogenesis

The circulatory system is comprised of a complex network of arteries, veins and capillaries that ensures sufficient distribution of oxygen throughout the body. Creating an intricate and organized network of vessels involves multiple steps that are tightly regulated. Development of the circulatory system in humans begins at approximately E30 and begins with vasculogenesis, the production of *de novo* vessels that arise from endothelial precursers (Hirakow, 1983; Carmeliet and Jain, 2011). In response to stimuli, endothelial cells undergo a series of steps involving proliferation, migration, and differentiation to organize themselves into tube-like structures. The sprouting of new vessels from existing ones, or angiogenesis, follows vascularization to continue growth of a network of arteries and veins.

Angiogenesis is primarily induced by VEGF, however, multiple signals are involved in this process, including angiopoietin, placental growth factor-1, and PDGF. Matrix metalloproteinases (MMPs) and plasminogen activator inhibitors are also required to support angiogenesis by facilitating the breakdown of the extra-cellular matrix (Page-McCaw *et al.*, 2007; Pepper, 2001). Local hypoxic environments during embryogenesis stimulate the production of VEGF, which binds to tyrosine kinase receptors VEGFR1 and VEFGR2 (Lee *et al.* 2001; Parast *et al.,* 1998). VEGFR2 is the predominant mediator of the VEGF cellular response, which involves regulating proliferation, survival, migration and permeability of vessels. The role of VEGFR1 stimulation still remains unclear, however, VEGFR1 appears to facilitate angiogenesis by recruiting bone marrow derived cells towards the site of angiogenesis or by inducing the release of MMPs into the extra-cellular matrix (Rafii *et al.*, 2003; Hiratsuka *et al.*, 2002). Angiogenesis also requires the removal of pericytes from surrounding endothelium that helps destabilize and mobilize vessels for proliferation and growth of new branches. Mesenchymal cells then migrate and differentiate along the new vessel into mature pericytes for stability.

In adults, vessels remain quiescent and rarely form new branches, except under circumstances of tissue regeneration and wound healing. In cancer, the induction of angiogenesis is often referred to as 'turning on a switch' that continuously sprouts growth of new vessels (Folkman, 1995). As a result, the organization of new blood vessels is aberrant, where capillary sprouting occurs too soon and there is excess branching of distorted and enlarged vessels, which are often leaky (Nagy *et al.*, 2010; Baluk *et al.*, 2005). Induction of tumour angiogenesis occurs during the early stages of cancer development in response to tumour hypoxia and nutrient deprivation (Shweiki *et al.*, 1992; Izuishi *et al.*, 2000). As with embryogenesis, tumour angiogenesis is primarily induced through increased expression of several factors, such as VEGF, that help sustain tumour angiogenesis. Moreover, the leakiness of tumour vessels initiates an inflammatory response and recruits several bone marrow derived cells, such as macrophages and myeloid cells. Recruitment of these cells often exacerbate angiogenesis by secreting more growth factors, including VEGF and PDGF, into the tumour microenvironment (Pander *et al.*, 2011; Lin *et al.*, 2006; Shojaei *et al.*, 2007). Fibroblasts within the external environment contribute to angiogenesis as well, through

the release of extra-cellular matrix proteins, such as MMPs, into the extra-cellular matrix (Forough *et al.*, 2006).

Activation of endogenous mechanisms that inhibit angiogenesis have been reported as well, to prevent excess proliferation of new blood vessels. Up-regulation of thrombospondin 1 within tumours down-regulates the VEGF-induced cellular response by interacting with endothelial receptors, including VEGFR2 (Rodriguez-Manzaneque *et al.*, 2001). Secreted factors that breakdown extra-cellular matrix components can also support inhibition of angiogenesis. Tumstatin and endostatins are both by-products of collagen breakdown that interacts with integrins. Tumstatin inhibits angiogenesis through interaction with integrins, preventing their association with other growth factors that promote angiogenesis (Sudhakar *et al.*, 2003).

Not surprisingly, inhibiting angiogenesis has been the focus of many therapeutic developments. Without adequate vascularization, most tumours will not exceed a few cubic millimetre in volume and remain clinically silent (Folkman, 1966). Avastin is a monoclonal antibody that specifically targets VEGF and was first approved by the Food and Drug Administration in 2001 for use in combination with chemotherapy for metastatic colon cancer (Zondor and Medina, 2004). Initial usage demonstrated decreased vascular proliferation and permeability that ultimately helped decrease tumour growth. This approval was extended for use in metastatic breast cancer but has since been revoked in 2011 as results from subsequent studies not only demonstrated resistance, but appeared to support a more aggressive tumour behaviour (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009). While the mechanism of resistance is still not well understood, it appears as though inhibiting one angiogenic factor simply promotes the activity of other pro-angiogenic factors, and may ultimately act as a mechanism for the positive selection of more malignant transformed cells. For example, inhibition of VEGF leads to an up-regulation of placental growth factor 1, which in turn leads to an influx of bone marrow derived cells to the site (Fischer *et al.*, 2007). Bone marrow derived cells can then continue supporting angiogenesis through release of their own growth factors, including VEGF and PDGF.

1.3.2. Epithelial-mesenchymal transition

During development, multicellular organisms undergo a series of processes involving the delamination, invagination, branching and multilayering of cells. This requires epithelial cells to undergo several inter- and intracellular changes to transition towards a more mobile mesenchymal phenotype, or EMT (Thiery and Sleeman, 2006). EMT first occurs at the blastula stage, where the epithelial epiblast gives rise to the mesoderm and continues at several subsequent stages, including formation of the primitive streak, gastrulation and neural crest development. A number of factors activate EMT through a variety of signaling pathways, and each event requiring EMT is regulated by a particular subset of factors. For example, TGF-β is the primary activator for EMT events during development of the circulatory system, whereas hepatocyte growth factor is primarily involved in somatogenesis, and the fibroblast growth factor (FGF) is an inducer of EMT in gastrulation (Wang *et al.*, 2005; Mizuno *et al.*, 1998; Yamaguchi *et al.*, 1994).

Loss of E-cadherin is considered a hallmark of EMT. E-cadherin is a cell adhesion protein that mediates interactions between the extra-cellular matrix and the intra-cellular cytoskeleton through α-catenin or β-catenin complexes (Ozawa and Kemler, 1992). E-cadherin also mediates contact between adherin junctions and the cytoskeleton, which promotes formation of junctions in between cells, called desmosomes (Gosavi *et al.*, 2011). Few transcriptional repressors have been identified as direct factors that down-regulate E-cadherin. The zinc finger transcriptional repressors snail and smad-interacting protein 1 (SIP1) inhibit E-cadherin transcription by directly binding to E2 boxes located near the transcriptional start site (Batlle *et al.*, 2000; Comijn *et al.*, 2001). Snail appears to have a more prominent role in the initiation of EMT, while SIP1 and other repressors, including E12/E47 and Zeb1, are important for maintaining the mesenchymal state (Perez-Moreno *et al.*, 2001).

Concurrent with the down-regulation of epithelial cell markers, such as E-cadherin, is an up-regulation of mesenchymal cell markers. Acquisition of a mesenchymal phenotype requires the reorganization of the actin cytokeleton and cell surface molecules. This enables cells to lose contact with their neighbouring cells and increase plasticity to allow for mobility. The intermediate filament vimentin is a typical

mesenchymal cell cytoskeletal protein, and is an important regulator of cell motility and for maintaining structural integrity of the mesenchymal phenotype (Mendez *et al.*, 2010). Up-regulation of vimentin is dependent on expression of other factors, such as snail or SIP1. vimentin also appears to be required for induction of cell migration and it may do so through the up-regulation of axl, a tyrosine receptor kinase previously identified as regulator of cell migration (Fridell *et al.*, 1998; Vuoriluoto *et al.*, 2011).

EMT has now been recognized as a mechanism that potentiates pathophysiological processes, including the metastasis of cancer, and much of the evidence stems from changes in E-cadherin expression. A correlation between decreased levels of E-cadherin expression and increased incidences of metastasis have been observed in many types of human cancers (Rasbridge, 1993; Oka, 1993; Zhou, 2002; Lim, 2010; Lewis-Tuffin, 2010). Moreover, a significant down-regulation of E-cadherin is observed within the dissociated cells of adenocarcinomas, compared to solitary cells within the originating tumour site (Hlubek *et al.*, 2001). Lastly, the specific manipulation of E-caherin expression favours a more invasive cellular phenotype. Transfection of E-cadherin cDNA into E-cadherin null carcinoma cells prevents invasiveness, whereas anti-E-cadherin antibodies that render E-cadherin non-functional induce invasiveness (Perl *et al.*, 1998; Imhof *et al.*, 1983).

Interestingly, several parallels have been observed between the molecular mechanisms of EMT in development and in tumour progression. While the complete range of EMT activators in tumour cells have not been completely identified, several growth factors appear to be responsible for induction of EMT. TGF-β, a key regulator in development of the circulatory system, is also a prominent mediator of EMT in tumours. In both development of the embryonic heart and in carcinoma cells, the up-regulation of slug through activation by TGF-β down-regulates E-cadherin expression (Wang *et al.*, 2005; Romano and Runyan, 2000; Janda *et al.*, 2002). Induction of the Ras-MAPK pathways through FGF signalling activates either snail or slug to inhibit E-cadherin expression, similar to the FGF-induced EMT that occurs during gastrulation. Other activators of EMT aside from growth factors have been reported, such as factors within the immune system and microRNAs (Reiman *et al.*, 2010). The miR-429 promotes inhibition of E-cadherin, thus maintaining a mesenchymal cell phenotype (Chen *et al.*,

2011). Altogether, there is an increasing amount of evidence that supports a role for EMT in the progression of cancer .

1.4. HIF1 and tumour metastasis

All solid tumours develop regions of hypoxia, where oxygen availability is limited due to the uncontrolled growth of tumour cells which exceeds that of the existing vasculature, coupled with an increase in oxygen consumption by tumour cells to support tumour growth (Tannock, 1972). Similar to its role in development, hypoxia also coordinates molecular mechanisms directly involved in angiogenesis, cell survival, glucose metabolism and invasion of tumours. Not surprisingly, the cellular response to hypoxia is predominately translated through the HIF1 transcription factor. Over-expression of HIF1α is common in several types of cancer, including breast, prostate, gastrointestinal, bladder, malignant melanoma, colon, esophageal, non-small cell lung and pancreatic cancers (Zhong *et al.*, 1999; Talks *et al.*, 2000). Moreover, an increase in HIF1α is associated with increased mortality with some cancers. Thus, HIF1 has been the subject of a significant amount of research to uncover the mechanistic details behind the cellular response to tumour hypoxia.

A number of consequences have been identified as a result of increased HIF1α expression and HIF1 activity. The most notable consequence of HIF1 activity is the regulation of angiogenic factors to increase tumour vascularization. HIF1 regulates angiogenesis, either directly, through VEGF, VEGFR1 and VEGFR2, or indirectly, through PDGF (Forsythe *et al.*, 1996; Gerber *et al.*, 1997; Elvert *et al.*, 2003; Yoshida *et al.*, 2006). VEGF was first described as a direct HIF1 target in a liver carcinoma cell line and was directly associated with increased vascularity and tumour growth within a xenograft mouse model (Forsythe *et al.*, 1996, Maxwell *et al.*, 1997). EPO and transferrin are also direct targets of HIF1 transcription involved in regulation of red blood cell production and iron transport, respectively (Wang and Semenza, 1993; Rolfs *et al.*, 1997).

Increased HIF1 activity also directly associated with increased invasiveness and metastasis of tumour cells. Snail is a direct target of the HIF1 transcriptional complex,

which provides an indirect mechanism for HIF1 regulation of both vimentin and E-cadherin (Imai *et al.*, 2003). Twist, a bHLH transcription factor that regulates gastrulation and mesoderm during development, is also directly up-regulated by HIF1 transcription in tumour cells (Yang *et al.*, 2008). Over-expression of HIF1α promotes EMT through a decrease in E-cadherin and an increase in vimentin. Moreover, an increase in cellular migration and invasiveness was observed in tumour cells, both *in vivo* and *in vitro*. Knockdown of twist demonstrated contrasting results, where a decrease in vimentin and increase in E-cadherin expression were observed, as well as a decrease in cellular invasiveness and migration. There is also increasing evidence for the role of CXCR4 in facilitating metastasis, whose physiological role is to direct migration of leukocytes towards sites of inflammation or tissue injury (Fulton, 2009). HIF1 directly regulates both CXCR4 and its chemokine ligand stromal derived factor 1 (Staller *et al.*, 2003; Hitchon *et al.*, 2002). Exposure of MCF7 human breast cancer cells to stromal derived factor 1 induces actin filament rearrangement and pseudopodia formation (Mueller *et al.*, 2001). Moreover, constitutive activation of CXCR4 in MCF7 cells and in a mouse mammary tumour model promotes tumour growth and metastasis (Rhodes *et al.*, 2010).

In addition to angiogenesis and invasion, HIF1 activity also up-regulates genes involved in metabolism. Hypoxia favours the transition from oxidative metabolism towards glucose metabolism as a more efficient means of producing ATP when oxygen availability is limited. HIF1 directly up-regulates expression of hexokinase, phosphofructokinase and pyruvate kinase, all of which are enzymes required for glycolysis (Riddle *et al.*, 2000; Semenza *et al.*, 1994). While glycolysis yields less ATP per molecule of glucose compared to oxidative metabolism, cells can adujust to compensate for this decrease by enhancing glucose up-take. HIF1 also directly up-regulates expression of the glucose transporter to facilitate glucose transport into the cell. Interestingly, glycolysis remains active in tumour cells, even after oxygen levels increase. This may be in part due to the increased activity of oncogenes, including cMyc and Ras, that also promotes glycolysis (Osthus *et al.*, 2000; Yalcin *et al.*, 2009). Increased glycolysis also suppresses the release of apoptosis-initiating factors from the mitochondria, which may also explain why glycolysis is maintained in tumour cells

(Bonnet *et al.*, 2007). Whether or not tumour cells rely soley on glycolysis for energy production still remains unclear.

Resistance to apoptosis is another hallmark of cancer in which HIF1 activity appears to be involved. While some studies suggest that HIF1 induces apoptosis, either through stabilization of p53 or up-regulation of the pro-apoptotic gene BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), others suggest that HIF1 may provide resistance to apoptosis (Chen *et al.*, 2003; Hansson *et al.*, 2002; Sowter *et al.*, 2001; Akakura *et al.*, 2001). Whether HIF1 activity either promotes or represses apoptosis, appears to depend on the pre-existing genetic background of the cell.

Tumour growth and invasion is a complex and multistep process, and HIF1 appears to regulate a number of processes that support this. Thus, targeting the response to hypoxia itself, rather than the consequences that result from hypoxia, may be a better approach to treating cancer. Inhibiting angiogenesis through treatment with anti-angiogenic therapy eventually fails and ultimately enhances the hypoxic response by increasing regions of tumour hypoxia that may support an even more aggressive phenotype (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009). Thus, targeting factors that promote both invasion and angiogenesis may be more effective at combating tumour progression. HIF1α itself has been the target of therapeutic development. Two HIF1α inhibitors, PX-478 and YC-1, have demonstrated promising therapeutic results in laboratory studies, however, the Food and Drug Administration has yet to approve any therapy that directly inhibits HIF1 activity (Welsh *et al.*, 2004, Koh *et al.*, 2008).

1.5. Retinoblastoma protein

RB was identified in 1986 as the first tumour suppressor whose inactivation directly results in development of retinoblastoma cancer (Friend *et al.*, 1986). RB is a member of the 'pocket' family of proteins, which also includes p107/RB-like protein 1 and p130/RB-like protein 2 (Du and Pogoriler, 2006). All members share some structural and functional similarities, however, p107 and p130 are more similar to each other compared to RB. Each member has a defined 'pocket' region comprised of two domains, domain A and domain B, separated by a spacer region. This domain

specifically interacts with LXCXE motifs that were originally identified in viral oncoproteins from the adenovirus, SV40 large T antigen and human papilloma virus (Vousden, 1993; Moran, 1993). RB also interacts with the LXCXE motif of endogenous proteins including cyclin D, histone deacytelase (HDAC) 1, HDAC2, BRG1 and AhR (Dowdy *et al.*, 1993; Dunaief *et al.*, 1994; Singh *et al.*, 1995; Brehm *et al.*, 1998). RB is an essential protein as RB null mice die at E13.5, displaying aberrant cell cycle progression patterns, increased apoptosis and defects in differentiation (Jacks *et al.*, 1992).

The most well characterized function of RB is its role in cell cycle progression through regulation of the E2 family of transcription factors (E2F) (Cobrinik, 2005). E2F transcription factors primarily regulate transcription of genes required for cell division, including those for DNA replication and repair. Activation of RB itself is regulated through cycling between hypo- and hyper-phosphorylated states, mediated by protein phosphatase 1 (PP1) and cyclin/cyclin-dependent kinase (CDK) complexes, respectively (Lees *et al.*, 1991). In the G_0/G_1 stage of the cell cycle, prior to DNA replication, RB exists in its hypo-phosphorylated state and is able to interact with E2F, preventing transcription. The p130 and p107 pocket proteins are also involved at this stage, forming a complex with other E2F transcription factors and repress E2F-mediated transcription (Smith *et al.*, 1998). As the cell cycle progresses toward the G_1/S checkpoint, cyclin-kinases become active and phosphorylate RB, dissociating E2F-RB-interaction and freeing E2F for transcription. Release of E2F transcription factors by RB appears to be temporally regulated as well, since over-expression of E2F or loss RB results in a prolonged S phase and shortened G_1/S checkpoint, respectively.

Several mechanisms for repression of E2F activity by RB have been proposed. RB was first suggested to physically interfere with E2F interaction with the transcriptional machinery (Ross *et al.*, 1999). This was originally demonstrated *in vitro* with purified RB and naked DNA promoter sequences, however, this method has never been demonstrated *in vivo*. Interestingly, the RB-interaction domain overlaps with the transactivation-domain of E2F, thus RB-binding may prevent co-activator association with E2F (Helin *et al.*, 1993). There is also considerable evidence suggesting that RB mediates repression of E2F transcription through the recruitment of chromatin modifiers. The hypo-phosphorylated form of RB interacts with HDAC1 within the switching

defective/sucrose non-fermentable (SWI/SNF) complex and recruits HDAC1 to E2F promoters, resulting in increased affinity of DNA to histones through histone deacetylation (Zhang *et al.*, 2000). Conversely, E2F transcription factors bind to the histone acetylase p300/CBP (Morris *et al.*, 2000). RB-interaction with DNA methylation enzyme suppressor of variegation 3-9 homologue 1 (SUV39H1) and DNA methyltransferase (DNMT) 1 have also been reported (Nielsen *et al.*, 2001; Roberston *et al.*, 2000)

RB also suppresses programs for apoptosis, as extensive cell death is observed in the central nervous system, skeletal muscles and lens of *RB* -/- mice (Clarke *et al.*, 1992, Jacks *et al.*, 1992). Moreover, several genes involved in apoptosis pathways are direct targets of E2F transcription, including apoptotic protease activating facor 1, a key regulator of p53-induced apoptosis pathways. RB can suppress apoptosis through inhibition of E2F-mediated transcription of apoptotic protease activating factor 1, decrease the amount of p53-induced apoptosis. This mechanism appears to be tissue specific, as apoptotic protease activating facor 1 knock-out in an RB null mice rescues apoptosis within the central nervous system but not within the peripheral nervous system or in skeletal muscle cells (Guo *et al.*, 2001). RB itself is a target of caspase cleavage, which promotes tumour necrosis factor (TNF)-induced apoptosis in fibroblasts (Chau *et al.*, 2002). Moreover, RB can also repress apoptosis through a caspase-independent mechanism through a direct interaction with Ableson tyrosine kinase 1 (ABL1) in mouse embryonic liver and central nervous system, and through c-Jun N-terminal kinase (JNK) in human embryonic kidney cells (Borges *et al.,* 2007; Shim *et al.*, 2000). Altogether, this demonstrate a clear role for RB-dependent control of apoptosis, however, it appears to be both tissue and lineage specific.

A role for RB in differentiation has also been observed, as RB null mice display defects in both muscle and hematopoiesis differentiation (Jacks *et al.*, 1992). The inhibitor of DNA binding (ID) family of bHLH transcription factors negatively regulates other bHLH transcription factors involved in differentiation. ID2 forms a complex with purine box factor 1 (PU1) to prevent transcription of genes for macrophage differentiation. RB will promote macrophage differentiation by physically interacting with ID2, preventing formation of a PU1-ID2 complex and allowing PU1-mediated expression of macrophage specific genes (Iavarone *et al.*, 2004). RB can also promote

differentiation through the regulation of chromatin modifiers. In undifferentiated myoblasts, a myogenic differentiation antigen (MyoD)-HDAC1 complex prevents transcription of muscle-specific genes. Induction of differentiation results in HDAC1 association with RB, liberating MyoD for activation of muscle specific genes (Puri *et al.*, 2001).

In addition to the canonical roles of RB in cell cycle regulation, apoptosis and differentiation, a role for RB has been indentified a variety of other cellular functions. RB interacts with regulators of chromatin structure to promote cellular senescence, a permanent exit from the cell cycle to prevent the continued use of aging cells. During a state of senescence, an increase in heterochromatin is observed at the promoter regions of E2F genes. Moreover, a decrease in heterochromatic regions is observed following removal of RB (Narita *et al.*, 2003). RB may also promote silencing of E2F transcription during senescence through its association with DMNT1 in complex with E2F1 (Robertson *et al.*, 2000). In relation to its role in differentiation, RB appears to control many aspects of stem cell function. Embryonic stem cells are in a continuous state of cell proliferation for the purpose of self-renewal and propagation of differentiated cells that give rise to a broad range of cell types. RB exists in its hyper-phosphorylated state within stem cells, thus allowing for transcription of E2F-responsive genes independently of cell cycle progression (Stead *et al.*, 2002; White *et al.*, 2005). The rate of cell division also occurs at a faster rate in stem cells, and has a much shorter G_1 phase. In a similar manner, hyper-phosphorylation of RB may also help prevent stem cells from entering a state of senescence. In addition to cell cycle arrest in response to DNA damage, RB also appears to initiate mechanisms of DNA repair. A variety of DNA damaging agents induce RB de-phosphorylation and promote RB-dependent cell cycle arrest (Harrington *et al.*, 1998; Knudsen *et al.*, 2000). Moreover, RB de-phosphorylation appears to be a result of attenuated cyclin D activity, which is dependent on expression of mismatch repair proteins (Lan *et al.*, 2001).

1.5.1. Retinoblastoma protein and cancer

RB was first identified in retinoblastoma tumours, which are malignancies of the retina (Friend *et al.*, 1986). Onset of this cancer follows the ‘two hit’ hypothesis: one RB null allele is inherited followed by loss of function of the other copy. Heterozygous mice

for RB are susceptible to development of pituitary and thyroid tumours (Hu *et al.*,1994; Jacks *et al.*,1992). Furthermore, tissue specific loss of RB in mice results in tumour development in the brain, prostate and lung, demonstrating that loss of RB plays a key role in tumour development (Marino *et al.*, 2000; Marino *et al.*, 2003; Mayhew *et al.*, 2005; Meuwissen *et al.*, 2003). With respect to humans, loss or inactivation of RB has been reported in several types of cancer, including cervical, liver, small cell lung, melanoma, and breast, among others. Although a considerable amount of research has uncovered a variety of roles for RB in normal cell function, how each function contributes to tumourigenesis has yet to be clearly defined.

In mice, inactivation of RB is essential for initiation of small cell lung carcinoma (Meuwissen *et al.*, 2003). Considering the role of RB in proliferation and differentiation, loss of RB might promote cancer development through a de-differentiation process and allowing for re-entry into the cell cycle. It may do so through chromatin remodelling, since loss of RB decreases the amount of heterochromatin and up-regulates expression of cell cycle genes. Loss of RB in liver cells allows for cell cycle re-entry, however this does not hold true for neuronal and muscle cells (Mayhew *et al.*, 2005; Slack *et al.*, 1998; Camarda *et al.*, 2004). Tumour development following loss of RB may also depend on the pre-existing cellular content. When RB function is maintained in mesenchymal progenitors in a p53 mutant background, RB functions with runt-related transcription factor 2 (RUNX2) to potentially give rise to osteoblastoma (Walkley *et al.*, 2008). When RB function is lost in p53-mutant mesenchymal progenitors, an up-regulation in the E2F-repressed peroxisome proliferator-activated receptor gamma subunit is observed, which may then potentially give rise to hibernomas (Calo *et al.*, 2010).

While loss of RB occurs within the early stages of some cancers to promote tumour development, other cancer types display loss or inactivation of RB at later stages. Maintenance of Rb function may be beneficial for some tumours since RB suppresses apoptosis, which would support survival of cancer cells. Moreover, repression of E2F target genes involved in DNA repair may promote genomic instability, which is another favourable trait of tumour cells. Nevertheless, maintenance of RB can still maintain tumour suppressive function in terms of cell cycle arrest and maintenance

of a de-differentiation or senescence state, and loss of RB at later stages may participate in mechanisms of tumour progression.

1.5.2. Retinoblastoma protein and HIF1

Not surprisingly, hypoxia induces cell cycle arrest in an effort to conserve energy when oxygen availability is limited (Ludlow *et al.*, 1993; Amellem *et al.*, 1996; Krtolica *et al.*, 1996; Seim *et al.*, 2003). Hypoxia decreases cyclin A and cyclin D levels, resulting in increased hypo-phosphorylated RB and G_1 cell cycle arrest in both normal and cancer cells (Ludlow *et al.*, 1993; Krtolica *et al.*, 1996). Moreover, HIF1α is required for an increase in hypo-phosphorylated RB, as *HIF1α* -/- cells show a significant decrease in CDK activity and subsequently a decrease in hyperphosphorylated RB (Goda *et al.*, 2003). When cells are placed back under normoxic conditions, cyclin levels increase concomitant with an increase in hyper-phosphorylated RB. Thus, RB activity appears to be hypoxia sensitive and potentially inhibit growth of tumours by promoting cell cycle arrest.

There is also evidence to suggest that RB is involved in modulating angiogenesis, a process that facilitates tumour growth and invasion. As previously described, hypo-phosphorylated RB binds to E2F proteins, inhibiting transcription of E2F target genes. E2F transcription factors regulate several genes associated with angiogenesis, either directly or indirectly, which include basic fibroblast growth factor (bFGF), FGF receptor 3, MMP16 and VEGFB (Stanelle *et al.*, 2002). RB appears to be indirectly involved in expression of these genes, as over-expression of cyclin D results in an increase in hyper-phosphorylated RB and a subsequent increase in bFGF expression. Thus, the active hypo-phosphorylated RB may negatively regulate angiogenesis by repressing transcription of E2F target genes that promote angiogenesis. RB also promotes angiogenesis through other transcription factors, including ID2. As previously mentioned, ID2 is a regulator of differentiation of the macrophage cell lineage (Iavarone *et al.*, 2004). In pituitary tumours, loss of ID2 in an RB mutant background results in aberrant VEGF expression, and subsequent aberrant vascularization (Lasorella *et al.*, 2005).

Recent evidence suggests that RB can modulate angiogenesis through direct association with the HIF1 transcription factor complex. In some cancers, such as hepatocellular and astrocytoma carcinomas, a correlation has been observed between the loss of RB and an increase in invasiveness and metastasis. In the case of astrocytomas, loss of RB in low grade anaplastic astrocytomas occurs before or near the same time as activation of the HIF1 target gene VEGF, increased hyperplasia, and increased progression towards glioblastoma multiforme, the highest grade of astrocytoma (Brat *et al.*, 2002).

RB has previously been identified as a negative regulator of thyroid hormone receptor transcription through interaction with the thyroid receptor/retinoblastoma interacting protein 230 (TRIP230) (Chang *et al.*, 1997). TRIP230 has also been identified as a co-activator for the HIF1 transcription complex (Beischlag *et al.*, 2004). TRIP230 is recruited to the promoter regions of HIF1 target genes VEGF and EPO in a hypoxic-dependent manner and is essential for transcription, as TRIP230 knockdown significantly reduces VEGF expression. Thus, TRIP230 may serve as a mediator for RB regulation of HIF1 transcription of angiogenic gene programs under hypoxia, and as a result, may ultimately contribute to increased metastasis of tumours.

1.6. TRIP230

The TRIP230 (GMAP210/TRIP11) protein was first identified from cellular extracts of patients with Sjögrenis syndrome, an autoimmune disease in which antibodies are produced against the golgi complex (Rios *et al.*, 1994, Rodriguez *et al.*, 1982). Structural analysis reveals TRIP230 is made up of an extended coil-coiled domain, comprised of two leucine zippers and a basic proline/glycine rich COOH terminal (Infanti *et al.*, 1999). Prior to it's cloning and characterization, activity of a putative 210 kDa protein thought to be TRIP230 was first identified in the golgi body, where TRIP230 co-localizes with the golgi body through its N-TAD (Rios *et al.*, 1994, Infanti *et al.*,1999). Over-expression of TRIP230 leads to enlargement of the golgi apparatus and fragmentation throughout the cytoplasm, suggesting that TRIP230 plays a role in reformation and repositioning of the golgi apparatus. Moreover, TRIP230 recruits γ-tubulin and has also been found to bind to lipid-associated protein, which may

explain how TRIP230 stabilizes the golgi apparatus (Rios *et al.*, 2004). Stabilization of the golgi apparatus is required due to the continous transport of membrane vessicles. TRIP230 associates with microtubules as well, serving as a link between the golgi apparatus and the ends of microtubules, and to help establish polarity by organizing the distribution of organelles within the cell (Ramos-Morales *et al.*, 2001). This also helps for intracellular transport of secretory protein, as over-expression of TRIP230 blocks transport between the endoplasmic reticulum and the golgi apparatus (Pernet-Gallay *et al.*, 2002). A role for TRIP230 has also been recognized in motility within cilium motors and spermatoid tail development (Follitt *et al.*, 2008, Kierszenbaum *et al.*, 2011).

TRIP230 has also been associated with disease, including cancer. A translocation within chromosome 14 creates a fusion protein between PDGF receptor β and TRIP230, which has been observed in hematologic malignancies (Abe *et al.*, 1997). Mouse models for skeletal dysplasia show increased swelling and stress in the endoplasmic receptor, which may be due to a deficiency in TRIP230 (Smits *et al.*, 2010). TRIP230 also interacts with alien/thyroid hormone-interacting protein 15 (TRIP15), a co-repressor protein of factors involved in cell cycle regulation and thyroid hormone receptor-mediated expression (Kab *et al.*, 2007, Tenbaum *et al.*, 2003). Up-regulation of alien inhibits endogenous expression of the E2F1 gene, whose expression is primarily up-regulated during the G_1, G_1/S and G_2 phase, resulting in reduced cell proliferation (Tenbaum *et al.*, 2007). Alien also interacts with RB, however, it has yet to be demonstrated whether all three work together in complex and whether it plays a role in disease (Escher *et al.*, 2007). Previous work has established TRIP230 as a necessary co-activator for thyroid hormone receptor-mediated gene transcription, where TRIP230 translocates from the golgi complex to the nucleus in response to hormone stimulation (Lee *et al.*, 1995; Chen *et al.*, 1999). This transcriptional activity is repressed by RB through a direct interaction with TRIP230 (Chang *et al.*, 1997). TRIP230 has also been established as an ARNT-interacting protein and a necessary co-activator for ARNT-mediated transcription of both HIF1α and AhR target genes (Beischlag *et al.*, 2004). As previously mentioned, TRIP230 may then serve as a mediator for RB regulation of HIF1-mediated transcription of angiogenesis under hypoxia, and as a result, may ultimately contribute to increased metastasis of tumours.

1.7. Objectives

The role of HIF1α/2α as master regulators of the hypoxic response is well established, as is it's role in tumour progression. HIF1 activity is typically up-regulated in several types of cancer in response to tumour hypoxia and activates transcription of genes involved in both angiogenesis and metastasis (Zhong *et al.*, 1999; Talks *et al.*, 2000; Forsythe *et al.*, 1996; Gerber *et al.*, 1997; Elvert *et al.*, 2003; Yoshida *et al.*, 2006; Imai *et al.*, 2003; Yang *et al.*, 2008; Staller *et al.*, 2003; Hitchon *et al.*, 2002). Little is known about the mechanisms behind HIF1-mediated transcription, however, evidence demonstrates a role for RB in the regulation of HIF1 target genes associated with both angiogenesis and cell invasion through a direct interaction with TRIP230 (Appendix A-D). Both Hepa-1 and MCF7 cells transfected with RB and TRIP230 showed a significant decrease in hypoxia-responsive reporter gene activity, compared to cells transfected with RB and a TRIP230 mutant lacking the RB-interaction domain (Appendix B). Furthermore, RB is recruited to the regulator regions of HIF1 target genes VEGF and EPO, in addition to HIF1α, ARNT and TRIP230, in a hypoxic-dependent manner (Appendix D). Lastly, knockdown of RB in non-invasive MCF7 cells leads to a dramatic increase in hypoxia-dependent cell invasion, as demonstrated in a matrigel invasion assay (Appendix C). Altogether, this data suggests that loss of RB promotes hypoxia-induced tumour metastasis. ***It is therefore hypothesized that RB attenuates HIF1-mediated transcription through a direct interaction with TRIP230 and that loss of RB supports the deregulation of HIF1 target genes involved in tumour progression, which ultimately contributes to increased invasiveness and metastasis of tumour cells*** (Figure 2).

The objective of this study was to better define the role of RB in HIF1-mediated transcription in response to hypoxia. This study specifically focused on the protein-protein interactions within the putative ARNT-TRIP230-RB complex, and how these interactions contribute to angiogenesis and invasiveness. To achieve this objective, the following specific aims were explored:

1. To further characterize the invasive phenotype following loss of RB in MCF7 human breast cancer cells using siRNA knockdown techonology. Small interference RNAs (siRNA) are short RNA duplexes that selectively target mRNA for degradation

through an associated RNA Induced Silencing Complex, resulting in reduced expression of a gene of interest. In this study, siRNA complimentary to regions within RB1 messenger RNA were used to knock down expression of the *RB1* gene in cell culture. Briefly, double stranded RB-specific RNA was transfected into cells and was targeted for degradation by the RNAse II class endoculclease Dicer to produce smaller fragments of double stranded RNA, or siRNA. A complex is formed between siRNA and an RNA-induced Silencing Complex, which then specifically annealed to and degraded transcripts of the *RB1* gene.

2. To perform a comprehensive analysis of the RB-TRIP230 interaction using glutathione-s-transferase (GST) pull-down analysis to identify the essential amino acids within TRIP230 required for RB-interaction. First, the minimal RB-interaction domain was identified using successive truncations of the defined TRIP230 RB-interacting domain expressed as glutathione-s-transferase fusion proteins. The minimal RB-interaction domain was then subjected to site-directed mutagenesis to indentify the individual amino acids essential for RB-interaction. Assuming the interaction between RB and TRIP230 was charge-dependent, individual residues would be mutated to non-polar amino acids which should disrupt protein-protein interactions. The information gleaned from this study would then allow for the design of a transgenic TRIP230 mutant mouse that does not interact with RB in order to establish the role of RB in hypoxia-dependent processes in an animal model.

3. To establish the existence of an ARNT-TRIP230-RB multi-meric complex using GST pull-down analysis with a GST-ARNT fusion moiety. TRIP230 has previously been identified as a component of the HIF1 complex through a direct interaction with ARNT, and not HIF1α (Beischlag *et al.*, 2004). Moreover, RB attenuates HRE reporter gene activity through a direct interaction with TRIP230 (Appendix B). It has yet to be established, however, whether or not ARNT, TRIP230 and RB exist as part of a multi-meric complex. Identification of an ARNT-TRIP230-RB trimeric complex would provide additional support to the hypothesis that RB attenuates HIF1 transcription through interaction with TRIP230.

Addressing these specific aims will further our understanding of hypoxia-induced HIF1-regulation of gene expression and establish a role for RB within this pathway. This

research will increase our understanding of gene regulation and the biological significance of co-regulators involved in this process, which are essential for the appropriate and controlled regulation of transcription. Furthermore, this research will provide further insight into the molecular mechanisms required for tumour progression and may yield potential novel therapeutic targets. RB is lost or inactivated in 20-30% of all breast tumours, and as a result, may contribute to the malignancy of a large proportion of breast cancers (Pietilainen *et al.*, 1995, Borg *et al.*, 1992). HIF1-mediated transcription is also active during the progression of cardiovascular disease, and thus this work may uncover a role for RB in a number of other biological processes.

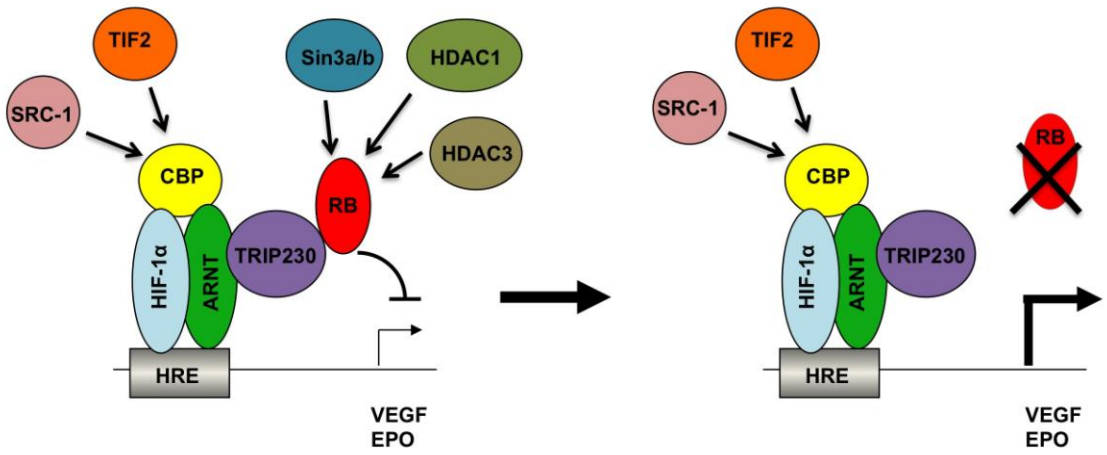

Figure 2. The proposed role of RB in HIF1-mediated transcription

RB attenuates HIF1-mediated transcription through a direct interaction with TRIP230. When RB is lost, expression of HIF1 target genes becomes deregulated.

2. Materials and Methods

2.1. Cell Culture, transfection and nuclear extraction preparation

2.1.1. Cell culture

MCF7 and MDA-MB-231 human breast cancer cells were maintained in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and 4.5 g/l L-glutamine (DMEM; BioWhittaker, Lonza, Cat. No. 12-604F) with 10% fetal bovine serum (FBS HyClone, PeRBlio, Thermo Fisher Scientific Inc., Cat. No. SH30396), 100 units/ml potassium penicillin-100 µg/ml streptomycin sulphate (BioWhittaker, Lonza, Cat. No. 17-602E), at 37°C, 20% O_2 and 5% CO_2. MCF7 cells were additionally supplemented with 0.01 µg/ml bovine pancreas insulin solution (Sigma Aldrich, Cat. No. 10516-5ML). LnCAP human prostate cancer cells were maintained in Roswell Park Memorial Institute (RPMI) Medium with L-glutamine (BioWhittaker, Lonza, Cat. No. 12-702F12) with 10% fetal bovine serum (FBS HyClone, PeRBlio, Thermo Fisher Scientific Inc., Cat. No. SH30396), and 100 units/ml potassium penicillin-100 µg/ml streptomycin sulphate (BioWhittaker, Lonza, Cat. No. 17-602E), at 37°C, 20% O_2 and 5% CO_2.

Human embryonic kidney (HEK)-293T cells for transient transfection were maintained in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and 4.5 g/l L-glutamine (DMEM; BioWhittaker, Lonza, Cat. No. 12-604F) with 10% fetal bovine serum (FBS HyClone, PeRBlio, Thermo Fisher Scientific Inc., Cat. No. SH30396), 100 units/ml potassium penicillin-100 µg/ml streptomycin sulphate (BioWhittaker, Lonza, Cat. No. 17-602E), at 37°C, 20% O_2 and 5% CO_2

2.1.2. siRNA transfection

Cells were cultured in the conditions described above until approximately 70% confluent prior to siRNA transfection. Cells were reverse transfected with 10 - 30 nM of either scrambled (SCX) siRNA (DS Scrambled negative control siRNA, Integrated DNA Technologies Inc.) or RB siRNA (HSC.RNAI.N000321.9.1 for siRB1, HSC.RNAI.N000321.9.4 for siRB2, HSC.RNAI.N000321.9.6 for siRB3, HSC.RNAI.N000321.10.8 for siRB4, HSC.RNAI.N000321.10.9 for siRB5, Integrated DNA Technologies Inc.) using 0.3% (v/v) Lipofectamine RNAiMAX (Invitrogen Inc.) according to the manufacturer's protocol. Cells were allowed to incubate in transfection mix for 5 h at 37°C, 20% O_2 and 5% CO_2, after which the transfection mix was removed and replaced with complete media for a total of 24 h.

2.1.3. pCMV-RB expression plasmid transfection

HEK-293T cells were maintained in conditions described above until approximately 50% confluent for a 100 mm plate prior to transfection. Approximately 20 µg of pCMV-RB expression plasmid was precipitated with 2M $CaCl_2$ in 600 µl 2x 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Buffered Saline solution (140 mM NaCl, 1.5 mM Na_2PO_4, 50 mM HEPES) and added drop wise directly to cells in media. Cells were maintained at 37°C, 20% O_2 and 5% CO_2 for 48 h prior to harvesting.

2.1.4. Nuclear extraction preparation

Nuclear extractions were performed as previously described (Andrews and Faller, 1991). MCF7 cells were maintained in conditions described above until approximately 90% confluent prior to harvesting. Cells were pooled into 2 ml cold Dulbeccos Phosphate Buffered Saline (DPBS, BioWhittaker, Lonza, Cat. No. 17-512F) and centrifuged for 10 min at 3200 rpm at 4°C. Pellets were resuspended in 400 µl cold Buffer A solution (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM Dithiothreitol (DTT), 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.3 µM Aprotinin, 10 µM Bestatin, 10 µM E64, 10 µM Leupeptin) and allowed to swell on ice for 10 min. Cells were then vortexed for 10 sec and centrifuged for 15 min at 4400 rpm at 4°C. Pellets were resuspended in 2-3x pellet volume of cold Buffer C solution (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM

Ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.3 µM Aprotinin, 10 µM Bestatin, 10 µM E64, 10 µM Leupeptin, 25% glycerol) and incubated on ice for 20 min. Nuclear extracts were then separated from cellular debris by centrifugation for 2 min at maximum speed and protein concentration was estimated by the Bradford assay (see below).

2.2. GST pull-down assays

2.2.1. GST construct preparation

GST-TRIP230 deletion constructs were prepared by polymerase chain reaction (PCR) amplification (0.2 mM deoxyribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.25 mM per primer, 1x PCR reaction buffer) from a full length TRIP230 clone. A total of 10 ng of DNA template was used in a 100 µl reaction amplified by cycling between 95°C for 15 sec, 54°C for 15 sec, and 72°C for 2 min (Veriti 96 Well Thermal Cycler, Applied Biosystems). Eleven deletion constructs were amplified from amino acids 1091 to 4493, each of which removed nucleotides encoding for approximately 20 amino acids deleted from the RB-interaction domain (Figure 3). Constructs were named in reference to the N-terminal TRIP230 amino acid that each construct was amplified with primer pairs used to generate each construct (Table 1). The amplified regions were each cloned into pGEX-5X-1 within BamH1 and XhoI restriction sites (Appendix A). All GST-TRIP230 constructs were confirmed by sequencing.

Four GST-ARNT constructs were generated: GST-PAS A encodes amino acids 157 – 252, GST-PAS B encodes amino acids 343 – 479, GST-PAS A-B encodes amino acids 157 – 479, and GST-N-PAS A-B encodes amino acids 2 – 479 (Figure 4A). Each region was amplified by PCR as described above from a full length mouse ARNT cDNA clone using primer pairs listed (Table 2). The amplified regions were each cloned into pGEX-5X-1 in the BamHI and XhoI restriction sites (Appendix A).

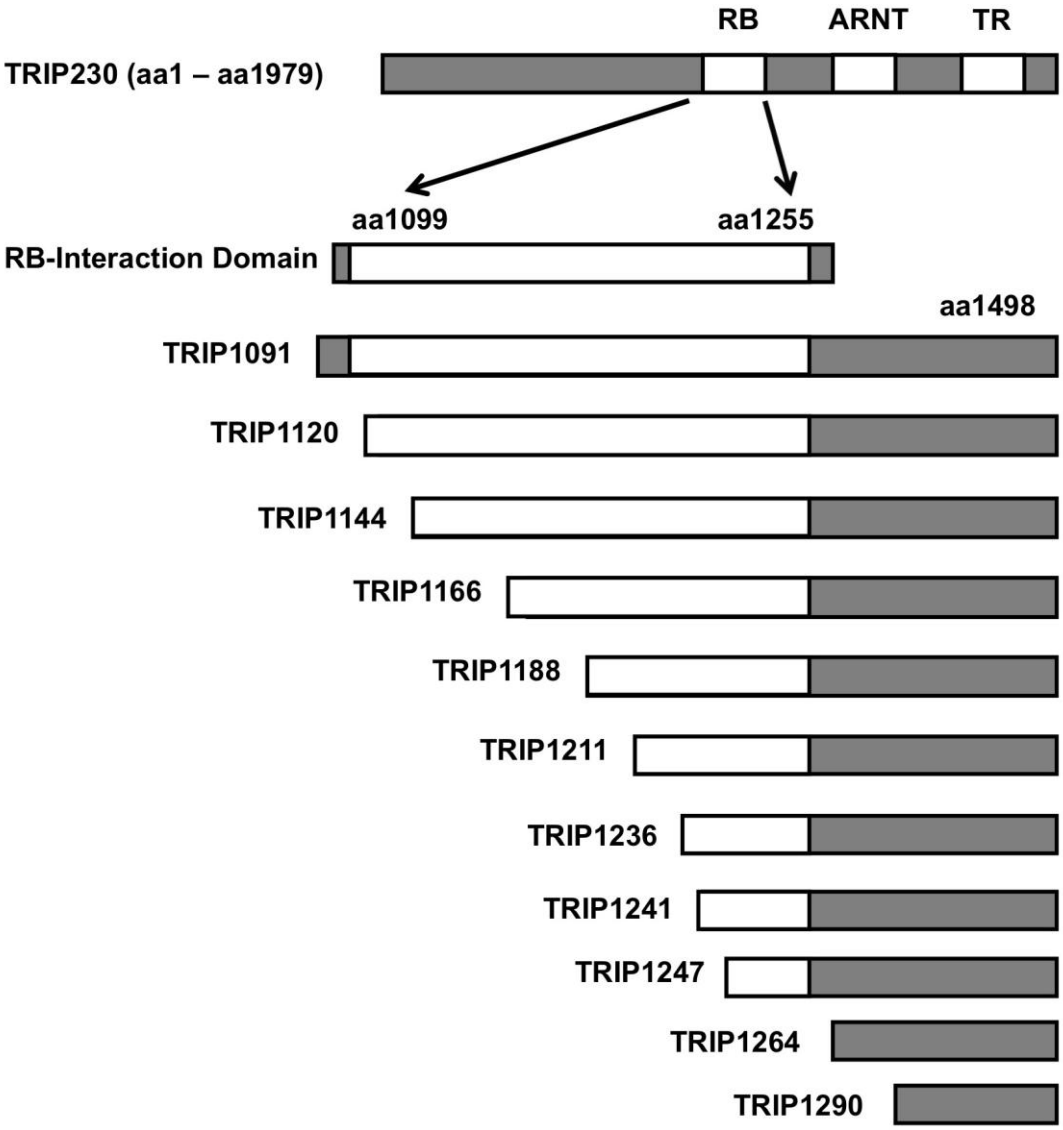

Figure 3. Schematic of the GST-TRIP230 deletion fusion moiety constructs

A total of 11 successive 20-amino acid deletion constructs of the TRIP230 RB-interaction domain were amplified from full-length TRIP230 and cloned into pGEX-5X-1. Constructs were named in reference to the N-terminal TRIP230 amino acid that each construct was amplified from. The full length TRIP230 schematic depicts the RB-interaction domain (RB), the ARNT interaction domain (ARNT), and the thyroid receptor interaction domain (TR).

Table 1. List of primer pairs for PCR amplification of TRIP230 deletion constructs

GST-TRIP230 Construct	Primer Pair
TRIP1091	Forward: 5’ CGGGGATCCTGCAGGCTTATGCTATGGAA 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1120	Forward: 5’ CGGGGATCCACAAAATGATGGATATTGTT 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1144	Forward: 5’ CGGGGATCTCCACTAGATTTGAAAGTAGTGGC 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1166	Forward:5’ CGGGATCATTCGAGAAAAAGACATCGAA 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1188	Forward: 5’ CGGGGATCCAAACATCCAGCACTGGTAAT 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1211	Forward: 5’ CGGGGATCCGTGACAAGTTAAAACAGCAA 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1241	Forward: 5’ CGGGGATCCAGCTTCAGGAAGAGCTTCAC 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1236	Forward: 5’ CGGGGATCCAACACGAGTCAGCCCAGCTT 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1247	Forward: 5’ CGGGGATCCACCAACTTCAAGCACAGGTT 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1264	Forward: 5’ CGGGGATCCAACTGGACTATACTGGCCTG 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’
TRIP1290	Forward: 5’ GCGGATCCCACAAGTTCAGCACAGCATTGGG 3’ Reverse: 5’ CCGCTCGAGGTGGCAGCAATCGAATC 3’

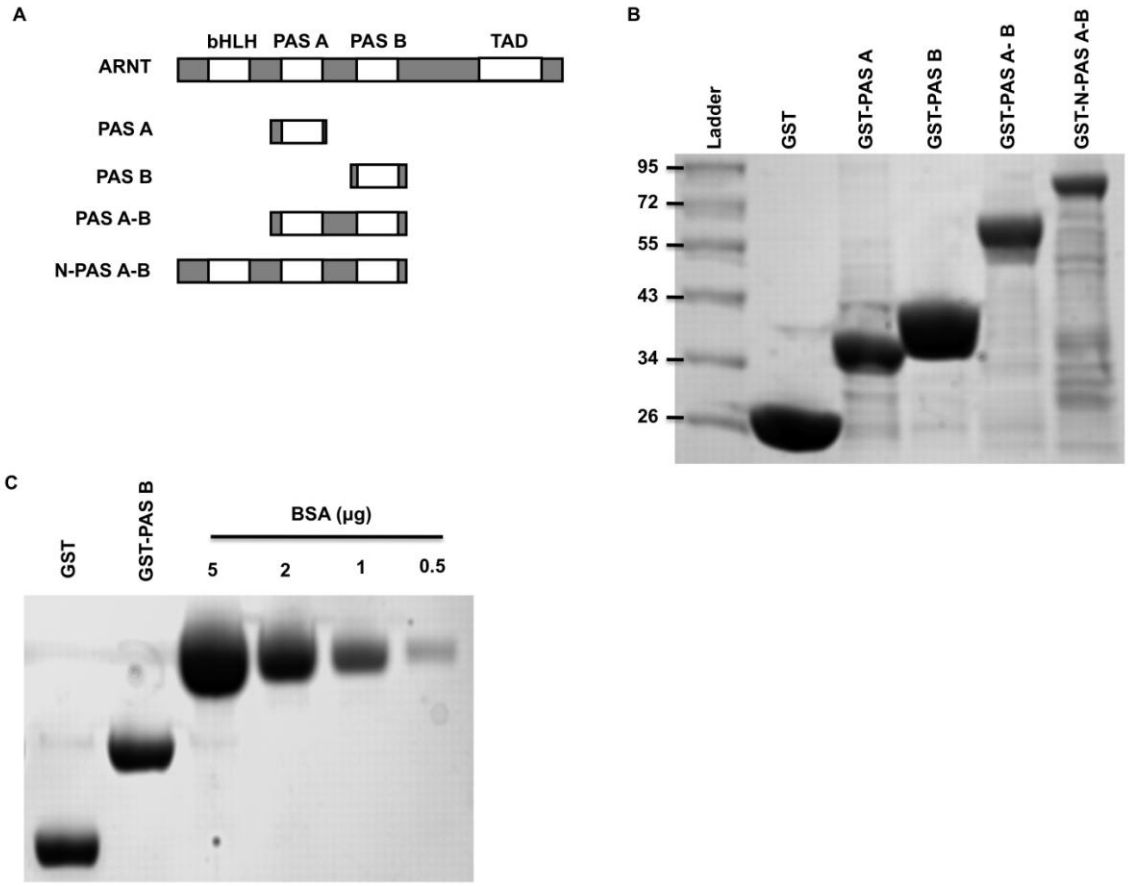

Figure 4. Schematic of the GST-ARNT fusion moiety constructs

(A) The PAS A, PAS B, PAS A-B, and N-PAS A-B domains were PCR amplified from full-length ARNT cDNA and cloned into pGEX-5X-1. The full length ARNT schematic of depicts the bHLH, PAS A, PAS B, and TAD domains. (B) Coomassie-stained gel of expressed GST constructs eluted from a 10 µl aliquot of glutathione agarose beads. (C) Protein concentrations were estimated by comparing to bovine serum albumin (BSA) standards as shown.

Table 2. *List of primer pairs for PCR amplification of GST-ARNT constructs*

GST-ARNT Construct	**Primer Pair**
PAS A	Forward: 5’ GCGGATCCCTTTCCTCACTGATCAGGAACTG 3’ Reverse: 5’ GCCTCGAGGGAAGACTGCTGGCCTTCCTT 3’
PAS B	Forward: 5’ GCGGATCCTGGTAACTAGTTCTCCCAACT 3’ Reverse: 5’ GCCTCGAGGATGGTGTTGGACAGTGTAGGCCG 3’
PAS A-B	Forward: 5’ GCGGATCCCTTTCCTCACTGATCAGGAACTG 3’ Reverse: 5’ GCCTCGAGGATGGTGTTGGACAGTGTAGGCCG 3’
N-PAS A-B	Forward: 5’ GCGGATCCCGACTACAGCTAACCCAGAA 3’ Reverse: 5’ GCCTCGAGGATGGTGTTGGACAGTGTAGGCCG 3’

2.2.2. GST protein expression

GST-TRIP230 constructs and GST alone were expressed in BL21 bacterial cells for 4 h at 30°C with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when bacteria cell cultures reached an OD600 between 0.4 and 0.5. Bacteria cultures were then centrifuged and cell pellets were resuspended in Lysis Buffer (50 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) pH 8, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Igpal, 1 mM DTT, 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.3 µM Aprotinin, 10 µM Bestatin, 10 µM E64, 10 µM Leupeptin). Bacteria cells were then sonicated on ice for 60 sec with a microtip probe (duty 30%, input 3; Branson Sonifier 450, Branson Ultrasonics Corporation, Model P/S Bio), centrifuged and recombinant proteins were isolated from the supernatant on glutathione-agarose beads (Sigma Aldrich, Cat No. G4510-5ML). Recombinant proteins were then eluted off the beads using 10 mM reduced glutathione and quantified using the RC DC protein assay (see below; BioRad, Cat. No. 500-0121).

GST-ARNT constructs and GST alone were prepared as described above, except recombinant proteins were expressed at 37°C for 4 h but were not eluted from the beads following affinity purification. Concentration of recombinant protein was estimated from a 20 µl aliquot was boiled off in 30 µl of 2x sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol), fractioned by SDS-polyacrylamide gel electrophoresis (PAGE) (see below) and stained with Coomassie staining. Briefly, gel was fixed in Fixing solution (50% Methanol, 10% CH_3COOH, 40% H_2O) for 30 min at room tempurature. Fixing solution was removed and gel stained with 10 volumes of Coomassie Blue Stain (50% Methanol, 0.05% Coomassie Brilliant Blue, 10% CH_3COOH, 40% H_2O) for 1 h at room temperature. The gel was destained with 10 volumes of 1x Destain solution (5% Methanol, 7% CH_3COOH, 88% H_2O). Protein concentrations were estimated by comparing to BSA standards (Figure 2C).

2.2.3. Protein determination assays

For the Bradford protein assay, samples were diluted 1:2 and the following seven standards were made from a 2 mg/ml stock solution of BSA (BioRad, Cat. No. 500-0206): 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125

mg/ml. A 20 µl aliquot of each was mixed with 1 ml of 1x Quick Start™ Bradford Dye Reagent (Biorad, Cat. No. 500-0205) and allowed to incubate for 5 min at room temperature. Absorbance was measured at wavelength 595 nm with a Biorad SmartSpec™ Plus Spectrophotometer (BioRad, Serial No. 273 BR 04787).

The RC DC protein assay was performed using the RC DC™ Protein Assay RC Reagents kit (BioRad, Cat. No. 500-0119) following the manufacturer's microfuge tube assay protocol. Briefly, a set of six standards were made from a 2 mg/ml stock solution of BSA: 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, and 0.2 mg/ml. A 25 µl aliquot of each sample and standard was mixed with 125 µl of RC Reagent I and allowed to incubate at room temperature for 1 min. Each sample and standards were then mixed with 125 µl of RC Reagent II and centrifuged for 5 min at max speed at room temperature. Supernatants were discarded and pellets were allowed to dry for approximately 5 minutes. Pellets were then resuspended in 127 µl of Reagent A' (prepared by mixing 5 µl of DC Reagent S (BioRad, Cat No. 500-0115) for each 250 µl of DC Reagent A (BioRad, Cat. No. 500-0113) required) and allowed to incubate for 5 minutes at room temperature. Each sample and standard was then incubated with 1 ml DC Protein Assay Reagent B (BioRad, Cat. No. 500-0114) for 15 min at room temperature and absorbance was measured at wavelength 750 nm.

2.2.4. Pull-down assay and peptide construction

For pull-down assays, 25-50 µg of GST protein was mixed at 4°C overnight with either 100 ng of purified Rb protein (Prolias Technologies, Cat. No. p2007-1) or 250 µg nuclear extract harvested from MCF7 cells maintained in 1% O_2 and 5% CO_2 for 5 h at 37°C. Recombinant proteins were isolated from the reaction mixture by mixing with 15 - 30 µl 50% slurry glutathione agarose beads for 2 h at room temperature. Beads were then washed 6x with Protein-protein interaction (PPI) Buffer (20 mM HEPES, 300 mM NaCl, 4 mM $MgCl_s$, 1 mM EDTA, 10% glycerol, 0.1% Igpal, 1mM DTT, 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.3 µM Aprotinin, 10 µM Bestatin, 10 µM E64, 10 µM Leupeptin), eluted off with 10 mM reduced glutathione and fractioned by SDS-PAGE gel, transferred to polyvinylidene fluoride (PVDF) membrane and analyzed by Western blot for the presence of RB using an anti-RB1 antibody (1:2000; mouse monoclonal IgG; Cell Signaling Techonology; Cat. No. 9309). The detection was

done using horseradish peroxidase conjugated anti-mouse IgG (1:10 000; GE Healthcare, Cat. No. RPN2124) and ECL detection kit (Pierce/Thermo Scientific, Cat. No. PIA34075).

For pull-down assays, approximately 50 µl of GST or GST-PAS B beads were mixed with 500 µg of MCF7 nuclear extract in PBS at 4°C for 2 h, washed twice with 0.5x Radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris pH 8, 1 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 140 mM NaCl, 1% Triton-X100, 0.02% Na-deoxycholate, 0.1% SDS), then twice with 1x RIPA buffer, and boiled off in 2x SDS sample buffer. Eluted samples were fractioned by SDS-PAGE, transferred to PVDF membrane and analyzed by Western blot for the presence of RB (as described above) and TRIP230 with an anti-TRIP230 antibody (1:1500; mouse polyclonal IgG; a gift from Dr. Y. Chen, Univ. of Texas, San Antonio). Detection of TRIP230 was done using horseradish peroxidase conjugated anti-mouse (1:2500; GE Healthcare, Cat. No. RPN2124) and ECL detection kit (GE Healthcare, Cat. No. RPN2132). Blots were imaged using a Syngene Dyversity blot imaging system.

For pull-down assay with blocking peptides, one peptide corresponding to critical residues within TRIP230 (TRIP1584), two peptides corresponding to critical residues within ARNT (ARNT392 and ARNT415) for the TRIP230-ARNT interaction, and a SCX negative control peptide were synthesized by GeneScript, and conjugated with a cell membrane transduction trans-activator of transcription (TAT) domain and a fluorescent tag (Table 3; Figure 5). The pull-down assay was done as described above except GST proteins were pre-incubated overnight at 4°C with 50 µM of peptide.

Table 3. *List of ARNT- and TRIP230-interacting peptides*

Peptide	**Amino Acid Position**	**Sequence**
TRIP1584	1584-1596	**LRNHLL**ESE**D**SY**T**
ARNT392	392-401	LGKNI**V**EGFC
ARNT415	415-424	VVKL**K**GQVLS
SCX	n/a	AHAHKGNVGIELFC

Note: Bold letters refer to critical residues (Partch et al., 2009)

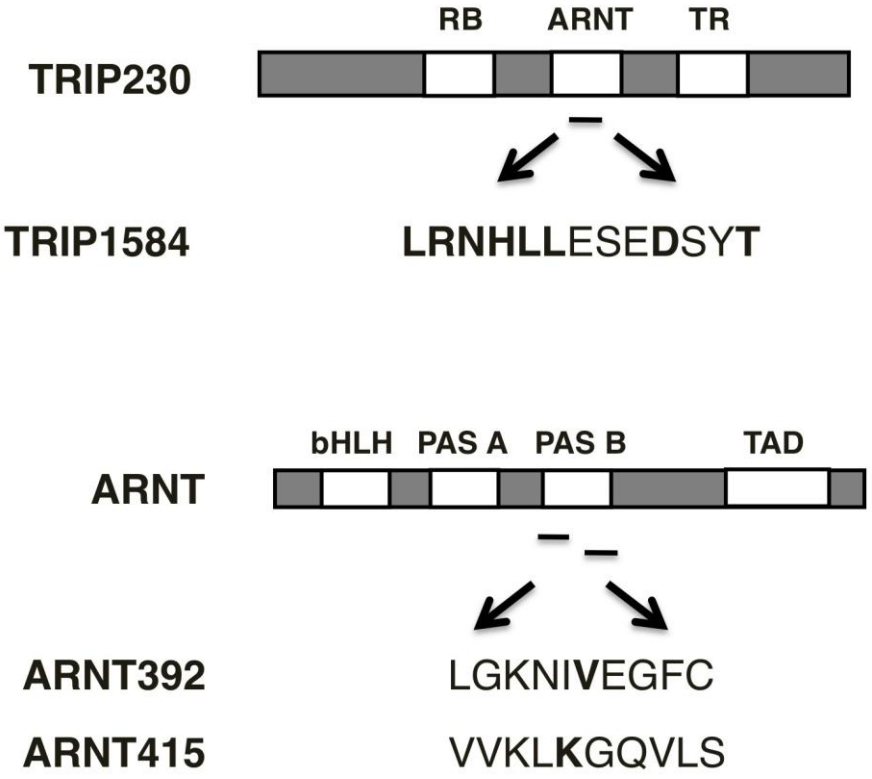

Figure 5. Schematic of the ARNT- and TRIP230-interacting peptides

An ARNT interacting peptide (TRIP1584) corresponds to a specific LRNHLL motif within TRIP230 that is required for ARNT-TRIP230 interaction. Two TRIP230-interacting peptides (ARNT392 and ARNT415) corresponds to important amino acids within ARNT required for the ARNT-TRIP230 interaction. Letters in bold refer to critical residues (Partch *et al.*, 2009).

2.3. Quantitative Real Time-Polymerase Chain Reaction

Cells were incubated under hypoxic conditions (1% O_2 and 5% CO_2) for 24 h at 37°C. Total RNA was isolated using TRI reagent (Sigma, Cat. No. T9424-200ML) according to the manufacturer's protocol. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Part No.4368814) according to the manufacturer's protocol (1x RT buffer, 1x RT random primers, 4 mM dNTP, 50 units MultiScribe™ reverse transcriptase). A total of 2 µg of RNA was used to make cDNA in a 20 µl reaction by incubating sequentially at 25°C for 5 min, 37°C for 120 min, then 85°C for 5 min (Veriti 96 Well Thermal Cycler, Applied Biosystems). For real-time-polymerase chain reaction (RT-PCR) experiments, analysis was done using StepOnePlus System (Applied Biosystems, Part No. 4376600) in 15 µl reaction volume per sample (0.3 µM primer pair, 1x ROX reference Dye LSR, 1x SYBR Advantage qPCR Premix). Samples were diluted 1:30 and standards were made from hypoxic SCX treated samples using the following dilutions: 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000. The primer pairs listed were used to quantify mRNA levels of VEGF, CXCR4, vimentin, RB, and 36B4 (Table 4).

Table 4. *List of primer pairs for RT-PCR analysis*

Protein	**Primer Pair**
VEGF	Forward: 5’ AGCTGAGAACGGGAAGCTGTG 3’ Reverse: 5’ ACAGACGTTCCTTAGTGCTGG 3’
CXCR4	Forward: 5’ CAGTGGCCGACCTCCTCTT 3’ Reverse: 5’ GGACTGCCTTGCATAGGAAGTT 3’
vimentin	Forward: 5’ TTCCAAACTTTTCCTCCCTGAACC 3’ Reverse: 5’ TCAAGGTCATCGTGATGCTGAG 3’
RB	Forward: 5’ GCGGGATCCATGCCGCCCAAAACCCCCCGAAAA 3’ Reverse: 5’ GCGACTAGTTCATTTCTCTTCCTTGTTTGA 3’
36B4	Forward: 5’ CCACGGTGCTGAACATGCT 3’ Reverse: 5’ TCGAACACCTGCTGGATGAC 3’

2.3.1. Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0. For RT-PCR experiments, statistical significance was determined between using a Two-Way ANOVA with Bonferroni post-tests comparison under a 95% confidence interval. Comparisons were made between normoxia versus hypoxia and SCX versus siRB groups. Values are presented as means ± standard error of the mean (SEM). A P value < 0.05 was considered to be significant.

2.4. Western blotting

MCF7 cells were incubated under hypoxic conditions (1% O_2 and 5% CO_2) for 48 h at 37°C. Cells were then harvested and protein concentration was quantified using the RC DC protein assay previously describe above. Equal amounts of proteins from each sample were resolved on an 8% SDS-PAGE (8% acrylamide/bis, 1 M Tris, 5 mM SDS, 0.1% ammonium persulfate, 0.01% tetramethylethylenediamine) in 1x Running buffer (1 M Tricine, 1 M Tris-Cl, 50 mM SDS), and then transferred to a PVDF membrane in transfer buffer (25 mM Tris-Cl, 250 mM Glycine, 0.1% SDS). Upon completion of transfer, the membrane was activated with 100% methanol, blocked for 1 h at room temperature with blocking solution (5% powdered milk, 0.1% Triton, 140 mM NaCl, 2.5 mM KCl, 2.5 mM Tris), and then probed with primary antibody in blocking solution overnight at 4°C. The following primary antibodies were used: anti-RB1 (1:3000; rabbit polyclonal IgG; Santa Cruz Biotechnology Inc., Cat. No. SC-7905), anti-fusin (1:1500; rabbit polyclonal IgG; Santa Cruz Biotechnology Inc., Cat. No. SC-9046), anti-E-cadherin (1:1500; mouse monoclonal IgG; Santa Cruz Biotechnology Inc., Cat. No. SC-8426), anti-vimentin (1:1500; rabbit polyclonal IgG; Santa Cruz Biotechnology Inc., Cat. No. SC-5565) or anti-tubulin antibodies (1:1500; mouse monoclonal IgG; Santa Cruz Biotechnology Inc., Cat. No. SC-8035). Blots were then probed with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (1:2500; Santa Cruz Biotechnology Inc., Cat No. SC-2004 and SC-2005, respectively) for 1 h in blocking solution at room temperature, and protein was detected with an ECL detection kit (GE Healthcare, Cat. No. RPN2132). Blots were imaged using a Syngene Dyversity blot imaging system.

3. Results

3.1. Loss of RB leads to enhanced expression of HIF1-regulated genes involved in angiogenesis and EMT

RB has previously been identified as an attenuator for TR-mediated transcription through a direct interaction with TRIP230 (Chang *et al.*, 1997). There is now evidence to suggest that RB acts as negative regulator of HIF1-mediated transcription through a direct interaction with TRIP230. Both Hepa-1 and MCF7 cells transfected with RB and TRIP230 showed a significant decrease in HRE reporter gene activity, compared to cells transfected with RB and a TRIP230 mutant lacking the RB-interaction domain (Appendix B). Chromatin immuno-precipitation analysis in MCF7 human breast cancer cells revealed that RB, in addition to HIF1α, ARNT, and TRIP230, associates with the regulatory regions of HIF1 target genes VEGF and EPO in a hypoxic dependent manner (Appendix C, Beischlag *et al.*, 2004). Moreover, siRNA knockdown of RB in MCF7 cells leads to a dramatic increase in hypoxic-dependent cell invasion, as demonstrated in a matrigel invasion assay (Appendix D). This change in cellular phenotype clearly demonstrates a role for RB in the hypoxic response that has yet to be completely defined.

To further characterize the invasive phenotype in MCF7 cells due to loss of RB, changes in expression of HIF1 target genes were examined following siRNA-mediated knockdown of RB, measured at either the mRNA level by RT-PCR or at the protein level by Western blot. Several HIF1 target genes have established roles in angiogenesis and EMT, both of which are processes that promote metastasis (Forsythe *et al.*, 1996; Yang *et al.*, 2008; Staller *et al.*, 2003; Imai *et al.*, 2003). Using siRNA knockdown technology to significantly reduce expression of the *RB1* gene, in combination with RT-PCR and Western blot analysis, would help identify HIF1 target genes involved in either angiogenesis or EMT directly affected by loss of RB. This would ultimately provide insight into how the hypoxic response may contribute to increased metastasis of tumour

cells following loss of RB.

3.1.1. Assessment of RB-specific knockdown efficiency

A total of five RB-specific siRNAs were individually assessed for knockdown efficiency of the *RB1* gene in MCF7 cells (Figure 6). Cells were transfected with either siSCX control or siRB for 24 h, followed by exposure to either hypoxia (2% O_2) or normoxia (20% O_2) for an additional 24 h. Two RB-specific siRNAs, siRB4 and siRB5, demonstrated an 80% and 70% decrease in RB mRNA accumulation, respectively, in both hypoxic and normoxic conditions (Figure 6A). Changes in RB mRNA accumulation, as well as for changes in mRNA accumulation for subsequent RT-PCR experiments, were normalized to constitutively active and non-hypoxia inducible 36B4 gene expression. Both siRB4 and siRB5 also demonstrated a similar decrease in RB protein expression (Figure 6B). Both siRB4 and siRB5 were selected for subsequent knockdown experiments and used simultaneously to eliminate the possibility of mistaking any idiosyncratic responses that may result from one particular siRNA for a bona fide biological effect.

3.1.2. Effect of RB knockdown on angiogenesis master regulator VEGF

Tumour cells release angiogenesis-stimulating factors to induce growth of blood vessels that support tumour growth and metastasis. Without adequate vascularization, most tumours will not exceed a few cubic milimeters and remain clinically silent (Folkman, 1966). VEGF is a primary regulator of angiogenesis and is a direct target of HIF1 transcription (Foresythe *et al.*, 1996). Changes in VEGF mRNA accumulation was monitored by RT-PCR in MCF7 cells following siRNA transfection as described above. Changes in mRNA accumulation were measured after subsequent exposure to hypoxia (2% O_2) for 8 h, 12 h or 24 h. A significant increase in VEGF mRNA accumulation was observed in both siRB treated cells (70% for siRB4 and 62% for siRB5) following a 24 h exposure to hypoxia (Figure 7). Moreover, an increase was not observed under normoxic conditions, demonstrating that these effects are specific to hypoxia. VEGF is a secreted protein, thus changes in expression at the protein level could not be assessed by Western blot.

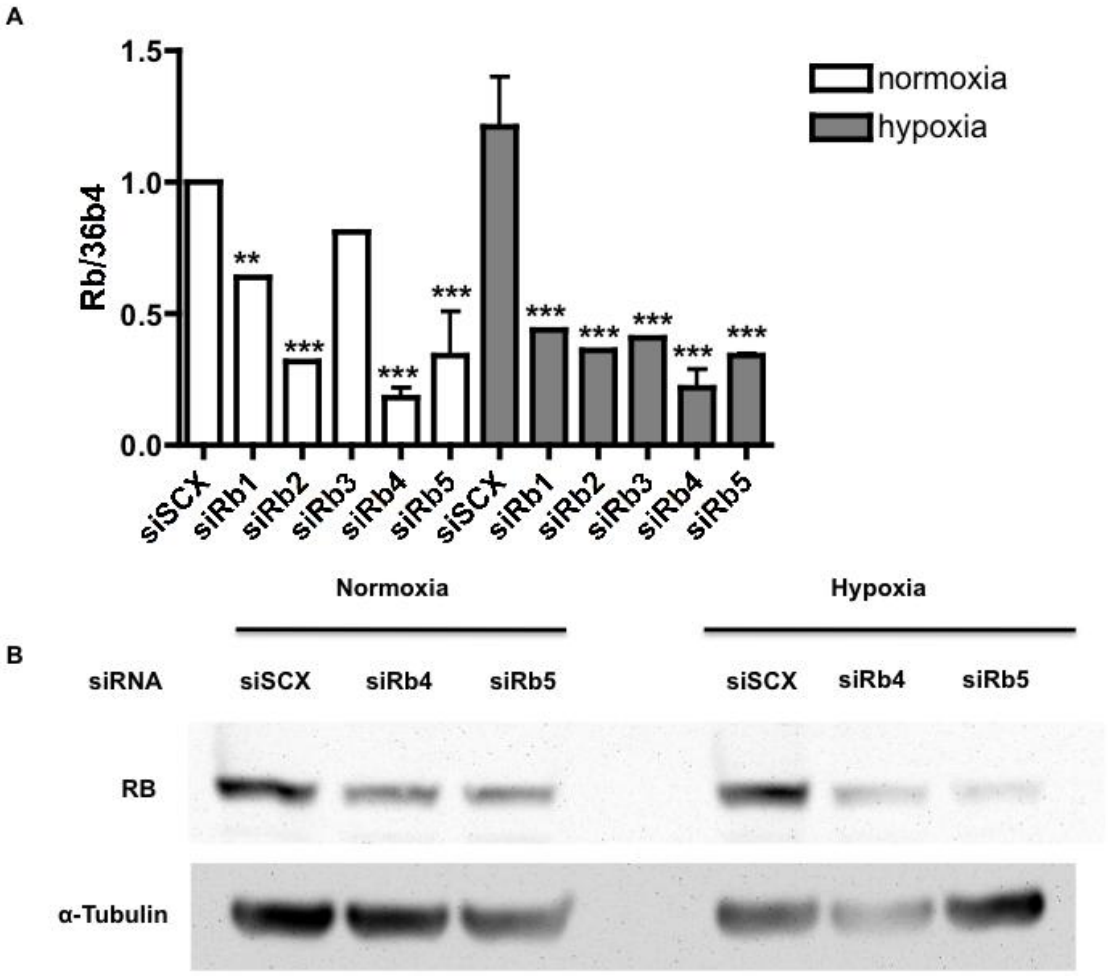

Figure 6. RB-specific siRNAs siRB4 and siRB5 demonstrate efficient knockdown of *RB1* gene expression

MCF7 cells were transfected with either siSCX or siRB for 24 h, followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions for an additional 24 h. (A) A total of 5 RB-specific siRNA were individually assessed for knockdown efficiency of the *RB1* gene by RT-PCR after isolation and reverse transcription of total RNA. Open bars represent normoxia and closed bars represent hypoxia. Error bars represent ± S.E.M. **p<0.01, ***p<0.001, N = 3. (B) RB protein expression levels assessed by Western blot following 24 h exposure to either normoxia or hypoxia.

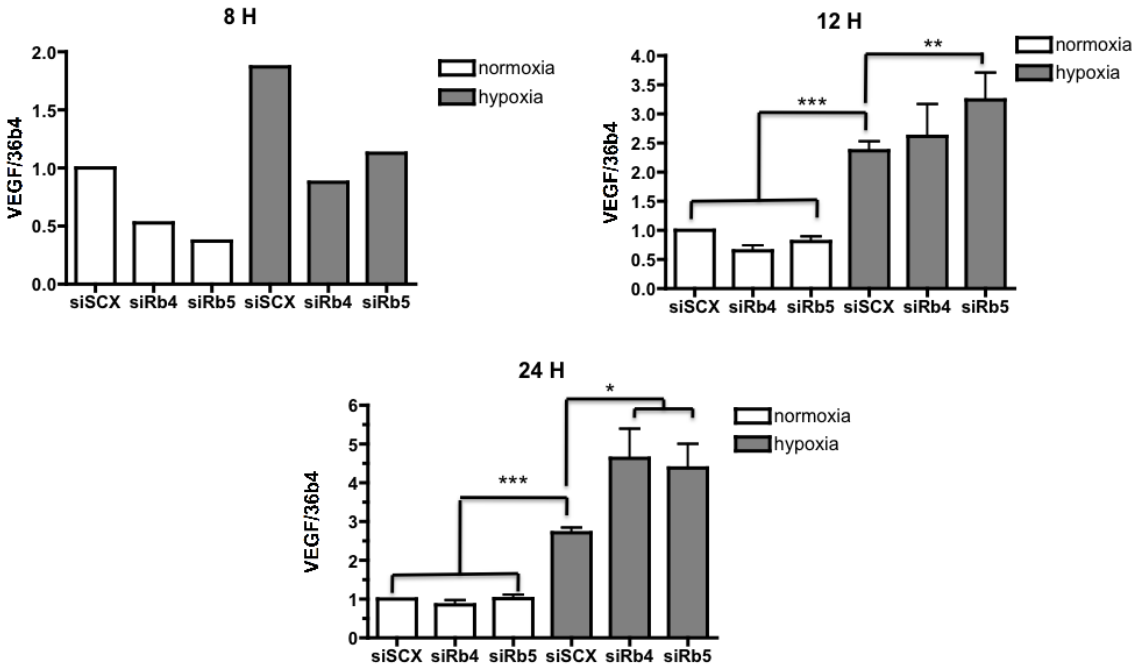

Figure 7. Loss of RB results in a hypoxic-dependent increase in VEGF mRNA accumulation

MCF7 cells were transfected with either siSCX or siRB4 and siRB5 for 24 h followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions. (A) VEGF mRNA accumulation was assessed by RT-PCR following 8 h (N=1), 12 h (N=2) and 24 h (N=3) exposure to hypoxia. Open bars (white) represent normoxia and closed bars (grey) represent hypoxia. Error bars represent ±SEM. *p<0.05, **p<0.01, ***p<0.001.

3.1.3. Effect of RB knockdown on expression of metastatic markers E-cadherin, CXCR4 and vimentin

EMT is a process in which cells transition from a sendentary epithelial-like cellular phenotype towards a more mobile mesenchymal-like phenotype and is also a requirement for metastasis to occur. Loss of epithelial cell marker E-cadherin is considered a hallmark of EMT, and is a process regulated by HIF1 target genes such as twist and snail (Yang *et al.*, 2008; Imai *et al.*, 2003). Expression of E-cadherin was monitored by Western blot in MCF7 cells following siRNA transfection as described above, followed by exposure to hypoxia (2% O_2) for 24 h or 48 h. Interestingly, an increase in E-cadherin protein expression was observed in siRB transfected cells exposed to 24 h of hypoxia, with trace amounts of expression observed under normoxia (Figure 8A). An overall increase E-cadherin protein expression was observed under both normoxia and hypoxia following 48 h compared to 24 h (Figure 8A-B). A slight decrease was observed under hypoxia compared to normoxia samples, but levels did not appear to decrease further in siRB treated cells (Figure 8B). Whether additional exposure to hypoxia was required to observe a greater decrease in E-cadherin in siRB transfected cells compared to siSCX was not addressed. Nonetheless, these results demonstrate that the role of E-cadherin in MCF7 cells still remains unclear.

Increased expression of another HIF1 target gene, the chemokine receptor CXCR4, has also been associated with EMT (Stellar *et al.*, 2003). Expression of CXCR4 was monitored at both the mRNA and protein level in MCF7 cells following siRNA transfection as described above. Changes in mRNA accumulation were measured after subsequent exposure to either hypoxia (2% O_2) or normoxia (20% O_2) at 8 h, 12 h and 24 h time points, whereas changes in protein expression were measured after 48 h exposure to hypoxia. A significant increase in CXCR4 mRNA accumulation was observed in both siRB treated cells (2.5 fold for siRB4 and 2 fold for siRB5) following a 24 h of exposure to hypoxia (Figure 9A). This increase was not observed under normoxic conditions, demonstrating that these effects are also specific to hypoxia. More importantly, a hypoxic-dependent increase was also observed at the protein level following 48 h exposure to hypoxia (Figure 9B)

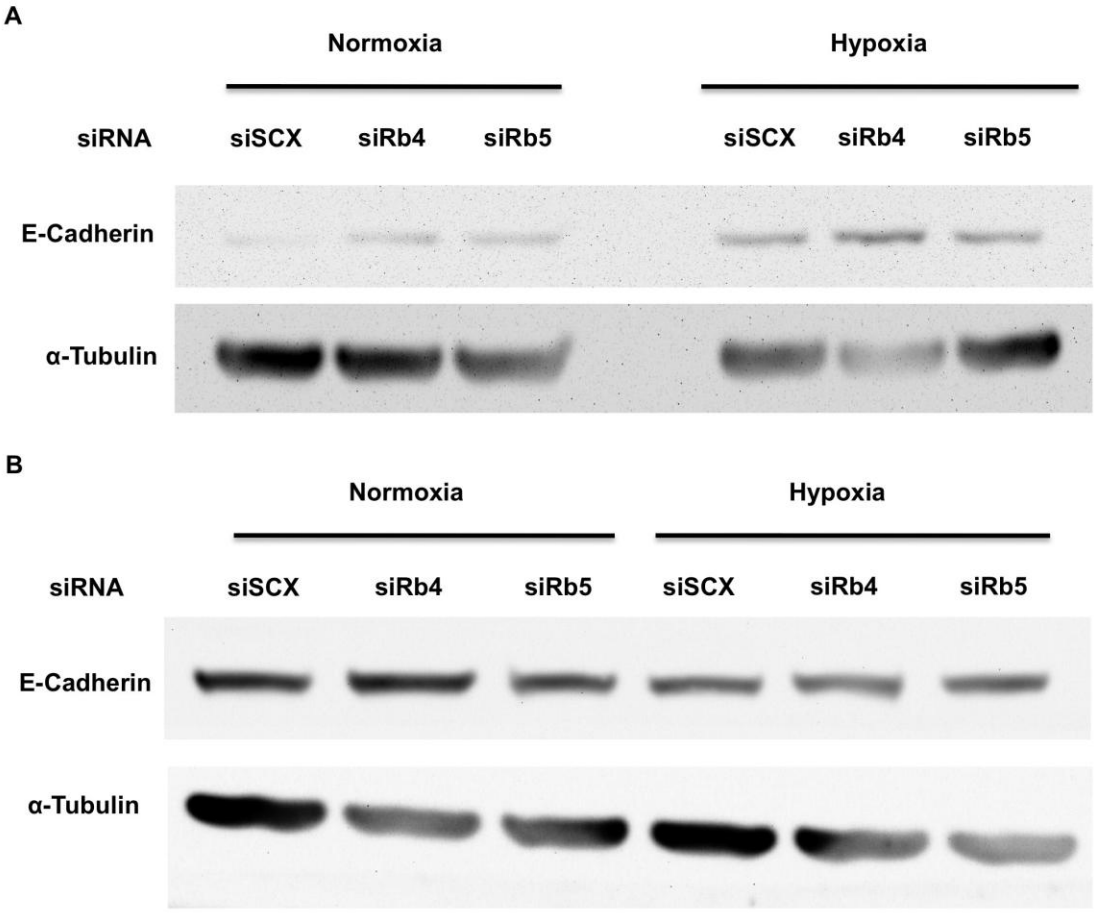

Figure 8. Loss of RB does not result in an additional hypoxic-dependent decrease in E-cadherin protein expression

MCF7 cells were transfected with either siSCX and siRB4 or siRB5 for 24 h, followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions. (A) E-cadherin protein expression levels were assessed by Western blot following 24 h exposure to hypoxia, and (B) following 48 h exposure to either normoxia or hypoxia.

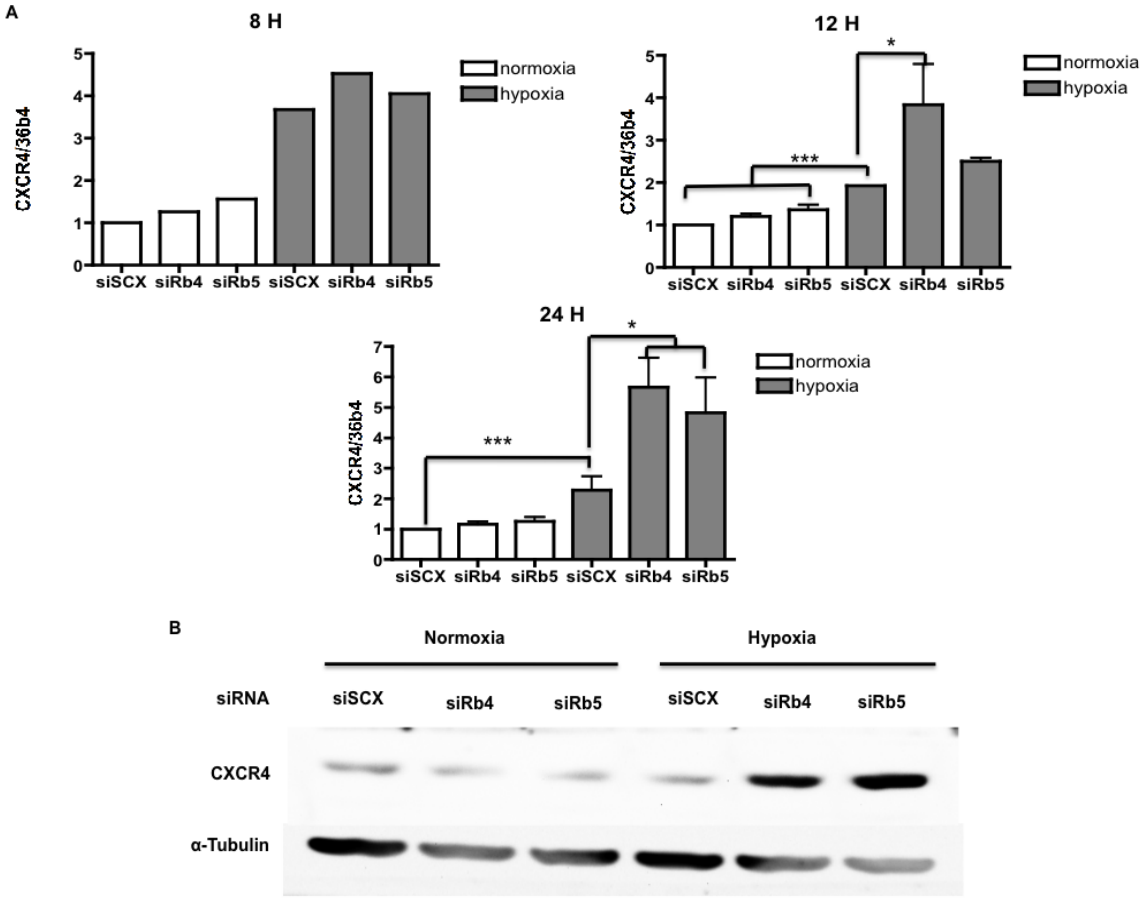

Figure 9. Loss of RB results in a hypoxic-dependent increase in CXCR4 mRNA accumulation and protein expression

MCF7 cells were transfected with either siSCX or siRB4 and siRB5 for 24 h, followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions. (A) CXCR4 mRNA accumulation was assessed by RT-PCR following 8 h (N=1), 12 h (N=2) and 24 h (N=3) exposure to hypoxia. Open bars (white) represent normoxia and closed bars (grey) represent hypoxia. Error bars represent ±SEM. *p<0.05, **p<0.01, ***p<0.001. (B) CXCR4 protein expression levels assessed by Western blot following 48 h exposure to either normoxia or hypoxia.

Concurrent with a decrease in epithelial markers within cells undergoing EMT, is an increase in mesenchymal markers such as vimentin. Vimentin is a member of the intermediate filament family of cytoskeletal proteins that helps maintain the structural integrity of healthy tissue but promotes an invasive phenotype when over-expressed in cancer cells (Mendez *et al.*, 2010). A 3-fold hypoxic-dependent increase in mRNA accumulation was observed in siRB treated cells compared to siSCX following 24 h, however, no obvious increase in protein expression following 48 h exposure to hypoxia (Figure 10A-B). While up-regulation of vimentin expression is associated with HIF1α activity, the relationship between the HIF1 transcription and vimentin expression in MCF7 cells still remains unclear.

3.1.4. Effect of RB knockdown on VEGF, CXCR4 and vimentin expression in MDA-MB-231 and LNCaP cells

A hypoxic-dependent increase in VEGF and CXCR4 mRNA accumulation (60% and 28%, respectively) was also observed following siRB knockdown and 24 h exposure to hypoxia in RB-positive MDA-MB-231 human breast cancer (Figure 11A). In addition, a hypoxic-dependent increase in mRNA accumulation of VEGF, CXCR4 and vimentin in LNCaP human prostate cancer cells (40%, 80%, and 42%, respectively) (Figure 11B). Changes in expression at the protein level have yet to be investigated, however, these results suggest that the role of RB in the hypoxic response may not be exclusive to MCF7 cells.

Taken together these results further support the hypothesis that RB attenuates the expression of HIF1 target genes that promotes angiogenesis and EMT. Loss of RB supports the hypoxic-dependent up-regulation of the angiogenic factor VEGF and the pro-metastatic factors CXCR4 and vimentin, which may ultimately contribute to increased invasive phenotype that supports metastasis. Furthermore, the concurrent hypoxic-dependent decrease in E-cadherin expression and the increase in vimentin expression following loss of RB, is indicative of EMT.

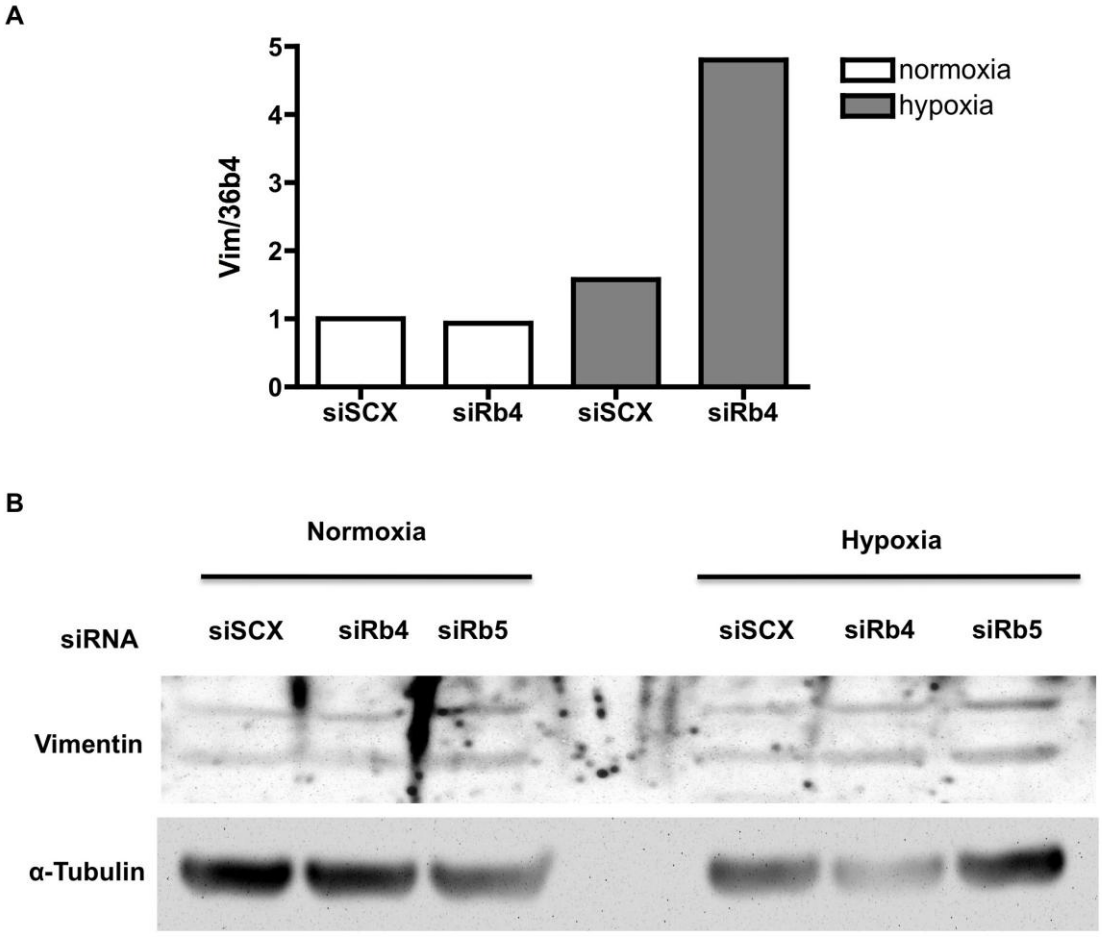

Figure 10. Loss of RB results in a hypoxic-dependent increase in vimentin mRNA accumulation but no change in protein expression

MCF7 cells were transfected with either siSCX or siRB4 and siRB5 for 24 h followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions. **(A)** Vimentin mRNA accumulation was assessed by RT-PCR following 24 h exposure to hypoxia. Open bars (white) represent normoxia and closed bars (grey) represent hypoxia. N=1. **(B)** Vimentin protein expression levels were assessed by Western blot following 48 h exposure to either normoxia or hypoxia.

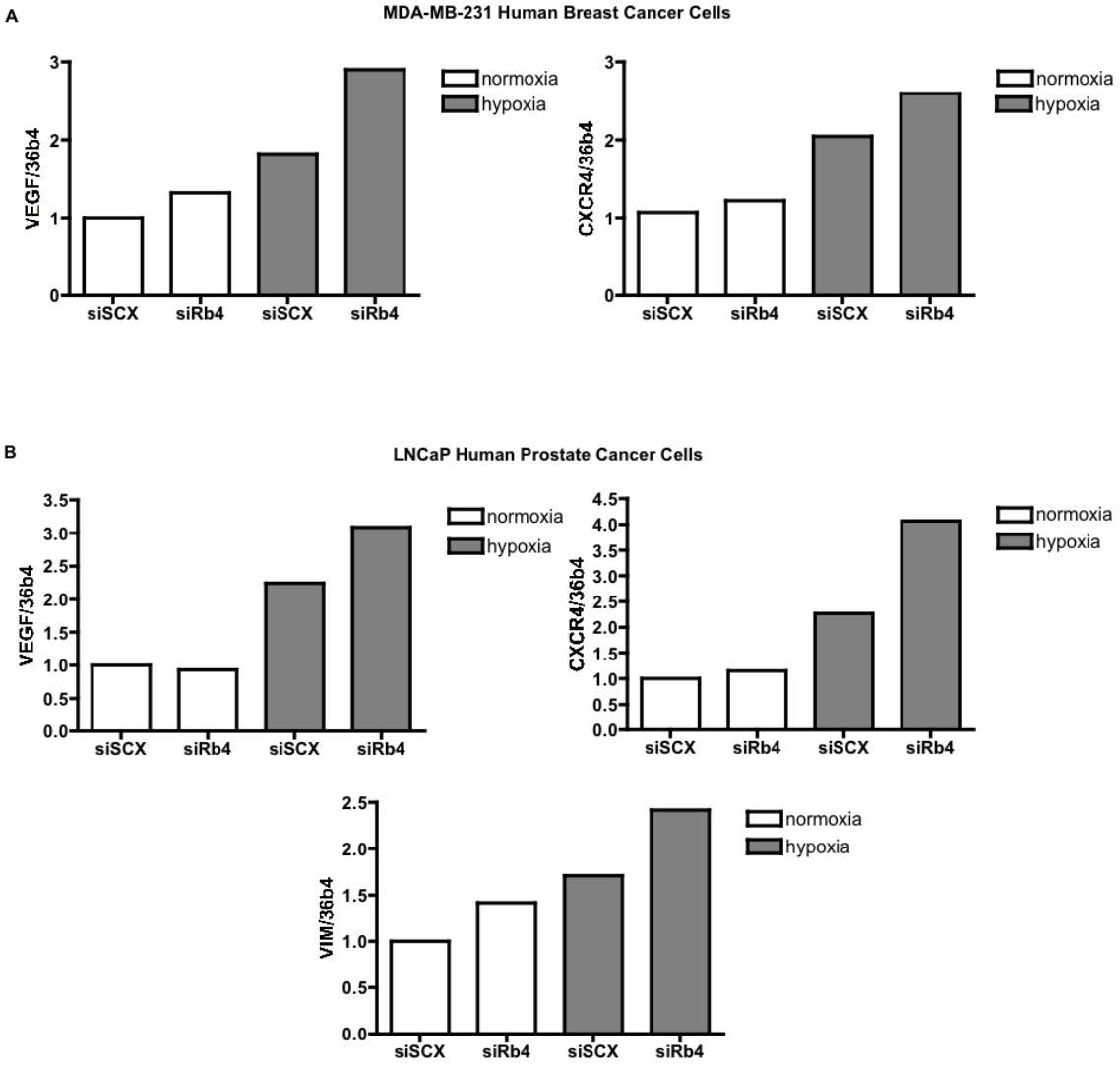

Figure 11. Loss of RB results in a hypoxic-dependent increase in HIF1 target gene mRNA accumulation in MDA-MB-231 human breast cancer and LNCaP human prostate cancer cells

Cells were transfected with either siSCX or siRB4 and siRB5 for 24 h followed by exposure to either normoxic (20% O_2) or hypoxic (2% O_2) conditions. VEGF, CXCR4 and vimentin mRNA accumulation were assessed by RT-PCR following a 24 h exposure to hypoxia in (A) MDA-MB-231 human breast cancer cells, and (B) LNCaP human prostate cancer cells. Open bars (white) represent normoxia and closed bars (grey) represent hypoxia. N=1.

3.2. ARNT, TRIP230 and RB exist as part of a multi-meric complex

As described earlier, previous work has demonstrated that both RB and TRIP230 are recruited to the regulatory regions of HIF1 target genes VEGF and EPO (Appendix D). TRIP230 associates with the HIF1 transcription factor complex through a direct interaction with ARNT, and does not interact with HIF1α (Beischlag *et al.*, 2004). Moreover, RB attenuates TRIP230-mediated HRE reporter gene activity (Appendix B). It had yet to be established, however, if ARNT, TRIP230 and RB exist as part of a multi-meric complex. Establishing the existence of an ARNT-TRIP230-RB complex would further support the hypothesis that RB does not repress HIF1-mediated transcription by preventing TRIP230 interaction, but rather attenuates transcription through a direct interaction with the HIF1 complex through TRIP230.

3.2.1. Generation and expression of GST-ARNT fusion moiety constructs

To assess the possibility of a multi-meric complex, a GST pull-down assay was performed using a GST-ARNT recombinant protein to isolate RB and TRIP230 from nuclear extracts of MCF7 cells exposed to hypoxia. The following regions were amplified from mouse ARNT cDNA and cloned into a GST-5X-1 expression vector: the PAS A domain encodes amino acids 157 – 252, the PAS B domain encodes amino acids 343 – 479, the PAS A-B domains encodes amino acids 157 – 479, and the N-PAS A-B encodes amino acids 2 – 479 (Figure 4A). GST proteins were expressed and purified from bacterial cells using glutathione agarose beads (Figure 4B). Protein concentrations were measured from a 20 µl aliquot of beads against a BSA standard (Figure 4C). High expression levels were achieved with pGEX alone and PAS B, but not with the other constructs. Since the PAS B domain of ARNT has been identified as the primary interaction domain of TRIP230, attempts at increasing expression of the other constructs were not pursued and GST alone was used as the only control in subsequent GST-ARNT pull-down experiments (Partch *et al.*, 2009).

3.2.2. Assessment of GST-PAS B pull-down of TRIP230 and RB

Approximately 30 µl of glutathione agarose beads bound with GST-PAS B and

GST alone bound to gluthatione agarose beads were mixed with 500 µg of MCF7 nuclear extract from cells exposed to hypoxia in PBS overnight at 4°C. Beads were washed twice in 0.5x RIPA (70 mM NaCl), followed by two washes in 1x RIPA (140 mM NaCl). Proteins were eluted off the beads by boiling in 2x SDS sample buffer and resolved by SDS-PAGE. The GST-PAS B fusion moiety was capable of pulling down TRIP230, with trace amounts observed in the GST alone sample (Figure 12A). Under these same conditions, GST-PAS B was also capable of pulling down RB, however, there was considerable amount of RB detected in the pGEX alone sample. Mixing GST protein and nuclear extract in Lysis buffer (400 mM NaCl, Figure 12B) or eluting off protein with 10 mM reduced glutathione (Figure 10C) resulted in no detectable RB in either samples. Increasing the stringency of washes with Lysis buffer (Figure 12D) or using less GST-protein (Figure 12E) reduced the amount RB within both GST-PAS B and GST samples but produced a slightly stronger interaction in the GST-PAS B. Using less GST-protein in combination with less nuclear extract (250 µg), the amount of RB within the GST alone sample was reduced but a weak RB interaction was still maintained with the GST-PAS B construct (Figure 12F). The pull-down repeated again under less stringent washing conditions, to one wash with 70 mM NaCl 0.5x RIPA buffer followed by four washes in 140 mM NaCl 1x RIPA. Also, RB was detected using an anti-RB mouse monoclonal antibody, rather than an anti-RB rabbit polyclonal antibody used in previous experiments. Under these conditions, a strong RB interaction was achieved with GST-PAS B, with minimal non-specific interaction detected with GST alone, as well as simultaneous pull-down of TRIP230 with the GST-PAS B (Figure 12G). These results suggest that TRIP230, RB and ARNT can exist in a single multi-meric complex, however, the role of RB within this complex still remains unclear.

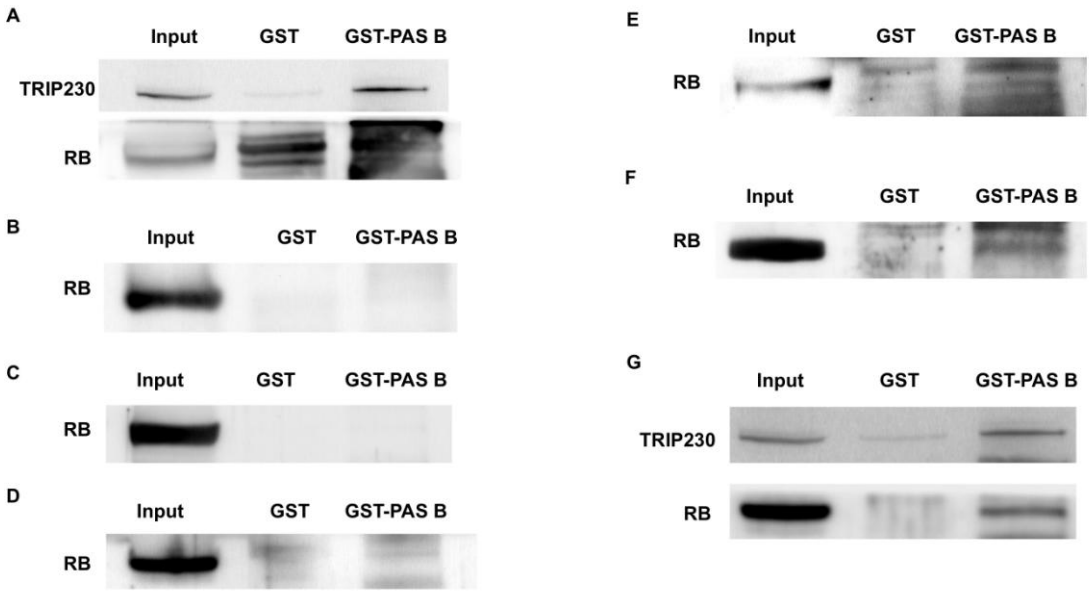

Figure 12. TRIP230 and RB from MCF7 nuclear extract interact with a GST-PAS B construct

(A) Approximately 30 µl of glutathione agarose beads bound with GST proteins were mixed with 500 µg of MCF7 nuclear extract from cells exposed to hypoxia in PBS overnight at 4°C. Beads were then washed four times with increasing stringency of RIPA buffer. Protein was eluted off by boiling in 2x SDS sample buffer and resolved by SDS-PAGE. GST-PAS B was capable of pulling down TRIP230, with trace amounts observed in the GST alone sample, however, similar amounts of RB was detected in both GST alone and GST-PAS B samples. (B) GST protein and nuclear extracts were mixed in Lysis buffer. (C) GST proteins were eluted off with 10 mM reduced glutathione. (D) Increased stringent washes with Lysis buffer. (E) Using less GST protein. (F) Using less GST protein in combination with less nuclear extract (250 µg). (G) Decreased stringent washes with RIPA buffer and detection of RB with a mouse monoclonal antibody.

3.2.3. An ARNT-interacting peptide blocks GST-PAS B pull-down of TRIP230 and RB

In addition to identifying the TRIP230-interaction domain of ARNT, the minimal region of TRIP230 required for ARNT interaction was also identified (Partch *et al.*, 2009). More specifically, an LRNHLL motif located within this region mediates interaction with the ARNT PAS-B domain. Since TRIP230 is a necessary co-activator for HIF1-mediated transcription and specifically interacts with ARNT, presumably disrupting this interaction would result in decreased HIF1 activity. This could potentially minimize the HIF1 response associated with angiogenesis and EMT and thus serve as basis for an anti-cancer therapy.

A GST pull-down assay using a GST-PAS B fusion moiety to pull-down TRIP230 was performed following the established protocol as previously described, except GST-fusion proteins were first pre-incubated overnight at 4°C with 50 μM of peptide corresponding to the LRNHLL motif of TRIP230 conjugated with a cell membrane transduction TAT domain and a fluorescent tag (TRIP1584) (Figure 5A). The peptide was capable of disrupting the interaction between GST-PAS B and TRIP230, while a negative control peptide had no effect (Figure 13A). Blocking the GST-PAS B-TRIP230 interaction also blocked recruitment of RB to the GST-PAS B construct (Figure 13B). The pull-down assay was repeated using two different TRIP230-interacting peptides, ARNT 315 and ARNT 492, that specifically block essential TRIP230 amino acids required for ARNT interaction (Figure 5B). Neither of these peptides alone were capable of disrupting the GST-PAS B-TRIP230 interaction (Figure 13C). Nonetheless, the TRIP1584 ARNT-interacting peptide establishes the efficacy of a blocking peptide capable of disrupting GST-PAS B-TRIP230 interaction. Moreover, loss of RB recruitment to the GST-PAS B construct now establishes the existence of an ARNT-TRIP230-RB multi-meric complex and that TRIP230 is required for RB recruiment to ARNT.

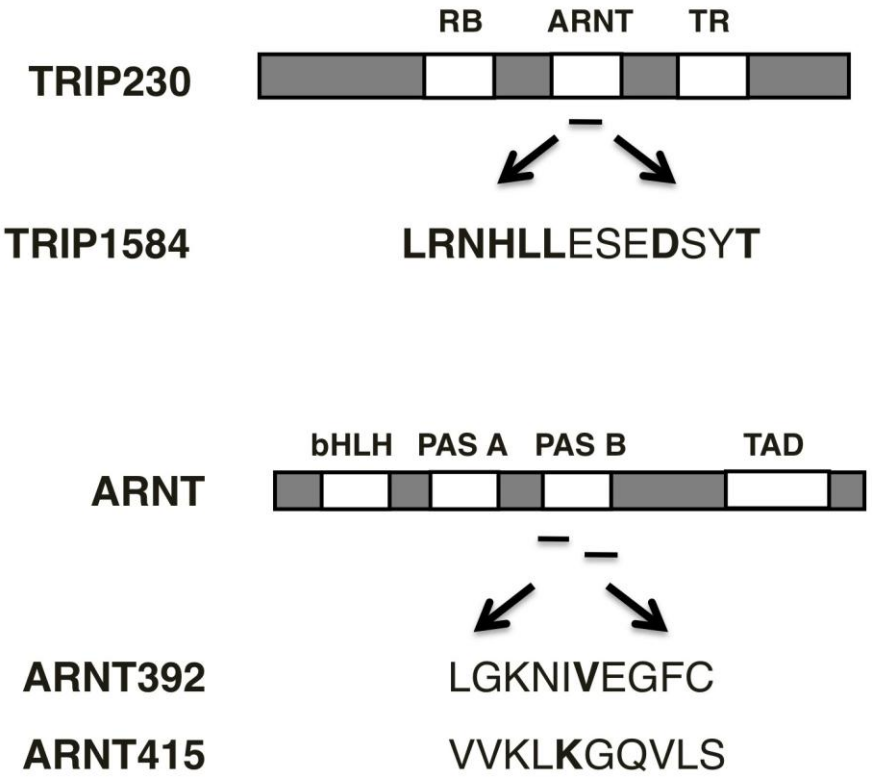

Figure 5. Schematic of the ARNT- and TRIP230-interacting peptides

An ARNT interacting peptide (TRIP1584) corresponds to a specific LRNHLL motif within TRIP230 that is required for ARNT-TRIP230 interaction. Two TRIP230-interacting peptides (ARNT392 and ARNT415) corresponds to important amino acids within ARNT required for the ARNT-TRIP230 interaction.

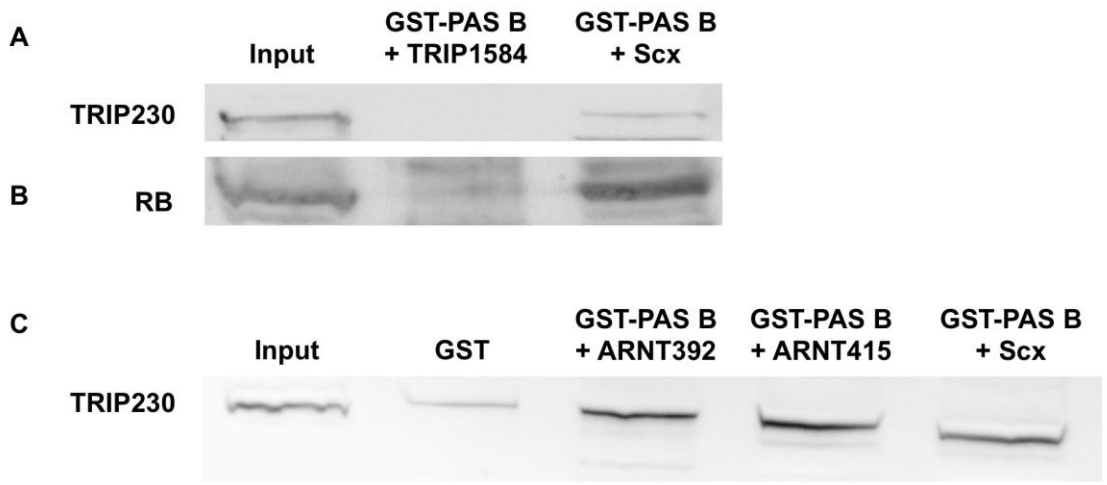

Figure 13. An ARNT-interacting peptide is capable of blocking ARNT-TRIP230-RB complex formation

Pull-down of TRIP230 with GST-PAS B construct was performed following the established protocol previously described, except GST proteins were first pre-incubated overnight at 4°C with 50 μM of an ARNT or TRIP230 interaction peptide, or a negative control peptide. (A) The TRIP1584 ARNT-interacting peptide was capable of disrupting interaction between GST-PAS B and TRIP230, while the negative control peptide had no effect. (B) Disruption of ARNT-TRIP230 interaction causes a disassociation between ARNT and RB. (C) Two TRIP230-interacting peptide (ARNT392 and ARNT415), each blocking amino acids essential for ARNT interaction, were unable to disrupt interaction between GST-PAS B and TRIP230.

3.3. The C-terminal region within the RB-interaction domain of TRIP230 contains the minimal interaction domain

The results presented above demonstrate an association between loss of RB and the deregulation of HIF1 target genes involved in angiogenesis and invasion (Figure 7-10). Furthermore, knockdown of RB in MCF7 cells leads to a dramatic increase in hypoxia-dependent cell invasion, as demonstrated in a matrigel invasion assay (Appendix C). Altogether, this work has set the foundation for investigating the role of RB in HIF1-mediated tumour progression in an animal model. To address this, a transgenic mouse expressing a mutant TRIP230 will be created where the specific amino acids of TRIP230 required for RB-interaction will be mutated. This approach should mimic the loss of RB, while minimally disrupting the structure and function of TRIP230, as a mutant lacking the RB-interaction domain still has co-activation capabilities (Appendix B). This mouse will then be crossed with an existing brain cancer mouse model, the *Nfl;p53 cis-/+* mouse, or breast cancer mouse model, the MMTV-PyMT^{634Mul}, to investigate whether disrupting TRIP230-RB-interaction increases the progression and hyper-vascularization of tumours.

3.3.1. Generation and expression of GST-TRIP230 fusion moiety deletion constructs

A 125 amino acid RB-interaction domain of TRIP230 has already been identified within amino acids 1099 and 1255 (Chang *et al.*, 1997). To identify the specific amino acids required for RB-interaction within this domain, the minimal interaction region was first identified by GST pull-down analysis to create a smaller and more manageable region to perform alanine scanning analysis using site directed mutagenesis. An initial set of six successive 20 amino acid deletion mutants of the RB-interaction domain were PCR amplified from full length TRIP230 up until amino acid 1498, and cloned into a pGEX-5X-1 expression vector: TRIP1120, TRIP1144, TRIP1166, TRIP1188, TRIP1211, and TRIP1241 (Figure 3). In addition, a TRIP1091 construct containing the full-length interaction domain and a TRIP1264 construct lacking the entire interaction domain were developed to serve as controls. Constructs were named in reference to the N-terminal TRIP230 amino acid.

Low levels of GST-TRIP230 proteins were detected following the standard protocol described above for expressing GST constructs (Figure 14A). Bacterial cells are particularly durable due to the additional protection provided by the cell wall and thus optimizing the cell lysing procedure may increase recovery of GST protein from bacteria cells. Multiple freeze thaw cycles of pellets prior to sonication could not increase recovery (Figure 14C), nor could resuspending cell pellets in a high salt Lysis buffer prior to sonication (Figure 14D). GST protein was detected in the pellet of the TRIP1120 sample, which would suggest the possibility of GST proteins forming inclusion bodies, but this may also have been a result of the high salt lysing conditions. Purified GST-TRIP230 protein was not assessed since increased protein expression was not achieved.

Modifications were then applied to enhance expression and increasing GST protein yield. Inducing expression during the exponential growth phase of bacteria is important for achieving high levels of expression but if induced too early, protein may degrade, resulting in low yields. Expression was assessed at the later stages of the exponential growth phase (OD600 of 0.6 and 0.9) using either 0.1 mM, 0.5 mM and 1.0 mM IPTG for either 1, 4 or 8 H. None of these conditions significantly enhanced expression, which suggested that degradation of GST proteins is not occurring (Figure 15A). Purified GST-TRIP230 protein was not assessed since increased protein expression was not achieved. Expression was then assessed when induced at an earlier stage of the growth cycle and at a lower temperature to slow down the bacterial growth rate, providing more time for protein expression. Sufficient levels were achieved for most of the constructs when expression was induced between an OD600 of 0.4 and 0.5 with 1 mM IPTG for 4 hours at 30°C (Figure 15B). GST-TRIP230 protein eluted from a 20 µl aliquot of beads following purification demonstrated a relatively pure yield of protein (Figure 15C). Increased expression was not observed with the TRIP1091, TRIP1144 and TRIP1241 constructs, thus multiple rounds of expression were performed and pooled together to obtain enough protein for pull-down experiments.

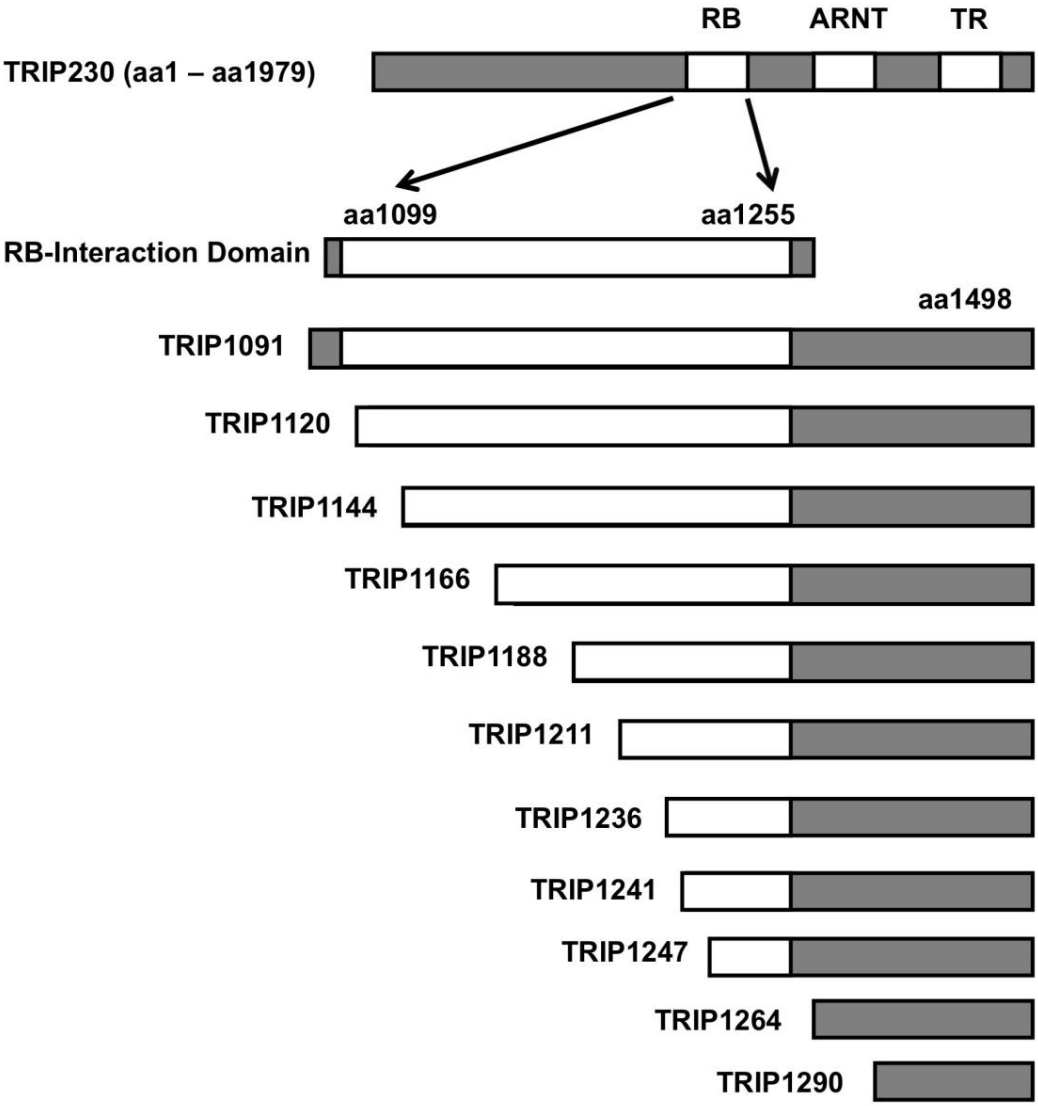

Figure 2. Schematic of the GST-TRIP230 deletion fusion moiety constructs

A total of 11 successive 20-amino acid deletion constructs of the TRIP230 RB-interaction domain were amplified from full-length TRIP230 and cloned into pGEX-5X-1. Constructs were named in reference to the N-terminal TRIP230 amino acid that each construct was amplified from. The full length TRIP230 schematic depicts the RB-interaction domain (RB), the ARNT interaction domain (ARNT), and the thyroid receptor interaction domain (TR).

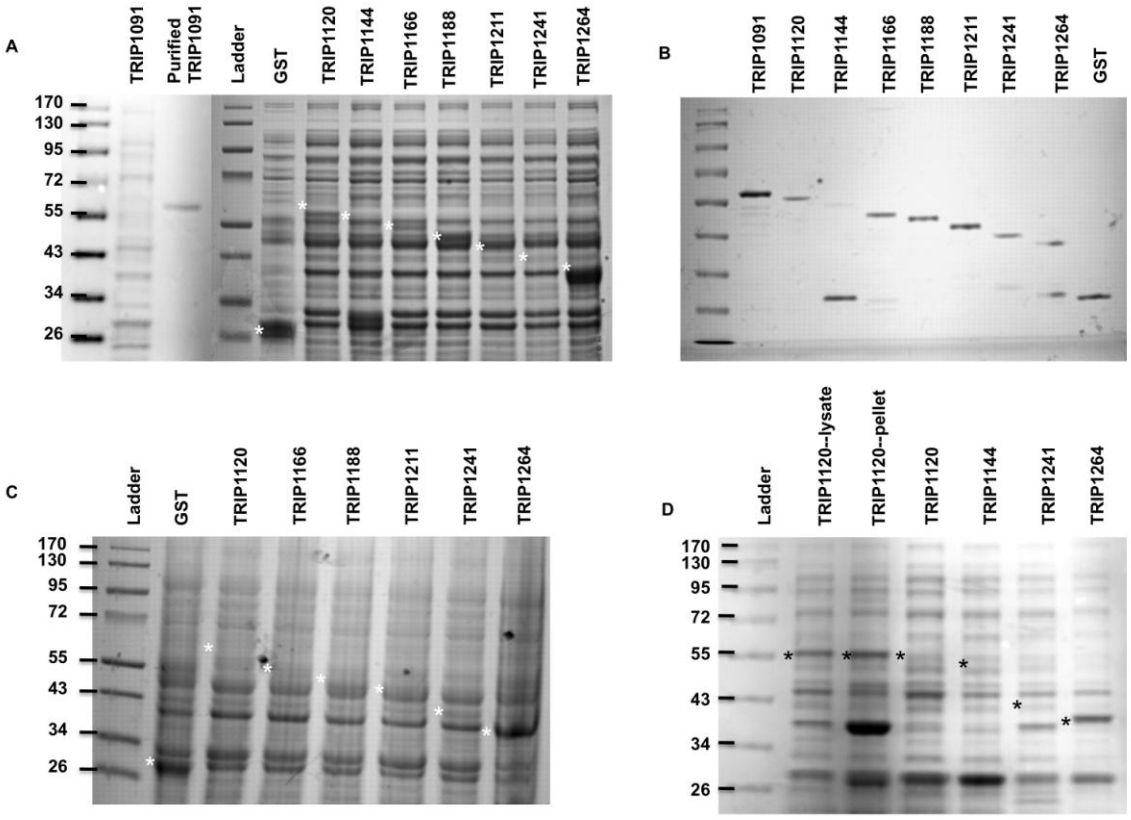

Figure 14. GST-TRIP230 protein recovery following multiple freeze cycles of bacterial cell pellets and high salt bacterial cell lysing conditions

Recombinant pGEX-5X-1 plasmids were individually transformed into BL21 bacterial cells. A 2 ml overnight bacterial cell culture was subcultured into 25 ml of LB-amp and allowed to expand. GST proteins were subsequently expressed following IPTG induction for an additional period of time. Bacterial cells were then lysed by sonication and a 20 µl aliquot of bacterial cell lysate from each sample was resolved by SDS-PAGE and protein expression was assessed by Coomassie staining. (A) Bacterial cultures were grown for 4 h at 37°C and protein expression was induced with 0.5 mM IPTG for an additional 1 h at 37°C. Bacterial cells were then lysed by sonication in 1 ml PBS. (B) A 1 µg sample of purified GST-TRIP230 protein resolved by SDS-PAGE and assessed by Coomassie staining. (C) Bacterial cell pellets underwent multiple freeze thaw cycles prior to sonication. (D) Bacterial cells were resusupended in high salt Lysis buffer prior to sonication. GST protein was not lost within the cell lysate pellet following sonication.

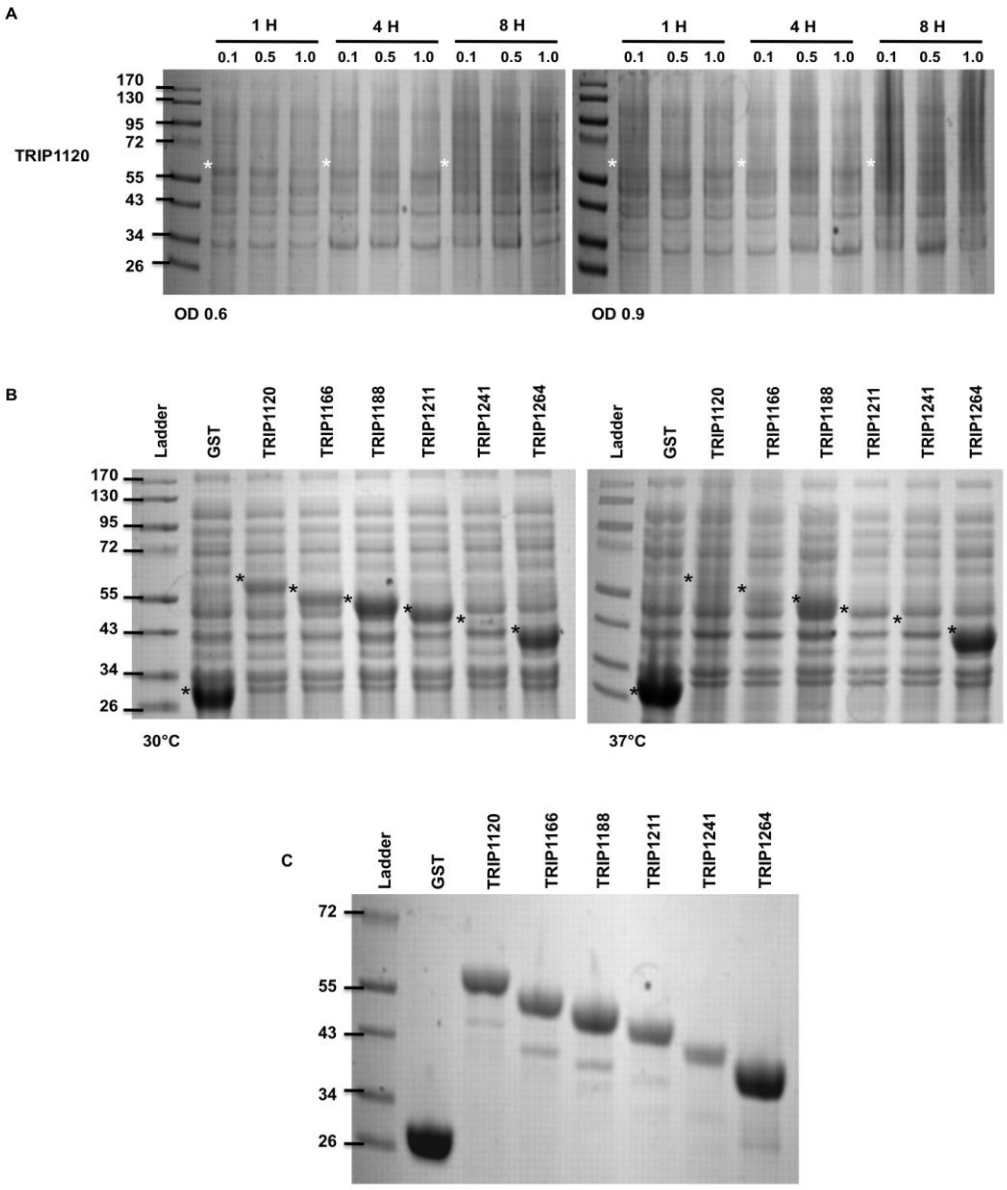

Figure 15. GST-TRIP230 protein expression following IPTG induction at different concentrations, exponential growth phases and temperature

(A) Protein expression induced at later stages of the exponential growth phase (OD600 of 0.6 and 0.9) using either 0.1 mM, 0.5 mM and 1.0 mM IPTG. (B) Protein expression induced at earlier stage of the exponential growth phase (OD600 of 0.4-0.5) and at a 30°C. (C) Purified GST-TRIP230 protein was assessed from a 20 µl aliquot of beads boiled in 2x SDS sample buffer.

3.3.2. Assessment of GST-TRIP230 deletion construct pull-down of RB

Preliminary experiments were performed using TRIP1091, TRIP1120 and TRIP1264 constructs. Equi-molar concentrations of GST-TRIP230 and GST alone bound to gluthathione agarose beads were mixed with 250 µg of MCF7 nuclear extract from cells exposed to hypoxia in PBS overnight at 4°C. Beads were then washed and protein was eluted off by boiling in 2x SDS sample buffer, then resolved by SDS-PAGE. Interaction was observed with TRIP1120 but not TRIP1091 or the pGEX and TRIP1264 controls (Figure 16A). These results suggested that the first 20 amino acids of the RB-interaction domain are not required for interaction, thus TRIP1091 was only used in subsequent experiments when sufficient protein levels could be expressed. When repeated using all GST-TRIP230 constructs, interaction was observed with TRIP1120, TRIP1166, TRIP1188, and weak interaction with TRIP1211 (Figure 16B). No interaction was observed with TRIP1144 and TRIP1241, however, significantly less GST protein was detected in both of these samples. When repeated using 300 mM NaCl Lysis buffer washing conditions, RB-interaction was lost with TRIP1211 (Figure 16C). Again, since interaction was still retained with TRIP1120 and TRIP1166, TRIP1144 was only used in subsequent experiments when sufficient protein levels could be expressed.

In an attempt to enhance RB-interaction and more clearly define the minimal interaction domain, the pull-down was repeated using cell lysate from 293T cells transfected with an RB expression plasmid (Figure 17A). The pull-down was repeated using 300 mM NaCl Lysis buffer washing conditions, revealing a similar interaction pattern as with MCF7 nuclear extract with only a slightly enhanced signal (Figure 16B vs Figure 16C). Using increased amounts of GST protein in addition to 293T cell lysates revealed interaction with both TRIP1211 and TRIP1241 constructs (Figure 17C). Since these results were inconsistent with the TRIP1120/TRIP1166/TRIP1188 interaction pattern previously observed, these interactions were considered non-specific.

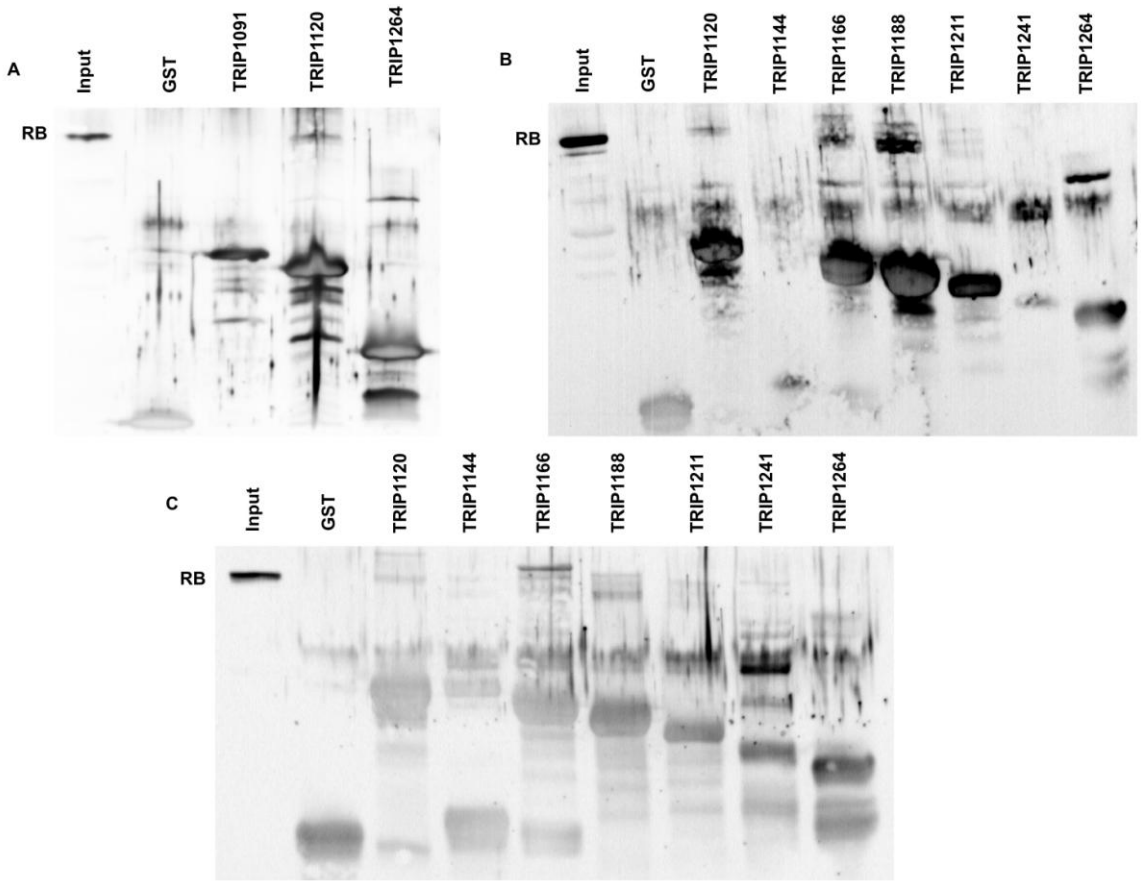

Figure 16. RB-interaction with GST-TRIP230 deletion constructs using MCF7 nuclear extract demonstrates inconsistent results

GST proteins were mixed overnight at 4°C with MCF7 nuclear extract from cells exposed to hypoxia. Beads were then washed and protein was eluted off by boiling in 2x SDS sample buffer and resolved by SDS-PAGE. (A) TRIP1091, TRIP1120, TRIP1264 and GST alone were mixed with 250 µg of MCF7 nuclear extract in PBS and washed three times with PBS. GST-TRIP1091 was no longer used in subsequent pull-down experiments. (B) RB-interaction was assessed with the remaining TRIP230 constructs under the same conditions. (C) Beads were washed using 300 mM NaCl Lysis buffer.

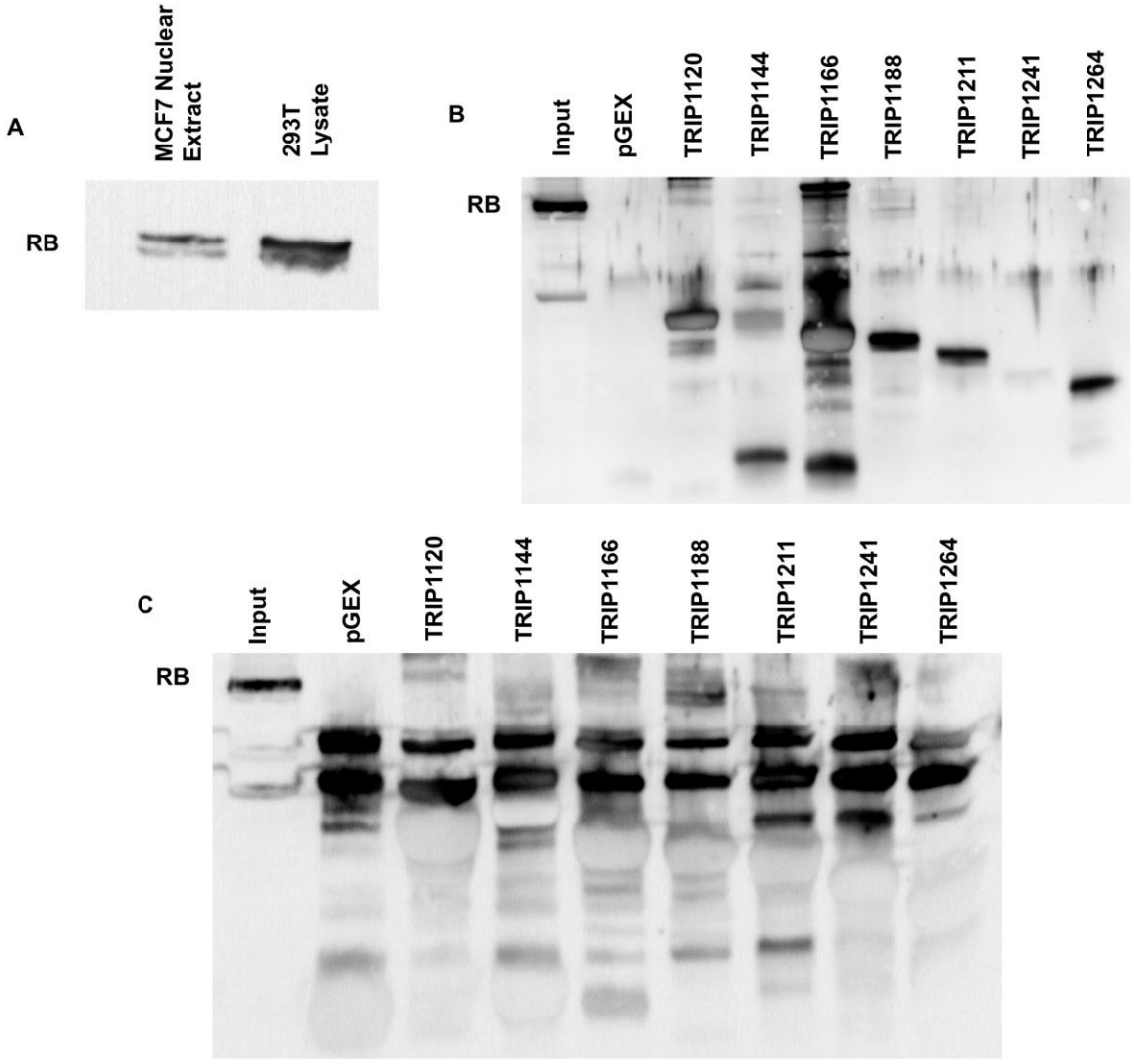

Figure 17. RB-interaction with GST-TRIP230 deletion constructs using cell lysate from 293T cells transfected with an RB expression plasimid demonstrates inconsistent results

GST proteins were mixed with MCF7 nuclear extract from cells exposed to hypoxia overnight at 4°C. Beads were then washed and protein was eluted off by boiling in 2x SDS sample buffer and resolved by SDS-PAGE. **(A)** 293T cells were transiently transfected with an RB expression vector. The amount of RB in 30 µg of MCF7 nuclear extract from cells exposed to hypoxia and 30 µg of cell lysate from 293T cells were compared. **(B)** GST-TRIP230 constructs were mixed with cell lysate from 293T cells transfected with an RB expression plasmid. **(C)** Pull-down was repeated using increased amounts of GST protein.

Purified sources of RB protein were also explored to help reduce the amount of background noise. *In vitro* translation is one tool for synthesizing a pure source of protein within a cell free system. Attempts to *in vitro* translate RB from full-length cDNA were not successful, as a full-length RB protein could not be synthesized (Figure 18A). Purified full length RB was then purchased and the pull-down assay was repeated using 140 mM NaCl RIPA buffer washes as originally described using nuclear extracts. Using 0.1 µg per reaction as recommend by the manufacturer's protocol, produced a significantly weaker RB-interaction (Figure 18B). Increasing this amount to 0.3 µg could produce a clear interaction pattern (Figure 18C). Interestingly, purified RB appeared to interact more readily with the TRIP1264 control. This was a cause for concern, since TRIP1264 does not contain amino acids within the putative RB-interaction domain (Chang *et al.*, 1997).

The question was then raised as to whether or not the antibody was detecting a species of the same size that was being pulled-down. The pull-down was repeated using 300 mM NaCl Lysis buffer washing conditions as previously described using nuclear extracts. Detection using a mouse monoclonal RB antibody significantly reduced background noise, however, RB-interaction was barely detectable (Figure 19A). Decreasing the stringency of washes using 300 mM NaCl PPI buffer improved interaction (Figure 19B), however, results of repeated experiments no longer demonstrated a consistent and logical interaction pattern (Figure 19C).

In order to limit the use of glutathione agarose beads used per reaction, GST proteins remained on the beads throughout the experiment. Since each GST-TRIP230 deletion construct did not express at the same efficiency, the volume of protein-bound beads varied per construct for each pull-down experiment. Interestingly, those constructs that did not express well (for example TRIP1144 and TRIP1241) and thus required more beads per reaction, displayed the strongest interaction with RB in several experiments (see Figure 19). This observation raised the possibility that non-specific interaction of RB was occurring on the beads alone and that the inconsistencies observed might be a result of varying concentrations of protein-bound beads used per reaction. Elutes boiled off from beads alone mixed with nuclear extracts were then assessed by western blot and demonstrated that RB was in fact interacting with the

beads alone (Figure 20). This prompted a modification to the protocol where GST-proteins were eluted off the beads prior to mixing with nuclear extracts overnight at 4°C.

GST proteins were then added back onto fresh beads by mixing for 2 h at room temperature. Modification of the TRIP1241 construct was also prompted upon closer inspection of the unique amino acids found within this construct, which revealed two consecutive negatively charged amino acids located near the N-terminus that could possibly be interfering with folding (Figure 21A). Two modified constructs were then made that were either 6 amino acids longer (TRIP1236) or 6 amino acids shorter (TRIP1247). The TRIP1247 construct that excluded those two amino acids expressed at significantly higher levels compared to TRIP1236 and thus replaced the TRIP1241 construct in subsequent pull-down experiments (Figure 21B).

Pull-down experiments were then repeated following the modified protocol using nuclear extracts and 300 mM NaCl PPI buffer washes. While interaction appeared weak with the longer GST-TRIP230 constructs, strong interaction was consistently observed with TRIP1247 (Figure 22A). Weak interactions observed with the larger constructs may be due to other factors within the extract interfering with RB's ability to bind to the GST construct. Purified RB demonstrated a more consistent banding pattern with the larger fragments in addition to TRIP1247 (Figure 22B), however, this also increased the occurrence of non-specific binding occurring with the pGEX and TRIP1264 controls (Figure 22C). To confirm that TRIP1264 was not a part of the TRIP230 RB-interaction as previously demonstrated, the TRIP1290 construct was constructed and assessed following the same protocol. RB-interaction with the TRIP1290 construct appeared minimal and at the same intensity as the pGEX and TRIP1264 controls (Figure 23). Taken together, these results suggest that the minimal interaction domain may lie within amino acids 1247 and 1255 of the TRIP230 RB-interaction domain, however, further experiments will be needed for conformation.

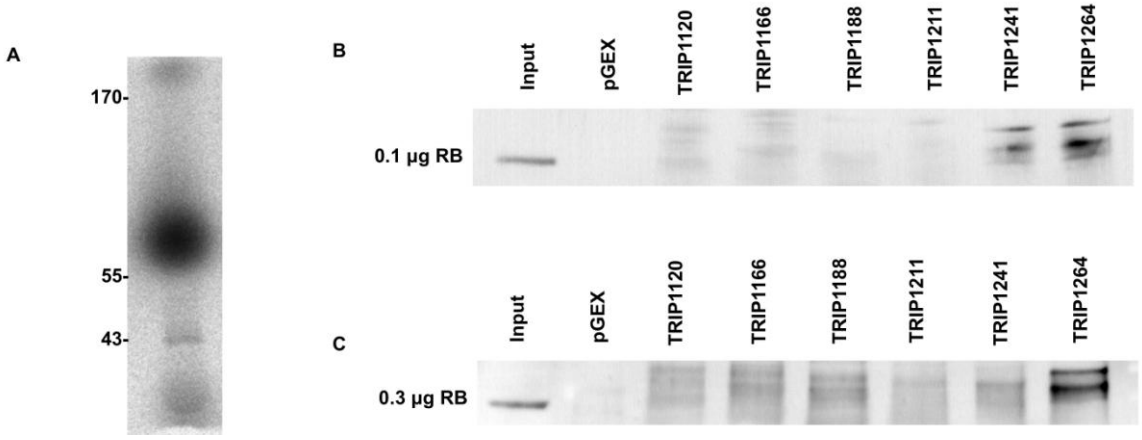

Figure 18. RB-interaction with TRIP230 deletion constructs using purified RB demonstrates inconsistent results

Pull-down experiments with GST-TRIP230 constructs were repeated using purified sources of RB. **(A)** Attempts to *in vitro* translate RB from full length cDNA were unsuccessful as a full length RB protein could not be synthesized. Pull-down assay was repeated using 0.1 µg **(B)** and 0.3 µg **(C)** of purchased purified full length RB.

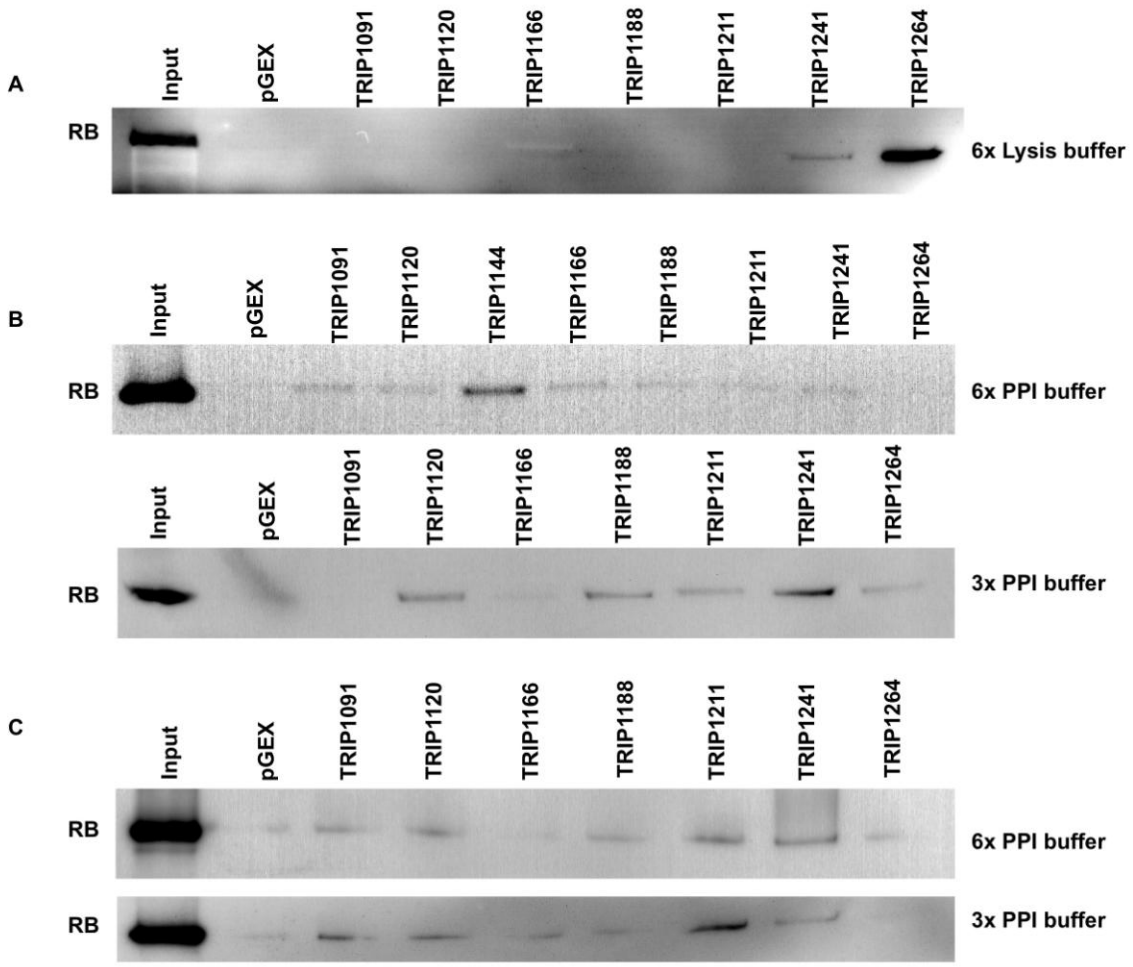

Figure 19. Pull-down assay with GST-TRIP230 deletion constructs repeated using a mouse monoclonal antibody

(A) Pull-down experiments with GST-TRIP230 constructs were repeated with 6 washes of 300 mM NaCl Lysis buffer washes (6x Lysis buffer). Detection for RB-interaction was assessed by Western blot with an anti-RB mouse monoclonal antibody. **(B)** Pull-down experiments were repeated except washed either 6 times or 3 times 300 mM NaCl PPI buffer washing conditions (6x or 3x PPI buffer). **(C)** Results of repeated experiments with 300 mM NaCl PPI buffer washing conditions.

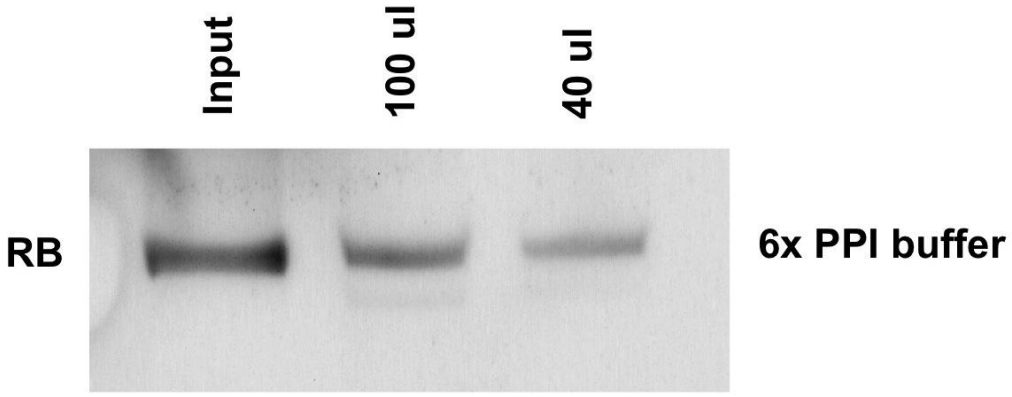

Figure 20. RB interacts with unbound glutathione agarose beads

Elutes from glutathione agarose beads mixed alone with nuclear extracts and washed with 300 mM NaCl PPI buffer. RB-interaction was assessed by Western blot.

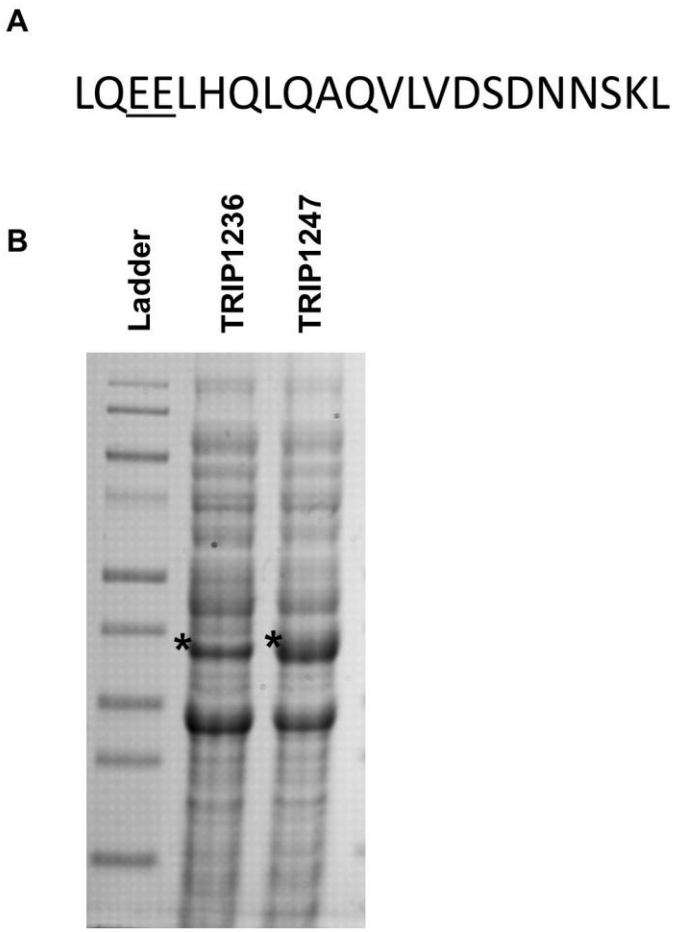

Figure 21. Expression of TRIP1236 and TRIP1247 constructs

(A) Unique amino acids within the GST-1241 construct. The underlined glutamic acid residues are negatively charged at physiological pH. **(B)** Recombinant pGEX-5X-1 plasmids were transformed and expressed in BL21 bacteria following the established protocol. A 20 µl aliquot of bacterial cell lysate from each sample was resolved by SDS-PAGE and protein expression was assessed by Coomassie staining.

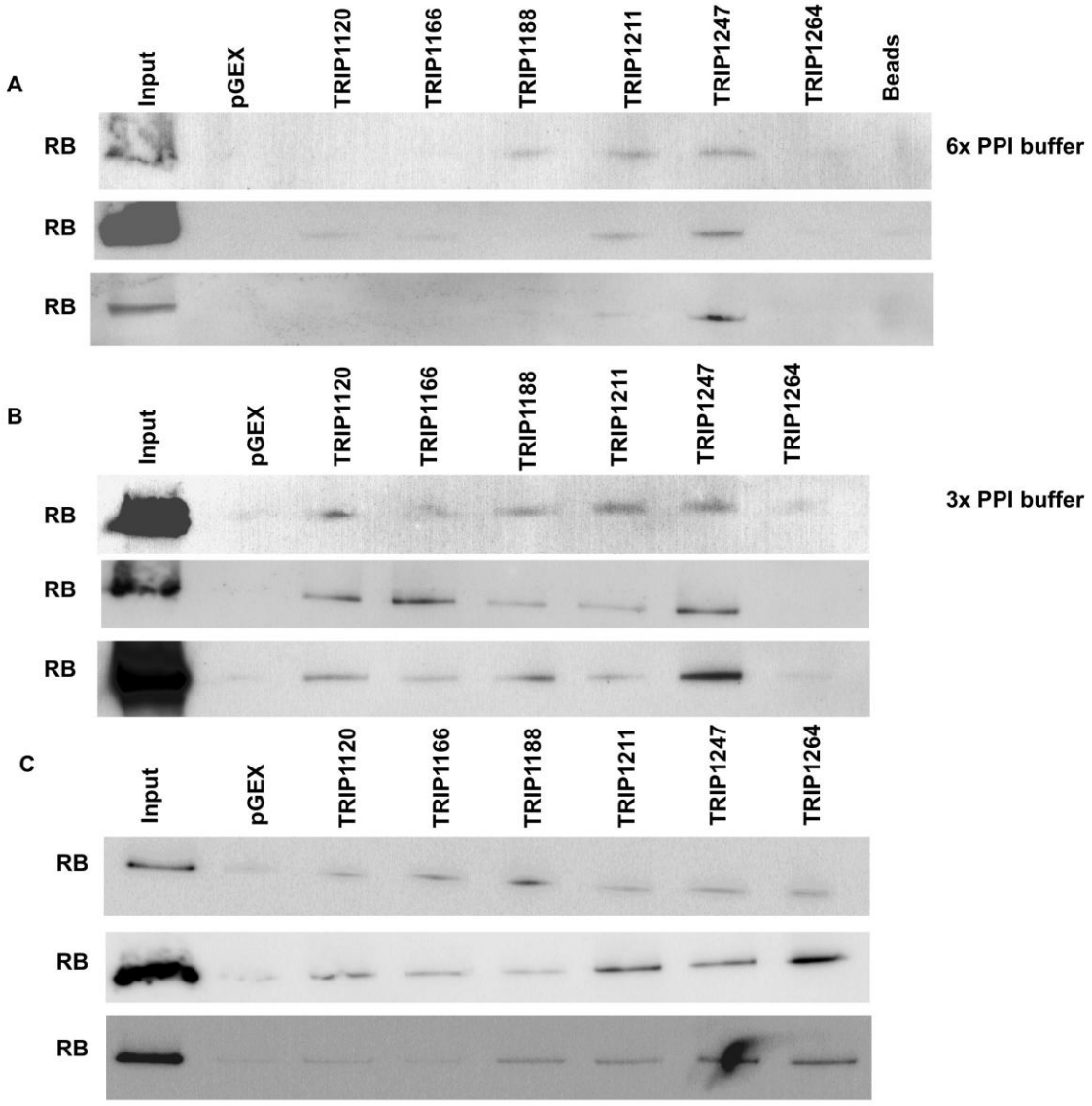

Figure 22. RB-interaction with TRIP230 deletion constructs demonstrates consistent interaction with the TRIP1247 construct

Pull-down experiments with GST-TRIP230 constructs were repeated using unbound GST protein mixed with either **(A)** MCF7 nuclear extract or **(B)** purified RB. **(C)** Pull-down experiments using purified RB were also repeated using 10 mM reduced glutathione to elute proteins from beads.

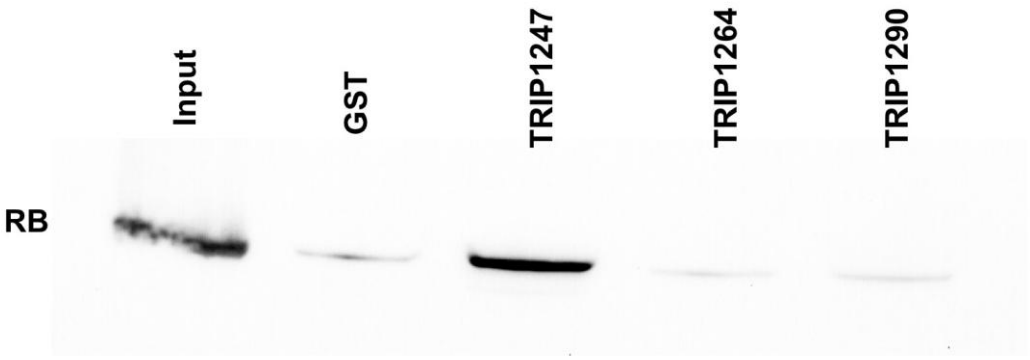

Figure 23. The TRIP230 RB-interaction domain does not extend past amino acid 1264

Pull-down experiments were repeated with MCF7 nuclear extract and the addition of a TRIP1290 construct, cloned and expressed as previously described.

4. Discussion

4.1. Establishing EMT in tumour cells is complex

The process of EMT was first recognized during development, where cells undergo a broad spectrum of changes to organize tissue and give rise to multiple components of the body. Parallels are now being observed between the molecular mechanisms required for EMT in development and those involved in invasion of tumour cells. This would suggest that tumour cells activate a similar EMT process to promote a more mobile cellular phenotype that supports invasion and metastasis into the surrounding tissue. While a number of *in vitro* studies have demonstrated that aberrant regulation of a small number of EMT-associated proteins in tumour cells promote a more invasive phenotype, observing similar patterns of aberrant regulation within human tumours does not conclusively demonstrate the occurrence of EMT (Hlubek *et al.*, 2001; Perl *et al.*, 1998; Janda *et al.*, 2002). Moreover, the ability to identify definitive markers of EMT has been proven difficult due to the genomic instability of tumour cells, thus the occurrence of EMT cannot be simply based on the deregulation of a few EMT-associated proteins alone.

It has been suggested that the acquisition of a metastatic phenotype in tumour cells may simply be a consequence of genomic instability, and not due to the intent activation of EMT. The accumulation of mutations may allow tumour cells to revert back to a de-differentiated state, rather than undergo a trans-differentiation process like EMT, resulting in a more mobile cellular phenotype (Klymkowsky and Savagner, 2009). While the genomic instability of tumour cells can lead to mutations within EMT-associated proteins, it is highly unlikely that mutation alone can account for all the necessary changes in cell morphology and gene expression patterns required for a more mobile phenotype.

It has been well established that external signals from within the tumour microenvironment heavily influence the metastatic potential of tumour cells. Growth factors such as TGF-β and FGF are secreted by neighbouring stromal cells, which activate signalling pathways in tumour cells similar to those required for EMT in development (Wang *et al.*, 2005; Romano *et al.*, 2000; Ciruna *et al.*, 2001; Boyer *et al.*, 1997). Hypoxia is one of many external signals within the tumour microenvironment that promotes tumour growth and invasion. Previous work has provided evidence to suggest that RB attenuates the HIF1-mediated hypoxic response, and that loss of RB supports a more invasive cellular phenotype in MCF7 human breast cancer cells. In this study, siRNA knockdown of RB in MCF7 cells was performed to identify HIF1 target genes involved in angiogenesis and EMT that are directly regulated by RB (Figures 7-10). This would help further characterize the invasive phenotype observed in MCF7 cells due to loss of RB, and ultimately provide insight into how the hypoxic response contributes to metastasis. The HIF1 target gene CXCR4 has been identified as an EMT-associated protein directly up-regulated following loss of RB in MCF7 cells exposed to hypoxia (Figure 9). It has previously been reported that exposure to hypoxia does not increase invasiveness in MCF7 cells (Lundgren *et al.*, 2009). Thus, this data provides a rationale for the increase in invasiveness observed in MCF7 cells exposed to hypoxia following loss of RB. However, to establish that CXCR4 is a contributor to the observed invasive phenotype, this data would have to be supported by a functional assay. Repeating the siRb knockdown invasion assay coupled with the knockdown of CXCR4 should decrease the observed invasive phenotype if either of these proteins are mediators of EMT. If no decrease in invasion is observed, then it is likely that CXCR4 alone is not sufficient for invasion to occur and requires other factors to drive EMT in MCF7 cells.

To demonstrate more conclusively that EMT is occurring, further characterization of the invasive phenotype in MCF7 cells is required. Monitoring for changes in expression of other epithelial cell markers, such as keratin, and other mesenchymal cell markers, such as fibronectin, N-cadherin and fibroblast surface protein 1, will strengthen the argument that loss of RB promotes a hypoxic-dependent EMT. Changes in expression of factors required to breakdown extra-cellular matrix components to facilitate invasion can also be assessed. An increase in factors such as MMPs or collagenase can be assessed using techniques such as in-gel zymography, an SDS-PAGE technique

that specifically detects for enzymatic degradation of a substrate co-polymerized with the polyacrylamide gel. An increase in extra-cellular matrix degradation factors following loss of RB in MCF7 cells would result in increased substrate degradation within the polyacrylamide gel that can be visualized by Coommassie staining.

Changes in cell polarity are another primary indicator of EMT, as epithelial cells have a distinct apical-basolateral polarization characterized by the asymmetric organization of cell surface molecules and the actin cytoskeleton. Additional immunohistochemical analysis should be performed to detect for changes in distribution of adherin junction proteins, such as cadherins and integrins, or organization of the actin cytoskeleton between siRB-treated MCF7 cells exposed to either normoxic or hypoxic conditions. If EMT is occurring as a result of RB knockdown, a hypoxic-dependent reorganization in cell polarity proteins should be observed.

4.2. Characterizing EMT through loss of E-cadherin

Decreased expression of E-cadherin is considered a hallmark of EMT, however, the detailed mechanisms involved with down-regulation of E-cadherin during EMT remains unclear. A decrease in E-cadherin expression in response to hypoxia has previously been reported in cancer cells, including human clear cell renal carcinoma (Esteban *et al.*, 2006), LNCaP human prostate cancer cells (Imani *et al.*, 2003), as well as in MCF7 cells (Lundgren *et al.*, 2009). In this study, expression of E-cadherin was monitored by Western blot to determine whether or not loss of E-cadherin was occurring in siRB-treated MCF7 cells. A slight decrease in E-cadherin was observed in cells exposed to 2% O_2 for 48 h, with no additional decrease observed in siRB knockdown samples (Figure 8). This was not expected since several HIF1 targets, such as snail, are direct repressors of E-cadherin expression. Lundgren and colleagues reported a significant decrease in E-cadherin expression in MCF7 cells, however, this was observed following a 72 h exposure to 0.1% O_2. Previously studies have reported that maximal expression of HIF1α occurs at 0.5% O_2, whereas half-maximal expression occurs between 1.5-2% O_2. It could be suggested that exposure to more severe hypoxic conditions may be required for greater loss of E-cadherin in siRB-treated MCF7 cells,

however, personal observation has not established a difference in HIF1α activity within MCF7 cells between 2% O_2 and 0.5% O_2 (Dr. Beishclag, personal communication)

Interestingly, a recent study reported observing an increase in mesenchymal markers Zeb1, fibroblast-specific protein 1 and slug, prior to observing a decrease in E-cadherin within a pancreatic cancer mouse model (Rhim *et al.*, 2012). This would suggest that perhaps siRB-treated MCF7 cells require exposure to hypoxia for a period longer than 48 h to observe a greater loss in E-cadherin expression. Moreover, it appears as though only a small subset of cells within a tumour metastasize, since not every cell within a tumour has the same genetic background and is exposed to the same external environment. Thus, observing a decrease in markers that are typically expressed in MCF7 cells, like E-cadherin, would be more difficult if EMT is only occurring within a small population of cells, compared to observing an increase in markers not typically expressed, like vimentin. A longer exposure to hypoxia may then provide more time for additional cells to under EMT and allow for a greater decrease in E-cadherin expression to occur.

Clinical data has not consistently observed a loss of E-cadherin with aggressive forms of breast cancer, which makes characterizing EMT through loss of E-cadherin even more complex. Some studies have recognized an association between aberrant E-cadherin expression and a more advanced staged tumour for breast cancer (Rasbridge *et al.*, 1993, Oka *et al.*, 1993), whereas others have observed no such correlation (Bukholm *et al.*, 1997). MCF7 cells used for this study originate from the ductal epithelial cells of breast tissue, which normally express E-cadhrin. Interestingly, loss of E-cadherin has been reported more frequently with infiltrating lobular carcinoma compared to invasive ductal carcinoma (Moll *et al.*, 1993). Thus, taking all these factors into consideration, a minimal decrease in E-cadherin expression observed in siRB-treated MCF7 cells exposed to hypoxia does not necessarily imply that an EMT-like process is not occurring. Additional experiments, such as those described above, will provide further insight into the mechanisms driving invasion in MCF7 cells following loss of RB.

4.3. Establishing a general mechanism for HIF1-mediated tumour progression processes regulated by RB

Expression of HIF1 target genes were assessed following loss of RB in other human cancer cell lines exposed to hypoxia, to determine whether or not the role of RB in the hypoxic response was exclusive to MCF7 cells. A hypoxic dependent increase in VEGF and CXCR4 expression was observed in both MDA-MB-231 and LNCaP cells, as well as in vimentin expression in LNCaP cells (Figure 11). While these results are similar to those observed in MCF7, more data is required to conclusively demonstrate a role for RB in the HIF1-mediated hypoxic response within these cell lines. There are several factor that may render it difficult to establish a general mechanism for HIF1-mediated invasion and metastasis regulated by RB. Since the genetic background of each tumour is so diverse, not every cancer will express the same signature of HIF1 target gene or will have the same requirements for inducing cell invasion. For example, in contrast to MCF7 cells, MDA-MB-231 cells do not express E-cadherin but continuously express vimentin, however, exposure to hypoxia alone does not change their invasive potential (Lombaerts *et al.*, 2006; Lundgren *et al.*, 2009). This would suggest that additional genetic alterations may be required to enhance the hypoxic response of MDA-MB-231 cells and increase migration. Interestingly, MDA-MB-231 cells do express RB, which may be involved in regulating process for cell invasion. To test this, the invasive potential of MDA-MB-231 cells in response to hypoxia following loss of RB could be assessed using a matrigel invasion assay.

Not all cells respond to hypoxia in the same fashion, adding an additional level of complexity when establishing loss of RB under hypoxia as a general mechanism for tumour progression (Chi *et al.*, 2006). Oxygen tension and threshold response to hypoxia vary among cells since not every cell is exposed to the same concentration of oxygen. For example, cells within the lung are exposed to far greater concentrations of oxygen compared to cells within the bone marrow (Simone and Keith, 2008). Moreover, inappropriate activation of the hypoxic response may also result from pre-existing genetic or epigenetic alterations. For example, mutations within the VHL tumour suppressor protein in cancer cells is common, resulting in the hypoxic-independent activation of HIF1-mediated transcription (Zhong *et al.*,1999; Maxwell *et al.*, 1999). Thus, cells that express a non-functional VHL protein, such as ECC-1 human endometrial

cancer cells, will have a decreased hypoxic response (Beischlag lab, unpublished data). Conversely, cells mutant for the p53 tumour suppressor protein and estrogen receptor-negative breast cancer cells have increased hypoxia sensitivity.

4.4. The role of RB in HIF1 transcriptional regulation

Results from this study provide additional support to the hypothesis that RB attenuates the HIF1-mediated transcriptional response through a direct interaction with TRIP230. RB is recruited to the HRE regions of HIF1 target gene in a hypoxic dependent manner and loss of RB leads to the significant up-regulation of HIF1 target genes VEGF and CXCR4 (Appendix D; Figure 7; Figure 9). Moreover, both Hepa-1 and MCF7 cells transfected with RB and TRIP230 showed a significant decrease in HRE reporter gene activity, compared to cells transfected with RB and a TRIP230 mutant lacking the RB-interaction domain (Appendix B). A previous study reported that RB interacts with the HIF1 complex through HIF1α to enhance transcription (Budde *et al.*,2003). RB is involved with the transcriptional activation of genes required for muscle and macrophage differentation, however, RB does not associate directly with regulatory regions of target genes to enhance transcription (Iavarone *et al.*, 2004; Puri *et al.*, 2001). Rather, RB facilitates transcriptional activation by releasing other factors that do associate with regulatory regions of target genes. Budde and colleagues based their conclusions solely on luciferase assays performed with a constitutively active RB expression plasmid. Factors required for transcription that are over-expressed in cells behave differently when initiating transcription within a reporter gene assay. Moreover, these observations were made under normoxic conditions, when HIF1α does not associate with the regulatory of target genes, which makes these results difficult to interpret.

Another study demonstrated that loss of RB contributes to EMT in a hypoxic-independent manner in MCF7 cells (Arima *et al.*, 2008). Loss of RB in MCF7 cells produced a morphological change towards a fibroblast-spindle shape, which is characteristic of EMT, and re-organized the actin filaments to produce filapodia-like and lamellipodia-like structures. Moreover, RB promotes up-regulation of E-cadherin through an associated with E-cadherin promoter, and loss of RB resulted in the translocation of

E-cadherin and β-catenin from the cell membrane towards the cytoplasm. Since E-cadherin is not a HIF1 target gene, this would suggest that RB is functioning through a different transcription factor. EMT can be initiated through signals other than hypoxia, such as TGF-β or FGF signaling, thus loss of RB could regulate other factors that influence EMT in a hypoxic-independent manner.

4.5. Existence of an ARNT-TRIP230-RB multi-meric complex supports a role for RB in HIF1-mediated transcription

Initial studies have demonstrated that TRIP230 and RB are recruited to the regulator regions of HIF1 target genes (Appendix D). Moreover, RB attenuates HRE-reporter gene activity through a direct interaction with TRIP230 (Appendix B). TRIP230 has previously been identified as an ARNT interacting protein, however it had yet to be established whether ARNT, TRIP230 and RB exist as part of a multi-meric complex (Beischlag *et al.*, 2004). This would provide additional evidence supporting RB as an attenuator for HIF1-mediated transcription. A GST pull-down assay revealed the simultaneous pull-down of TRIP230 and RB with a GST-PAS B fusion moitey, which suggests the existence of a ARNT-TRIP230-RB mutlimeric complex (Figure 12G). This alone, however, does not clearly demonstrate relationship between TRIP230 and RB interaction within this complex. While it has previously been demonstrated that RB is not an ARNT interacting protein, this does not rule out the possibility that a protein other than TRIP230 is involved RB recruitment to ARNT (Elferink *et al.*, 2001). Addition of an ARNT-interacting peptide reveals that RB association with GST-PAS B is lost when TRIP230 interaction with GST-PAS B is blocked (Figure 13). If an additional protein was involved with RB association to ARNT, the GST-PAS B would still be capable of pulling down RB (Figure 24). This now demonstrates that TRIP230 is required for RB recruitment to ARNT, thus establishing the existence of an ARNT-TRIP230-RB multi-meric complex.

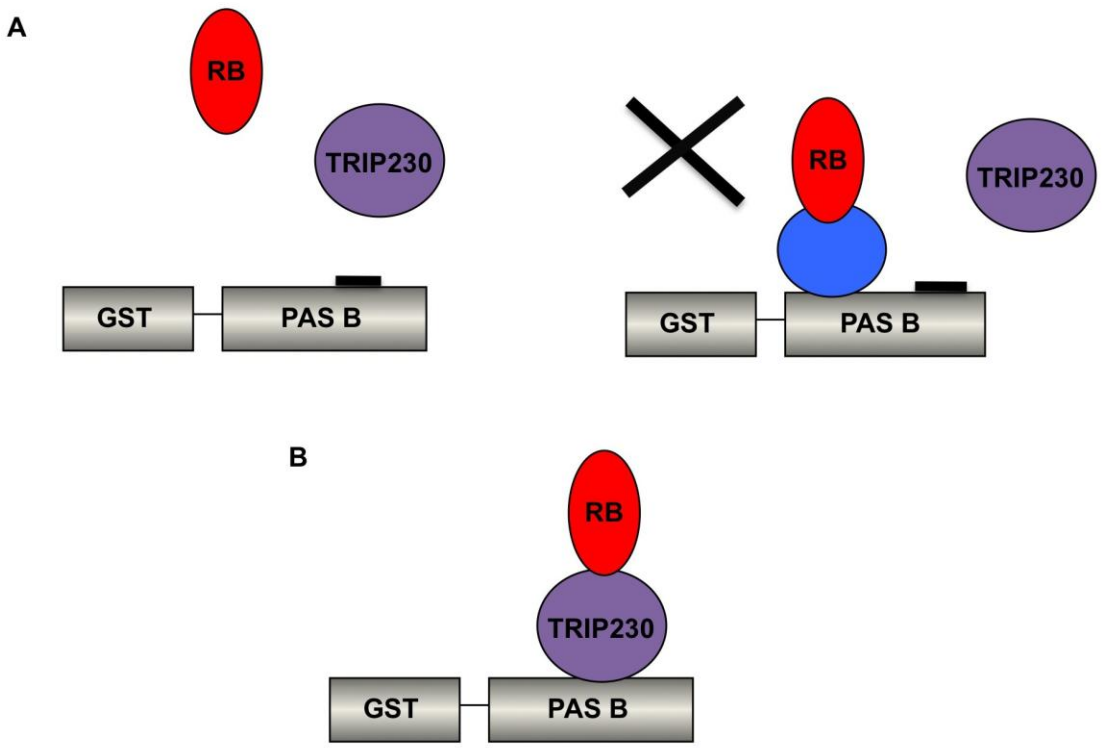

Figure 24. TRIP230 is required for RB recruitment to ARNT

A GST-PAS B construct is capable of pulling down both RB and TRIP230, however, this does not clearly establish the relationship between TRIP230 and RB interaction within this complex. (A) Addition of an ARNT-interacting peptide capable of disrupting the GST-PAS B-TRIP230 interaction blocks recruitment of RB to GST-PAS B. This eliminates the possibility of an additional protein involved in RB recruitment, as RB interaction would still be maintained with GST-PAS B. (B) Altogether, this establishes the existence of an ARNT-TRIP230-RB multi-meric complex.

4.6. Establishing an ARNT-interacting peptide as an inhibitor of the HIF1-mediated hypoxic response

TRIP230 has been established as a necessary co-activator for HIF1 transcription through an interaction with ARNT (Beischlag *et al.*, 2004). Presumably, a decrease in the HIF1-mediated hypoxic response could be achieved by disrupting the TRIP230-ARNT interaction. This study demonstrates that an ARNT-interacting peptide is capable of disrupting ARNT-TRIP230 interaction, causing a dissociation of RB-interaction (Figures 11). This peptide can now be applied to cell culture to demonstrate if loss of TRIP230-ARNT interaction can decrease the HIF1-mediated hypoxic response *in vivo* following loss of RB. It would be expected that disrupting this interaction in siRB treated MCF7 cells would decrease CXCR4 and VEGF expression under hypoxia. SiRNA knockdown of TRIP230 has already demonstrated a decrease in hypoxic induction of VEGF expression (Beischlag *et al.*, 2004). Moreover, applying this peptide to siRB treated MCF7 cells exposed to hypoxia decreases invasion in a matrigel invasion assay (Appendix E). Thus, it is expected that the TRIP230-interacting peptide will produce a similar cellular phenotype as observed following loss of RB. This peptide can now be applied to animal models for the potential development of a novel therapy to inhibit tumour progression.

Two TRIP230-interacting peptides were applied to the GST-PAS B pull-down assay, but were not successful at disrupting the ARNT-TRIP230 interaction (Figure 11C). Each peptide blocked a previously identified essential amino acid in ARNT required for TRIP230-ARNT interaction (Partch *et al.*, 2009). These essential amino acids are not a part of a previously defined protein-protein interaction motif, unlike the LNRNHLL motif within TRIP230, which is a variant of the LXXLL motif. Mutating these essential amino acids only decreased interaction, rather than eliminate interaction completely, which may explain why these peptides did not disrupt ARNT-TRIP230 interaction. Alternatively, there may have been not enough amino acids within the peptide to form a stable interaction with ARNT. Nonetheless, future studies conducted to assess the consequences of disrupting the ARNT-TRIP230 interaction on HIF1-mediated transcription will be conducted using the ARNT-interacting peptide.

4.7. Establishing the C-terminal portion as the minimal RB-interaction domain of TRIP230

Using a set of GST fusion moieties containing successive deletion mutants of the TRIP230 RB-interaction domain, consistent interaction was observed with the 1247 construct and suggets that minimal RB-interaction domain is within amino acids 1247 and 1255 (Figure 22A-B). Additionally, there was a consistent interaction observed with the TRIP1264 negative control, which was a cause of concern (Figure 22C). To confirm if the minimal RB-interaction domain is within amino acids 1247 and 1255, the pull-down could be repeated using a GST-fusion moiety containing a mutant RB-interaction domain lacking the unique amino acids found within the TRIP1247 construct. If the TRIP1247 construct does contain the true minimal RB-interaction domain, then a disruption in TRIP230-RB-interaction would be expected. Alternatively, the pull-down could be repeated using a peptide that specifically interacts with the unique amino acids found within the TRIP1247 construct.

The purpose of identifying the essential amino acids required for TRIP230-RB-interaction was to eventually make a transgenic mouse model that expresses a TRIP230 mutant for the specific amino acids required for RB-interaction. This mouse will then be crossed with an existing brain cancer mouse model, the *Nfl;p53 cis-/+* mouse, or breast cancer mouse model, the MMTV-PyMT^{634Mul}, to investigate whether disrupting TRIP230-RB-interaction increases the progression and hyper-vascularization of tumours. The advantage of using these models is that they are both responsive to hypoxia and the occurrence of tumour progression is modest (Kalliomaki *et al.*, 2008). Thus, an increase in the progression of tumours under a TRIP230 mutant background would be more easily detected. Altering the specific amino acids required for TRIP230-RB-interaction would minimally disrupt the endogenous structure and function of TRIP230. If the minimal interaction domain cannot be identified, a transgenic mouse model could also be constructed with a TRIP230 mutant lacking the entire RB-interaction domain. A blast search performed on the RB-interaction domain identified homology with other centromere interacting proteins, which suggests that this region may also function in centromere positioning. Thus, removal of the entire RB-interacting domain may interefere with other TRIP230 functions. Nonetheless, it could still demonstrate

increased invasiveness of tumour cells and ultimately help establish a role for RB in the hypoxic response under a breast or brain cancer mouse model.

RB contains a characteristic 'pocket' region, comprised of an A domain and a B domain, that mediates interaction with other proteins, including E2F transcription factors. The shallow grooves within the B domain interact with an LXCXE motif found within a majority RB-interacting protein, where the X donates any amino acid (Singh *et al.*, 2005). The L and the C typically form H-bonds through their backbone carbonyl group, where the E forms an H-bond through its OH group in its R group. Surrounding amino acids also contribute H-bonds and van der waals forces to the interaction, but are not required. While the LXCXE is conserved in several proteins, there are some LXCXE-like motifs that can also interact with RB. They include the LXFXE motif of tax, the LXSXE motif of protein phosphatase 1 (PP1), and the IXCXE motif of HDAC1 (Singh *et al.*, 2005; Kehn *et al.*, 2005; Dunaief *et al.*, 2002). Other RB-interacting proteins, like plasminogen activator inhibitor-2, contain a PENF motif, however, this domain typically interacts with C-domain outside of the pocket region (Singh *et al.*, 2005).

A yeast two-hybrid assay demonstrated that TRIP230 primarily interacts with the A and B domains of RB's 'pocket' region (Chang *et al.*, 1997). TRIP230 does not contain a conserved LXCXE domain and a blast search did not reveal sequence similarity with any proteins of similar function. It has been observed that the LXCXE and LXCXE-like motifs follow a consensus hydrophobicity pattern: hydrophobic-hydrophobic-hydrophobic-hydrophilic-hydrophilic. Within the TRIP1247 construct is a VLVDSD region, where the LXCXE-like motif could potentially be LXDXD. This follows the pattern of hydrophobic-hydrophobic-hydrophobic-hydrophilic-hyrdophilic-hydrophilic, which is very similar, although not exact, to the consensus hydrophobicity pattern. If the subsequent experiments described above can demonstrate that TRIP1247 does in fact contain the minimal interaction domain, alanine scanning will confirm whether or not these are the essential amino acids required for interaction.

5. Concluding remarks

Research has begun to elucidate the molecular mechanisms behind the adaptive response to changes in cellular oxygen concentration, and the HIF1 transcription factor complex has been identified as an essential regulator of oxygen homeostasis. Previous work has demonstrated a role for RB as an attenuator for the HIF1 hypoxic response (Appendix A-D). Furthermore, loss of RB contributes to a more invasive phenotype that promotes invasion in MCF7 human breast cancer cells (Appendix C). In an effort to further characterize this invasive phenotype, siRNA knockdown of RB in MCF7 cells exposed to hypoxia has identified CXCR4 and VEGF as HIF1 target genes involved in metastasis directly regulated by RB (Figure 7 and 9). Moreover, a concomitant increase in vimentin and decrease in E-cadherin was also observed, which reflects the occurrence of EMT (Figure 8 and 10). Similar results were observed in MDA-MB-231 and LnCAP cells, suggesting that the role of RB in HIF1-mediated transcription is not exclusive to MCF7 cells (Figure 11). While additional experiments are required to further establish the occurrence of EMT following loss of RB, these results uncover a potential role for RB in the HIF1 hypoxic response that contributes to increased invasiveness of cancer cells.

Previous characterization of TRIP230 has established it as an essential co-activator for HIF1 transcriptional activity and functions through a direct interaction with the ARNT subunit (Appendix B). An additional focus of this study was on the specific protein-protein interactions within the HIF1 complex, and how these interactions contribute to metastasis. The results from a GST pull-down assay in this study revealed the simultaneous interaction of TRIP230 and RB with the PAS B domain of ARNT (Figure 12G). Furthermore, disrupting the ARNT-TRIP230 interaction with an ARNT-interacting peptide blocks RB-interaction association with ARNT, which establishes the existence of an ARNT-TRIP230-RB multi-meric complex and demonstrates a possible mechanism for inhibiting the HIF1-mediated hypoxic response that can serve as the basis for an anti-cancer therapy (Figure 13).

Lastly, a more comprehensive analysis was performed on the specific requirements for the TRIP230-RB interaction. Consistent RB interaction was observed between amino acids 1247 and 1255 of the TRIP230 RB-interaction domain, however, additional experiments are required to confirm these results (Figure 21). Nonetheless, this opens up the possibility for creating a TRIP230-mutant transgenic mouse that does not interact with RB, to further establish the role of RB in hypoxic-dependent mechanisms of tumour progression within an animal model.

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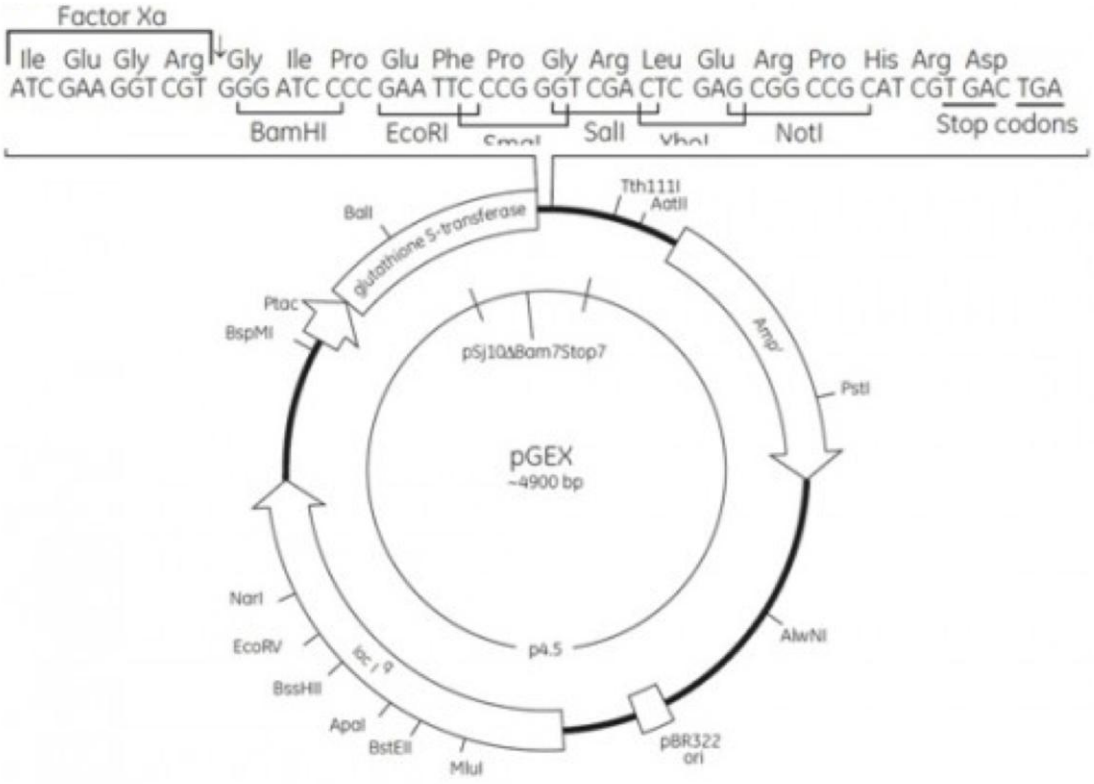
Appendices

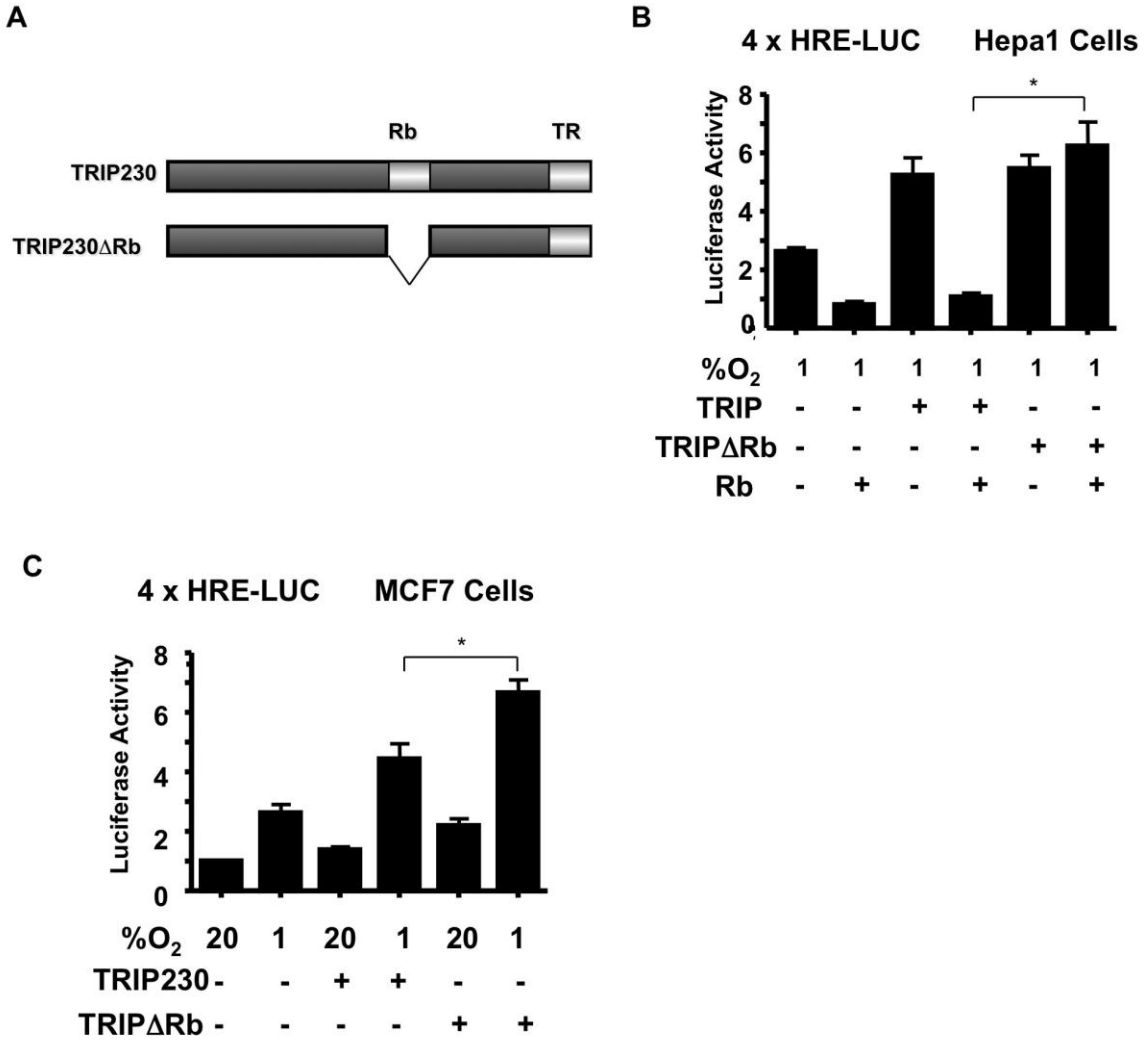
Appendix A.

Multiple cloning site of the pGEX-5X-1 vector.



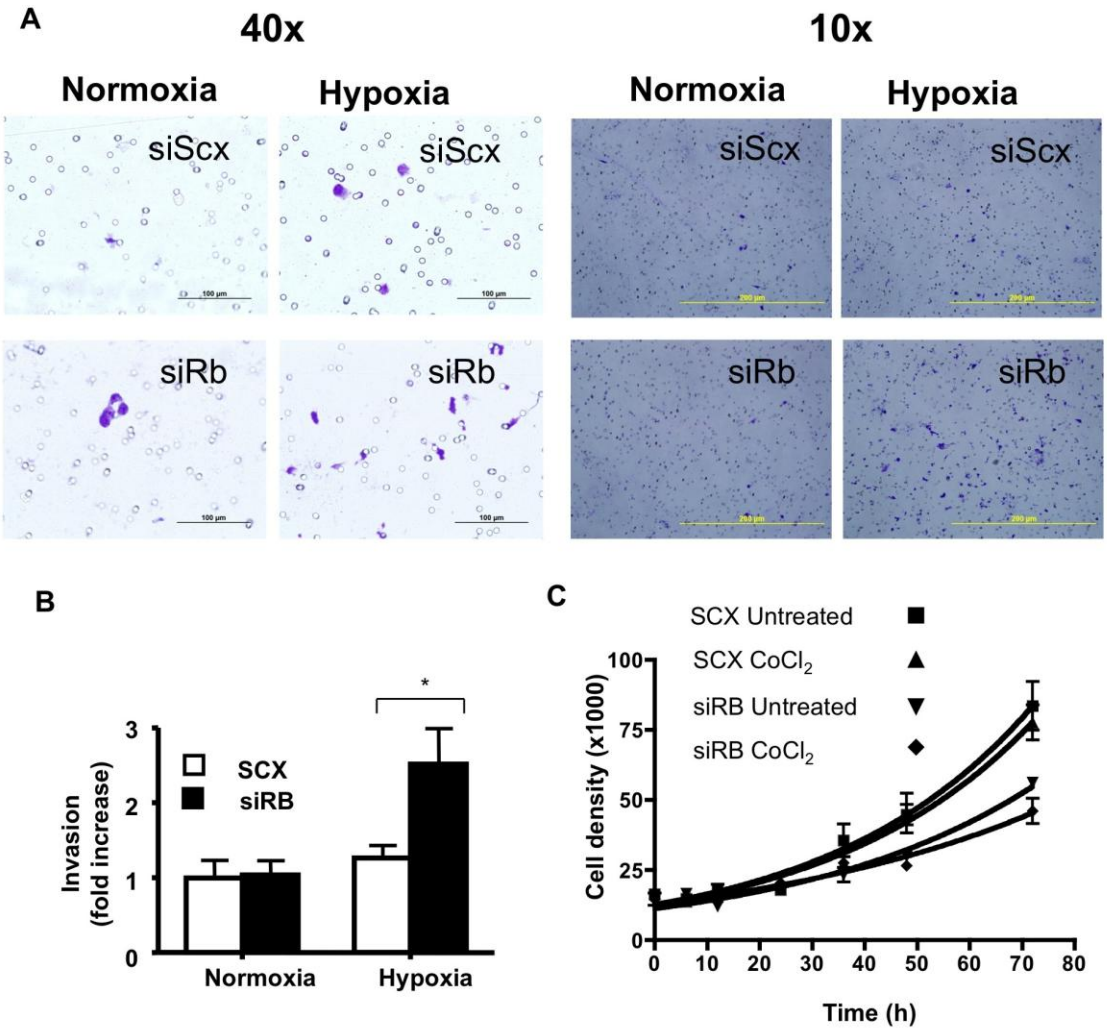
Appendix B.

RB attenuates TRIP230-mediated co-activation of ARNT-dependent transcriptional activity. (A) A schematic of the TRIP230ΔRB mutant. **(B)** RB-negative Hepa-1 cells and **(C)** RB-positive MCF7 cells were transfected with a hypoxia responsive 4xHRE-driven luciferase construct as a reporter and subjected to 1% O_2 for 24 h. Whole cell lysates were assayed for luciferase activity. *p<0.05. (This work was completed by other members of the Beischlag lab and used with permission from Dr. Timothy Beishclag)



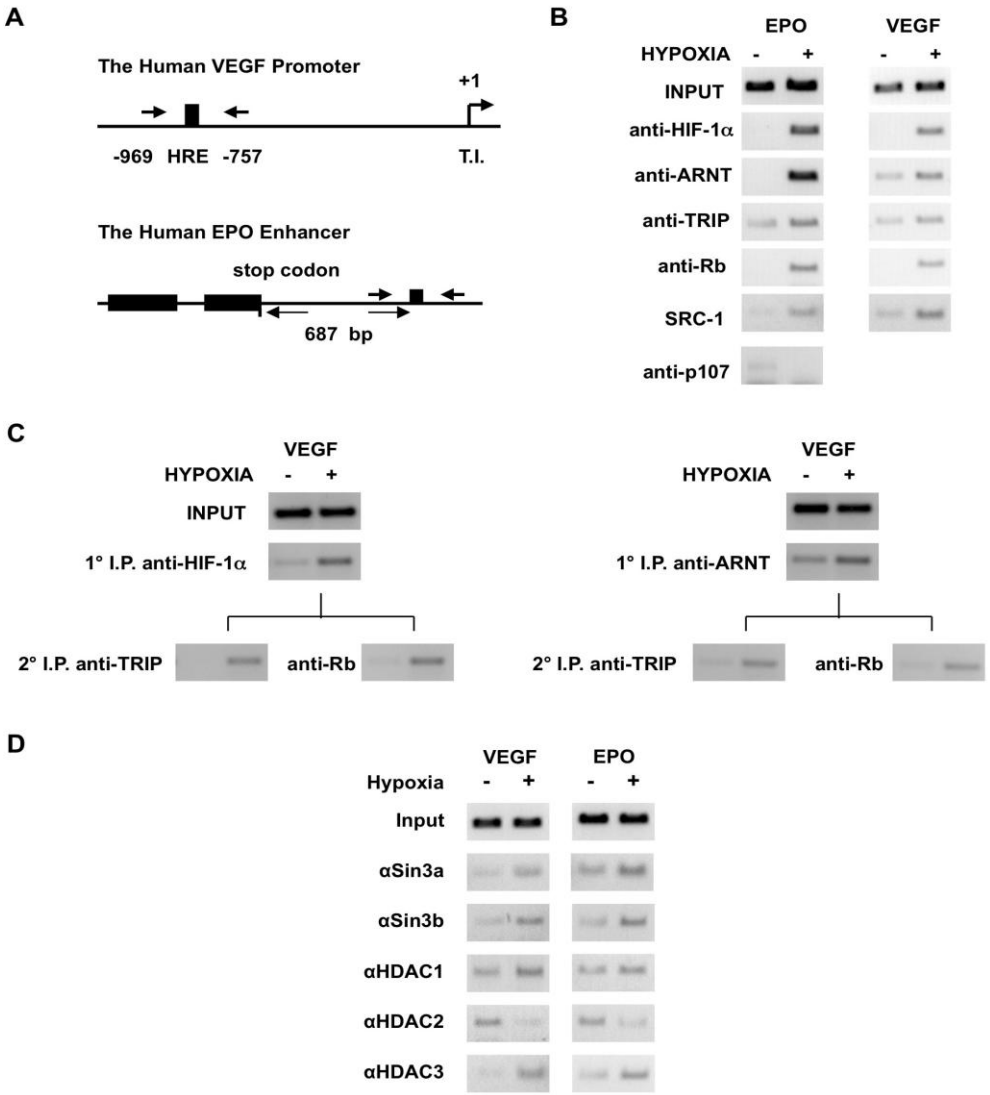
Appendix C.

Loss of RB promotes hypoxia-dependent invasiveness in MCF7 cells in a matrigel invasion assay. MCF7 cells were transfected with siSCX or siRB as described in the Materials and Methods section. Twenty-four hours after siRNA transfection, the cells were trypsinized, resuspended and subjected to the matrigel invasion assay. The plates were incubated in normoxia (20%O_2) or hypoxia (2% O_2) at 37^oC for 24 h **(A)** Photomicrographs of matrigel-embedded MCF7 cells and **(B)** numerical representation of relative invasion under varying conditions. **(C)** Knock-down of RB in MCF7 cells does not alter cell proliferation in response to hypoxia. Cells were transfected with siRNA as described above. Twenty-four h after transfection, cells were treated with vehicle or 100 µM $CoCl_2$ to activate HIF1α. * $p < 0.01$. (This work was completed by other members of the Beischlag lab and used with permission from Dr. Timothy Beishclag)



Appendix D.

HIF1α, ARNT, TRIP230, RB and RB-associated repressor proteins occupy hypoxia responsive regulatory regions of HIF1-regulated genes is a hypoxia-dependent fashion. (A) A schematic of the VEGF proximal promoter and EPO enhancer and the placement of oligonucleotides used for PCR amplification. **(B)** The status of HIF1α, ARNT, TRIP230, and RB at the VEGF promoter and EPO enhancer in MCF7 cells as assayed by chromatin immunoprecipitation assay (ChIP) and PCR. **(C)** Sequential ChIP of the proximal VEGF promoter and EPO enhancer using either anti-HIF1α or anti-ARNT affinity purified antibodies followed by immunoprecipitation with anti-TRIP230 and anti-RB antibodies. **(D)** ChIP of RB-repressor complex proteins in MCF7 cells. (This work was completed by other members of the Beischlag lab and used with permission from Dr. Timothy Beishclag)



Appendix E.

An ARNT-interacting peptide inhibits siRB-mediated hypoxia-dependent invasiveness in MCF7 cells in a matrigel invasion assay. MCF7 cells were transfected with siSCX or siRB and the TRIP1584 ARNT-interacting peptide. Twenty-four hours after siRNA transfection, the cells were trypsinized, resuspended and subjected to the matrigel invasion assay. The plates were incubated in normoxia (20%O_2) or hypoxia (2% O_2) at 37^{o}C for 24 h. The graph represents a numerical representation of relative invasion under varying conditions. (This work was completed by other members of the Beischlag lab and used with permission from Dr. Timothy Beishclag)