The Role of the Retinoblastoma Protein on Hypoxia-Inducible Factor Dependent Tumor Cell Transformation: Microarray Validation

by

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Abstract

Intratumoral hypoxia results in tumour cell adaptations mediated by the hypoxia inducible factor 1-α (HIF1-α) and its dimerization partner the aryl hydrocarbon receptor nuclear translocator (ARNT). This process is attenuated by the retinoblastoma protein (Rb) via its association with the thyroid hormone receptor/retinoblastoma interacting protein (TRIP230). This study's aim was to examine the role of Rb on HIF1 tumour cell transformation. By interrogating the transcriptome of human MCF-7 and LNCaP cells using gene expression microarrays, we developed a list of 21 common HIF1 target genes further up-regulated following loss of Rb. Real-time PCR, immuno-blotting and immuno-cytochemistry were used to validate mRNA and protein levels of genes. Wound healing assays were used to measure cell migration following loss of Rb and hypoxia. Results show loss of Rb exacerbates the expression of HIF1 genes associated with neuroendocrine differentiation; however no change in cell migration was observed.

Keywords: hypoxia; hypoxia inducible factor; retinoblastoma protein; TRIP230; tumour cell; transformation; invasion; neuroendocrine differentiation

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To my Family who taught me to laugh, and dream big

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Table of Contents

Approval ii
Abstract iii
Dedication iv
Acknowledgements v
Table of Contents vi
List of Tables viii
List of Figures ix
List of Acronyms x

Chapter 1. Introduction 1
1.1. Tumour microenvironment 3
1.1.1. Oxygen Homeostasis and Hypoxia 3
1.2. Basic Helix-Loop-Helix and Hypoxia-Inducible Factor 4
1.2.1. HIF1-α 6
1.2.2. HIF2-α and HIF3-α 9
1.2.3. HIF1-β/ARNT 10
1.3. TRIP230 11
1.4. The Retinoblastoma Protein 12
1.4.1. Retinoblastoma protein and HIF1 13
1.5. Epithelial to Mesenchymal Transition and Neuroendocrine Differentiation 14
1.5.1. EMT 14
1.5.2. NED 15
1.6. Objective of the study 16
1.6.1. Specific aims 17

Chapter 2. Materials and Methods 19
2.1. Cell Culture 19
2.2. Microarray Collection and Analysis 19
2.3. Reverse Transcription and Quantitative Real-Time PCR 20
2.3.1. Hypoxia Treatment 20
2.3.2. RNA Extraction 21
2.3.3. Reverse Transciption PCR 21
2.3.4. Quantitative Real-Time PCR 22
2.3.5. Statistical Analysis 23
2.4. Immuno-blotting 23
2.4.1. Hypoxia Treatment 23
2.4.2. Whole Cell Lysate Collection and Protein Quantification 24
2.4.3. Immuno-blotting 25
2.5. Immuno-cytochemistry 26
2.5.1. Slide Preparation and Hypoxia Treatment 26
2.5.2. Immuno-staining 26
2.6. Wound Healing Assay 27

Chapter 3. Results **28**
3.1. Determination of hypoxia inducible genes further regulated by Rb in MCF-7 and LNCaP cells 28
3.2. MCF-7 Microarray Gene Validation 30
3.2.1. Assessment of MCF-7 shSCX and shRb cells gene expression following hypoxia 32
3.2.2. Translational validation of genes up-regulated in MCF-7 shSCX and shRb cells at various time points in a hypoxic environment 35
3.2.3. Assessment of MCF-7 shSCX and shRb mouse tumour cells 38
3.3. LNCaP microarray gene validation 41
3.3.1. Assessment of LNCaP shSCX and shRb gene expression following exposure to hypoxia 41
3.3.2. Translational validation of the genes up-regulated in LNCaP shSCX and shRb cells following exposure to various time points of hypoxia 43
3.3.3. Loss of Rb and hypoxia causes morphological changes in LNCaP cells. 48
3.4. The role of Rb and hypoxia on tumour cell migration 49

Chapter 4. Discussion **53**

Chapter 5. Conclusion **62**

References **64**
Appendix A. MCF-7 top 30 up-regulated genes induced greater than 2-fold by a combination of loss of Rb and hypoxia when compared to negative controls. 80
Appendix B. LNCaP top 30 up-regulated genes induced greater than 2-fold by a combination of loss of Rb and hypoxia when compared to negative controls. 82
Appendix C. Supplementary image for Figure 3.4. 84
Appendix D. Supplementary images for Figure 3.6. 85
Appendix E. Supplementary images for Figure 3.12. 86
Appendix F. MCF-7 gene ontology analysis showing key networks, molecular, cellular and physiogical functions and top transcripton factors regulated by hypoxia and Rb loss. The top list (A.) shows the pathways that are regulated by genes up-regulated under hypoxia and Rb loss. The bottom list (B.) shows the pathways that are regulated by genes down-regulated under hypoxia and Rb loss. 87
Appendix G. LNCaP gene ontology analysis showing key networks, molecular, cellular and physiogical functions and top transcripton factors regulated by genes that are up-regulated under hypoxia and Rb loss. 89

List of Tables

Table 2.1 List of Oligos used for MCF-7 and LNCaP Quantitative PCR 22

Table 2.2 List of 1° Ab's used for MCF-7 immuno-blotting 25

Table 2.3 List of 1° Ab's used for LNCaP immuno-blotting 25

Table 3.1 LNCaP and MCF-7 common list of up-regulated genes under hypoxia, and following loss of Rb. 29

List of Figures

Figure 1.1 A schematic representation of HIF1-α structure and post-translational modification under normoxic conditions 8

Figure 1.2 Schematic representation of HIF1-β/ARNT structure 10

Figure 1.3 The role of Rb in HIF1 mediated gene transcription 18

Figure 3.1 Schematic illustration of the network of genes with modified expression under hypoxia and loss of Rb in MCF-7 cells. 31

Figure 3.2 Schematic illustration of the network of genes with modified expression under hypoxia and loss of Rb in MCF-7 cells. 32

Figure 3.3 Loss of Rb together with hypoxia in MCF-7 cells results in an increased expression of genes involved in cell invasion, and differentiation. 34

Figure 3.4 Loss of Rb and hypoxia leads to an increase in S1PR4 and NDRG1 protein level in MCF-7 cells. 36

Figure 3.5 Exposure to hypoxic condition causes a decrease in E-cadherin protein level. 37

Figure 3.6 Exposure to hypoxia causes a decrease in LOXL2 protein levels. 38

Figure 3.7 Hypoxia and Rb loss results in changes in CXCR4 and S1PR4 protein levels in CB-17 SCID female mice. 40

Figure 3.8 Hypoxic conditions and loss of Rb in LNCaP cells results in an increased expression level of genes involved in metastasis and neuroendocrine differentiation. 42

Figure 3.9 Loss of Rb increases protein levels of HIF1 target genes involved in metastasis and neuroendocrine differentiation. 44

Figure 3.10 Exposure to hypoxic conditions causes a decrease in E-cadherin protein level. 45

Figure 3.11 KISS1R expression increases following loss of Rb and exposure to hypoxia. 47

Figure 3.12 Loss of Rb together with exposure to hypoxic conditions leads to morphological changes in LNCaP cells. 49

Figure 3.13 Loss of Rb and hypoxia does not have an affect on LNCaP migration ability 51

Figure 3.14 Hypoxia and loss of Rb does not play a large role in MCF-7 migration ability 52

List of Acronyms

36B4	Ribosomal protein 36B4
AHR	Aryl hydrocarbon
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASCL2	Achaete scute-like bHLH transcription factor 2
ATP	Adenosine triphosphate
bHLH	Basic helix loop helix
BRCA1	Breast cancer 1 early onset
BRCA2	Breast cancer 2 early onset
C-TAD	C-terminal transactivation domain
CDK	Cyclin dependent kinase
CH1	Cysteine/histidine-rich 1
CO_2	Carbon dioxide
CXCR4	Chemokine receptor 4
DNA	Deoxyribonucleic acid
EMT	Epithelial to mesenchymal transition
ENO1	Enolase 1
ENO2	Enolase 2
EPAS-1	Endothelial PAS domain protein -1
EPO	Erythropoietin
FIH-1	Factor inhibiting HIF1
FSH	Follicle stimulating hormone
GAL3ST1	Galactose-3-O-sulfontransferase
GLUT1	Glucose transporter 1
GMAP-210	Golgi microtubule associated protein 210
HIF	Hypoxia inducible factor
HIF(1,2,or 3)-α	Hypoxia inducible factor (1,2, or 3)-α
HIF1	Hypoxia inducible factor 1
HIF1-β	Hypoxia inducible factor 1-β
HRE	Hypoxia response element

HRF	HIF related factor
HTR5A	5-hydroxytryptamine receptor 5A
IGF-1	Insulin like growth factor 1
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IPA	Ingenuity pathways assist
IPAS	Inhibitory PAS domain protein
KISS1R	Kisspeptin receptor
KP	Kisspeptin
LH	Luteinizing hormone
LNCaP	Lymph node carcinoma of the prostate
LOXL2	Nuclear associated lysyl hydroxylase 2
MCF-7	Michigan cancer foundation 7
MET	Mesenchymal to epithelial transition
mRNA	Messenger ribonucleic acid
N-TAD	N-terminal transactivation domain
NDRG1	N-myc downstream regulated 1
NE	Neuroendocrine
NED	Neuroendocrine differentiation
NFκB	Nuclear factor-κ B
O_2	Oxygen
ODD	Oxygen dependent degradation domain
PAS	Period-Aryl hydrocarbon receptor nuclear translocator-Single minded
PDGF	Platelet derived growth factor
PER	Period
PH	Prolyl hydroxylase
PHD	Prolyl hydroxylase domain
PLOD2	Procollagen-lysine 2-oxoglutarate 5-dioxygenase 2
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
S1PR4	Sphingosine 1 phosphate receptor 4
SCX	Scrambled

sh	Short hairpin
SIM	Single minded protein
STC1	Stanniocalcin 1
T3	Triiodothyronine
TAD	Transactivation domain
TGF-β	Transforming growth factor β
TNFα	Tumour necrosis factor α
TR	Thyroid hormone receptor
TRIP230	Thyroid hormone receptor/retinoblastoma interacting protein
VEGF	Vascular endothelial factor
vHL	von Hippel Lindau

Chapter 1.

Introduction

Cancer refers to groups of diseases that can affect all parts of the body and is characterized by epigenetic changes or changes in the DNA sequence of the genome of cancer cells. These changes can be inherited (germ line mutations) increasing chances of developing certain types of cancer as is seen in women carrying the BRCA1 and BRCA2 gene mutation having a 80-90% chance of developing breast cancer in their lifetime (Gayther, Warren, Mazoyer, & Russelll, 1995; King, Marks, & Mandell, 2015). In addition, these changes can occur as a result of many different factors including environmental exposures, viruses, and lifestyle habits (Bernard & Christopher, 2014; Danaei et al., 2005) resulting in somatic mutations and sporadic tumours. However, mammalian cells have multiple ways to detect these mutations and either fix them or destroy the affected cells. The problem arises when the body is unable to repair the DNA damage and several genes become "defective"; if these defective genes include tumor-supressor genes, oncogenes, or DNA stability genes, which control a cells ability to detect mutations or defects, tumourigenesis can occur (Vogelstein & Kinzler, 2004).

Tumourigenesis is not a straightforward process and involves multiple steps. It entails cancer cells developing biological capabilities including the 6 hallmarks of cancer and 2 emerging hallmarks (Hanahan & Weinberg, 2011). These hallmarks include: 1) resisting cell death, 2) evading growth suppressors, 3) enabling replicative immortality, 4) sustaining proliferative signalling, 5) inducing angiogenesis, 6) activating invasion and metastasis, and the two new possible hallmarks: reprogramming of energy metabolism, and evading immune destruction (Hanahan & Weinberg, 2011). The tumours microenvironment is one key factor that influences cancer cells abilities to develop biological adaptations to increase survival and lead to tumourigenesis. The tumour microenvironment is composed of proliferating cancer cells, blood vessels, inflammatory

cells, and many non cancerous cells (Whiteside, 2008). All of these non-cancerous cells and their surroundings allow the cancer cells to communicate with themselves, quiescent adjacent cells, and non cancerous cells, leading to cancer cell division, suppressed immune cell function and resistance to therapeutic intervention (Bernard & Christopher, 2014). Aside from cell signalling, another crucial aspect of the tumour microenvironment allowing cancer progression is the tumour vasculature. When tumours grow bigger than a few millimetres in diameter they start to become hypoxic and deprived of nutrients, consequently leading to the angiogenic switch allowing the tumour to continue progressing (Weis & Cheresh, 2011). Characteristic of this switch is the development of new vasculature by increasing levels of the vascular endothelial growth factor (VEGF), and certain chemokines including the chemokine receptor 4 (CXCR4) (Chan & Giaccia, 2007).

This angiogenic switch is mediated largely by the hypoxia inducible factor (HIF) transcriptional complex, composed of a highly regulated α-subunit and it's constitutively expressed β-subunit (HIF-α and ARNT respectively) (Wang, Jiang, Rue, & Semenza, 1995). Not only is the HIF complex capable of increasing the levels of genes associated with increasing angiogenesis, it is also associated with tumour growth and metastasis (Maxwell, Pugh, & Ratcliffe, 2001). Given that most tumours develop regions of hypoxia, and that hypoxia leads to decreased efficacy of chemotherapy and radiation (*Oxygen Biology and Hypoxia*, 2007), targeting the HIF pathway to prevent tumour growth and metastasis may be a viable treatment option.

Since the hypoxia inducible factor 1-α (HIF1-α) in complex with hypoxia inducible factor1-β (HIF1-β or aryl hydrocarbon receptor nuclear translocator (ARNT)) increases the expression of genes involved in angiogenesis and metastasis (Semenza, 2001; Staller et al., 2003), this study looks to validate genes that are regulated by the HIF pathway. More specifically at the genes regulated by the HIF1 complex that are further controlled by the retinoblastoma protein (Rb), a tumour suppressor protein. This will allow us to gain additional insight into how the tumour generates a more aggressive phenotype under hypoxic conditions and possibly leading to novel therapeutic targets.

1.1. Tumour microenvironment

A tumours microenvironment is quite diverse, and interactions between the two are crucial for adaptive changes in the tumour. The tumour microenvironment makes up the biochemical and cellular composition of the tissue(s) surrounding a tumour. The microenvironment is composed of many different components including: a heterogeneous population of cancer cells, non-cancer cells, soluble proteins, blood vessels, peritumoural lymphatic vessels, structural matrices and a scale of different oxygen tensions, glucose concentrations, pH levels and interstitial pressure (Bernard & Christopher, 2014; Lee et al., 2011).

Chronic inflammation and neovascularization are two critical environments that promote and enhance tumour growth and metastasis (Rüegg, 2006). In response to initial tumour hypoxia and necrosis, tumour cells and other cells of the microenvironment release growth factors and cytokines that recruit monocytes and macrophages (Lorusso & Rüegg, 2008). This release of growth factors also results in tumour cell motility, active tumour cell endothelium and chronic inflammation (Lorusso & Rüegg, 2008). In response to chronic inflammation the tumour cells release tumour necrosis factor (TNFα), interleukins (IL-1, IL-6 and IL-8) and chemokines (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006), many of which are regulated by the nuclear factor-kappa B (NF-κB) (Karin, 2006). This, in turn leads to increased tumour cell invasion, and angiogenesis processes also heavily controlled by hypoxia (Harris, 2002).

1.1.1. Oxygen Homeostasis and Hypoxia

Levels of oxygen (O_2) in human cells are very heterogeneous and can vary from 21% O_2 at the upper airway to 1% O_2 at the cortico medullary junction of the kidney (Semenza, 2010). Therefore normoxia levels (normal O_2 levels) are monitored in a tissue specific manner and range from 110 mmHg in the alveoli to 9.9-19 mmHg in cells and 30 mmHg in the prostate (Carreau, Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011). The proper level of oxygen in all aerobic forms of life is required for production of adenosine triphosphate (ATP), a molecule required for many biological functions. Therefore, fluctuations in O_2 levels activates various O_2 sensing systems (Cash, Pan, & Simon,

2007). During oxygen consuming reactions, formation of reactive oxygen species (ROS); superoxide anion, hydrogen peroxide, and hydroxyl radicals can cause DNA damage, oxidize protein and damage cellular membranes (Bunn & Poyton, 1996; Cash et al., 2007; Galanis et al., 2008). Interestingly intracellular ROS levels also increases during hypoxia leading to diverse functional responses such as increased gene expression (Cash et al., 2007; Guzy & Schumacker, 2006).

Hypoxia is when cells, tissues or whole organisms are lacking physiological levels of oxygen (below 2%), which can be chronic, acute or cycling. Hypoxia can occur as a result of many different factors including low O_2 partial pressure in arterial blood, decreased O_2 carrying capacity of the blood, decreased tissue perfusion, changes in diffusion distances and pathways, and inability to use O_2 due to intoxication (Höckel & Vaupel, 2001). Hypoxic levels play key roles in many physiological and pathophysiological pathways in the human body including embryogenesis, wound healing, ischemic diseases, diabetes, inflammatory disorders, and cancer (Dayan, Mazure, Brahimi-Horn, & Pouysségur, 2008; Knighton, Silver, & Hunt, 1981).

One of the main sensors of oxygen levels in metazoans are the hypoxia-inducible factor1-α and 2-α (HIF1-α/2-α). In response to low oxygen levels HIF1 increases the transcription of genes including erythropoietin (EPO), VEGF, glucose transporter 1 (GLUT1), enolase 1 (ENO1), lactate dehydrogenase A and phosphoglycerate kinase 1, which increase O_2 delivery and decrease O_2 consumption (Semenza, 1998). The components of HIF1 will be discussed in further detail below.

1.2. Basic Helix-Loop-Helix and Hypoxia-Inducible Factor

Hypoxia-inducible factor 1 (HIF1) was first discovered as a protein required for transcription of the human erythropoietin gene (Greer, Metcalf, Wang, & Ohh, 2012; Semenza & Wang, 1992). Upon activation, HIF1 (HIF1-α/HIF1-β) binds to cis-acting hypoxia response elements (HREs) on the promoter or enhancer regions of DNA of specific genes causing changes in transcript levels (Semenza, 2010).

Hypoxia-inducible factors (HIFs) are DNA binding transcription factors belonging to the basic Helix-Loop-Helix (bHLH)/ PER-ARNT-SIM (PAS) family of transcriptional regulators (Kewley, Whitelaw, & Chapman-Smith, 2004). bHLH transcription factors are important in responding to environmental pollutants, and low oxygen tensions. They are involved in many essential biological processes including neurogenesis, myogenesis and cell proliferation and differentiation (Atchley & Fitch, 1997; Kewley et al., 2004; Massari & Murre, 2000). The bHLH proteins can be divided into three main groups based on their structural domains: 1) containing only the bHLH domain, 2) containing a leucine zipper adjacent to the bHLH motif, and 3) containing a period (PER)/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM) (PAS) domain adjacent to the bHLH (Kewley et al., 2004).

These groups can be further broken down into classes, class I-VII, based on their tissue distribution, DNA binding specificity, and dimerization capabilities (Massari & Murre, 2000). Class I are E-box binding proteins and include E12, E47, HEB, E2-2 and Daughterless. Class II includes MyoD, myogenin, Atonal, and NeroD/BETA2. These proteins heterodimerize with E proteins and are tissue restricted (Massari & Murre, 2000). Both class I and class II can be associated with group 1 and can be negatively regulated by class V proteins including Id and emc which lack a basic region. Group 2 includes class III proteins including the Myc family of transcription factors, TFE3, SREBP-1 and Mi. Class IV proteins can dimerize with one another or with Myc and include Mad, Max, and Mxi. Class VI proteins contain a proline in their basic region and include Hairy and Enhancer of split. Lastly, class VII proteins can be categorized into group 3, and contain the PAS domain. This class includes the aryl hydrocarbon receptor (AHR), the aryl hydrocarbon receptor nuclear translocator (ARNT), HIF-α, and Single minded (SIM) and Period (PER) proteins (Massari & Murre, 2000).

Members of the bHLH-PAS family (group 3 bHLH proteins) are characterized by a DNA binding domain, a PAS domain (consisting of two side by side degenerate repeats of amino acids, PAS A and PAS B) and specific regulatory and transcriptional control elements (Kewley et al., 2004; Partch & Gardner, 2010). This bHLH-PAS family of transcription factors can be further separated into two groups: group 1 includes AHR, HIF, and SIM which can detect and respond to certain signals including aromatic

hydrocarbons, and hypoxia respectively (Ema et al., 1996; Lindebro, Poellinger, & Whitelaw, 1995; Wang et al., 1995), and group 2 which dimerizes with group 1 to form active transcription factor complexes, and includes ARNT (the best characterized group 2 protein) and circadian rhythm proteins BMAL1 and BMAL2 (Hoffman, Reyes, Chu, Sander, Conley, Brooks, & Hankinson, 1991; Kewley et al., 2004). The N-terminal domain of the bHLH transcription factor holds the primary dimerization interface while the PAS domain defines partner choice (Kewley et al., 2004). Although homodimerization of bHLH-PAS proteins can occur, the more functional unit consists of heterodimerization, where one partners expression is highly regulated and one partner is broadly expressed, as is the case for hypoxia inducible factors (HIFs) (Crews, 1998). HIFs are a heterodimer of an oxygen sensitive α-subunit including three isoforms, HIF1-α, HIF2-α, HIF3-α, and a constitutively expressed β-subunit (HIF1-β or ARNT) (Maxwell et al., 2001). Although HIF3-α is considered an isoform of HIF-α, it has been found to lack the oxygen dependent degradation domain, and therefore is not sensitive to varying oxygen tensions (Hara, Hamada, Kobayashi, Kondo, & Imura, 2001).

1.2.1. HIF1-α

Out of the three identified HIF-α isoforms, HIF1-α is the most widely expressed, and can be found in many different cell types. HIF1-α consists of a bHLH-PAS (containing two PAS domains: PAS A and PAS B) domain at the amino terminal end which is required for heterodimerization. In addition they have a transcriptional activation and an oxygen dependent regulatory domain at the carboxy terminus (Jiang, Rue, Wang, Roe, & Semenza, 1996). HIF1-α also has two hypoxia inducible transactivation domains (TADs) in the C-terminal. The N-terminal TAD (N-TAD) is located between amino acids 532 and 585 and the C-terminal TAD (C-TAD) is located between amino acids 776 and 826 (Ruas, Poellinger, & Pereira, 2002). While the N-TAD overlaps with the oxygen dependent degradation (ODD) domain making it continuous with protein stability, the C-TAD also functions in a hypoxia inducible fashion and interacts with the cysteine/histidine-rich 1 (CH1) domain of the p300/CBP coactivator (Lando, Peet, Whelan, Gorman, & Whitelaw, 2002b; Ruas et al., 2002). In addition, HIF1-α also

contains a N-terminal and C-terminal nuclear localization signal located at amino acid 17-74 and 718-721 respectively (Kallio et al., 1998).

Oxygen plays a significant role in regulating HIF1-α activity due to its ODD domain, and this regulation comes mostly at the post translational level since mRNA levels of HIF1-α don't change under normoxic or hypoxic conditions (Huang, Arany, Livingston, & Bunn, 1996) (Figure 1.1). Under conditions of normoxia, prolyl hydroxylases (PHs) or more specifically prolyl hydroxylase domain (PHD) containing proteins hydroxylate HIF1-α. PHD 1, 2 and 3 with the help of: 2-oxoglutarate, iron and oxygen, hydroxylate HIF1-α at Pro-564, and PHD 1 and 2, hydroxylate HIF1-α at Pro-402 (Epstein, Gleadle, McNeill, & Hewitson, 2001). Alternatively PHD1, PHD2, and PHD3 can also be called HIF prolyl hydroxylases 3, 2 and 1 respectively (Bruick & McKnight, 2001). Both of the proline residues Pro-402 and Pro-564 are located within the strongly conserved amino acid motif LXXLAP (Epstein et al., 2001; Masson, Willam, Maxwell, Pugh, & Ratcliffe, 2001). This prolyl hydroxylation of Pro-402 and Pro-564 creates a binding site for the von Hippel Lindau (vHL) protein, an E3 ubiquitin ligase, on HIF1-α. This leads to HIF1-α polyubiquitination and sequential degradation by the 26S proteasome (Iwai et al., 1999; Kaelin, 2008; Salceda & Caro, 1997). Concurrently, a negative regulator of the HIF1-α C-TAD, factor inhibiting HIF1 (FIH-1) is hydroxylating the β-carbon of an asparagine residue (Asn-803) which is located up from the FIH-1 binding site on HIF1-α (Lando, Peet, Gorman, Whelan, Whitelaw, & Bruick, 2002a; Lando, Peet, Whelan, Gorman, & Whitelaw, 2002b; Mahon, Hirota, & Semenza, 2001). The asparagine hydroxylation silences the HIF1-α C-TAD by preventing its interaction with the CH1 domain of p300/CBP, further preventing the assembly of transcriptional coactivator complexes. (Lando, Peet, Whelan, Gorman, & Whitelaw, 2002b). Like the PHDs, FIH-1 is also a 2-oxoglutarate and Fe(II)-dependent oxygenase (Lando, Peet, Gorman, Whelan, Whitelaw, & Bruick, 2002a).

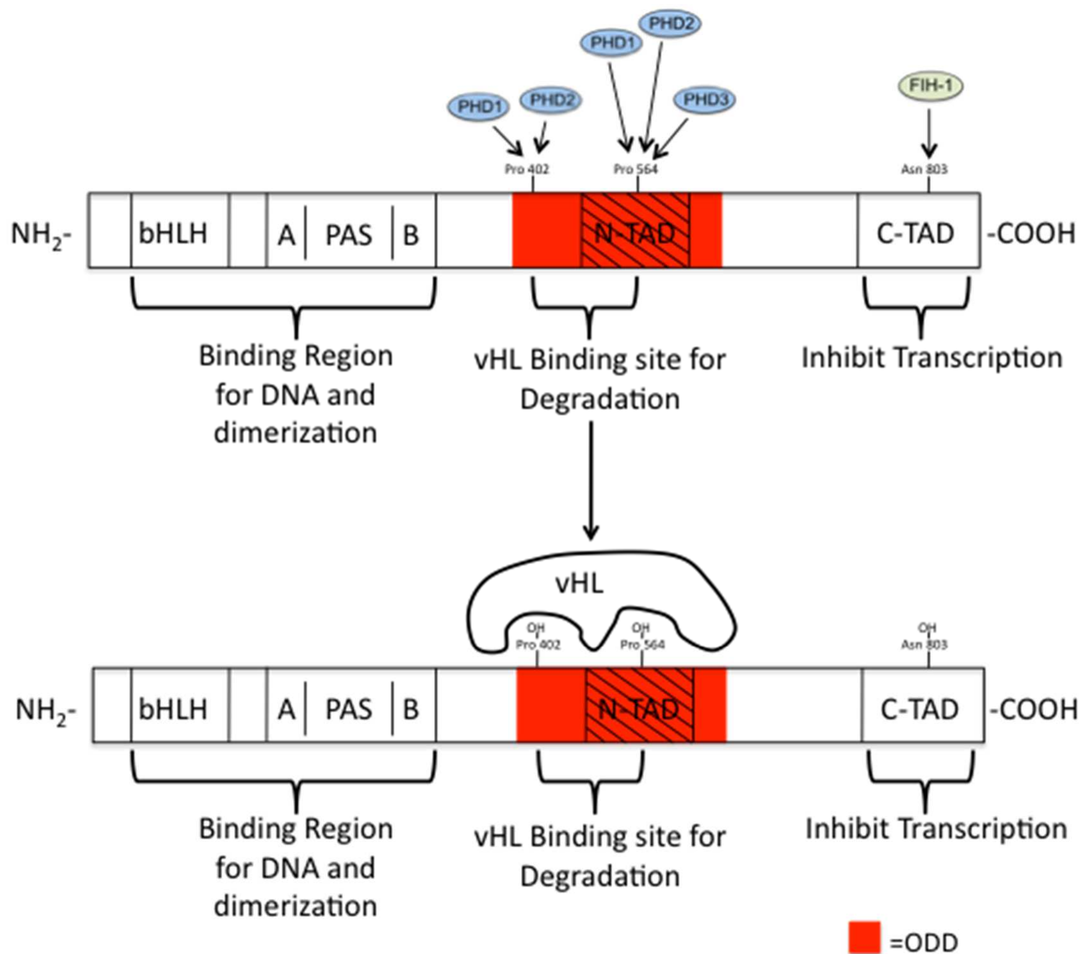


Figure 1.1 A schematic representation of HIF1-α structure and post-translational modification under normoxic conditions
Under normoxic conditions (the presence of normal physiological O_2) HIF1-α is hydroxylated at proline residues 402 and 564 by prolyl hydroxylases 1, 2 and 3 and by a factor inhibiting HIF1 at an asparagine residue 803 (top pane). The hydroxylation at proline residues 402 and 564 allows for the binding of the von Hippel Lindau protein, which targets HIF1-α for proteosomal degradation (bottom pane).

Alternatively, HIF1-α can also be regulated by other biological signals and pathways including ROS, insulin and insulin like growth factors, growth factors (PDGF, and TGF-β), coagulation factors, cytokines, and mechanical stress (Kietzmann & Görlach, 2005). The binding of these mediators to their membrane receptors might activate various kinase pathways such as the mitogen-activated protein kinases, phosphatidylinositol 3-kinase and the serine-threonine kinases (Hirota & Semenza, 2005; Stiehl, Jelkmann, Wenger, & Hellwig-Bürgel, 2002).

1.2.2. HIF2-α and HIF3-α

HIF2-α is also known as endothelial PAS-1 (EPAS-1) (Tian, McKnight, & Russell, 1997), HIF-like factor (HLF) (Ema et al., 1997), HIF-related factor (HRF) (Flamme et al., 1997) and member of PAS family 2 (MOP2) (Hogenesch et al., 1997). HIF2-α is expressed in multiple tissues, but preferentially in endothelial cells and shares 48% sequence identity with HIF1-α; with the highest sequence conservation at the bHLH, PAS A and PAS B regions (Tian et al., 1997). Like HIF1-α, EPAS-1 can dimerize with ARNT and carry out transcriptional responses under hypoxic conditions by binding to the HREs in the promoter or enhancer regions of hypoxia inducible genes (Tian et al., 1997). EPAS-1 is regulated by both the N-TAD and C-TAD domains similar to HIF1-α (O'Rourke, Tian, Ratcliffe, & Pugh, 1999). One difference between HIF1-α and EPAS-1 expression noted by Wiesener et al. is that under normoxic levels, EPAS-1 expression is higher and can be induced by less severe hypoxia compared to HIF1-α (Wiesener et al., 1998).

HIF3-α also shares amino acid sequence identity with HIF1-α and EPAS-1 at the N-terminal bHLH/PAS domain, and at the C-terminus hypoxia responsive domain 1 with HIF1-α (Gu, Moran, Hogenesch, Wartman, & Bradfield, 1998). However, unlike HIF1-α and EPAS-1, which contain both a N-TAD and C-TAD, HIF3-α only contains an N-TAD region and lacks the C-TAD region (Hara et al., 2001). Due to HIF3-α being a truncated form of the other two HIFα isoforms (HIF1-α and HIF2-α), and lacking the ODD domain, HIF-3α levels are not affected in normoxic conditions (Hara et al., 2001). HIF3-α may also function as a regulator of HIF1-α and EPAS-1 gene expression, as at low levels of ARNT expression, HIF3-α suppresses HRE driven gene expression (Hara et al., 2001). This suppression might be due to competition for ARNT between the HIFα isoforms (Hara et al., 2001). Or due to direct binding of HIF3-α with HIF1-α causing interference with DNA HRE binding as was with the inhibitory PAS (IPAS) domain proteins (Makino et al., 2001). IPAS is a splice variant of the HIF3-α locus and its binding to HIF1-α decreases vascularisation in mouse corneas (Makino, 2002). Similarly, HIF3-α4, another splice variant of HIF3-α is capable of binding to both HIF1-α and ARNT and like IPAS inhibits HIF1 binding to HREs on DNA (Maynard et al., 2005). Altogether, although HIF3-

α shares similarities to HIF1-α and EPAS-1 in structure, it may work to deregulate HIF1 activity compared to the other two HIFα isoforms.

1.2.3. HIF1-β/ARNT

HIF1-β/ARNT belongs to the family of bHLH/PAS proteins and is the dimerization partner for the AHR, HIFα and SIM proteins (Hoffman et al., 1991; Keith, Adelman, & Simon, 2001; Kewley et al., 2004). Like HIFα, the bHLH/PAS domain is located near the amino terminal end of ARNT followed by the PAS domain which consists of two repeating 50 amino acid repeats (PAS-A and PAS-B) (Hankinson, 1995). ARNT also contains a TAD in the C terminus which is required for the binding of coactivators such as CBP/p300 (A. Kobayashi, Numayama-Tsuruta, Sogawa, & Fujii-Kuriyama, 1997). Unlike HIFα, ARNT has no N terminus TAD (Whitelaw, Gustafsson, & Poellinger, 1994) (Figure 1.2). The bHLH/PAS domain of ARNT is required for its dimerization ability and function in response to xenobiotics and hypoxia (Fukunaga, Probst, Reisz-Porszasz, & Hankinson, 1995; Reisz-Porszasz, Probst, Fukunaga, & Hankinson, 1994; Wood, Gleadle, Pugh, Hankinson, & Ratcliffe, 1996).

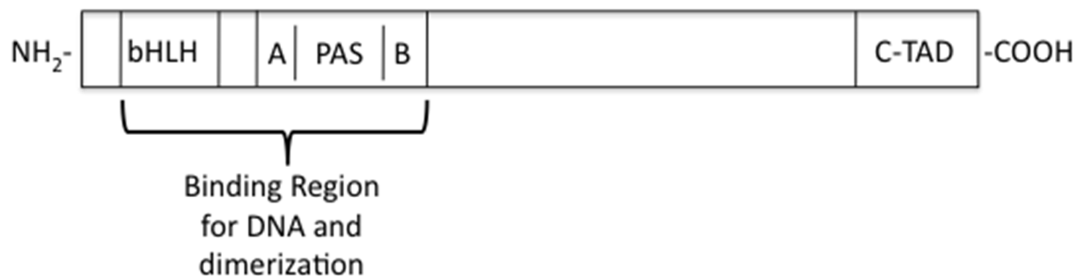


Figure 1.2 Schematic representation of HIF1-β/ARNT structure
ARNT is composed of a bHLH/PAS domain at the amino terminus and a C terminus transactivation domain at the carboxy terminus.

Dissimilar to HIFα which is localised to the cytoplasm, ARNT is expressed abundantly in the nucleus (Eguchi, Ikuta, Tachibana, Yoneda, & Kawajiri, 1997; Hord & Perdew, 1994). However it was found that different ARNT homologues are found in different tissues, such as ARNT2 being mainly found in the brain, and kidney (Hirose et al., 1996).

1.3. TRIP230

The thyroid hormone receptor/retinoblastoma protein-interacting protein 230 (TRIP230) also known as the Golgi microtubule-associated protein 210 (GMAP-210) is a ubiquitously expressed protein (Chang et al., 1997). GMAP-210 was identified as a cis-Golgi membrane protein by using human autoantibodies to the golgi apparatus with the serum of a patient with Sjögren's syndrome (Rios et al., 1994). Structurally, TRIP230 is a coiled coil protein that associates with both the golgi apparatus and microtubules (Pernet-Gallay et al., 2002; Rios et al., 1994). Through its carboxy terminus GMAP-210 is able to bind to the negative ends of microtubules and to the golgi membrane via its amino terminus, which helps maintain the integrity of the golgi apparatus (Infante, Ramos-Morales, Fedriani, Bornens, & Rios, 1999). Over expression of TRIP230 results in an enlargement of the golgi apparatus, disrupts microtubule networks (Infante et al., 1999) and blocks the anterograde and retrograde transport of membrane proteins between the golgi and endoplasmic reticulum (Pernet-Gallay et al., 2002).

Apart from TRIP230's role in golgi integrity, Chang and colleagues found that TRIP230 has the ability to bind to both the thyroid hormone receptor (TR) in the presence of triiodothyronine (T3) and to the retinoblastoma protein (Rb) independently (Chang et al., 1997). TRIP230's ability to enhance TR mediated transcription is negatively regulated by Rb (Chang et al., 1997). Additionally, Chang and his colleagues determined that the TRIP230 protein is 1,978 amino acids long with a molecular mass of 230 kDa (Chang et al., 1997). TRIP230 also contains a LXXLL motif in its C-terminal thyroid receptor domain (Beischlag et al., 2004). Which corresponds with TRIP230 being a transcriptional coactivator, as LXXLL motifs are involved in both receptor specific and ligand specific assembly of coactivator complexes and are bona fide indicators of transcriptional co-regulatory proteins (McInerney et al., 1998). This is due to the spacing between LXXLL motifs and their structural features which guides the specific formation of coactivator components required for transcription factors (McInerney et al., 1998).

As well as being a TR coactivator, TRIP230 regulates AHR, and HIF1 mediated transcriptional activity via its ability to bind to ARNT, the AHR and HIFα dimerization partner (Beischlag et al., 2004). The ARNT/TRIP230 interaction occurs in a distinct site

from the LXXLL motif interaction site of TRIP230 and TR (Beischlag et al., 2004). This interaction of TRIP230 with ARNT is essential to both AHR and HIF1 transcriptional activation independently and transcript levels of genes induced by both pathways respectively is considerably decreased in the absence of TRIP230 (Beischlag et al., 2004). Furthermore, TRIP230 was found to be indispensible for hypoxia inducible transcription (Beischlag et al., 2004)

1.4. The Retinoblastoma Protein

The retinoblastoma (Rb) tumour suppressor gene was the first tumour suppressor gene to be cloned. Loss of function or mutation of Rb has been implicated in many human cancers including retinoblastoma, small cell lung carcinoma, osteosarcoma, breast carcinoma and prostatic small cell carcinoma (Classon & Harlow, 2002; Tan et al., 2014). Rb encodes a nuclear phosphoprotein, which is regulated by cyclin dependent kinases (CDKs) that consequently control Rb's binding with E2F transcription factors (Cance, Brennan, Dudas, Huang, & Cordon-Cardo, 1990; Classon & Harlow, 2002). In particular, Rb binds to E2F1, 2, 3, 4 and 5 and inhibits E2F transcriptional activity or recruits transcriptional repression complexes to E2F sites on promoters (Lipinski & Jacks, 1999). This binding occurs in the Rb pocket domain, consisting of two packed subdomains A and B that are connected by a spacer region (Genovese, Trani, Caputi, & Claudio, 2006). Rb also contains an LXCXE conserved domain which acts as its core binding site to other proteins and is needed for biological processes such as cellular transformation (Genovese et al., 2006).

Other biological roles of Rb include differentiation, apoptosis, and regulation of cell cycle progression (Du & Pogoriler, 2006). Cell cycle control is mediated by the phosphorylation status of Rb/p105 and the Rb family proteins, Rb2/p130 and p107 (Du & Pogoriler, 2006; Genovese et al., 2006). This control is mediated through their ability to bind E2Fs and recruit co-repressor proteins capable of modifying histones and chromatin (Rubin, 2013). During the G1 cell cycle phase, Rb is hypophosphorylated, which allows its binding to E2Fs 1-3. This in return inhibits E2F activation of genes that stimulate DNA synthesis which are required at the G1/S transition phase. Therefore, this interaction prevents and controls cells from transitioning into the S phase (Du &

Pogoriler, 2006; Goodrich, Wang, Qian, Lee, & Lee, 1991). The other two E2Fs, E2F 4 and 5 are capable of binding with Rb and preferentially bind to p130. Unlike E2F 1-3, they act as repressive E2Fs and lack the cdk binding or the nuclear localization sequences which exist in the N-terminus of the activating E2Fs (Du & Pogoriler, 2006). p130 binds E2F 4 and 5 in quiescent cells at the G0 stage while p107 binds E2F4 in the G1 phase, and Rb binds to E2F4 in the S phase (Genovese et al., 2006). When p130 is hypophosphorylated it remains bound to E2F4 causing the cells to remain in the G0 stage. When Rb becomes phosphorylated by CDKs, it releases its depression of E2Fs, causing increases in E2F activation of genes required in the S phase (Chau & Wang, 2003).

In addition to controlling cell cycle, Rb and E2Fs also function in regulating apoptosis (Chau & Wang, 2003; Masselli & Wang, 2006). This may be done through E2F1s interaction with the Ras-PI3 kinase-Akt and p53 signalling pathways (Hallstrom & Nevins, 2003). Likewise, loss of Rb may also play a role in regulating differentiation, such that if cells do not exit the cell cycle, they wouldn't be able to differentiate (Du & Pogoriler, 2006).

1.4.1. Retinoblastoma protein and HIF1

Aside from its canonical role in cell cycle control, Rb can also bind to other transcriptional activators including HIF1-α (Budde, Schneiderhan-Marra, Petersen, & Brüne, 2005). Budde and colleagues showed that HIF1-α recruits Rb to HREs on DNA promoter or enhancer regions, and increases transcriptional levels (Budde et al., 2005). Induction of HIF1-α by hypophosphorylated Rb interferes with Rbs mediated regulation of E2Fs due to hypophosphorylated Rb not being available to bind to E2Fs (Budde et al., 2005). Alternatively, hyperphosphorylated Rb also controls HIF1 transcriptional activity via its interaction with TRIP230 (Chen, Chen, Chen, Sharp, & Lee, 1999; Labrecque et al., 2014). TRIP230 enhanced TR mediated transcription is attenuated by Rb binding (Chang et al., 1997), a phenomenon that was also observed in TRIP230 enhanced HIF1 transcription (Labrecque et al., 2014). The Rb dual role on HIF1 mediating transcription according to its phosphorylation status illustrates the complexity of Rbs ability to control transcriptional regulation in response to hypoxia.

1.5. Epithelial to Mesenchymal Transition and Neuroendocrine Differentiation

1.5.1. EMT

Epithelial to mesenchymal transition (EMT) is a reversible process in which epithelial cells: that are normally in close contact with their neighbours, posses an apicobasal axis of polarity, and are separate from adjacent tissues by a basal lamina, undergo multiple biochemical changes (Kalluri & Weinberg, 2009; Thiery, Acloque, Huang, & Nieto, 2009). These changes allow the epithelial cells to posses a phenotype typical of a mesenchymal cell. This means that the epithelial cells are now more migratory, invasive, and resistant to apoptosis (Kalluri & Weinberg, 2009). EMT is an important process occurring in many different biological processes including embryonic development, organ formation, wound healing, organ fibrosis, and tumour progression (Kalluri & Weinberg, 2009; Michael Zeisberg, 2009; Thiery et al., 2009). As mentioned previously, one of the hallmarks of cancer include activating invasion and metastasis, processes that are facilitated in part by EMT. Before a tumour cell can metastasize, it needs to lose its cell polarity and detach from its basement membrane (EMT). This is followed by entering and exiting the circulation at a new site and forming new tumours which may be by regaining an epithelial phenotype through the mesenchymal to epithelial transition (MET) (Kalluri & Weinberg, 2009). An important step in EMT playing a crucial role in cancer progression is the loss of E-cadherin (a cell-cell adhesion molecule) expression (Perl, Wilgenbus, Dahl, Semb, & Christofori, 1998).

E-cadherin repression can occur through many different mechanisms including factors binding directly to its promoter and hence repressing activity, or by factors repressing transcription indirectly (Thiery et al., 2009). Examples of E-cadherin promoter repressors include SNAIL, ZEB, E47 and KLF8, and indirect repressors include TWIST, Goosecoid, E2.2, and FoxC2 (Thiery et al., 2009; Yang & Weinberg, 2008). These repressors are further controlled by signalling pathways including the transforming growth factor β (TGFβ) and Notch pathways, which can up-regulate Hey1 or SNAIL resulting in E-cadherin repression (Huber, Kraut, & Beug, 2005). Hypoxia has also been found to play a role in decreasing E-cadherin expression by enhancing production of E-

cadherin signalling pathway regulators including TGFβ, Notch, and NFκB; which as previously mentioned, up-regulate Hey1 or SNAIL (Jiang, Tang, & Liang, 2011). Moreover, hypoxia can regulate the expression of E-cadherin transcription factors including TWIST, SIP1 AND ZEB1, and inflammatory cytokines such as tumour necrosis factor-α (TNFα), IL-6 and IL-1 which also stabilize and induce SNAIL (Jiang et al., 2011). This highlights an important role for hypoxia and HIF1 in increasing one of the hallmarks of cancer progression.

1.5.2. NED

The stromal compartment of the human prostate is composed of many different cell types including nerves, fibroblasts, endothelial cells and smooth muscle cells; while the epithelial compartment contains neuroendocrine (NE) cells (Sun, Niu, & Huang, 2009) which can act in secretory and autocrine/paracrine fashions (Vashchenko & Abrahamsson, 2005). One characteristic of NE cells is that they contain and secrete serotonin, histamine, calcitonin, chromogranin A, neuropeptide Y, alpha-human chorionic gonadotropin, somatostatin and many other peptides (di Sant'Agnese, 1992; Sun et al., 2009). Another feature of NE cells is that they do not express androgen receptors, or prostate specific antigen (PSA); therefore they do not respond to androgen deprivation therapy (J. Huang et al., 2006).

In terms of prostate cancer, neuroendocrine differentiation (NED) refers to the presence of NE cells scattered in prostatic adenocarcinomas (Sun et al., 2009). NED in prostatic tumours occurs in small cell carcinoma, carcinoid/carcinoid like tumours and focal NED in typical prostatic adenocarcinomas (di Sant'Agnese, 1998a), and can be distinguished from benign tissue by looking for NE cell markers such as chromagranin A and neuron specific enolase (NSE/ENO2) (Nelson et al., 2006).

As a result of NE cells not responding to androgen deprivation therapy, NED in prostate tumour cells creates a tumour that grows in androgen independent conditions (Huang et al., 2005) and is more proliferative and aggressive (Vashchenko & Abrahamsson, 2005). This aggressiveness is in part due to the NE prostate cancer cells being able to escape apoptosis by having increased expression of Bcl-2 a proto-

oncogene involved in overriding apoptosis (McDonnell et al., 1992), and survivin an inhibitor of apoptosis (Xing, Qian, Bostwick, Bergstralh, & Young, 2001). NED in prostate cancer also increases the tumours proliferative properties by different signalling pathways including bombesin/GRPs deregulation of cell cycle, serotonin binding to its receptors, and parathyroid hormone related protein stimulating tumour growth via paracrine signalling (di Sant'Agnese, 1998b; Nelson et al., 2006). This is problematic as androgen deprivation therapy is one of the main treatments for patients with prostate cancer. However, since NE cells and NED prostatic tumours don't react to this type of treatment the patient is eventually left with androgen independent tumours that are associated with low life expectancy (Nelson et al., 2006).

1.6. Objective of the study

Tumours, in response to their low oxygen tension microenvironment undergo adaptive changes in order to increase survival including angiogenesis, and altered glucose metabolism. These changes are mediated by the bHLH/PAS domain HIF1-α family of transcription factors and its dimerization partner ARNT (Semenza, 2004). We have found that hypoxic conditions, in combination with loss of Rb leads to an exacerbation of HIF1 activity in human breast and prostate cancer cell lines (Labrecque et al., 2014). Rb mediates this via its interaction with TRIP230, a transcriptional coactivator of ARNT (Beischlag et al., 2004). To determine the extent to which Rb regulates hypoxia inducible genes, we interrogated the MCF-7 and LNCaP transcriptome using gene expression microarrays with the Agilent Human genome wide expression 44K array.

The objective of this study was to take a sample of the most highly expressed genes from the MCF7 and LNCaP microarray gene lists known to have an association with invasion, transformation and metastasis and validate their expression at the mRNA and protein levels. Additionally, I sought to determine if the physiological alterations observed after loss of Rb under hypoxia resulted in a more migratory phenotype.

1.6.1. Specific aims

1. Validate the microarray genes that are up-regulated in response to both loss of Rb and hypoxia in both MCF-7 and LNCaP cell lines by QPCR and immuno-blotting.

2. Determine if both the loss of Rb and hypoxia leads to cell migration.

Given that Rb attenuates hypoxia induced gene expression via its interaction with TRIP230, we hypothesize that loss of Rb will result in an increased proliferative, migratory, and invasive phenotype in MCF-7 and LNCaP cancer cells (Figure 1.3).

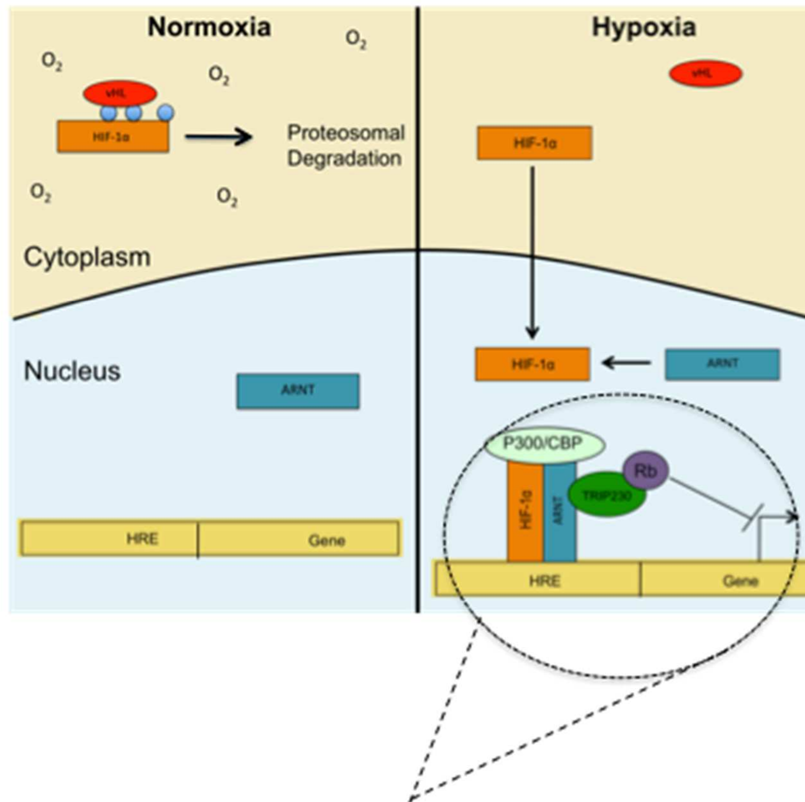


Figure 1.3 The role of Rb in HIF1 mediated gene transcription
In the presence of hypoxia, the HIF1 transcriptional response is attenuated by Rb. However, loss of Rb results in an exacerbated HIF1 transcriptional response increasing the tumour cells ability to become metastatic, invasive, increase proliferation and go through neuroendocrine differentiation. * = phosphate groups

Chapter 2.

Materials and Methods

2.1. Cell Culture

MCF-7 human breast adenocarcinoma cells were obtained from Cedarlane Labs (Burlington, ON), tested for intact Rb and stable shSCX and shRb cell lines were made by Mark Labrecque, another member of the Beischlag lab. Cells were cultured and maintained in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, 4.5 g/L L-glutamine, and sodium pyruvate (DMEM; CORNING cellgro) supplemented with 10% (v/v) fetal bovine serum (FBS Gibco) and 1% 100 units/mL potassium penicillin-100 μg/mL streptomycin sulphate (BioWhittaker, Lonza) at 37 °C, 20% O_2, and 5% CO_2. Cells were split at a 1/5 ratio for passaging and at other specified dilutions for experiments.

LNCaP human prostate cancer cells were obtained from Dr. Michael Cox (Vancouver Prostate Centre), tested for intact Rb and stable shSCX and shRb cell lines were made by Mark Labrecque, another member of the Beischlag lab. Cells were cultured and maintained in Roswell Park Memorial Institute1640 medium with L-glutamine (RPMI; CORNING cellgro) supplemented with 10% fetal bovine serum (FBS Gibco) and 1% 100 units/mL potassium penicillin-100 μg/mL streptomycin sulphate (BioWhittaker, Lonza) at 37 °C, 20% O_2, and 5% CO_2.

2.2. Microarray Collection and Analysis

MCF-7 shSCX and shRb and LNCaP shSCX and shRb cell lines were pre-treated with 24 h of hypoxia and mRNA was isolated. Probes were made from RNA

derived from the MCF-7 and LNCaP shSCX and shRb cells. The whole human genome was interrogated using the Agilent 44K Human Genome-wide expression array at the Laboratory for Advanced Genome Analysis (Vancouver Prostate Centre, Vancouver, Canada). Gene lists were generated from triplicate experiments and genes from the shRb hypoxia treated data sets that were up or down regulated ($P<0.05$) were selected. Hypoxia inducible genes sensitive to loss of Rb ($P<0.05$) and Rb regulated genes further up-regulated with exposure to hypoxia ($\geq$2.0 compared to the control or the hypoxia SCX control group.) were selected for further analysis. The microarray resulted in a total of 207 genes up-regulated and 94 genes down-regulated after exposure to hypoxia and loss of Rb in the MCF-7 cell line, and a total of 184 genes up-regulated and 93 genes down-regulated after exposure to hypoxia and loss of Rb in the LNCaP cell line.

Bioinformatics was carried out at the Laboratory for Advanced Genome Analysis, (Vancouver Prostate Centre) and analysis was done using the Ingenuity Pathway Assist (IPA) software. IPA analysis consisted of identifying the top network maps for diseases and biological programs regulated by hypoxia inducible genes further up-regulated with loss of Rb. A cut-off score of 1.5 fold induction (MCF-7) and 2 fold induction (LNCaP) was used to determine the genes involved in the networks and pathways detrmined by IPA analysis.

2.3. Reverse Transcription and Quantitative Real-Time PCR

2.3.1. Hypoxia Treatment

MCF-7 shSCX and shRb cells were split individually to 90% confluency in 2 Multiwell™ 6-well cell culture plates. The following day, one of the plates was placed into a hypoxia chamber set at 37 °C, 1% O_2, and 5% CO_2, while the other plate was left at 37 °C, 20% O_2, and 5% CO_2 for 24 h. Nitrogen was used to flush out oxygen in the hypoxic chambers.

LNCaP shSCX and shRb cells were split individually to 90% confluency in 2 Multiwell™ 6-well cell culture plates. The following day, one of the plates was placed into a hypoxia chamber set at 37 °C, 1% O_2, and 5% CO_2, while the other plate was left at 37

°C, 20% O_2, and 5% CO_2 for 24 h. Nitrogen was used to flush out oxygen in the hypoxic chambers.

2.3.2. RNA Extraction

After 24 h of either normoxia (20% O_2) or hypoxia (1% O_2) plates were taken out of the incubators, the culture media was removed, and cells were washed once with 1 mL of phosphate buffered saline solution. Cells were then collected in 750 μL of TRI Reagent® (Sigma) and transferred to appropriately labelled 1.5 mL microcentrifuge tubes. Then 200 μL of chloroform (Caledon) was added to each tube, after which the tubes were shaken vigorously by placing a rack on top of the one containing the tubes with the cell/TRI/chloroform mixture for 15-20 seconds. Next, the samples were centrifuged at 11,000 x g for 10 min at 4 °C, and approximately 200 μL of the top aqueous layer was transferred to a new, clean microcentrifuge tube on ice. 200 μL of Isopropanol was added to each tube (a 1:1 ratio) and the tubes were inverted several times before being centrifuged at 11,000 x g for 10 min at 4 °C. The supernatant was then carefully poured out and the pellet was washed in 750 μL of ice cold 75% Ethanol. At this stage the samples were either stored at – 80 °C or centrifuged at 14,000 x g for 15 min at 4 °C. The ethanol was poured out with caution and the tubes were pulsed down, and excess ethanol was removed. The pellet was then allowed to air-dry until all ethanol was evaporated after which it was re-suspended in 20 μL RNase and DNase free water (Sigma).

2.3.3. Reverse Transciption PCR

RNA concentration was determined using the NanoDrop Lite (Thermo Scientific) and 2 μg of RNA was transcribed for each sample using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems cat. No. 4368814) containing: 1 x RT Buffer, 1 x dNTP, 1 x random primers and 50 units MultiScribe™ reverse transcriptase for the 20 μL total reaction volume following the manufacturers guidelines. The reaction was carried out using the Veriti, 96 well Thermo Cycler (Applied Biosystems) with the following conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C until storage at - 20 °C.

2.3.4. Quantitative Real-Time PCR

Each cDNA sample was diluted at 1:15 and 4 μL of sample was added to 16 μL of master mix containing 1x Power SYBR Green (Applied Biosystems cat. No. 4472908), 3.0 pm primers and RNase and DNase free water for a total reaction volume of 20 μL. Standards were made from SCX normoxia for 36B4 and Rb hypoxia for all other targets using the following dilutions: 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000. The samples were placed in a MicroAmp® Fast Optical 96-well reaction plate (Applied Biosystems) and subjected to quantitative real-time PCR: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min and ending with 1 cycle of 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec in a StepOne Plus™ Real-Time PCR system (Applied Biosystems). Triplicate reactions were performed for each sample and data were averaged and normalized to the mean expression of the endogenous control gene, 36B4. The primer sequences used to quantify mRNA levels of 36B4, ASCL2, CXCR4, ENO2, GAL3ST1, HTR5A, KISS1R, NDRG1, PLOD2, S1PR4, and STC1 are listed in Table 2.1 and were obtained from Integrated DNA Technologies. All experiments were repeated in triplicate.

Table 2.1 List of Oligos used for MCF-7 and LNCaP Quantitative PCR

Gene	Gene Name	Oligo Sequences
36B4	Ribosomal protein 36B4	Forward: 5'-CCA CGG TGC TGA ACA TGC T-3' Reverse: 5'-TCG AAC AAC TGC TGG ATG AC-3'
ASCL2	Achaete-scute family bHLH transcription factor 2	Forward: 5’-AGC TGG TTA GGG GGC TAC TGA G-3’ Reverse: 5’-CCT TAT GGG GCC AGC TCC AAG-3’
CXCR4	Chemokine (C-X-C motif) receptor 4	Forward: 5'-TCT GTG ACC GCT TCT ACC -3' Reverse: 5'-AGG ATG AGG ATG ACT GTG G-3'
ENO2	Enolase 2	Forward: 5’-AGC CAT CGA CAA GGC TGG CTA C-3’ Reverse: 5’-TGG ACC AGG CAG CCC AAT CAT C-3’
GAL3ST1	Galactose-3-O-sulfotransferase 1	Forward: 5’-GAA GAC GCA CAA GAC GGC CA-3’ Reverse: 5’-AAG GCG AAC TTG AGC CGG TG-3’
HTR5A	5-hydroxytryptamine receptor 5A, G protein-coupled	Forward: 5’- GGC GGA CCG TGA ACA CCA T-3’ Reverse: 5’- ACT CTC CGC TGT CAT CTC TC TGG-3’
KISS1R	KISS1 receptor	Forward: 5’-CGT TCG GTG CAG TTT CGT TGT GAA-3’ Reverse: 5’- CTG GAA TGA TCC AGA AAG TCC TGT G-3’
NDRG1	N-myc downstream regulated 1	Forward: 5’-CGC CAG CAC ATT GTG AAT GAC-3’ Reverse:- 5’-TTT GAG TTG CAC TCC ACC ACG-3’

Gene	Gene Name	Oligo Sequences
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	Forward: 5'- GCG TTC TCT TCG TCC TCA TC-3' Reverse: 5'-GTG TGA GTC TCC CAG GAT GC-5'
S1PR4	Sphingosine-1-phosphate receptor 4	Forward: 5'-TCC ACC TTC AGC CTG CTC TTC A-3' Reverse: 5'-CCC TGC TGC GGA AGG AGT AG-3'
STC1	Stanniocalcin 1	Forward: 5'-CCA TGA GGC GGA GCA GAA T-3' Reverse: 5'-TGA GGC AAC GAA CCA CTT CA- 3'

2.3.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.0 software. Statistical significance was determined by using One-way ANOVA with a Tukey multiple comparisons test. Comparisons were made between SCX normoxia and Rb Hypoxia, Rb normoxia and Rb hypoxia, and SCX hypoxia and Rb hypoxia. All values are presented as means $\pm$ standard error of the mean (SEM). A P-value < 0.05 was considered to be significant. For the mouse protein tissue samples, a t-test was used and comparisons were made between SCX and Rb, values are presented as means $\pm$ standard error of the mean (SEM).

2.4. Immuno-blotting

2.4.1. Hypoxia Treatment

MCF-7 shSCX and shRb cells were split individually into 10cm plates. The plates were then placed into a hypoxia chamber set at 37 °C, 1% O_2, and 5% CO_2, or left at 37 °C, 20% O_2, and 5% CO_2, from 2 – 7 days. After 2-7 days in hypoxia or normoxia whole cell lysates were collected.

LNCaP shSCX and shRb cells were split individually into 10cm plates. The plates were then placed into a hypoxia chamber set at 37 °C, 1% O_2, and 5% CO_2, or left at 37 °C, 20% O_2, and 5% CO_2, from 2 – 7 days. After 2-7 days in hypoxia or normoxia whole cell lysates were collected.

2.4.2. Whole Cell Lysate Collection and Protein Quantification

Cells were kept on ice and were washed twice with 1x ice cold PBS. Following the washes 500 μL of lysis buffer (50 mM Tris pH 8, 400 mM NaCl, 0.5 M EDTA, 0.1% (v/v) glycerol, 0.1% IGEPAL® CA-630, autoclaved milliQ water; per 1 mL lysis buffer 20 μL of 100x complete protease inhibitor cocktail (P.I., Bioshop) and 0.34 μL of β-mercaptoethanol was added and cells were scraped off and collected in 1.7 mL tubes on ice. Samples were kept on ice and vortexed every 5 min for a total of 20 min, followed by centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatant was then collected in aliquots and stored at – 80 °C. Protein concentration was determined using the RC DC protein assay (BioRad) following the manufacturer's protocol. Standards were made for the following concentrations: 0.2 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL and 2.0 mg/mL. Samples were diluted 1:3 with lysis buffer for a total volume of 25 μL, 125 μL of Reagent I was added to each sample, standard and blank and vortexed. Samples were then let to sit for 1 min following which 125 μL Reagent II was added, each sample was vortexed and centrifuged at 15,000 x g for 5 min at room temperature. The supernatant was carefully poured out and tubes were inverted over absorbent paper until all liquid was gone. After this, 127 μL of Reagent A' (made from 5 μL of DC Reagent S and 250 μL of Reagent A) was added to each sample, vortexed, and let to sit for 5 min. Following another round of vortexing 1 mL of DC Reagent B was added to each sample followed immediately by vortexing and incubation for 15 min at room temperature. Concentrations were then read using a SmartSpec™ Plus Spectrophotometer (Bio-Rad) at 750 nm absorbancy.

A mouse xenograft pilot study was carried out at the BC Cancer Agency. MCF-7 shSCX and shRb cells pre-treated with hypoxia were injected under the mammary fat pad of overiectomized female CB-17 SCID mice. Mice were euthanized after 12 weeks and tumour samples were flash frozen in liquid nitrogen. Tumour protein collection was carried out in lysis buffer and homogenized by manual grinding. Protein isolation and concentration determination was carried out as above.

2.4.3. Immuno-blotting

Equal protein concentrations were denatured in 5x SDS sample buffer (33.33 mM Tris-HCl, pH 6.8, 5.3% glycerol, 1.0% SDS, 26.6 mM β-mercaptoethanol and 0.006 % bromophenol blue) at 100 °C for 5 min. The samples and PageRuler Plus Prestained Protein Ladder (Thermo Scientific) were then resolved on an 8% SDS-polyacrylamide gel in 1x Running Buffer (1 M Tricine, 1 M Tris-Cl, 50 mM SDS). The protein was transferred to a polyvinylidene fluoride (PVDF) membrane at 0.6 Amps for 2 h in Transfer Buffer (25 mM Tris-Cl, 250 mM Glycine, 0.1% SDS). Upon completion of transfer the membrane was re-activated with 100% Methanol for 30-60 sec, and blocked for 1 h at room temperature in 1x TBST (2.5 mM Tris, 140 mM NaCl, 2.5 mM KCl, 0.5% Tween20) with 5% non-fat powdered milk. The membrane was then probed with primary antibody in 1x TBST + 5% milk at 4 °C overnight. The list of antibodies and dilutions used are listed in Table 2.2 and Table 2.3 .

Table 2.2 List of 1° Ab's used for MCF-7 immuno-blotting

1° Ab	Dilution	Species	Supplier and Catalogue Number	2° Ab dilution
anti-fusin/CXCR4	1/500	rabbit polyclonal IgG	abcam; ab2074	1/5000
anti-LOXL2	1/1,500	rabbit polyclonal IgG	Sigma-Aldrich; SAB1100132	1/15,000
anti-NDRG1	1/1,000	rabbit polyclonal IgG	Santa Cruz Inc.; sc-30040	1/10,000
anti-Rb	1/1,500	rabbit polyclonal IgG	Santa Cruz Inc.; sc-7905	1/10,000
anti-S1PR4/EDG6	1/300	mouse polyclonal IgG	Millipore; MABC97	1/7,500
anti-α-tubulin	1/1,500	mouse polyclonal IgG	Santa Cruz Inc.; sc-8035	1/7,500

Table 2.3 List of 1° Ab's used for LNCaP immuno-blotting

1° Ab	Dilution	Species	Supplier and Catalogue Number	2° Ab dilution
anti-ENO2	1/200	mouse polyclonal IgG	Sigma-Aldrich; SAB1405758-50UG	1/7,500
anti-HTR5A	1/1,500	rabbit polyclonal IgG	Sigma-Aldrich; SAB2101110-50UG	1/10,000
anti-KISS1R	1/500	rabbit polyclonal IgG	Sigma-Aldrich; SAB2700212	1/7,500

anti-NDRG1	1/1,000	rabbit polyclonal IgG	Santa Cruz Inc.; sc-30040	1/10,000
anti-PLOD2	1/1,000	mouse polyclonal IgG	Abnova; H00005352-B01P	1/10,000
anti-Rb	1/1,000	rabbit polyclonal IgG	Santa Cruz Inc.; sc-7905	1/10,000
anti-α-tubulin	1/1,000	mouse polyclonal IgG	Santa Cruz Inc.; sc-8035	1/10,000

The blots were then washed three times in 1x TBST + 5% milk with gentle rocking for 10 min at room temperature and incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (table 2.2 and table 2.3) in 1x TBST + 5% milk for 1 h at room temperature while rocking. Next, they were washed three times with 1x TBST for 5 min with gentle rocking at room temperature. Protein was detected using Luminata^{TM} Crescendo Western HRP Substrate (Millipore) and imaged using the Dyversity 2D-image analysis system (Syngene).

2.5. Immuno-cytochemistry

2.5.1. Slide Preparation and Hypoxia Treatment

18 mm round slides were soaked in 1 N Nitric Acid overnight, then washed two-three times with mili-Q water and stored in 70% Ethanol. Before use, the slides were washed twice with sterile PBS and coated with polyethylenamine for 30 min at 37 °C. LNCaP shSCX and shRb cells were seeded at 62,500 cells/slide on 18 mm round glass slides in a 12-well plate. One of the plates was placed into a hypoxia chamber set at 37 °C, 1% O_2, and 5% CO_2, while the other plates was left at 37 °C, 20% O_2, and 5% CO_2 for 96 h.

2.5.2. Immuno-staining

Following 96 h of hypoxia or normoxia the slides were washed two times with ice cold PBS, and fixed with ice cold Methanol at -20 °C for 10 min. The slides were then washed two times with ice cold PBS and blocked in 5% Normal Donkey Serum (Jackson ImmunoResearch Laboratories) and 0.3 M Glycine in PBS for 30 min at room temperature. Next the slides were incubated with either PBS or KISS1R primary antibody (1/100 dilution; abcam; ab140839) overnight at 4 °C. Following which, the

slides were washed two times with ice cold PBS for 5 min at room temperature. Next, the slides were incubated with Alexa Flour® 680 donkey anti-rabbit IgG (1/1000, Life technologies; A10043) in PBS for 1 h at room temperature, then washed two times with ice cold PBS for 5 min. Slides were stained with Hoescht (1/10,000) borrowed from the Lee lab in PBS for 1 min, followed by two washes of ice cold PBS. Lastly, the slides were mounted using FlourSave™ (Calbiochem), and imaged at 60x using water and the Metamorph software.

2.6. Wound Healing Assay

MCF-7 and LNCaP shSCX and shRb cells were split into 2, 6-well plates. One of the plates was placed into hypoxia (1% O_2) while the other was left at normoxia (20% O_2) for 72 h. The cells were then dosed with 10 μg/ml of mitomycin 2 h before a wound was made using a 2 mm comb. The cells were then carefully washed once with PBS, and refreshed with complete media specific to the cell type. Images were taken using the Olympus CP Controller program, these images were considered time 0. All plates were placed back into the humidified incubator set at 20% O_2 levels and 37 °C for 96 h, after which they were imaged again. Following imaging at time 96 h, cells were fixed with methanol at room temperature for 2 min and stained with 1% Toluidine blue for 5 min and were re-imaged. A fine tip marker was used for drawing vertical lines onto the 6-well plates to ensure imaging was being done on the same area at each time point measured.

Migration was determined by measuring the difference in distances between the wound edges at time 0 and time 96 h using the imageJ 1.48v software.

Chapter 3.

Results

3.1. Determination of hypoxia inducible genes further regulated by Rb in MCF-7 and LNCaP cells

Previously, it was shown that loss of Rb leads to an exacerbated hypoxic response in both breast (MCF-7) cancer cells and prostate (LNCaP) cancer cell lines, mediated through its interaction with Trip230 (Labrecque et al., 2014). Loss of Rb results in an increase expression of genes involved in cell invasion and metastasis (Labrecque et al., 2014). To further examine the role of Rb in hypoxia mediated gene expression, we interrogated the transcriptome of MCF7 and LNCaP cells using the gene expression 44K microarray. MCF-7 and LNCaP cells stably transfected with either control (scrambled, SCX) or Rb-specific short hairpin RNA's (shRNA) were placed into either normoxia or hypoxia for 24 h. Next, total RNA was isolated and checked for integrity before being labelled with a Quick Amp Labelling Kit (Agilent), hybridized and scanned by the Agilent Human genome wide expression 44K array. Bioinformatics was carried out at the Laboratory for Advanced Genome Analysis, Vancouver Prostate Centre.

The microarrays resulted in a large list of genes that were both up-regulated and down-regulated after 24 h of hypoxia. Due to the large amount of genes found on the microarray lists, we narrowed down the up-regulated gene list to include only the top 30 genes up-regulated after exposure to hypoxia and loss of Rb. Genes were chosen on the parameters that they were up-regulated and induced greater than 2-fold following loss of Rb and hypoxia compared to the negative controls for both MCF-7 and LNCaP cells (appendix A and B). The up-regulated genes in both MCF-7 and LNCaP cell lines were compared and a list of 21 common genes was developed (Table 3.1). Of the 21 common genes, 12 genes appear on the top 30 list for MCF-7 cells and 6 genes are on

the top 30 list for LNCaP cells, indicating that the HIF1 pathway along with the loss Rb has an impact on increasing gene expression. In particular, hypoxia and loss of Rb in both cell lines increases similar genes associated with epithelial to mesenchymal transition, tumour cell invasion, and metastasis (Kijima et al., 2002; Zhou et al., 2015).

Table 3.1 LNCaP and MCF-7 common list of up-regulated genes under hypoxia, and following loss of Rb.
The common genes that are up-regulated and induced greater than 2-fold by loss of Rb and hypoxia compared to the negative controls in both LNCaP and MCF-7 cells and the known pathways they are associated with. The increased fold induction for LNCaP cells are denoted by the white boxes and MCF-7 cells by the grey boxes.

Gene Name	Associated Pathway	Gene Fold Induction (vs. shSCX-normoxia)		
		shRb-nomoxia	shSCX-hypoxia	shRb-hypoxia
ASB2	Tumor	1.0888582	1.6343423	3.6415098
		1.6650	23.845	34.7281
ASCL2	Metastasis	1.1362084	2.552587	5.5468407
		1.4282	1.6306	2.5558
CXCR4	Metastasis	1.2965798	2.651608	62.5567
		0.9313	2.8015	3.7357
CYP26A1		1.1462438	2.8671377	68.5052
		1.0561	1.6842	3.548
EFCAB3		1.1659759	1.1482776	2.4258952
		1.8567	4.0265	7.0963
FAM13A	Tumor	1.3270723	1.3372574	4.1334763
		1.5326	6.8521	12.6826
FAM167A		1.7861654	1.1764673	4.151946
		1.8161	1.3958	3.0273
GAD1	Metastasis	1.7789828	1.7294159	5.8401966
		0.9050	1.3728	3.5480
GAL3ST1		1.6424665	1.0873642	2.807558
		1.0588	3.3125	10.4002
LDLRAD1		2.624331	1.0060819	8.452955
		1.7046	3.7053	11.5997
NDRG1	Metastasis	1.1927673	6.8038516	18.600838
		1.8595	20.7463	34.488
PADI2		1.9904629	1.4207916	5.3511834
		1.6067	1.6850	3.4993
PCP4L1		1.9710473	2.5147488	21.017593
		2.1561	4.7972	6.6552
S1PR4	Epithelial to mesenchymal transition	1.0949008	4.339506	10.621089
		2.4918	5.804	14.001

Gene Name	Associated Pathway	Gene Fold Induction (vs. shSCX-normoxia)		
		shRb-nomoxia	shSCX-hypoxia	shRb-hypoxia
SCNN1B		1.0179839	1.1496718	3.3335135
		0.9725	1.6127	5.6789
SCNN1G		1.0980676	1.2999465	19.01704
		1.4867	3.1485	7.9785
STBD1		1.1973119	1.2994887	3.7748692
		1.5457	1.6992	3.2452
STC1	Metastasis	1.0974838	4.4176493	18.996122
		1.7672	8.5651	13.8954
TBC1D8B		1.1889718	1.2218432	3.18768
		1.7199	1.1729	3.3942
TLE6		1.2411373	1.2337842	3.18392
		3.6094	5.383	9.7791
TNNT1		1.1826651	1.4182757	3.1716626
		1.5205	1.9868	2.6982

3.2. MCF-7 Microarray Gene Validation

MCF-7 microarray analysis conducted by the Ingenuity Pathway Assist (IPA) software system was used to develop network maps based on the genes that were up-regulated under hypoxia and loss of Rb (Figure 3.1 and 3.2). The genes highlighted in yellow represent hypoxia inducible genes up-regulated by loss of Rb and were chosen if their fold induction was $\geq$ 1.5 compared to the controls. These networks suggest that many of the genes up-regulated after loss of Rb and under hypoxic conditions are involved in regulating key nodes including NFκB, PDGF BB, MEK and ERK1/2 which are associated with increasing epithelial to mesenchymal transition and promoting tumour metastasis (Hou, Lin, Hou, & Liu, 2014; Huber et al., 2005; 2004). From our common gene list (Table 3.1) 2 genes (STC1, and GAL3ST1) are highlighted in Figure 3.1 and are associated with tumour cell metastasis and migration (Yoon et al., 2014), and 3 genes (ASCL2, CXCR4, and CYP26A1) show up in Figure 3.2 and are associated with tumour cell metastasis, migration and epithelial to mesenchymal transition (Kijima et al., 2002; Zhou et al., 2015). The pathways associated with these nodes and common

genes highlight important roles for Rb and hypoxia in regulating key biological processes linked with tumour cell transformation according to the IPA analysis.

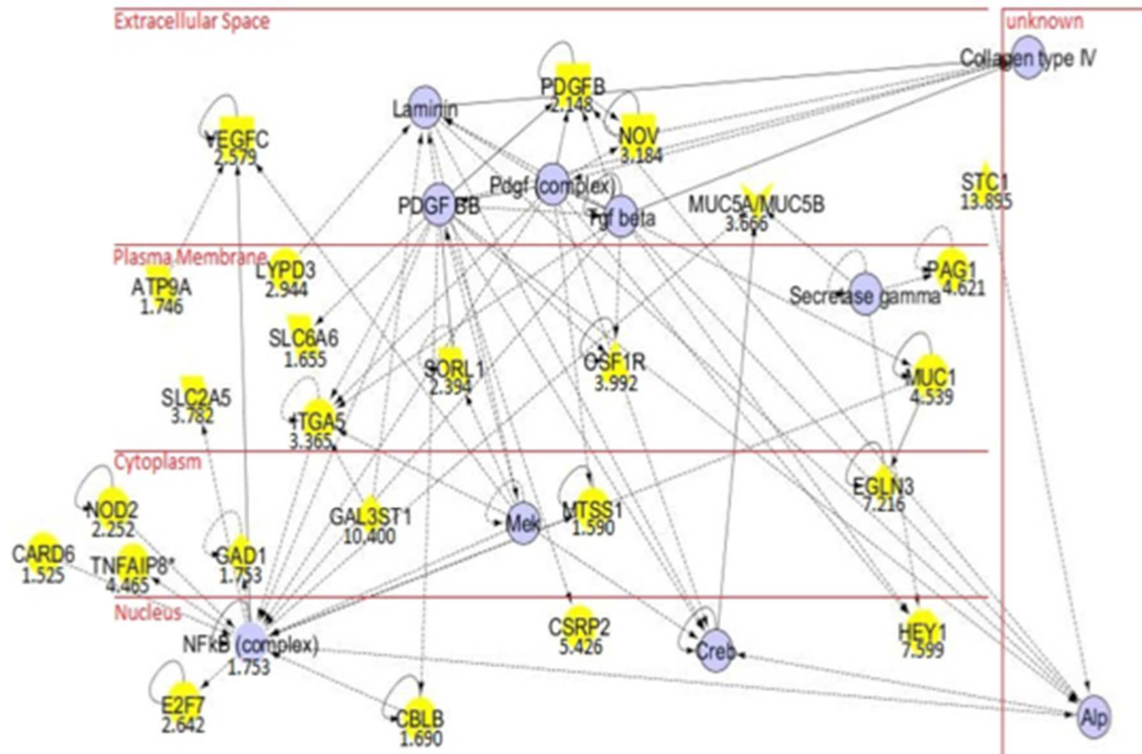


Figure 3.1 Schematic illustration of the network of genes with modified expression under hypoxia and loss of Rb in MCF-7 cells.

IPA analysis of the signaling pathways linked with organ development, and cellular movement. NFκB, Mek, and PDGF BB are nodes of this network. Hypoxia inducible genes up-regulated by the loss of Rb are presented in yellow. The genes presented in blue are absent from our MCF-7 microarray list but are suggested as part of the network by IPA analysis. (Gene ontology score = 47)

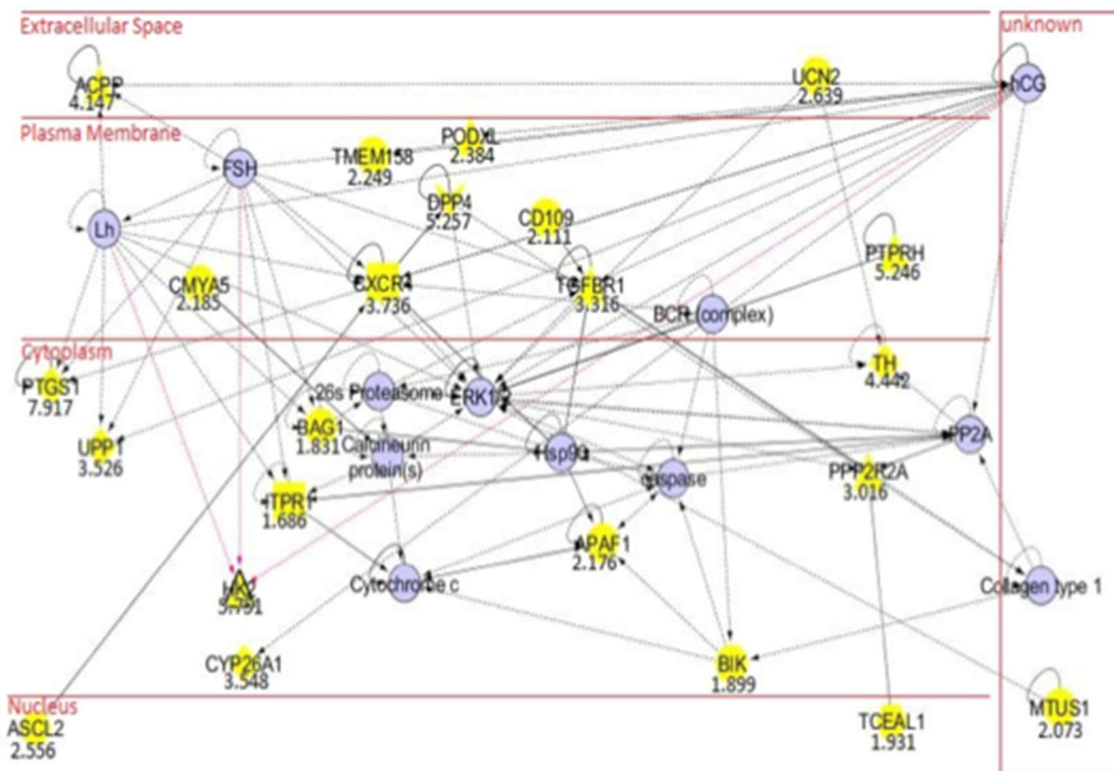


Figure 3.2 Schematic illustration of the network of genes with modified expression under hypoxia and loss of Rb in MCF-7 cells.

IPA analysis of the signalling pathways linked with drug metabolism, and cellular movement. A key node of this pathway is Erk 1/2. Hypoxia inducible genes up-regulated by the loss of Rb are presented in yellow. The genes presented in blue are absent from our MCF-7 microarray list but are suggested as part of the network by IPA analysis. (Gene ontology score = 41)

3.2.1. Assessment of MCF-7 shSCX and shRb cells gene expression following hypoxia

To validate the MCF-7 microarray data, genes from the common list (table 3.1) and Appendix A were chosen based on gene ontology analysis if there were involved in tumour cell transformation and metastasis. MCF-7 shSCX and shRb cells were seeded into two 6-well tissue culture plates; one plate was placed into hypoxia (1% O_2) while one was left at normoxia (20% O_2). Following 24 h, cDNA was made and samples were used for QPCR to determine relative mRNA expression levels of Rb, CXCR4, S1PR4, GAL3ST1, NDRG1, and STC1. All genes were normalized against mRNA expression levels of the ribosomal protein 36B4 (Figure 3.3). Following exposure to hypoxia

transcript levels of CXCR4, S1PR4, GAL3ST1, NDRG1, and STC1 all increase. However, this increase is further enhanced by the loss of Rb. These results confirm the data from our MCF-7 microarray and indicate that Rb plays a significant role in increasing expression of hypoxia-inducible genes. In particular, loss of Rb increases expression of genes that have been found to play a role in tumour metastasis and EMT (Kijima et al., 2002; Zhou et al., 2015).

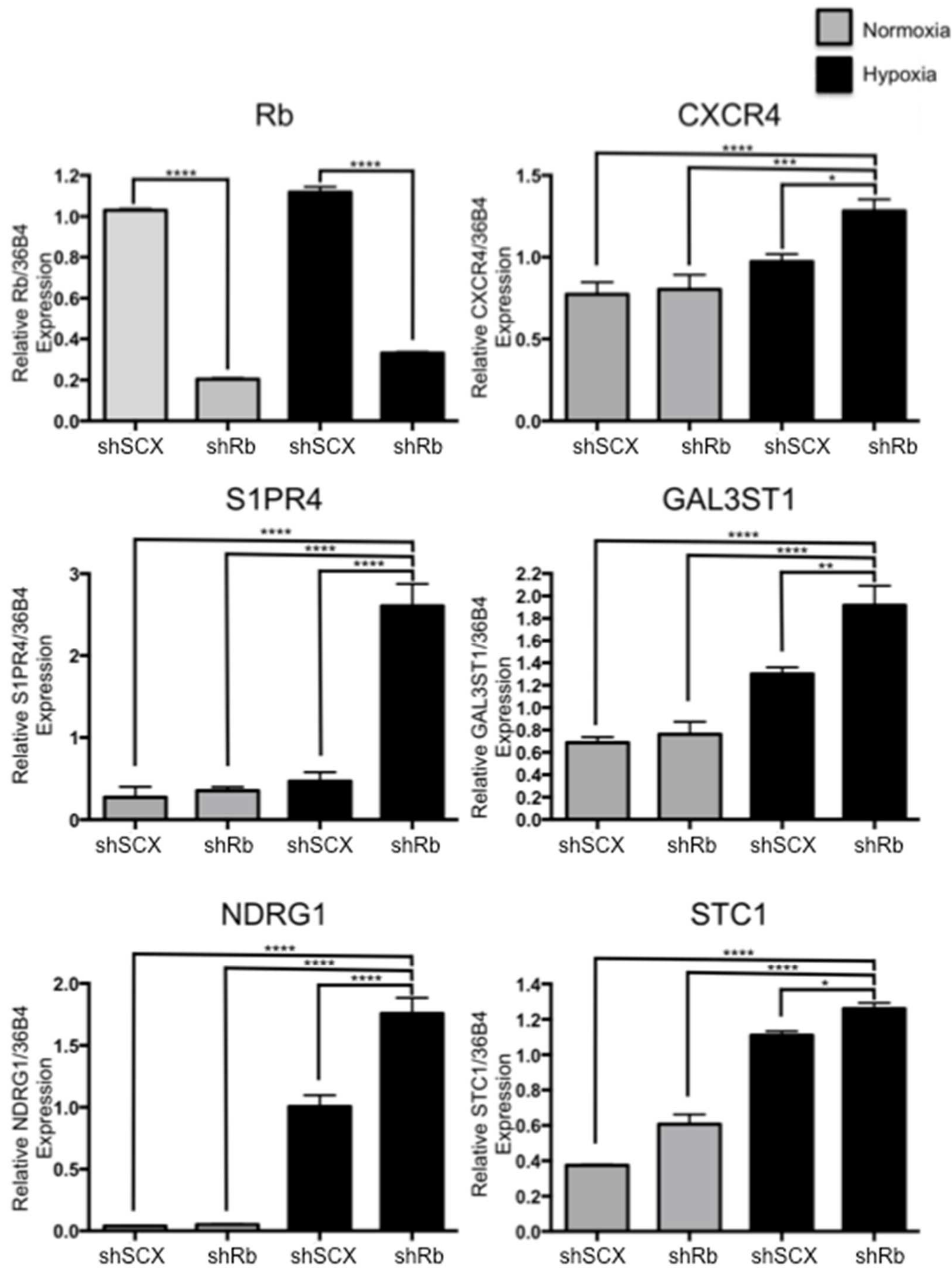


Figure 3.3 Loss of Rb together with hypoxia in MCF-7 cells results in an increased expression of genes involved in cell invasion, and differentiation.
MCF-7 shSCX and shRb cells were placed into hypoxia (1% O_2) or normoxia (20% O_2) for 24 h. Rb, CXCR4, S1PR4, GAL3ST1, NDRG1 and STC1 transcript levels were quantified by PCR. All mRNA levels were normalized against 36B4. Values are presented as means $\pm$ SEM (n=3). * $P<0.05$, ** $P<0.01$, *** $P<0.005$, **** $P<0.001$.

3.2.2. Translational validation of genes up-regulated in MCF-7 shSCX and shRb cells at various time points in a hypoxic environment

MCF-7 shSCX and shRb cells were seeded in 10 cm tissue cultures plates, and exposed to hypoxic conditions from 48 h up to 7 days. Protein levels were detected by immuno-blotting using anti-Rb, anti-NDRG1, and anti-S1PR4 antibodies. We checked the Rb protein level to ensure that Rb was still knocked down in our MCF-7 shRb cell line (Figure 3.4B). For the most part, Rb still seemed be knocked down in the MCF-7 shRb cell line, although the last lane (Rb, 7 days of hypoxia) shows Rb reappearance. Following loss of Rb there is a higher level of S1PR4 protein in normoxic conditions compared to the shSCX sample. Hypoxia causes an increase in S1PR4 protein levels in the shSCX samples however, the greatest increase occurs after 96 h of hypoxia and loss of Rb (Figure 3.4A). Similarly, exposure to hypoxia leads to an increase in NDRG1 protein in both MCF-7 shSCX and shRb samples with a significantly greater extent of protein accumulation occurring in the shRb samples compared to the shSCX samples after 5 and 7 days of hypoxia (Figure 3.4B). Protein levels of GAL3ST1 and STC1 were not validated, as we did not have a working antibody for them. Since S1PR4 and NDRG1 play a role in epithelial to mesenchymal transition (EMT) (Pyne & Pyne, 2013) and metastasis (Park, Adams, Lachat, Bosman, Pang, & Graham, 2000) respectively, loss of Rb under hypoxic conditions may be involved in increasing MCF-7 tumour cells ability to escape its microenvironment and metastasize to other organ sites.

Figure 3.4 Loss of Rb and hypoxia leads to an increase in S1PR4 and NDRG1 protein level in MCF-7 cells.

MCF-7 shSCX and shRb cells were exposed to normoxia (20% O_2) and hypoxia (1% O_2) from 48 h to 7 days. **A.** The top panel represents the quantified protein levels of SIPR4 and the bottom panel shows the representative immuno-blot used for quantification. **B.** The two top panels illustrate quantified levels of Rb and NDRG1 in MCF-7 shSCX and shRb cells and the bottom panel represents one of the representative immuno-blots used for quantification. α-tubulin was used as the loading control. Values for NDRG1 are presented as means ± SEM (n=2). * P<0.05, *** P<0.005.

To determine if the increased expression of S1PR4 and NDRG1 were concomitant with EMT and tumour cell transformation, we looked to see if there was a corresponding decrease in E-cadherin protein levels (an EMT marker) (Vincent-Salomon & Thiery, 2003) following loss of Rb and hypoxic exposure for 3-7 days (Figure 3.5). Under normoxic conditions loss of Rb results in lower E-cadherin expression. Decrease in E-cadherin is also observed in hypoxic samples. However, under the varying times points of hypoxia there is no greater decrease in E-cadherin between the shSCX and shRb samples. This indicates that Rb may play a role in controlling E-cadherin expression but under hypoxic conditions there is no further difference following Rb loss.

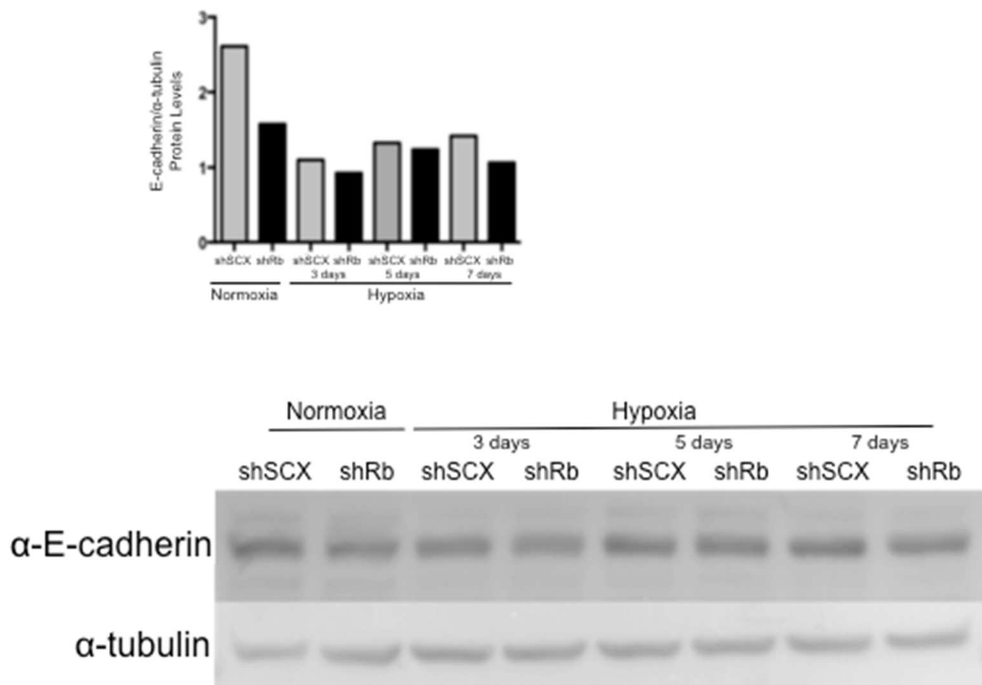


Figure 3.5 Exposure to hypoxic condition causes a decrease in E-cadherin protein level.
MCF-7 shSCX and shRb cells were exposed to normoxia (20% O_2) and hypoxia (1% O_2) for 3-7 days. The top panel illustrates the quantified protein levels of E-cadherin and the bottom shows the representative immuno-blots used for the quantification. α-tubulin was used as the loading control.

We looked at nuclear associated lysyl hydroxylase 2 (LOXL2) protein levels because it was on our list of the top 30 genes up-regulated by loss of Rb and hypoxia in MCF-7 cells (Appendix A). Interestingly LOXL2 levels are the highest in the shSCX and shRb normoxic samples (Figure 3.6). With exposure to hypoxia there is a decrease in LOXL2 levels with no difference between the shSCX and shRb cell lines. These results suggest that other factors may be regulating LOXL2 at the translational level.

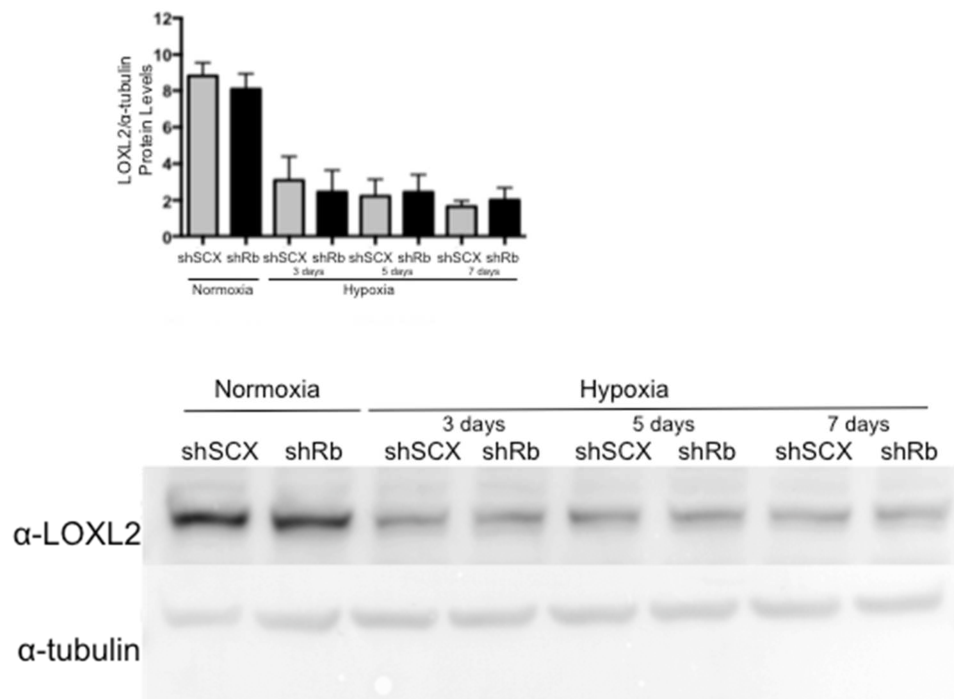


Figure 3.6 Exposure to hypoxia causes a decrease in LOXL2 protein levels.
MCF-7 shSCX and shRb cells were exposed to normoxia (20% O_2) and hypoxia (1% O_2) for various time points up to 7 days. The top panel illustrates the quantified protein levels of LOXL2 and the bottom shows one of the representative immuno-blotss for LOXL2 used for quantification. α-tubulin was used as the loading control. Values are presented as means $\pm$ SEM (n=3).

3.2.3. Assessment of MCF-7 shSCX and shRb mouse tumour cells

A xenograft pilot study was carried out in SCID mice using MCF-7 shSCX and shRb cells. MCF7 shRb and shSCX cells pre-treated with hypoxia were injected under the mammary fat pad of ovariectomized mice at the BC Cancer Agency. Animals were

euthanized after 12 weeks and tissues were harvested immediately afterwards and flash frozen in liquid nitrogen. The shRb mouse tumours were noticeably larger than the shSCX tumours (approximately 2-fold) (Beischlag lab, unpublished data).

The tumour samples were placed into lysis buffer for protein extraction. Mouse tumour protein levels were probed for human Rb, human CXCR4 and human S1PR4 using human-specific Rb, CXCR4 and S1PR4 polyclonal antibodies (Santa Cruz, Abcam and Millipore respectively). The first five lanes represent 5 different shSCX mouse tumour samples and the last four represent different shRb mouse tumour samples. Looking at the blot (Figure 3.7, bottom panel) we can see that the protein levels of CXCR4 varied between each sample. Nevertheless, an average of the shSCX and shRb quantified protein levels show that CXCR4 protein levels are significantly higher ($p<0.05$) in the mouse shRb tumour cells compared to the shSCX tissue samples (Figure 3.7, top panel). While S1PR4 protein expression was absent in most of the samples, one of the shRb hypoxia samples (lane 8) was seen to have S1PR4 expression (Figure 3.7, bottom panel). We also found that levels of Rb were re-appearing in the shRb mouse tumour samples (Figure 3.7, bottom panel) which may have impacted some of the CXCR4 and S1PR4 levels observed. Taken together, these results show that even with individual differences, the overall effect of hypoxia, and Rb knockdown in mice causes an increase in CXCR4 protein expression and possibly an increase in S1PR4 protein expression.

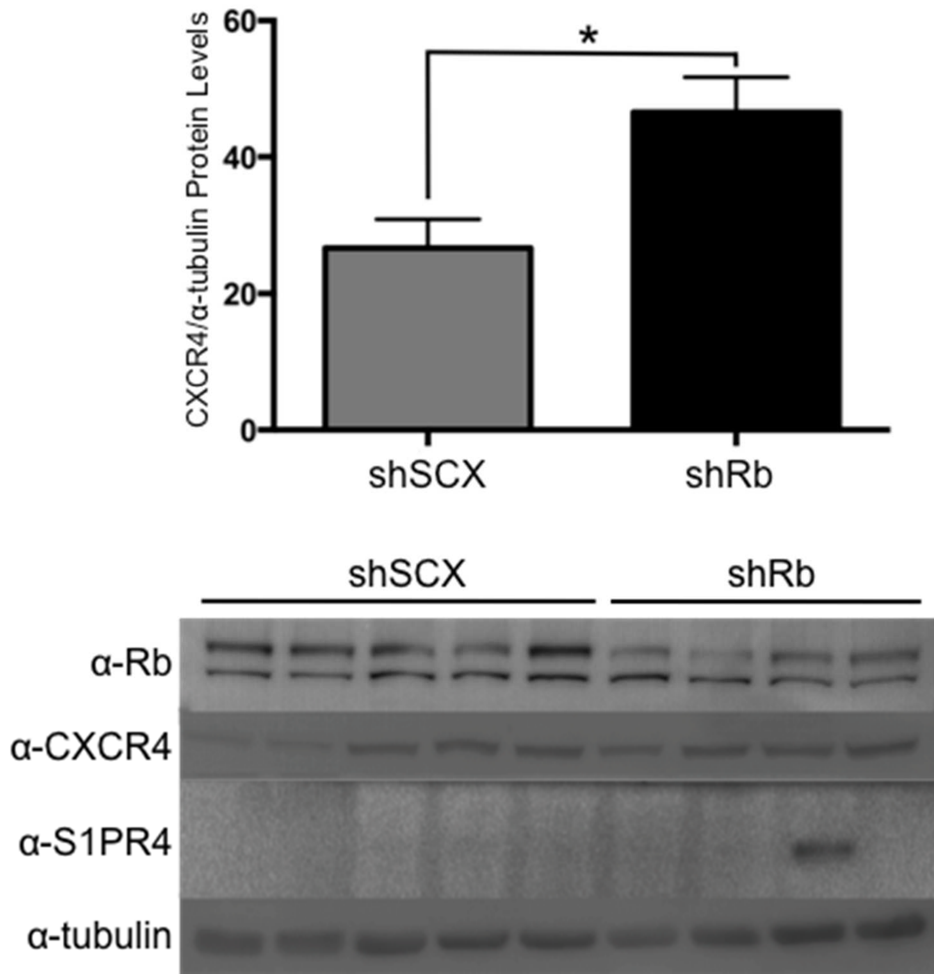


Figure 3.7 Hypoxia and Rb loss results in changes in CXCR4 and S1PR4 protein levels in CB-17 SCID female mice.
The top panel illustrates an average of shSCX and shRb quantified CXCR4 protein levels and the bottom panel represents the immuno-blot for S1PR4 and the immuno-blot used for the quantification of CXCR4. α-tubulin was used as the loading control. The first five lanes represent different shSCX mouse tumour samples and the last four represent different shRb mouse tumour samples. Values for the top panel are presented as means $\pm$ SEM (SCX, n=5; Rb, n=4). * $P<0.05$.

3.3. LNCaP microarray gene validation

3.3.1. Assessment of LNCaP shSCX and shRb gene expression following exposure to hypoxia

Many of the top up-regulated genes from the LNCaP tables are involved in neuroendocrine differentiation (NED) and tumour cell invasion and metastasis (Appendix B and Table 3.1). Therefore, genes from both of these lists were used for further validation of the microarray data.

LNCaP shSCX and shRb cells were seeded into two 6-well tissue culture plates and placed into either hypoxia (1% O_2) or maintained in normoxic conditions (20% O_2). Following 24 h of hypoxia or normoxia, cDNA was used in QPCR to determine relative mRNA expression levels of Rb, CXCR4, PLOD2, NDRG1, STC1, ASCL2, ENO2, and HTR5A (Figure 3.8). Levels of gene transcript were normalized against mRNA levels of the internal control, 36B4, and experiments were repeated three times. Transcriptional levels of PLOD2, NDRG1, STC1 and ASCL2 were all increased following exposure to hypoxia. However, the inductions were more significant following loss of Rb in a hypoxia-dependent fashion, confirming our array data. Also, with loss of Rb and hypoxic conditions there is an increase in the NED markers ENO2, and HTR5A. This identifies a novel role for Rb in increasing both cell invasiveness and NED in LNCaP cells in hypoxic environments.

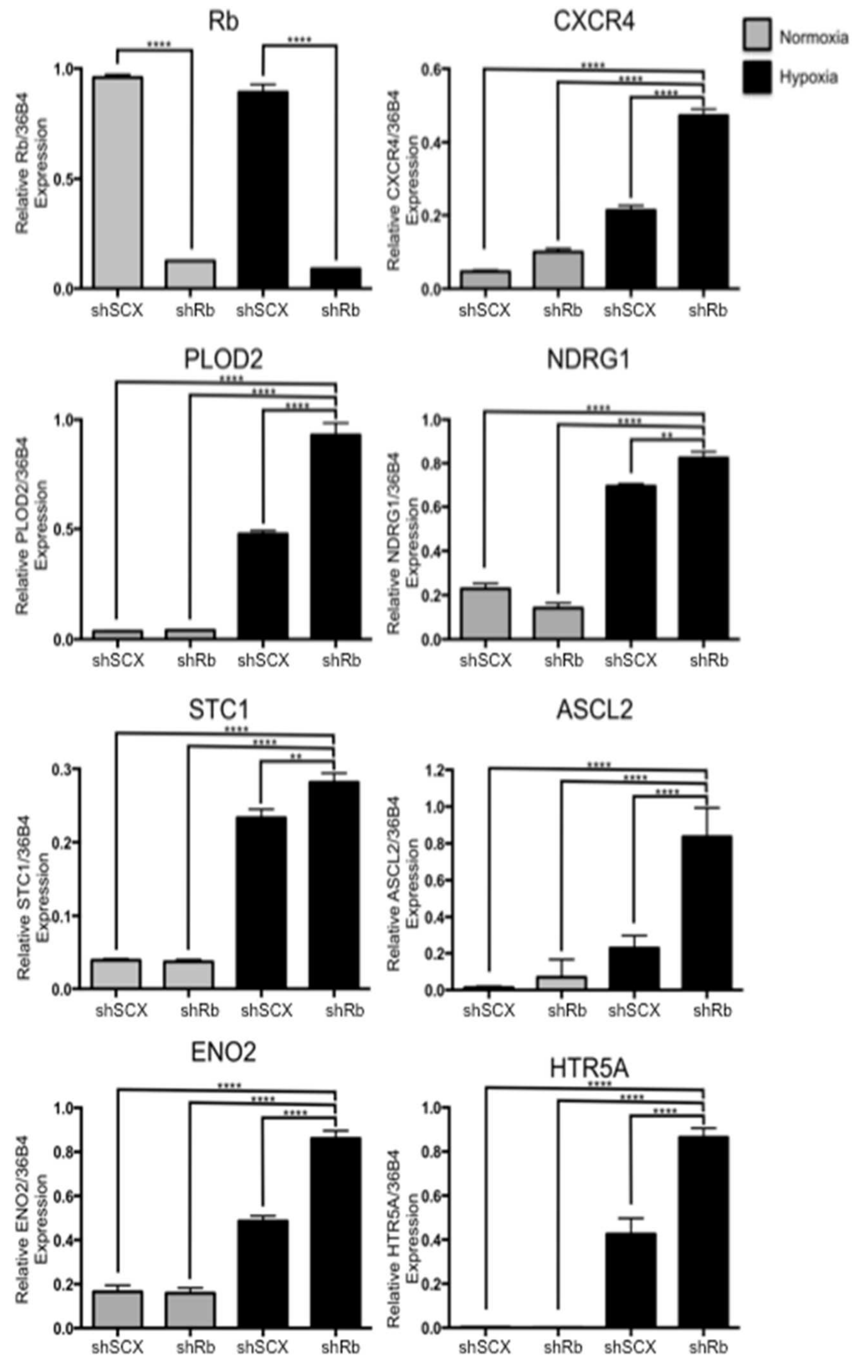


Figure 3.8 Hypoxic conditions and loss of Rb in LNCaP cells results in an increased expression level of genes involved in metastasis and neuroendocrine differentiation.
LNCaP shSCX and shRb cells were placed into hypoxia (1% O_2) or normoxia (20% O_2) for 24 h. Rb, CXCR4, PLOD2, NDRG1, STC1, ENO2, ASCL2 and HTR5A transcript levels were quantified by PCR. All mRNA levels were normalized against 36B4. Values are presented as means $\pm$ SEM (n=3). ** P<0.01,**** P<0.001.

3.3.2. Translational validation of the genes up-regulated in LNCaP shSCX and shRb cells following exposure to various time points of hypoxia

The results of Figure 3.6 agreed with our microarray data, however to further validate the increasing expression of PLOD2, NDRG1, HTR5A, ENO2, and KISS1R following loss of Rb, and induction of the HIF1 pathway, we also looked at their protein levels. LNCaP shSCX and shRb cells were seeded in 10 cm tissue cultures plates, and placed into normoxia (20% O_2) or hypoxia (1% O_2) for various exposure times ranging from 48 h up to 7 days. Whole cell lysates were collected and equal amounts of samples were run on an SDS-page gel. Exposure to 24 h of hypoxia causes an increase in the protein levels of genes associated with metastasis including PLOD2, and NDRG1 in both shSCX and shRb samples (Figure 3.9). This increase is enhanced following 48 h and 72 h of hypoxia, except that the shRb samples are expressing higher PLOD2 and NDRG1 protein levels compared to the shSCX samples. NDRG1 protein levels seem to increase the most following loss of Rb and hypoxic conditions lasting 96 h. On the other hand the protein levels of genes associated with NED, such as ENO2, KISS1R and HTR5A increase after 3 days of hypoxia and are further enhanced by loss of Rb (Figure 3.9). ENO2, KISS1R, and HTR5A protein levels are seen to continuously increase following further exposure times to hypoxia in the shRb samples. Protein levels of ASCL2 and STC1 were not validated as we did not have a working antibody for them. These results show that hypoxia, with the loss of Rb may promote a more invasive phenotype in LNCaP cells because these conditions lead to increased levels of genes seen to be involved in tumour cell metastasis (PLOD2 and NDRG1) (Gilkes, Bajpai, Chaturvedi, Wirtz, & Semenza, 2013a; Park et al., 2000), and NED (ENO2, HTR5A, and KISS1R) (Abrahamsson, 1999).

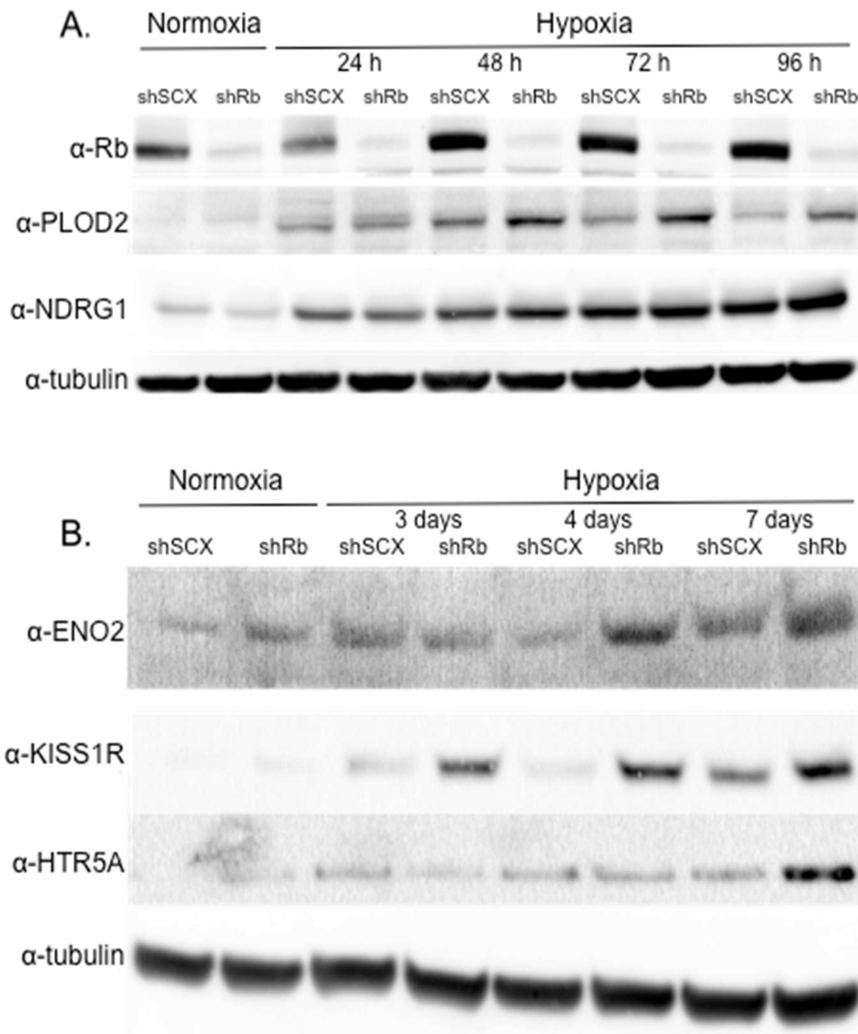


Figure 3.9 Loss of Rb increases protein levels of HIF1 target genes involved in metastasis and neuroendocrine differentiation.
LNCaP shSCX and shRb cells were placed into normoxia (20% O_2) or hypoxia (1% O_2) up to 7 days**. A.** Immuno-blot results representing Rb, PLOD2 and, NDRG1 protein levels **B.** Immuno-blot results representing ENO2, KISS1R and HTR5A protein levels. α-tubulin was used as the loading control.

We wanted to examine if the increase in hypoxia inducible genes, enhanced by loss of Rb resulted in decreased E-cadherin expression. Results show that E-cadherin protein level decreases following loss of Rb in both the normoxic and hypoxic samples, but following 5-7 days of hypoxia it decreases to a greater extent (Figure 3.10).

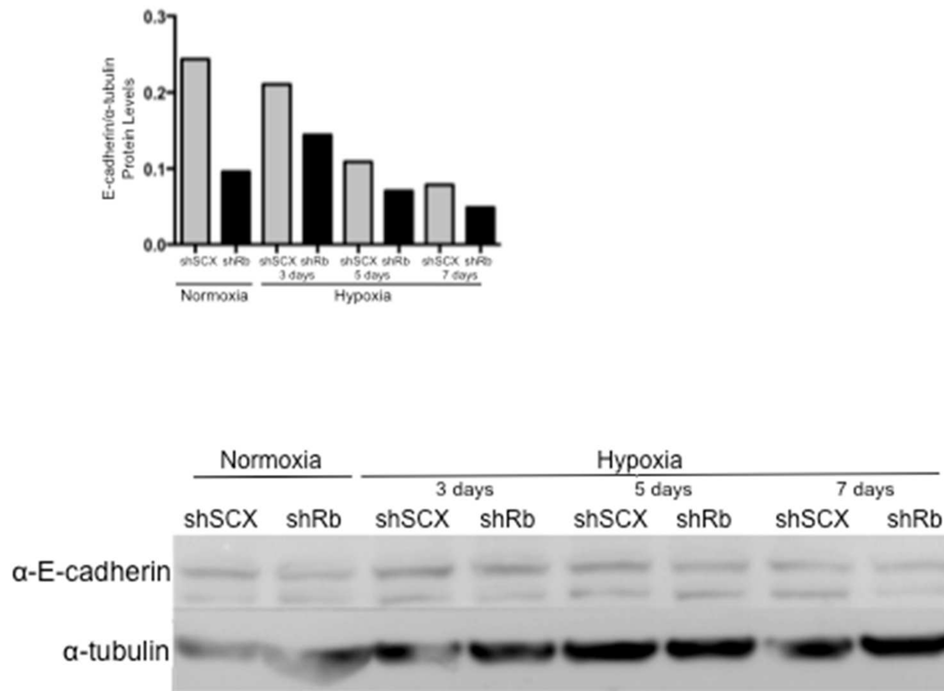


Figure 3.10 Exposure to hypoxic conditions causes a decrease in E-cadherin protein level.
LNCaP shSCX and shRb cells were exposed to normoxia (20% O_2) and hypoxia (1% O_2) from 3-7 days. The top panel illustrates the quantified protein levels of E-cadherin and the bottom shows the representative immuno-blots used for the quantification. α-tubulin was used as the loading control.

KISS1R is a G protein-coupled receptor that works with kisspeptin (KP) to enable the secretion of gonadotropin-releasing hormone (Millar & Babwah, 2015). This in turn regulates the neuroendocrine reproductive axis (Millar & Babwah, 2015), thus making KISS1R an attractive target to examine when investigating NED. From what we showed earlier, KISS1R protein levels increase greatly with the loss of Rb under hypoxic conditions. However, we wanted to investigate whether this increase is occurring due to a subset of cells or due to all cells expressing more KISS1R. To do this LNCaP shSCX and shRb cells were seeded at 62,500 cells/slides on 18 mm round glass slides and grown in 12-well plates. The slides were then either maintained in normoxia or exposed to hypoxic conditions for 96 h, and blocked with anti-KISS1R antibody (Abcam). The slides were imaged and analyzed using the Metamorph software. Figure 3.11A shows

that exposure to hypoxia causes an increase in KISS1R accumulation in most of the cell population, with a higher expression observed in hypoxic and Rb negative cells. Using the Metamorph software, we counted the amount of fluorescent foci seen in each sample and found that the shRB hypoxic cells had a significantly higher (approximately 2-fold, $P<0.005$) number compared to the shSCX hypoxic samplesand both normoxic samples (Figure 3.11B). Taken together this data shows that the effect of Rb loss under hypoxic conditions has an effect in increasing KISS1R translation in large cell populations, which may increase the chance of the tumour cells developing NED.

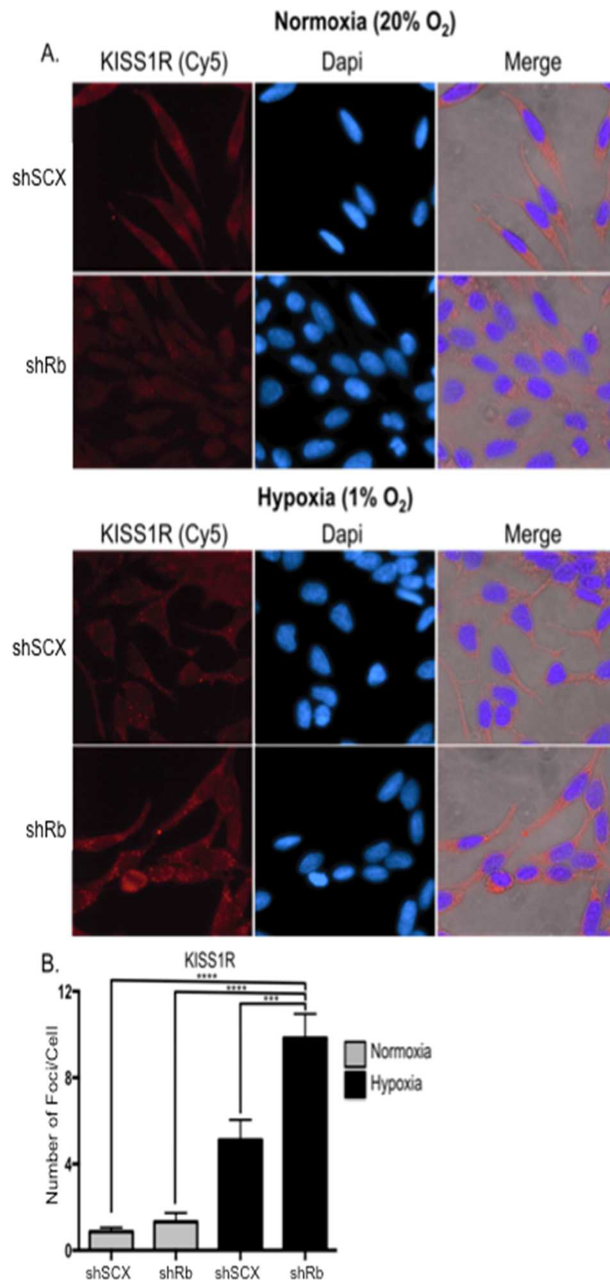


Figure 3.11 KISS1R expression increases following loss of Rb and exposure to hypoxia.

LNCaP shSCX and shRb cells were seeded at 62,500 cells/slide and placed into normoxia (20% O_2) or hypoxia (1% O_2) for 96 h. Slides were fixed with ice-cold methanol and probed with antibody specific to KISS1R. **A.** KISS1R levels were visualized using Alexa Flour® 680 donkey anti-rabbit IgG and Dapi staining was used for nuclei visualization. **B.** A graph representing the averaged quantified levels of fluorescence detected by the Cy5 filter for KISS1R levels from 6 experiments in LNCaP shSCX and shRb cells after exposure to either normoxia or hypoxia. Values are presented as means $\pm$ SEM (n=6). *** P<0.005, **** P<0.001.

3.3.3. Loss of Rb and hypoxia causes morphological changes in LNCaP cells.

From the previous figures we observed that hypoxia and Rb together cause a high increase in gene transcription and translation of genes involved in changing the LNCaP cells to become more invasive, and possibly go through NED. To see if any changes in the cells were occurring we seeded LNCaP shSCX and shRb cells on glass slides at 62,500 cells/slide. After which they were placed in either normoxia or hypoxia for 96 h. Following exposure to hypoxia or normoxia the slides were imaged using an Olympus inverted microscope. The LNCaP shRb slides that were exposed to hypoxia showed observable differences in cellular shape and structure compared to the LNCaP shSCX normoxia and hypoxia slides, and the LNCaP shRb normoxia slides (Figure 3.12). Instead of growing close together as the LNCaP cells normally do, the LNCaP shRb hypoxia cells grew further apart, and appeared to have long dendrite-like extensions. However, since we did not stain the cells for any neuronal markers, we cannot say for certain what is causing these morphological changes, only that they are occurring.

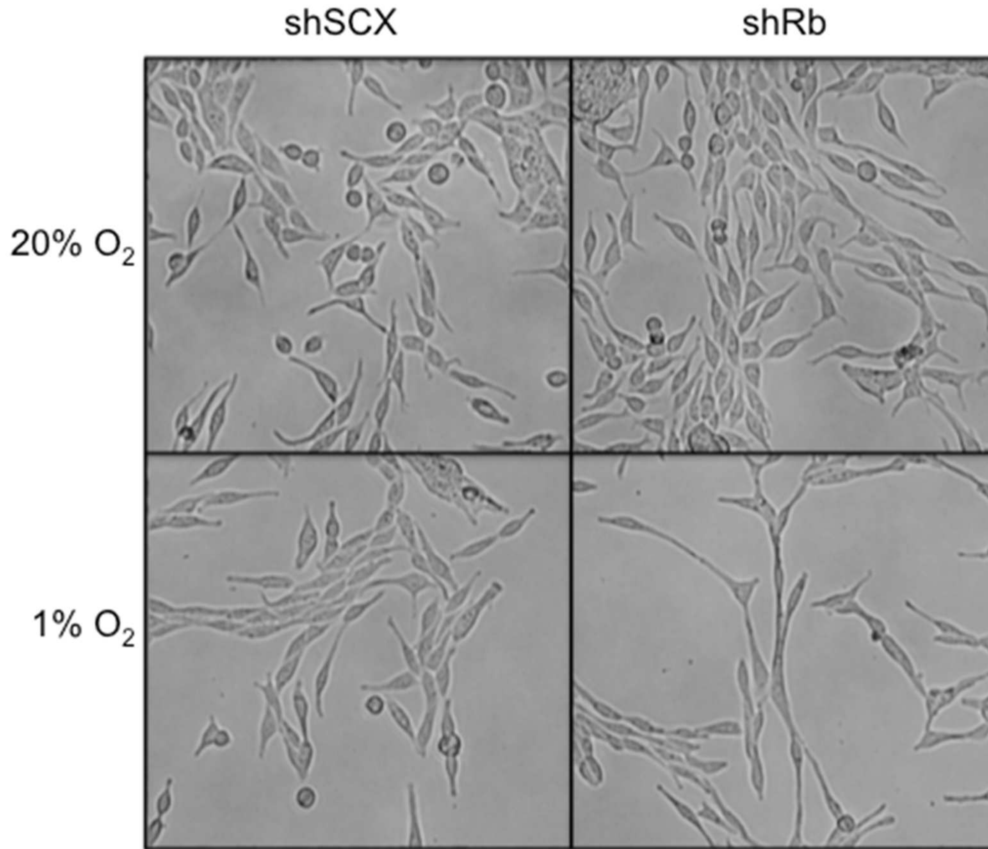


Figure 3.12 Loss of Rb together with exposure to hypoxic conditions leads to morphological changes in LNCaP cells.
LNCaP shSCX and shRb cells were seeded at 62,500 cells/slide and placed into hypoxia (1% O_2) or normoxia (20% O_2) for 96 h. Slides were then imaged at 40x using the Olympus CP Controller program.

3.4. The role of Rb and hypoxia on tumour cell migration

Now that we know loss of Rb is capable of increasing hypoxic gene induction, we wanted to examine if this actually caused the cells to change their phenotype to be more aggressive and gain the ability to migrate. LNCaP shSCX and shRb cells were seeded in two 6-well plates, one of the plates was placed into hypoxia for 72 h and the other was left at normoxia. Following 72 h of hypoxia or normoxia, cells were treated with 10 μg/ml of mitomycin for 2 h before a 2 mm horizontal scratch was made using a comb (Saraon et al., 2012). Initially images of the wound were taken after the wound was made (time 0

h), and 24 h, 48 h, 72 h, and 96 h afterwards. No difference in cell movement occurred until 96 h post wound addition. Therefore this time point along with time 0 h was used for all subsequent imaging. Cell movement occurred in both conditions and in both LNCaP short hairpin cell lines: shSCX normoxia, shRb normoxia, shSCX hypoxia and shRb hypoxia cells (Figure 3.13). There was no major difference with regards to migration pattern in each of the groups.

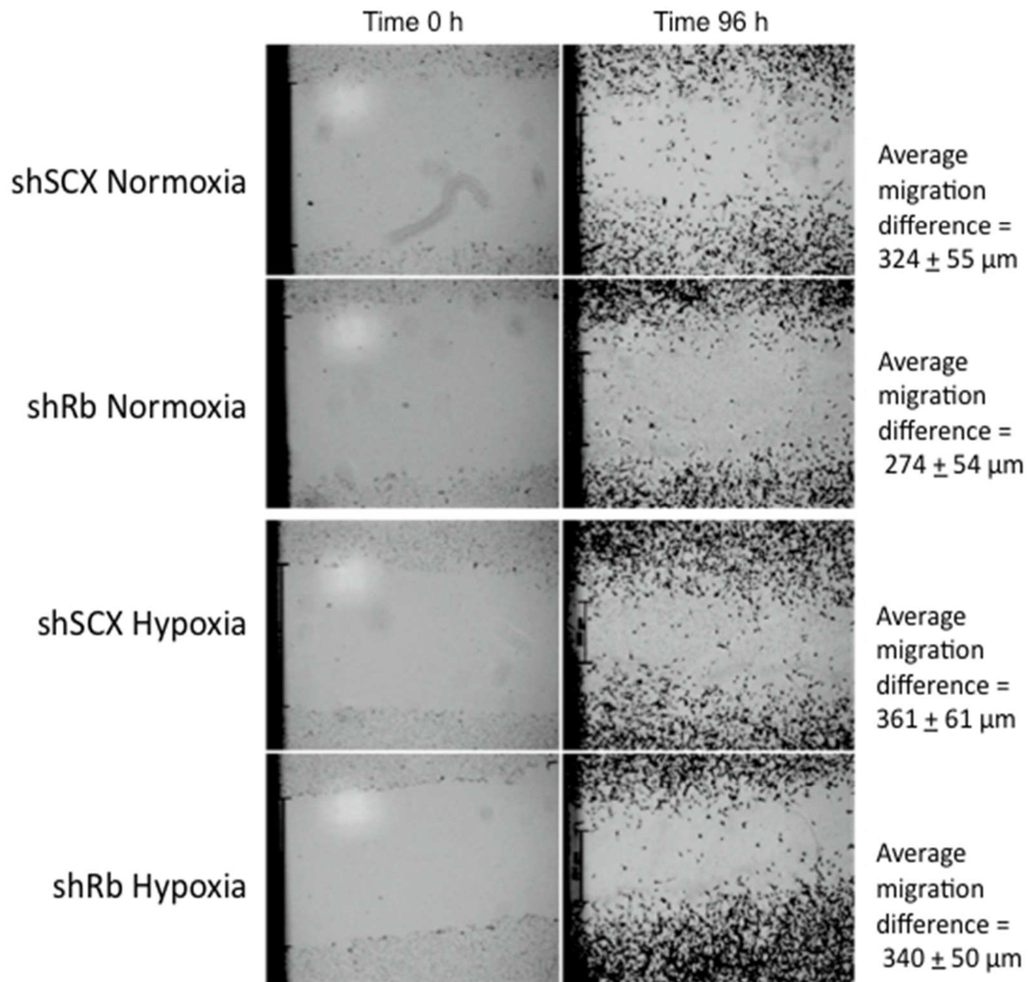


Figure 3.13 Loss of Rb and hypoxia does not have an affect on LNCaP migration ability
LNCaP shSCX and shRb cells were plated in 6-well tissues culture plates and placed into hypoxia (1% O_2) or maintained in normoxic (21%O_2) conditions for 96 h. After which a wound was made and cells were imaged at time 0 h and 96 h post wound. Migration was assessed based on the difference in gap distance (n=9).

Similar conditions were carried out for MCF-7 shSCX and shRb cells. Figure 3.14 shows that hypoxia decreased migration of MCF-7 shRb cells. However, the shRb normoxic conditions present a striking increase in migration distance compared to all the other conditions. Even in the hypoxic condition, the shRb cells seem to be migrating slightly more than the shSCX cells, although the migration is far less than the normoxic

conditions. This may be representing a role for Rb loss in increasing the migratory ability of MCF-7 cells. Validation from our microarray indicates that loss of Rb and hypoxia create tumour cells that are more invasive, and migratory, however, we were unable to see any changes in cell migration following observation with wound healing assays.

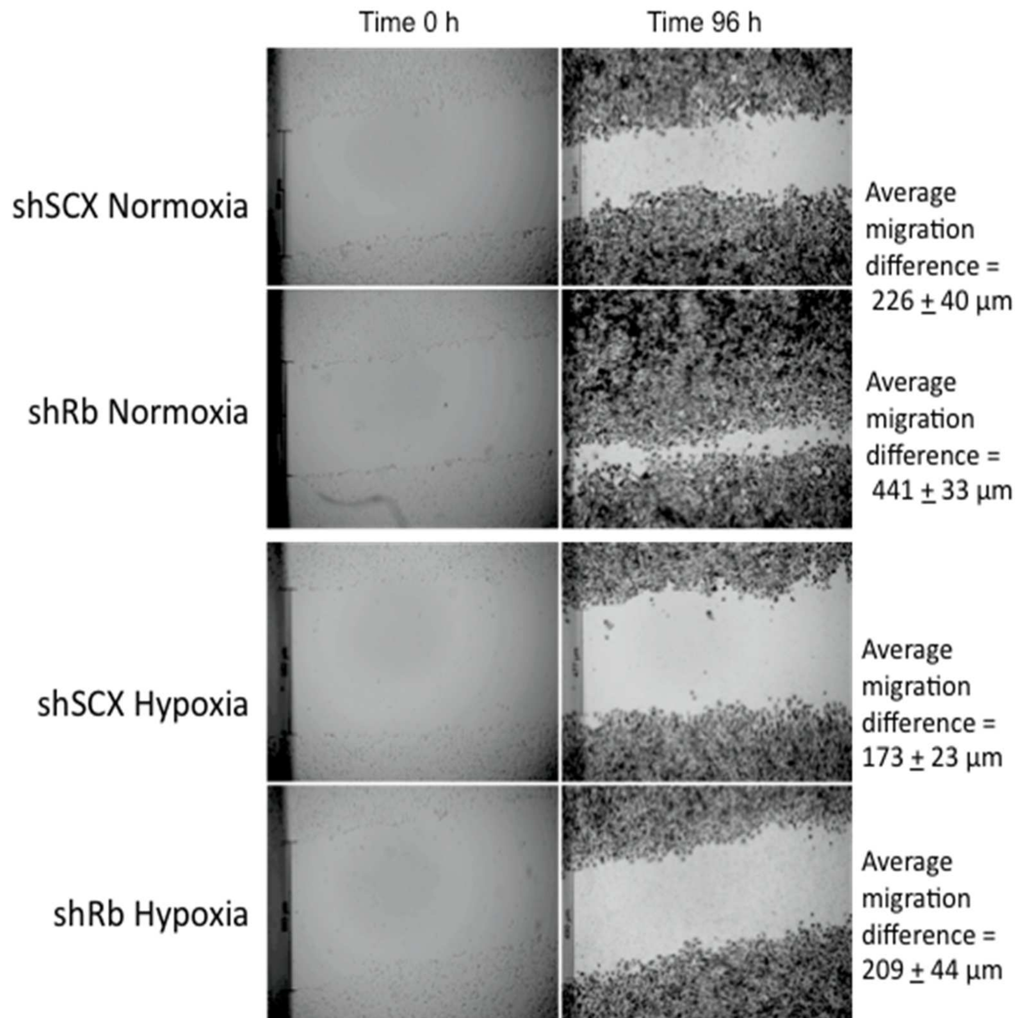


Figure 3.14 Hypoxia and loss of Rb does not play a large role in MCF-7 migration ability
MCF-7 shSCX and shRb cells were plated in 6-well tissues culture plates and placed into hypoxia (1% O_2) or maintained in normoxic (21%O_2) conditions for 96 h. After which a wound was made and cells were imaged at time 0 h and 96 h post wound. Migration was assessed based on the difference in gap distance (n=9).

Chapter 4.

Discussion

Due to the evolving nature of a tumour and its microenvironment, all solid tumours eventually develop regions of hypoxia. This activates the hypoxia-inducible factor complex, comprised of HIF1- or 2-α, its dimerization partner HIF1-β/ARNT, and TRIP230 (an ARNT co-activator). Consequently, activation of this complex increases expression of genes involved in gluconeogenesis, angiogenesis, cell proliferation, metastasis, invasion and epithelial to mesenchymal transition (Lundgren, Nordenskjöld, & Landberg, 2009; Semenza, 2002). Previously, we found that Rb is capable of attenuating the HIF1 response to hypoxia by interacting with TRIP230 (Labrecque et al., 2014), and loss of Rb results in exacerbated HIF1 gene induction. In this study we looked to validate the microarray results of MCF-7 human breast and LNCaP human prostate cancer cell lines with knocked down levels of Rb in normoxic or hypoxic conditions. Results of our microarray analysis show that Rb plays an important role in regulating the expression of many hypoxia inducible genes involved in EMT, tumour cell metastasis, and invasion in both cell lines, such as ASCL2, NDRG1, and S1PR4 (Table 3.1, Appendix A and Appendix B), as well as genes involved in neuroendocrine differentiation in the LNCaP cells. We combined the microarray data from the two cell lines and found 21 common genes that were up-regulated and induced 2-fold or greater by loss of Rb and hypoxia compared with the negative controls of shRb normoxic or shSCX hypoxic samples. Most of the 21 common genes are involved in tumour metastasis, migration, and epithelial to mesenchymal transition.

To validate the microarray of the MCF7 and LNCaP transcriptomes under our hypoxia-regulated Rb-knockdown paradigm, we selected top candidate genes from the common gene list (Table 3.1) and determined gene expression levels by QPCR. Genes for validation were picked based on gene ontology analysis if they were involved with

tumour cell transformation and metastasis. The results show that loss of Rb and hypoxic tumour microenvironments significantly increases expression of CXCR4, S1PR4, GAL3ST1, NDRG1 and STC1 compared to hypoxic induction alone in MCF-7 cells. Hypoxia and loss of Rb also significantly increases expression of CXCR4, PLOD2, NDRG1, STC1 and ASCL2 in LNCaP cells.

These findings demonstrate that loss of Rb is involved in the exacerbation of the HIF1 response by increasing the transcription of genes involved in tumour cell metastasis and migration (CXCR4, NDRG1, STC1 and ASCL2) and epithelial to mesenchymal transition (S1PR4, and ASCL2). CXCR4 is involved in cancer cell proliferation, invasion, metastasis and cell migration in lung cancer (Kijima et al., 2002), breast cancer (K. E. Luker & Luker, 2006) prostate cancer (Darash-Yahana et al., 2004) and brain metastasis (Salmaggi et al., 2009). This makes it an attractive candidate for possible therapeutics. Our data shows that CXCR4 mRNA levels increase as expected under hypoxic conditions, however this increase is significantly higher in the shRb samples compared to shSCX samples in both MCF-7 and LNCaP cell lines (Figure 3.3 and 3.8). Also, since we know CXCR4 levels are increased following hypoxia, and from our microarray data even more so after loss of Rb, we used it as a marker to determine changes in the mouse xenograft samples. Overall, CXCR4 protein levels are significantly higher (approximately 1.7 fold, $P<0.05$) in shRb hypoxic samples compared to shSCX hypoxic samples (Figure 3.7), this provides an *in vivo* model demonstrating the role of Rb control on hypoxic gene induction.

Another gene found to be involved in tumour metastasis is STC1. STC1 is a secreted glycoprotein that has been found to have altered expression in breast cancer (Chang et al., 2015). Chang and colleagues demonstrated that STC1 was capable of increasing cell invasion in the MDA-MB231 human breast cancer cell line, while not affecting cell proliferation (Chang et al., 2015). This highlights a possible role for STC1 in increasing tumour cell metastasis by causing the cells to become more migratory. This later role of STC1 was found to be true in glioma cells (Yoon et al., 2014). STC1 increased migration of glioma cells under a hypoxia driven pathway. This data corresponds with our observation of increased STC1 expression following Rb loss and

hypoxia in both MCF-7 and LNCaP cells and indicates the possibility of a transition to more invasive MCF-7 and LNCaP cells.

Furthermore, the NDRG1 also known as PROXY-1 gene is upregulated under hypoxic conditions in human breast cancer cells and human trophoblast cells (Park et al., 2000). An increase in NDRG1 gene expression corresponds with enhanced invasiveness of breast tumour cells and subsequently metastatic potential. Loss of Rb under hypoxic conditions significantly increased NDRG1 expression at the mRNA (Figures 3.3 and 3.8) and protein level (Figures 3.4 and 3.9) in MCF-7 and LNCaP cells. Although hypoxic conditions increased NDRG1 protein levels, the affect was more significant upon loss of Rb in both the MCF-7 cells and LNCaP cell lines.

Sphingosine-1-phosphate receptors 1-5 (S1PRs 1-5) with sphingosine-1-phosphate have been found to play roles in cancer progression specifically tumour growth, proliferation, migration, survival and response to therapeutics (Pyne & Pyne, 2010; Pyne & Pyne, 2013). S1PRs and in particular S1PR3 is involved in estrogen induced epidermal growth factor receptor transactivation and internalization which results in breast cancer cell stimulation and proliferative signalling (Sukocheva & Wadham, 2014) and S1PR4 is involved in stimulating the Erk 1/2 pathway in estrogen receptor negative breast cancer cells (Ohotski et al., 2012). Interestingly, when Wang and colleagues assessed expression and localization patterns of the S1PRs in different tissues and organ sites they found a lack of expression of S1PR4 in normal breast or prostate tissues and no difference in these levels compared with benign and malignant tissues (Wang et al., 2014). However, Ohotski and colleagues determined that high cytoplasmic S1PR4 levels in tissue samples from patients with estrogen negative breast cancer corresponded with shorter disease free and disease specific survival (Ohotski et al., 2012). Our data shows that loss of Rb significantly elevates expression of S1PR4 mRNA and protein levels under hypoxic conditions in estrogen positive MCF-7 human breast cancer cells. We also found S1PR4 expression in one of our MCF-7 shRb mouse tumour samples suggesting that S1PR4 is regulated by hypoxia and Rb. All together our results suggest that loss of Rb and hypoxia results in an exaggerated expression of S1PR4. If these conditions occur in breast cancer patient tumours it might result in

worse survival outcomes. Therefore these results highlight possible therapeutic targets for patients with breast cancer.

Another gene candidate, achaete scute-like 2 (ASCL2) that is involved in epithelial to mesenchymal transition (EMT) showed increased expression in LNCaP shRb cells under hypoxic conditions. ASCL2 is a basic helix-loop-helix transcription factor, and was found to be up-regulated in colorectal cancer (Jubb et al., 2006; Jubb, Hoeflich, Haverty, Wang, & Koeppen, 2011; Stange et al., 2010) and gastric cancer (Kwon et al., 2013) and is involved in increasing cell proliferation and metastasis. Tian and colleagues demonstrated that knockdown of ASCL2 expression led to decreased mRNA and protein levels of the mesenchymal cell markers N-cadherin, Snail and Slug and an increase in the epithelial cell marker E-cadherin indicating a role for ASCL2 in EMT (Tian et al. 2014). Alternatively, ASCL2 may function in increasing metastasis and invasion by increasing levels of CXCR4 (Zhou et al., 2015). Therefore, the increase in ASCL2 expression in LNCaP shRb cells we observed following hypoxic conditions may encourage tumour cell metastasis and invasion by promoting EMT and cell invasion/metastasis via further increases in CXCR4 levels.

The extracelluar matrix is an important component of the tumour microenvironment and is composed of many molecules and cells, which regulate its structure and function. Along with laminin, and heparan sulfate proteoglycans the basement membrane is also composed of collagen-type IV, which together make a fairly impenetrable barrier preventing tumour migration (Stetler-Stevenson, Aznavoorian, & Liotta, 2003). One way in which the collagen component is regulated is by procollagen lysyl hydroxylases, (PLOD1, PLOD2 and PLOD3) which are necessary for collagen production (Gilkes et al., 2013b). High levels of PLOD2 have been found in breast cancer biopsies and play a role in the stiffening of the ECM, promoting cell growth and metastasis under hypoxic conditions (Gilkes et al., 2013b). Aside from breast cancer tumours, other studies have shown that HIF1 is capable of increasing PLOD2 expression and consequently tumour cell metastasis in sarcomas (Eisinger-Mathason et al., 2013) and hepatocellular carcinomas (Noda et al., 2012). Our findings show that loss of Rb exacerbates HIF1 activation of PLOD2, which may enhance tumour cell metastatic potentials. Taken together these results indicate an important role for Rb in negatively

regulating hypoxic gene induction, preventing highly migratory and invasive tumour cell phenotypes.

To identify if the observed changes in gene expression had an effect on epithelial to mesenchymal transition, we looked at E-cadherin (an epithelial cell marker) protein levels (Figure 3.5 and 3.10). We found that loss of Rb under normoxic conditions decreased E-cadherin protein levels in both MCF-7 and LNCaP cell lines. However, following exposure to various time points of hypoxia, there was no greater observable changes in E-cadherin protein levels. Furthermore, increased levels of lysyl oxidase-like 2 (LOXL2) is also involved in increasing cell invasive properties by inducing EMT (Moon et al, 2013). LOXL2 gene expression level is regulated by hypoxia (Schietke et al., 2010) and is highly up-regulated in breast cancer cells (Moon et al., 2013). The MCF-7 microarray data suggested increased expression of LOXL2 following Rb loss and exposure to hypoxic conditions. However, when we assessed LOXL2 protein levels under normoxic and various hypoxic conditions we found that LOXL2 protein level was highest in normoxic conditions, decreased following hypoxia, and showed no difference between shSCX and shRb hypoxic samples (Figure 3.6).

Apart from increasing tumour cell's invasive, proliferative and migratory properties in LNCaP cells, Rb was also found to play an important role in attenuating the expression of genes involved in neuroendocrine differentiation (NED) (Figures 3.8 and 3.9). Usually NED is induced in LNCaP cells following androgen deprivation (Sun et al., 2009), yet we saw increases in NED markers including ENO2, HTR5A, and KISS1R without androgen deprivation. Neuroendocrine differentiation is detected in patients with prostatic carcinoma by looking for increased plasma neuroendocrine secretory products: chromagranin A and ENO2 (Abrahamsson, 1999; Cussenot, Villette, Cochand-Priollet, & Berthon, 1998). Thus elevated levels of chromogranin A and ENO2 was found to be correlated with androgen independence and poorer prognosis (Abrahamsson, 1999). Since ENO2 is one of the bona fide clinical markers of NED (Abrahamsson, 1999; Schmechel, Marangos, & Brightman, 1978), the increased levels of ENO2 mRNA and protein levels we found, strongly indicates the occurrence of NED in hypoxic cells lacking Rb.

Additionally prostatic neuroendocrince differentiation leads to prostate cancer cells being more sensitive to serotonin due to the increase in serotonin releasing NE cells and increased serotonin receptors (Dizeyi et al., 2004). This is problematic as serotonin and serotonin receptors (5-HTRs) are associated with playing a role in morphogenesis and tumour cell proliferation (Abrahamsson, 1999; Dizeyi et al., 2004). However, Abdul and his colleagues demonstrated the use of an antagonist for the HTR1A receptor in inhibiting growth affects in the PC-3 human prostate cell line (Abdul, Anezinis, Logothetis, & Hoosein, 1994). Indicating that tumour growth may be halted by inhibiting 5-HTRs. As we found elevated levels of HTR5A mRNA and protein levels in LNCaP shRb hypoxic cells indicating NED, this highlights a potential for HTR5A inhibitors being possible therapeutic agents in preventing the development of the aggressive phenotype of prostatic NE cells.

Another marker of NED is increased expression of the kisspeptin receptor, KISS1R (Giandomenico, 2010). The KISS1 gene was first identified as a metastasis suppressor gene in melanoma cells and as a potent ligand for KISS1R (Lee et al., 1996). Since then, decreased levels of KISS1 have also been found to be associated with bladder, breast, choriocarcinoma, ovarian and osteosarcoma metastatic tumours to name a few (Mead, Maguire, Kuc, & Davenport, 2007). Conversely, increased expression of KISS1 and KISS1R has been found to be a stimulator of breast cancer metastasis and tumour cell invasion (Marot et al., 2007; Zajac et al., 2011). This later role of KISS1R mediated metastasis may be due to the transactivation of epidermal growth factor receptor (EGFR) via direct interaction with KISS1R (Zajac et al., 2011). Since we observed high KISS1R expression patterns in LNCaP shRb cells after hypoxic exposure, our data is surprising as high KISS1 expression decreases prostate cancer cell migration and invasion, and suppress metastasis (Wang et al., 2012). However, Wang and colleagues suggest that the KISS1 suppression of prostate metastasis may be acting in a pathway independent from KISS1R (Wang et al., 2012).

Alternatively, KISS1 is capable of increasing hormone levels by stimulating the hypothalamic-pituitary gonadal (HPG) axis (Dhillo et al., 2013). KISS1R is expressed in gonadotropin releasing hormone (GnRH) neurons and upon activation by kisspeptins supports the secretion of GnRH which promotes the release of luteinizing hormone (LH)

and follicle stimulating hormone (FSH) from the pituitary (Rothman & Wierman, 2007). Subsequently stimulating the gonads to produce sex steroids, which in turn can also act as negative regulators on kisspeptin neurons inhibiting KISS1 and KISS1R expression (Gottsch, Clifton, & Steiner, 2006; Rothman & Wierman, 2007). Over stimulation of KISS1R with KISS1 analogs (KP-10 or KP-54) leads to an initial increase followed by a decrease in LH corresponding to a desensitization of KISS1/KISS1R signalling and consequently decreases in testosterone plasma levels (Matsui et al., 2012; Seminara, Dipietro, Ramaswamy, Crowley, & Plant, 2006; Thompson et al., 2006). Alternatively, release of LH and FSH can also be regulated through KISS1/KISS1R singlaling by using antagonists to block KISS1 stimulation (MacLean, Matsui, Suri, Neuwirth, & Colombel, 2014). This illustrates a huge potential for KISS1/KISS1R agonists and antagonists in controlling the HPG axis and NED in prostatic tumours.

We also noticed that when LNCaP cells lacking Rb were exposed to hypoxia, they developed observable changes in their cellular morphology compared to the LNCaP shSCX (hypoxic or normoxic) cells and LNCaP shRb normoxia cells. The LNCaP shRb hypoxia cells appeared to be more elongated containing long dendrite-like extensions and were sparsely spaced. These observable characteristics are similar to those of neuroendocrine cells, which have been observed to have long dendrite-like processes (Sun et al., 2009). While, the control cells were observably more tightly packed together and rounder in shape with shorter processes. These results suggest that in LNCaP cells Rb loss in combination with hypoxia leads to morphological changes in the cells that may correspond to NED; however, we did not check the cells for neuronal markers – especially, those associated with NED. Thus, we can not certainly conclude the morphology of the cells change, however it seems that this would be an avenue of research worth pursuing in the future to further establish the link between loss of Rb and NED. Although, ENO2 is a bona fide neuronal cell marker, and our data has already shown that it is significantly up-regulated under hypoxia following loss of Rb. In order to quantify the morphological changes, we can use imageJ to measure the length of the dendritic process and determine the difference in size between the cells.

Lastly, based on our microarray and validation data we would expect MCF-7 and LNCaP cells to exhibit a more migratory and invasive phenotype when placed into a

hypoxic environment and further amplified upon loss of Rb. However, we failed to find any increase in cell migration following treatment with hypoxia in both the MCF-7 and LNCaP cell lines. Although greater migration is observed in the normoxic samples compared to the hypoxic samples the migratory ability of the MCF-7 shRb cells appeared to be greater in comparison to the shSCX cells for their given oxygen tensions. Suggesting that Rb loss may still play a role in causing the tumour cells to become more migratory in MCF-7 cells. However, there did not seem to be any difference in migration in LNCaP shSCX and shRb cells in either conditions of normoxia and hypoxia. In addition, the hypoxic cells used to conduct the migration assays were taken out of hypoxia and placed back into normoxic conditions after the wound was made. This re-oxygenation may have been the reason why there was no significant change between the groups. As we do not know the half-life of the proteins that are initiating cell migration, this re-oxygenation may have caused a decrease in the proteins up-regulated by hypoxia and loss of Rb. On the other hand, the MCF-7 and LNCaP cells may not be becoming more migratory or invasive following loss of Rb under hypoxic conditions.

IPA analysis of the MCF-7 gene microarray identified NFκB, Mek, PDGF BB, and ERK 1/2 as key nodes involved in cellular movement. These nodes are regulated by hypoxia inducible genes that are up-regulated by the loss of Rb, conversely the nodes also regulate these genes. The identification of these nodes underlines the importance of hypoxia and Rb in cellular movement as NFκB, Mek, and ERK 1/2 all play a role in regulating processes involved in EMT (Bae et al., 2013; Huber et al., 2004; Xie et al., 2004). While PDGF BB drives metastasis and cell proliferation (Cheng et al., 2013; Heldin, 2013). All together, these networks bring attention to the importance of hypoxia and loss of Rb in promoting EMT and creating more invasive tumours.

Lastly, gene ontology analysis shows that Rb and hypoxia regulate key pathways and functions regulating cellular movement, growth, development, and morphology in MCF-7 cancer cells (Appendix F). These pathways are regulated both by genes that are up-regulated and down-regulated by loss of Rb and hypoxia. There are 25 up-regulated genes and 7 down-regulated genes that are involved in cellular growth and proliferation, and 19 up-regulated and 6 down-regulated genes involved in cellular movement. Taken together, this illustrates that Rb loss in relation to hypoxia, results in the expression or

inhibition of genes regulating cellular transformation. Similarly, gene ontology of LNCaP cells shows that loss of Rb and hypoxia are involved up-regulating genes involved in the regulation of systems and pathways regulating cellular development, growth, proliferation, signalling, and tumour morphology (Appendix G). From the LNCaP list of up-regulated genes after Rb loss and hypoxia, 17 appear to be involved in cellular development, 17 in cellular movement, 12 in cell-to-cell signalling, and 6 in tumour morphology. All together these tables show that Rb and hypoxia are key regulators of pathways concerning cancer progression.

Chapter 5.

Conclusion

We have shown that hypoxia in combination with loss of Rb leads to an increase in mRNA accumulation of genes that play a role in cellular movement through NFκB, Mek, PDGF BB, and Erk1/2 in human cancer cell lines. Furthermore, 21 of these genes were common to both breast (MCF-7) and prostate (LNCaP) cancer cell lines. These genes include CXCR4, S1PR4, GAL3ST1, NDRG1, STC1, and ASCL2 and are found to be associated with tumour cell migration, metastasis and EMT. Our gene ontology analysis suggested that when HIF1 activity is not attenuated by Rb, the global alterations in gene transcription would lead to increased cancer cell motility. However, we were unable to find any differences in cell migration in either MCF-7 or LNCaP shSCX and shRb cells exposed to normoxia or hypoxia. This may have been due to the cells being re-exposed to oxygen during the migration measurements. Further studies should include performing the wound healing assays again, this time keeping the cells under hypoxic conditions before and after the wound is made. Alternatively, we could also look at performing different assays such as matrigel invasion and trans-well migration assays to see if the cells are becoming more invasive and migratory. This would allow us to see if the cells are migratory, or confirm the results we found in which the MCF-7 and LNCaP cells do not become more migratory following loss of Rb under hypoxia.

In addition we found that Rb regulates the hypoxic induction of neuroendocrine markers in LNCaP cells despite them not being deprived of androgens. This illustrates that our hypoxia-regulated Rb-knockdown paradigm is capable of inducing neuroendocrine differentiation (NED) in LNCaP cells by increasing expression of ENO2, HTR5A and KISS1R. We also found that hypoxia and loss of Rb leads to observable changes in LNCaP cell morphology from looking like "typical" LNCaP cells, to exhibiting properties similar to those found in neuroendocrine cells such as long dendrite-like

extensions. However, we have not yet quantified these changes by examining the cells for the appearance of neuronal markers. Therefore, we can not say for certain that these observable morphological changes are due to the cells going through NED or if it is because they are becoming more migratory. Future experiments should include staining the LNCaP cells for specific neuronal markers such as NSE, NeuN and beta III tubulin.

Lastly, other future directions include further examination of alterations in the migratory and invasive properties of hypoxic MCF-7 and LNCaP cells resulting from loss of Rb by using siRNA to knock down potential gene targets identified in our microarray analysis. This will allow us to further identify the role Rb plays in regulating aggressive tumour phenotypes via the HIF1 pathway. Potentially leading to the development of novel therapeutic targets to prevent tumour invasion, metastasis, and neuroendocrine differentiation.

References

Abdul, M., Anezinis, P. E., Logothetis, C. J., & Hoosein, N. M. (1994). Growth inhibition of human prostatic carcinoma cell lines by serotonin antagonists. *Anticancer Research*, *14*(3A), 1215–1220.

Abrahamsson, P. A. (1999). Neuroendocrine differentiation in prostatic carcinoma. *The Prostate*, *39*(2), 135–148. http://doi.org/10.1002/(SICI)1097-0045(19990501)39:2<135::AID-PROS9>3.0.CO;2-S

Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., & Sethi, G. (2006). Inflammation and cancer: How hot is the link? *Biochemical Pharmacology*, *72*(11), 1605–1621. http://doi.org/10.1016/j.bcp.2006.06.029

Antonsson, C., Arulampalam, V., Whitelaw, M. L., Pettersson, S., & Poellinger, L. (1995). Constitutive function of the basic helix-loop-helix/PAS factor Arnt. Regulation of target promoters via the E box motif. *The Journal of Biological Chemistry*, *270*(23), 13968–13972.

Atchley, W. R., & Fitch, W. M. (1997). A natural classification of the basic helix–loop–helix class of transcription factors. *Proceedings of the National Academy of Sciences of the United States of America*, 5172–5176.

Bae, G.-Y., Choi, S.-J., Lee, J.-S., Jo, J., Lee, J., Kim, J., & Cha, H.-J. (2013). Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer. *Oncotarget*, *4*(12), 2512–2522.

Beischlag, T. V., Taylor, R. T., Rose, D. W., Yoon, D., Chen, Y., Lee, W.-H., et al. (2004). Recruitment of thyroid hormone receptor/retinoblastoma-interacting protein 230 by the aryl hydrocarbon receptor nuclear translocator is required for the transcriptional response to both dioxin and hypoxia. *The Journal of Biological Chemistry*, *279*(52), 54620–54628. http://doi.org/10.1074/jbc.M410456200

Bernard, W. S., & Christopher, P. W. (2014). World Cancer Report 2014. International Agency for Research on Cancer. World Health Organization. http://doi.org/10.1002/cncr.28233/full

Bruick, R. K., & McKnight, S. L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, *294*(5545), 1337–1340. http://doi.org/10.1126/science.1066373

Budde, A., Schneiderhan-Marra, N., Petersen, G., & Brüne, B. (2005). Retinoblastoma susceptibility gene product pRB activates hypoxia-inducible factor-1 (HIF-1). *Oncogene*, *24*(10), 1802–1808. http://doi.org/10.1038/sj.onc.1208369

Bunn, H. F., & Poyton, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiological Reviews*, *76*(3), 839–885.

Cance, W. G., Brennan, M. F., Dudas, M. E., Huang, C. M., & Cordon-Cardo, C. (1990). Altered expression of the retinoblastoma gene product in human sarcomas. *The New England Journal of Medicine*, *323*(21), 1457–1462. http://doi.org/10.1056/NEJM199011223232105

Carreau, A., Hafny-Rahbi, El, B., Matejuk, A., Grillon, C., & Kieda, C. (2011). Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of Cellular and Molecular Medicine*, *15*(6), 1239–1253. http://doi.org/10.1111/j.1582-4934.2011.01258.x

Cash, T. P., Pan, Y., & Simon, M. C. (2007). Reactive oxygen species and cellular oxygen sensing. *Free Radical Biology and Medicine*, *43*(9), 1219–1225. http://doi.org/10.1016/j.freeradbiomed.2007.07.001

Chan, D. A., & Giaccia, A. J. (2007). Hypoxia, gene expression, and metastasis. *Cancer and Metastasis Reviews*, *26*(2), 333–339. http://doi.org/10.1007/s10555-007-9063-1

Chang, A. C.-M., Doherty, J., Huschtscha, L. I., Redvers, R., Restall, C., Reddel, R. R., & Anderson, R. L. (2015). STC1 expression is associated with tumor growth and metastasis in breast cancer. *Clinical & Experimental Metastasis*, *32*(1), 15–27. http://doi.org/10.1007/s10585-014-9687-9

Chang, K.-H., Chen, Y., Chen, T.-T., Chou, W.-H., Chen, P.-L., Ma, Y.-Y., et al. (1997). A thyroid hormone receptor coactivator negatively regulated by the retinoblastoma protein. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 9040–9045.

Chau, B. N., & Wang, J. Y. J. (2003). Coordinated regulation of life and death by RB. *Nature Reviews Cancer*, *3*(2), 130–138. http://doi.org/10.1038/nrc993

Chen, Y., Chen, P. L., Chen, C. F., Sharp, Z. D., & Lee, W. H. (1999). Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(8), 4443–4448.

Cheng, J., Ye, H., Liu, Z., Xu, C., Zhang, Z., Liu, Y., & Sun, Y. (2013). Platelet-derived growth factor-BB accelerates prostate cancer growth by promoting the proliferation of mesenchymal stem cells. *Journal of Cellular Biochemistry*, *114*(7), 1510–1518. http://doi.org/10.1002/jcb.24492

Classon, M., & Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. *Nature Reviews Cancer*, *2*(12), 910–917.

http://doi.org/10.1038/nrc950

Crews, S. T. (1998). Control of cell lineage-specific development and transcription by bHLH–PAS proteins. *Genes & Development*, *12*(5), 607–620.

Cussenot, O., Villette, J. M., Cochand-Priollet, B., & Berthon, P. (1998). Evaluation and clinical value of neuroendocrine differentiation in human prostatic tumors. *The Prostate. Supplement*, *8*, 43–51.

Danaei, G., Vander Hoorn, S., Lopez, A. D., Murray, C. J. L., Ezzati, M., Comparative Risk Assessment collaborating group (Cancers). (2005). Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet*, *366*(9499), 1784–1793. http://doi.org/10.1016/S0140-6736(05)67725-2

Darash-Yahana, M., Pikarsky, E., Abramovitch, R., Zeira, E., Pal, B., Karplus, R., et al. (2004). Role of high expression levels of CXCR4 in tumor growth, vascularization, and metastasis. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *18*(11), 1240–1242. http://doi.org/10.1096/fj.03-0935fje

Dayan, F., Mazure, N. M., Brahimi-Horn, M. C., & Pouysségur, J. (2008). A Dialogue between the Hypoxia-Inducible Factor and the Tumor Microenvironment. *Cancer Microenvironment*, *1*(1), 53–68. http://doi.org/10.1007/s12307-008-0006-3

Dhillo, W. S., Chaudhri, O. B., Patterson, M., Thompson, E. L., Murphy, K. G., Badman, M. K., et al. (2013). Kisspeptin-54 Stimulates the Hypothalamic-Pituitary Gonadal Axis in Human Males. *The Journal of Clinical Endocrinology & Metabolism*. http://doi.org/10.1210/jc.2005-1468

di Sant'Agnese, P. A. (1992). Neuroendocrine differentiation in carcinoma of the prostate. Diagnostic, prognostic, and therapeutic implications. *Cancer*, *70*(1 Suppl), 254–268. http://doi.org/10.1002/1097-0142(19920701)70:1+<254::AID-CNCR2820701312>3.0.CO;2-E

di Sant'Agnese, P. A. (1998a). Neuroendocrine cells of the prostate and neuroendocrine differentiation in prostatic carcinoma: a review of morphologic aspects. *Urology*, *51*(5A Suppl), 121–124.

di Sant'Agnese, P. A. (1998b). Neuroendocrine differentiation in prostatic carcinoma: an update. *The Prostate. Supplement*, *8*, 74–79.

Dizeyi, N., Bjartell, A., Nilsson, E., Hansson, J., Gadaleanu, V., Cross, N., & Abrahamsson, P. A. (2004). Expression of serotonin receptors and role of serotonin in human prostate cancer tissue and cell lines. *The Prostate*, *59*(3), 328–336. http://doi.org/10.1002/pros.10374

Du, W., & Pogoriler, J. (2006). Retinoblastoma family genes. *Oncogene*, *25*(38), 5190–5200. http://doi.org/10.1038/sj.onc.1209651

Eguchi, H., Ikuta, T., Tachibana, T., Yoneda, Y., & Kawajiri, K. (1997). A Nuclear Localization Signal of Human Aryl Hydrocarbon Receptor Nuclear Translocator/Hypoxia-inducible Factor 1β Is a Novel Bipartite Type Recognized by the Two Components of Nuclear Pore-targeting Complex. *The Journal of Biological Chemistry*, *272*(28), 17640–17647. http://doi.org/10.1074/jbc.272.28.17640

Eisinger-Mathason, T. S. K., Zhang, M., Qiu, Q., Skuli, N., Nakazawa, M. S., Karakasheva, T., et al. (2013). Hypoxia-dependent modification of collagen networks promotes sarcoma metastasis. *Cancer Discovery*, *3*(10), 1190–1205. http://doi.org/10.1158/2159-8290.CD-13-0118

Ema, M., Morita, M., Ikawa, S., Tanaka, M., Matsuda, Y., Gotoh, O., et al. (1996). Two new members of the murine Sim gene family are transcriptional repressors and show different expression patterns during mouse embryogenesis. *Molecular and Cellular Biology*, *16*(10), 5865–5875.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., & Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(9), 4273–4278.

Epstein, A., Gleadle, J. M., McNeill, L. A., & Hewitson, K. S. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*.

Flamme, I., Fröhlich, T., Reutern, von, M., Kappel, A., Damert, A., & Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mechanisms of Development*, *63*(1), 51–60.

Fukunaga, B. N., Probst, M. R., Reisz-Porszasz, S., & Hankinson, O. (1995). Identification of functional domains of the aryl hydrocarbon receptor. *The Journal of Biological Chemistry*, *270*(49), 29270–29278.

Galanis, A., Pappa, A., Giannakakis, A., Lanitis, E., Dangaj, D., & Sandaltzopoulos, R. (2008). Reactive oxygen species and HIF-1 signalling in cancer. *Cancer Letters*, *266*(1), 12–20. http://doi.org/10.1016/j.canlet.2008.02.028

Gayther, S. A., Warren, W., Mazoyer, S., Russell, P. A., Harrington, P. A., Chiano, M., et al. (1995). Germline mutations of the BRCA1 gene in breast and ovarian cancer families provide evidence for a genotype–phenotype correlation. *Nature Genetics*, *11*(4), 428–433. http://doi.org/10.1038/ng1295-428

Genovese, C., Trani, D., Caputi, M., & Claudio, P. P. (2006). Cell cycle control and beyond: emerging roles for the retinoblastoma gene family. *Oncogene*, *25*(38), 5201–5209. http://doi.org/10.1038/sj.onc.1209652

Giandomenico, V. (2010). Molecular pathology of gastrointestinal neuroendocrine tumours - selected topics. *Diagnostic Histopathology*, *16*(5), 243–250. http://doi.org/10.1016/j.mpdhp.2010.03.005

Gilkes, D. M., Bajpai, S., Chaturvedi, P., Wirtz, D., & Semenza, G. L. (2013a). Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *The Journal of Biological Chemistry*, *288*(15), 10819–10829. http://doi.org/10.1074/jbc.M112.442939

Gilkes, D. M., Bajpai, S., Wong, C. C., Chaturvedi, P., Hubbi, M. E., Wirtz, D., & Semenza, G. L. (2013b). Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Molecular Cancer Research : MCR*, *11*(5), 456–466. http://doi.org/10.1158/1541-7786.MCR-12-0629

Goodrich, D. W., Wang, N. P., Qian, Y.-W., Lee, E. Y. H. P., & Lee, W.-H. (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell*, *67*(2), 293–302. http://doi.org/10.1016/0092-8674(91)90181-W

Gottsch, M. L., Clifton, D. K., & Steiner, R. A. (2006). Kisspepeptin-GPR54 signaling in the neuroendocrine reproductive axis. *Molecular and Cellular Endocrinology*, *254-255*, 91–96. http://doi.org/10.1016/j.mce.2006.04.030

Greer, S. N., Metcalf, J. L., Wang, Y., & Ohh, M. (2012). The updated biology of hypoxia-inducible factor. *The EMBO Journal*, *31*(11), 2448–2460. http://doi.org/10.1038/emboj.2012.125

Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L., & Bradfield, C. A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expression*, *7*(3), 205–213.

Guzy, R. D., & Schumacker, P. T. (2006). Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Experimental Physiology*, *91*(5), 807–819. http://doi.org/10.1113/expphysiol.2006.033506

Hallstrom, T. C., & Nevins, J. R. (2003). Specificity in the activation and control of transcription factor E2F-dependent apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(19), 10848–10853. http://doi.org/10.1073/pnas.1831408100

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, *144*(5), 646–674. http://doi.org/10.1016/j.cell.2011.02.013

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annual Review of Pharmacology and Toxicology*, *35*(1), 307–340. http://doi.org/10.1146/annurev.pa.35.040195.001515

Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., & Imura, N. (2001). Expression and Characterization of Hypoxia-Inducible Factor (HIF)-3α in Human Kidney:

Suppression of HIF-Mediated Gene Expression by HIF-3α. *Biochemical and Biophysical Research Communications*, *287*(4), 808–813. http://doi.org/10.1006/bbrc.2001.5659

Harris, A. L. (2002). Hypoxia |[mdash]| a key regulatory factor in tumour growth. *Nature Reviews Cancer*, *2*(1), 38–47. http://doi.org/10.1038/nrc704

Heldin, C.-H. (2013). Targeting the PDGF signaling pathway in tumor treatment. *Cell Communication and Signaling : CCS*, *11*(1), 97. http://doi.org/10.1186/1478-811X-11-97

Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., et al. (1996). cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). *Molecular and Cellular Biology*, *16*(4), 1706–1713.

Hirota, K., & Semenza, G. L. (2005). Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. *Biochemical and Biophysical Research Communications*, *338*(1), 610–616. http://doi.org/10.1016/j.bbrc.2005.08.193

Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., & Hankinson, O. (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science*, *252*(5008), 954–958.

Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y.-Z., Pray-Grant, M., et al. (1997). Characterization of a Subset of the Basic-Helix-Loop-Helix-PAS Superfamily That Interacts with Components of the Dioxin Signaling Pathway. *The Journal of Biological Chemistry*, *272*(13), 8581–8593. http://doi.org/10.1074/jbc.272.13.8581

Hord, N. G., & Perdew, G. H. (1994). Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator. *Molecular Pharmacology*, *46*(4), 618–626.

Hou, C.-H., Lin, F.-L., Hou, S.-M., & Liu, J.-F. (2014). Cyr61 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by Raf-1/MEK/ERK/Elk-1/TWIST-1 signaling pathway. *Molecular Cancer*, *13*(1), 236. http://doi.org/10.1186/1476-4598-13-236

Höckel, M., & Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *Journal of the National Cancer Institute*, *93*(4), 266–276. http://doi.org/10.1093/jnci/93.4.266

Huang, J., Yao, J. L., di Sant'Agnese, P. A., Yang, Q., Bourne, P. A., & Na, Y. (2006). Immunohistochemical characterization of neuroendocrine cells in prostate cancer. *The Prostate*, *66*(13), 1399–1406. http://doi.org/10.1002/pros.20434

Huang, J., Yao, J. L., Zhang, L., Bourne, P. A., Quinn, A. M., di Sant'Agnese, P. A., &

Reeder, J. E. (2005). Differential expression of interleukin-8 and its receptors in the neuroendocrine and non-neuroendocrine compartments of prostate cancer. *The American Journal of Physical Anthropology*, *166*(6), 1807–1815. http://doi.org/10.1016/S0002-9440(10)62490-X

Huang, L. E., Arany, Z., Livingston, D. M., & Bunn, H. F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *The Journal of Biological Chemistry*, *271*(50), 32253–32259.

Huber, M. A., Azoitei, N., Baumann, B., Grünert, S., Sommer, A., Pehamberger, H., et al. (2004). NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *Journal of Clinical Investigation*, *114*(4), 569–581. http://doi.org/10.1172/JCI21358

Huber, M. A., Kraut, N., & Beug, H. (2005). Molecular requirements for epithelial–mesenchymal transition during tumor progression. *Current Opinion in Cell Biology*, *17*(5), 548–558. http://doi.org/10.1016/j.ceb.2005.08.001

Infante, C., Ramos-Morales, F., Fedriani, C., Bornens, M., & Rios, R. M. (1999). GMAP-210, A Cis-Golgi Network-associated Protein, Is a Minus EndMicrotubule-binding Protein. *The Journal of Cell Biology*.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., et al. (1999). Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(22), 12436–12441. http://doi.org/10.1073/pnas.96.22.12436

Jiang, B. H., Rue, E., Wang, G. L., Roe, R., & Semenza, G. L. (1996). Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *The Journal of Biological Chemistry*, *271*(30), 17771–17778. http://doi.org/10.1074/jbc.271.30.17771

Jiang, J., Tang, Y.-L., & Liang, X.-H. (2011). EMT: A new vision of hypoxia promoting cancer progression. *Cancer Biology & Therapy*, *11*(8), 714–723. http://doi.org/10.4161/cbt.11.8.15274

Jubb, A. M., Chalasani, S., Frantz, G. D., Smits, R., Grabsch, H. I., Kavi, V., et al. (2006). Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia. *Oncogene*, *25*(24), 3445–3457. http://doi.org/10.1038/sj.onc.1209382

Jubb, A. M., Hoeflich, K. P., Haverty, P. M., Wang, J., & Koeppen, H. (2011). Ascl2 and 11p15.5 amplification in colorectal cancer. *Gut*, *60*(11), 1606–7– author reply 1607. http://doi.org/10.1136/gut.2010.231746

Kaelin, W. G., Jr. (2008). The von Hippel|[ndash]|Lindau tumour suppressor protein: O2 sensing and cancer. *Nature Reviews Cancer*, *8*(11), 865–873. http://doi.org/10.1038/nrc2502

Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., & Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *The EMBO Journal*, *17*(22), 6573–6586. http://doi.org/10.1093/emboj/17.22.6573

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation*, *119*(6), 1420–1428. http://doi.org/10.1172/JCI39104

Karin, M. (2006). Nuclear factor-|[kappa]|B in cancer development and progression. *Nature*, *441*(7092), 431–436. http://doi.org/10.1038/nature04870

Keith, B., Adelman, D. M., & Simon, M. C. (2001). Targeted mutation of the murine arylhydrocarbon receptor nuclear translocator 2 (Arnt2) gene reveals partial redundancy with Arnt. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(12), 6692–6697. http://doi.org/10.1073/pnas.121494298

Kewley, R. J., Whitelaw, M. L., & Chapman-Smith, A. (2004). The mammalian basic helix–loop–helix/PAS family of transcriptional regulators. *The International Journal of Biochemistry & Cell Biology*, *36*(2), 189–204. http://doi.org/10.1016/S1357-2725(03)00211-5

Kietzmann, T., & Görlach, A. (2005). Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Seminars in Cell & Developmental Biology*, *16*(4-5), 474–486. http://doi.org/10.1016/j.semcdb.2005.03.010

Kijima, T., Maulik, G., Ma, P. C., Tibaldi, E. V., Turner, R. E., Rollins, B., et al. (2002). Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells. *Cancer Research*, *62*(21), 6304–6311.

King, M.-C., Marks, J. H., & Mandell, J. B. (2015). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science*, 302(5645), 643-646. http://doi.org/10.1126/science.1088759

Knighton, D. R., Silver, I. A., & Hunt, T. K. (1981). Regulation of wound-healing angiogenesis-effect of oxygen gradients and inspired oxygen concentration. *Surgery*, *90*(2), 262–270.

Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K., & Fujii-Kuriyama, Y. (1997). CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *Journal of Biochemistry*, *122*(4), 703–710.

Kwon, O.-H., Park, J.-L., Baek, S.-J., Noh, S.-M., Song, K.-S., Kim, S.-Y., & Kim, Y. S. (2013). Aberrant upregulation of ASCL2by promoter demethylation promotes the growth and resistance to 5-fluorouracil of gastric cancer cells. *Cancer Science*, *104*(3), 391–397. http://doi.org/10.1111/cas.12076

Labrecque, M. P., Takhar, M. K., Jagdeo, J. M., Tam, K. J., Chiu, C., Wang, T.-Y., et al. (2014). A TRIP230-retinoblastoma protein complex regulates hypoxia-inducible factor-1α-mediated transcription and cancer cell invasion. *PLoS ONE*, *9*(6), e99214. http://doi.org/10.1371/journal.pone.0099214

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., & Bruick, R. K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes & Development*, *16*(12), 1466–1471. http://doi.org/10.1101/gad.991402

Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., & Whitelaw, M. L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*, *295*(5556), 858–861. http://doi.org/10.1126/science.1068592

Lee, H.-O., Silva, A. S., Concilio, S., Li, Y.-S., Slifker, M., Gatenby, R. A., & Cheng, J. D. (2011). Evolution of tumor invasiveness: the adaptive tumor microenvironment landscape model. *Cancer Research*, *71*(20), 6327–6337. http://doi.org/10.1158/0008-5472.CAN-11-0304

Lee, J. H., Miele, M. E., Hicks, D. J., Phillips, K. K., Trent, J. M., Weissman, B. E., & Welch, D. R. (1996). KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *Journal of the National Cancer Institute*, *88*(23), 1731–1737.

Lindebro, M. C., Poellinger, L., & Whitelaw, M. L. (1995). Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *The EMBO Journal*, *14*(14), 3528–3539.

Lipinski, M. M., & Jacks, T. (1999). The retinoblastoma gene family in differentiation and development. *Oncogene*, *18*(55), 7873–7882. http://doi.org/10.1038/sj.onc.1203244

Lorusso, G., & Rüegg, C. (2008). The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochemistry and Cell Biology*, *130*(6), 1091–1103. http://doi.org/10.1007/s00418-008-0530-8

Luker, K. E., & Luker, G. D. (2006). Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Letters*, *238*(1), 30–41. http://doi.org/10.1016/j.canlet.2005.06.021

Lundgren, K., Nordenskjöld, B., & Landberg, G. (2009). Hypoxia, Snail and incomplete epithelial-mesenchymal transition in breast cancer. *British Journal of Cancer*, *101*(10), 1769–1781. http://doi.org/10.1038/sj.bjc.6605369

MacLean, D. B., Matsui, H., Suri, A., Neuwirth, R., & Colombel, M. (2014). Sustained exposure to the investigational Kisspeptin analog, TAK-448, down-regulates testosterone into the castration range in healthy males and in patients with prostate cancer: results from two phase 1 studies. *The Journal of Clinical Endocrinology & Metabolism*, *99*(8), E1445–53. http://doi.org/10.1210/jc.2013-4236

Mahon, P. C., Hirota, K., & Semenza, G. L. (2001). FIH-1: a novel protein that interacts

with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & Development*, *15*(20), 2675–2686. http://doi.org/10.1101/gad.924501

Makino, Y. (2002). Inhibitory PAS Domain Protein (IPAS) Is a Hypoxia-inducible Splicing Variant of the Hypoxia-inducible Factor-3alpha Locus. *Journal of Biological Chemistry*, *277*(36), 32405–32408. http://doi.org/10.1074/jbc.C200328200

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., et al. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature*, *414*(6863), 550–554. http://doi.org/10.1038/35107085

Marot, D., Bieche, I., Aumas, C., Esselin, S., Bouquet, C., Vacher, S., et al. (2007). High tumoral levels of Kiss1 and G-protein-coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors. *Endocrine-Related Cancer*, *14*(3), 691–702. http://doi.org/10.1677/ERC-07-0012

Massari, M. E., & Murre, C. (2000). Helix-Loop-Helix Proteins- Regulators of Transcription in Eucaryotic Organisms. *Molecular and Cellular Biology*, 429–440.

Masselli, A., & Wang, J. Y. J. (2006). Phosphorylation site mutated RB exerts contrasting effects on apoptotic response to different stimuli. *Oncogene*, *25*(9), 1290–1298. http://doi.org/10.1038/sj.onc.1209161

Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., & Ratcliffe, P. J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *The EMBO Journal*, *20*(18), 5197–5206. http://doi.org/10.1093/emboj/20.18.5197

Matsui, H., Tanaka, A., Yokoyama, K., Takatsu, Y., Ishikawa, K., Asami, T., et al. (2012). Chronic administration of the metastin/kisspeptin analog KISS1-305 or the investigational agent TAK-448 suppresses hypothalamic pituitary gonadal function and depletes plasma testosterone in adult male rats. *Endocrinology*, *153*(11), 5297–5308. http://doi.org/10.1210/en.2012-1388

Maxwell, P., Pugh, C. W., & Ratcliffe, P. J. (2001). Activation of the HIF pathway in cancer. *Current Opinion in Genetics & Development*, *11*(3), 293–299. http://doi.org/10.1016/S0959-437X(00)00193-3

Maynard, M. A., Evans, A. J., Hosomi, T., Hara, S., Jewett, M. A. S., & Ohh, M. (2005). Human HIF-3alpha4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *19*(11), 1396–1406. http://doi.org/10.1096/fj.05-3788com

McDonnell, T. J., Troncoso, P., Brisbay, S. M., Logothetis, C., Chung, L. W., Hsieh, J. T., et al. (1992). Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Research*, *52*(24), 6940–6944.

McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T.-M., Krones, A., et al. (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. *Genes & Development*, *12*(21), 3357–3368. http://doi.org/10.1101/gad.12.21.3357

Mead, E. J., Maguire, J. J., Kuc, R. E., & Davenport, A. P. (2007). Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. *British Journal of Pharmacology*, *151*(8), 1143–1153. http://doi.org/10.1038/sj.bjp.0707295

Michael Zeisberg, E. G. N. (2009). Biomarkers for epithelial-mesenchymal transitions. *The Journal of Clinical Investigation*, *119*(6), 1429–1437. http://doi.org/10.1172/JCI36183

Millar, R. P., & Babwah, A. V. (2015). KISS1R: Hallmarks of an Effective Regulator of the Neuroendocrine Axis. *Neuroendocrinology*, *101*(3), 193–210. http://doi.org/10.1159/000381457

Moon, H.-J., Finney, J., Xu, L., Moore, D., Welch, D. R., & Mure, M. (2013). MCF-7 cells expressing nuclear associated lysyl oxidase-like 2 (LOXL2) exhibit an epithelial-to-mesenchymal transition (EMT) phenotype and are highly invasive in vitro. *The Journal of Biological Chemistry*, *288*(42), 30000–30008. http://doi.org/10.1074/jbc.C113.502310

Nelson, E. C., Cambio, A. J., Yang, J. C., Ok, J.-H., Lara, P. N., & Evans, C. P. (2006). Clinical implications of neuroendocrine differentiation in prostate cancer. *Prostate Cancer and Prostatic Diseases*, *10*(1), 6–14. http://doi.org/10.1038/sj.pcan.4500922

Noda, T., Yamamoto, H., Takemasa, I., Yamada, D., Uemura, M., Wada, H., et al. (2012). PLOD2 induced under hypoxia is a novel prognostic factor for hepatocellular carcinoma after curative resection. *Liver International*, *32*(1), 110–118. http://doi.org/10.1111/j.1478-3231.2011.02619.x

O'Rourke, J. F., Tian, Y. M., Ratcliffe, P. J., & Pugh, C. W. (1999). Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha. *The Journal of Biological Chemistry*, *274*(4), 2060–2071.

Ohotski, J., Long, J. S., Orange, C., Elsberger, B., Mallon, E., Doughty, J., et al. (2012). Expression of sphingosine 1-phosphate receptor 4 and sphingosine kinase 1 is associated with outcome in oestrogen receptor-negative breast cancer. *British Journal of Cancer, 106*(8), 1453–1459. http://doi.org/10.1038/bjc.2012.98

Park, H., Adams, M. A., Lachat, P., Bosman, F., Pang, S. C., & Graham, C. H. (2000). Hypoxia Induces the Expression of a 43-kDa Protein (PROXY-1) in Normal and Malignant Cells. *Biochemical and Biophysical Research Communications*, *276*(1), 321–328. http://doi.org/10.1006/bbrc.2000.3475

Partch, C. L., & Gardner, K. H. (2010). Coactivator recruitment: A new role for PAS domains in transcriptional regulation by the bHLH-PAS family. *Journal of Cellular*

Physiology, n/a–n/a. http://doi.org/10.1002/jcp.22067

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., & Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature*, *392*(6672), 190–193. http://doi.org/10.1038/32433

Pernet-Gallay, K., Antony, C., Johannes, L., Bornens, M., Goud, B., & Rios, R. M. (2002). The overexpression of GMAP-210 blocks anterograde and retrograde transport between the ER and the Golgi apparatus. *Traffic (Copenhagen, Denmark)*, *3*(11), 822–832.

Pyne, N. J., & Pyne, S. (2010). Sphingosine 1-phosphate and cancer. *Nature Publishing Group*, *10*(7), 489–503. http://doi.org/10.1038/nrc2875

Pyne, S., & Pyne, N. J. (2013). New Perspectives on the Role of Sphingosine 1-Phosphate in Cancer. In *Sphingolipids in Disease* (Vol. 216, pp. 55–71). Vienna: Springer Vienna. http://doi.org/10.1007/978-3-7091-1511-4_3

Reisz-Porszasz, S., Probst, M. R., Fukunaga, B. N., & Hankinson, O. (1994). Identification of Functional Domains of the Aryl Hydrocarbon Receptor Nuclear Translocator Protein (ARNT). *Molecular and Cellular Biology*, 6075–6086.

Rios, R. M., Tassin, A. M., Celati, C., Antony, C., Boissier, M. C., Homberg, J. C., & Bornens, M. (1994). A peripheral protein associated with the cis-Golgi network redistributes in the intermediate compartment upon brefeldin A treatment. *The Journal of Cell Biology*, *125*(5), 997–1013. http://doi.org/10.1083/jcb.125.5.997

Rothman, M. S., & Wierman, M. E. (2007). The role of gonadotropin releasing hormone in normal and pathologic endocrine processes. *Current Opinion in Endocrinology, Diabetes, and Obesity*, *14*(4), 306–310. http://doi.org/10.1097/MED.0b013e3281e2c9fc

Ruas, J. L., Poellinger, L., & Pereira, T. (2002). Functional analysis of hypoxia-inducible factor-1 alpha-mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein. *The Journal of Biological Chemistry*, *277*(41), 38723–38730. http://doi.org/10.1074/jbc.M205051200

Rubin, S. M. (2013). Deciphering the retinoblastoma protein phosphorylation code. *Trends in Biochemical Sciences*, *38*(1), 12–19. http://doi.org/10.1016/j.tibs.2012.10.007

Rüegg, C. (2006). Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions. *Journal of Leukocyte Biology*, *80*(4), 682–684. http://doi.org/10.1189/jlb.0606394

Salceda, S., & Caro, J. (1997). Hypoxia-inducible Factor 1α (HIF-1α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES. *The*

Journal of Biological Chemistry, *272*(36), 22642–22647. http://doi.org/10.1074/jbc.272.36.22642

Salmaggi, A., Maderna, E., Calatozzolo, C., Gaviani, P., Canazza, A., Milanesi, I., et al. (2009). CXCL12, CXCR4 and CXCR7 expression in brain metastases. *Cancer Biology & Therapy*, *8*(17), 1608–1614.

Saraon, P., Musrap, N., Cretu, D., Karagiannis, G. S., Batruch, I., Smith, C., et al. (2012). Proteomic Profiling of Androgen-independent Prostate Cancer Cell Lines Reveals a Role for Protein S during the Development of High Grade and Castration-resistant Prostate Cancer. *The Journal of Biological Chemistry*, *287*(41), 34019–34031. http://doi.org/10.1074/jbc.M112.384438

Schietke, R., Warnecke, C., Wacker, I., Schödel, J., Mole, D. R., Campean, V., et al. (2010). The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. *The Journal of Biological Chemistry*, *285*(9), 6658–6669. http://doi.org/10.1074/jbc.M109.042424

Schmechel, D., Marangos, P. J., & Brightman, M. (1978). Neurone-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature*, *276*(5690), 834–836. http://doi.org/10.1038/276834a0

Semenza, G. L. (1998). Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Current Opinion in Genetics & Development*, *8*(5), 588–594.

Semenza, G. L. (2001). Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. *Journal of Clinical Investigation*, *108*(1), 39–40. http://doi.org/10.1172/JCI13374

Semenza, G. L. (2002). Signal transduction to hypoxia-inducible factor 1. *Biochemical Pharmacology*, *64*(5-6), 993–998. http://doi.org/10.1016/S0006-2952(02)01168-1

Semenza, G. L. (2004). Hydroxylation of HIF-1: Oxygen Sensing at the Molecular Level. *Physiology*, *19*(4), 176–182. http://doi.org/10.1152/physiol.00001.2004

Semenza, G. L. (2010). Oxygen homeostasis. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, *2*(3), 336–361. http://doi.org/10.1002/wsbm.69

Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology*, *12*(12), 5447–5454.

Seminara, S. B., Dipietro, M. J., Ramaswamy, S., Crowley, W. F., & Plant, T. M. (2006). Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (Macaca mulatta): a finding with therapeutic implications. *Endocrinology*, *147*(5), 2122–2126. http://doi.org/10.1210/en.2005-1550

Sies, H., & Bruene, B. (2007). *Oxygen biology and hypoxia* (Vol. 435). Academic Press.

Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E. J., & Krek, W. (2003). Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature*, *425*(6955), 307–311. http://doi.org/10.1038/nature01874

Stange, D. E., Engel, F., Longerich, T., Koo, B. K., Koch, M., Delhomme, N., et al. (2010). Expression of an ASCL2 related stem cell signature and IGF2 in colorectal cancer liver metastases with 11p15.5 gain. *Gut*, *59*(9), 1236–1244. http://doi.org/10.1136/gut.2009.195701

Stiehl, D. P., Jelkmann, W., Wenger, R. H., & Hellwig-Bürgel, T. (2002). Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. *FEBS Letters*, *512*(1-3), 157–162.

Sukocheva, O., & Wadham, C. (2014). Role of sphingolipids in oestrogen signalling in breast cancer cells: an update. *The Journal of Endocrinology*, *220*(3), R25–35. http://doi.org/10.1530/JOE-13-0388

Sun, Y., Niu, J., & Huang, J. (2009). Neuroendocrine differentiation in prostate cancer. *American Journal of Translational Research*, *1*(2), 148–162.

Thiery, J. P., Acloque, H., Huang, R. Y. J., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell*, *139*(5), 871–890. http://doi.org/10.1016/j.cell.2009.11.007

Thompson, E. L., Murphy, K. G., Patterson, M., Bewick, G. A., Stamp, G. W. H., Curtis, A. E., et al. (2006). Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. *American Journal of Physiology. Endocrinology and Metabolism*, *291*(5), E1074–82. http://doi.org/10.1152/ajpendo.00040.2006

Tian, H., McKnight, S. L., & Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes & Development*, *11*(1), 72–82.

Vashchenko, N., & Abrahamsson, P. A. (2005). Neuroendocrine differentiation in prostate cancer: implications for new treatment modalities. *European Urology*, *47*(2), 147–155. http://doi.org/10.1016/j.eururo.2004.09.007

Vincent-Salomon, A., & Thiery, J. P. (2003). Epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Research*.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, *10*(8), 789–799. http://doi.org/10.1038/nm1087

W G Stetler-Stevenson, S Aznavoorian, A., & Liotta, L. A. (2003). Tumor Cell Interactions with the Extracellular Matrix During Invasion and Metastasis. *Dx.Doi.org*, *9*(1), 541–573. http://doi.org/10.1146/annurev.cb.09.110193.002545

Wang, C., Mao, J., Redfield, S., Mo, Y., Lage, J. M., & Zhou, X. (2014). Systemic distribution, subcellular localization and differential expression of sphingosine-1-phosphate receptors in benign and malignant human tissues. *Experimental and Molecular Pathology*, *97*(2), 259–265. http://doi.org/10.1016/j.yexmp.2014.07.013

Wang, G. L., Jiang, B. H., Rue, E. A., & Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(12), 5510–5514.

Wang, H., Jones, J., Turner, T., He, Q. P., Hardy, S., Grizzle, W. E., et al. (2012). Clinical and Biological Significance of KISS1 Expression in Prostate Cancer. *The American Journal of Physical Anthropology*, *180*(3), 1170–1178. http://doi.org/10.1016/j.ajpath.2011.11.020

Weis, S. M., & Cheresh, D. A. (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. *Nature Medicine*, *17*(11), 1359–1370. http://doi.org/10.1038/nm.2537

Whitelaw, M. L., Gustafsson, J. A., & Poellinger, L. (1994). Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. *Molecular and Cellular Biology*, *14*(12), 8343–8355. http://doi.org/10.1128/MCB.14.12.8343

Whiteside, T. L. (2008). The tumor microenvironment and its role in promoting tumor growth. *Oncogene*, *27*(45), 5904–5912. http://doi.org/10.1038/onc.2008.271

Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., et al. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood*, *92*(7), 2260–2268.

Wood, S. M., Gleadle, J. M., Pugh, C. W., Hankinson, O., & Ratcliffe, P. J. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *The Journal of Biological Chemistry*, *271*(25), 15117–15123.

Xie, L., Law, B. K., Chytil, A. M., Brown, K. A., Aakre, M. E., & Moses, H. L. (2004). Activation of the Erk Pathway Is Required for TGF-β1-Induced EMT In Vitro. *Neoplasia*, *6*(5), 603–610. http://doi.org/10.1593/neo.04241

Xing, N., Qian, J., Bostwick, D., Bergstralh, E., & Young, C. Y. (2001). Neuroendocrine cells in human prostate over-express the anti-apoptosis protein survivin. *The Prostate*, *48*(1), 7–15. http://doi.org/10.1002/pros.1076

Yang, J., & Weinberg, R. A. (2008). Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. *Developmental Cell*, *14*(6), 818–829. http://doi.org/10.1016/j.devcel.2008.05.009

Yoon, J. H., Kim, J., Kim, K. L., Kim, D.-H., Jung, S.-J., Lee, H., et al. (2014). Proteomic analysis of hypoxia-induced U373MG glioma secretome reveals novel hypoxia-dependent migration factors. *Proteomics*, *14*(12), 1494–1502. http://doi.org/10.1002/pmic.201300554

Zajac, M., Law, J., Cvetkovic, D. D., Pampillo, M., McColl, L., Pape, C., et al. (2011). GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. *PloS One*, *6*(6), e21599. http://doi.org/10.1371/journal.pone.0021599

Zhou, Z.-H., Rao, J., Yang, J., Wu, F., Tan, J., Xu, S.-L., et al. (2015). SEMA3F prevents metastasis of colorectal cancer by PI3K-AKT-dependent down-regulation of the ASCL2-CXCR4 axis. *The Journal of Pathology*, 236, 467-478. http://doi.org/10.1002/path.4541

Appendix A.

MCF-7 top 30 up-regulated genes induced greater than 2-fold by a combination of loss of Rb and hypoxia when compared to negative controls.

Gene Name	Associated Pathway	Fold Induction (vs. shSCX Normoxiai)		
		shRb Normoxia	shSCX Hypoxia	shRb Hypoxia
SEMA5B	Tumour	2.682	12.192	45.941
ASB2	Tumour	1.665	23.845	34.728
NDRG1	Metastasis	1.860	20.746	34.488
SPAG4	Metastasis	2.265	9.410	15.369
PLAC8	Epithelial to mesenchymal transition	4.101	3.989	14.666
FGD5	Tumour	1.237	3.406	14.606
S1PR4	Epithelial to mesenchymal transition	2.492	5.804	14.001
STC1	Metastasis	1.767	8.565	13.895
VTCN1	Metastasis	4.061	4.807	13.133
FAM13A	Tumour	1.532	6.852	12.683
LRP4	Tumour	0.832	7.778	12.173
LDLRAD1		1.705	3.705	11.600
PADI1		4.3484	1.5704	11.2928
EPHA3	Angiogenesis & epithelial to mesenchymal transition	3.587	1.933	10.687
GAL3ST1		1.059	3.313	10.400
TLE6	Tumour & progression	3.6094	5.383	9.779
LOXL2	Metastasis & epithelial to mesenchymal transition	2.285	4.520	9.537
UCA1	Tumour	5.198	1.820	9.318
WNT11	Metastasis	4.294	1.727	8.691
HOXA13	Tumour & progression	2.329	4.070	8.079
SCNN1G		1.469	3.149	7.979

PTGS1	Metastasis	4.613	1.649	7.917
HEY1	Epithelial to mesenchymal transition	1.165	4.024	7.599
EGLN3	Tumour & progression	1.144	5.362	7.216
EFCAB3		1.857	4.027	7.096
CPXM2		2.075	1.342	6.978
TARP	Epithelial to mesenchymal transition & progression	4.071	1.046	6.871
PCP4L1		2.156	4.797	6.655
KIAA1199	Epithelial to msenchymal transition & metastasis	3.199	1.449	6.638
HK2	Epithelial to mesenchymal transition	1.513	3.824	5.751

Appendix B.

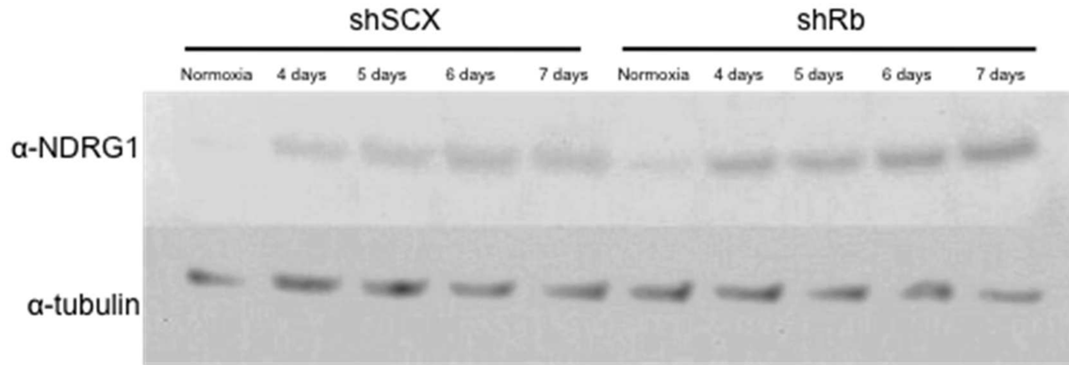
LNCaP top 30 up-regulated genes induced greater than 2-fold by a combination of loss of Rb and hypoxia when compared to negative controls.

Gene Name	Associated Pathway	Fold Induction (vs. shSCX Normoxiai)		
		shRb Normoxia	shSCX Hypoxia	shRb Hypoxia
HTR5A	Neuroendocrine differentiation (NED)	2.967	27.414	256.399
PLOD2	Metastasis (MET)	2.527	24.258	200.172
SLC16A3		1.731	24.852	200.172
ATP4A		2.457	12.534	157.926
PLA2G4D		1.155	10.047	97.723
NIM1		1.322	5.729	91.574
CYP26A1		1.146	2.867	68.505
CXCR4	MET	1.297	2.652	62.557
KISS1R	NED	1.641	8.513	61.942
ANGPTL4	Angiogenesis	1.921	3.627	53.166
GPR26	NED	1.016	9.839	53.004
MYBPC2		1.185	3.737	50.392
FOS	Cell proliferation, and transformation	1.474	1.413	49.014
PPFIA4	MET	1.606	8.315	39.759
CA9	Cell proliferation, and transformation	1.182	2.090	28.626
NFATC4		1.515	4.696	21.969
PFKFB4		1.531	2.938	21.816
PCP4L1	NED	1.971	2.515	21.018
RORA	NED	1.404	2.568	20.806
AMPD3		1.102	1.119	19.431
ALDOC	NED	1.456	4.845	19.334
ENO2	NED	1.795	2.606	19.093
SCNN1G		1.098	1.300	19.017
STC1	MET	1.097	4.418	18.996

NDRG1	MET	1.193	6.804	18.601
TSPEAR		5.972	1.714	17.314
TMEM45A		1.460	2.607	17.071
MAFF		1.227	2.017	16.515
ATP8B3		1.919	2.357	15.520
GPR146		1.355	2.137	15.098

Appendix C.

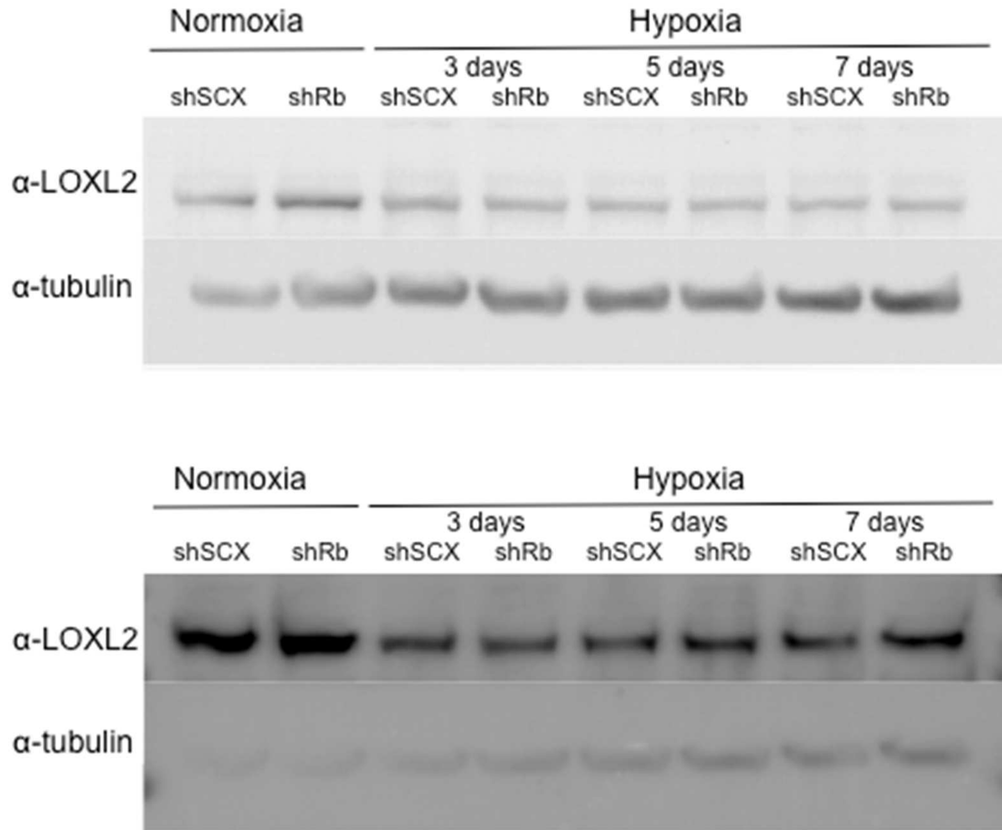
Supplementary image for Figure 3.4.



Loss of Rb and hypoxia leads to an increase in NDRG1 protein levels in MCF-7 cells.
MCF-7 shSCX and shRb cells were exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 4-7 days. This image represents one of the immuno-blots used for NDRG1 protein quantification.

Appendix D.

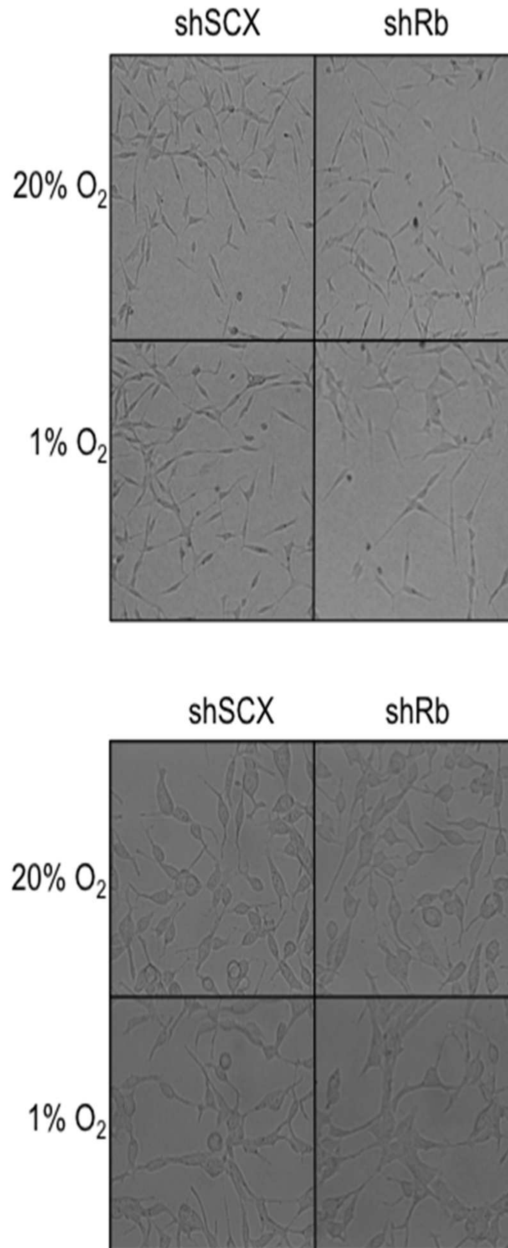
Supplementary images for Figure 3.6.



Exposure to hypoxic conditions causes a decrease in LOXL2 protein level.
MCF-7 shSCX and shRb cells were exposed to normoxia (20% O_2) and hypoxia (1% O_2) for various time points up to 7 days. These images represent the other two immuno-blots used for LOXL2 protein quantification.

Appendix E.

Supplementary images for Figure 3.12.



Loss of Rb and exposure to hypoxia for 4 days causes morphological changes in LNCaP cell structure.
LNCaP shSCX and shRb cells were placed in normoxia (20% O_2) or hypoxia (1% O_2) for 4 days. Hypoxia and loss of Rb led to observable morphological changes in LNCaP structure.

Appendix F.

MCF-7 gene ontology analysis showing key networks, molecular, cellular and physiogical functions and top transcripton factors regulated by hypoxia and Rb loss. The top list (A.) shows the pathways that are regulated by genes up-regulated under hypoxia and Rb loss. The bottom list (B.) shows the pathways that are regulated by genes down-regulated under hypoxia and Rb loss.

A.

Top Networks	Score	
Cellular movement, free radical scavenging, cellular growth and proliferation	48	
Cardiovascular system development and function, cellular movement, hematopoiesis	28	
Development disorder, neurological disease, tissue development	26	
Top Molecular and Cellular Function	**p-value**	**# Molecules**
Cellular development	1.90E-04 - 2.64E-02	25
Cell morphology	2.89E-04 - 2.64E-02	11
Cellular growth and proliferation	4.96E-04 - 2.90E-02	25
Antigen presentation	1.25E-03 - 2.20E-02	6
Cellular movement	1.25E-03 - 2.66E-02	19
Top Physiological System Development and Function	**p-value**	**# Molecules**
Embryonic development	5.83E-05 - 2.64E-02	21
Nervous system development and function	5.83E-05 - 2.64E-02	7
Organ development	5.83E-05 - 2.64E-02	17
Organismal development	5.83E-05 - 2.64E-02	22
Tissue development	5.83E-05 - 2.64E-02	24
Top Transcription Factors	**p-value of overlap**	
HIF1α	3.19E-10	
EPAS1	2.91E-06	
RARG	3.82E-04	

B.

Top Networks	Score	
Free radical scavenging, cell death, cellular growth and proliferation	21	
Inflammatory resonse, cell-to-cell signaling and interaction, cellular movement	8	
Cancer, cell cycle, cell-to-cell signaling and interaction	3	
Top Molecular and Cellular Function	**p-value**	**# Molecules**
Cellular development	1.31E-06 – 4.24E-02	8
Cellular movement	8.86E-06 – 4.14E-02	6
Cellular growth and proliferation	1.62E-04 – 4.24E-02	7
Cell death	1.68E-04 – 3.15E-02	5
Free radical scavenging	2.09E-04 – 5.16E-03	3
Top Physiological System Development and Function	**p-value**	**# Molecules**
Cardiovascular system development and function	1.24E-06 – 3.93E-02	8
Hematopoiesis	8.86E-06 – 4.24E-02	4
Immune cell trafficking	8.86E-06 – 4.04E-02	4
Hematological system development and function	1.11E-05 – 4.24E-02	4
Organismal development	2.78E-05 – 2.99E-02	9
Top Transcription Factors	**p-value of overlap**	
NFκBIE	4.97E-05	
ESR2	1.44E-04	
CITED4	1.08E-03	

Appendix G.

LNCaP gene ontology analysis showing key networks, molecular, cellular and physiogical functions and top transcripton factors regulated by genes that are up-regulated under hypoxia and Rb loss.

Top Networks	Score	
Cardiac arteriopathy, cardiovascular disease, hematological disease	44	
Organ morpohology, organismal development, reproductive system development and function	39	
Cellular development, cellular growth and proliferation, hematological system development and function	20	
Top Molecular and Cellular Function	**p-value**	**# Molecules**
Cell death and survival	1.33E-04 – 1.92E-02	10
Cellular movement	1.42E-04 – 2.13E-02	17
Cellular development	2.41E-004 – 2.16E-02	17
Cell-to-cell signaling and interaction	3.11E-04 – 2.13E-02	12
Cellular compromise	3.35E-04 – 2.92E-02	7
Top Physiological System Development and Function	**p-value**	**# Molecules**
Organ development	2.68E-05 – 2.16E-02	20
Digestive system development and function	3.69E-05 – 2.00E-02	9
Organ morphology	7.14E-05 – 2.00E-02	15
Reproductive system development and function	7.14E-05 – 1.92E-02	11
Tumour morphology	1.33E-04 – 1.97E-02	6
Top Transcription Factors	**p-value of overlap**	**Predictive activation state**
HIF1α	2.49E-15	Activated
EPAS1	2.30E-14	Activated
Farnesylthiosalicylic acid	1.83E-11	Inhibited