Regulation of the *Rgs4* Gene by the Hypoxia Inducible Factors

Sam Olechnowicz

Laboratory of Dr Daniel Peet
School of Molecular and Biomedical Sciences
University of Adelaide

DECLARATION

Name: Sam Olechnowicz

Program: PhD

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sam Olechnowicz and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission as been granted by the University to restrict access for a period of time.

Signature: …….

Date: ……..

Abstract

The transcriptional response to hypoxia is critically dependent on the Hypoxia Inducible Factors HIF-1 and HIF-2, which have roles not only in development and cellular adaptation to low oxygen levels, but also in diseases such as cancer. Although many HIF target genes have been well-characterised, there is strong evidence that the complete set of HIF responsive genes have not been described. This thesis describes the characterisation of a novel hypoxia responsive gene, *Rgs4*, found by a previous microarray study. *Rgs4* encodes the Regulator of G-protein Signalling 4 (RGS4 protein), which directly inhibits signalling from various G-protein coupled receptors. Hypoxic regulation of *Rgs4* mRNA is observed in neuroblastoma and pheochromocytoma cells derived from rat, mouse and human samples. This response is found to be mimicked by HIF pathway activating chemicals, and occurs in a manner consistent with direct HIF regulation of *Rgs4* transcription. However, hypoxic regulation of this gene is not observed in all cell types that *Rgs4* is expressed in. Reporter gene assays testing 32.9kb of the locus encompassing the *Rgs4* gene failed to detect a hypoxia responsive element, though bioinformatics analysis indicates that *Rgs4* is under the control of distant enhancers outside of the region tested. Further characterisation of the *Rgs4* hypoxic response could help to explain functions of HIF that are currently poorly characterised, such as its effect on catecholamine release and signalling, while the atypical nature of *Rgs4* regulation by HIF may provide a model to discover other as-yet unknown HIF interacting proteins and cell type specific HIF target genes.

Acknowledgements

I would like to acknowledge the guidance and support of my supervisors Dr. Dan Peet and Dr. Murray Whitelaw, who contributed greatly to discussions about the directions and relevance of this research, and set the standard for making the lab a welcoming place to work. I would also like to thank my parents Kathy and Jan, who have nurtured my curious nature and love of science throughout my entire life, and supported me during the difficult stages of completing this thesis. To Sarah, who the one who was always there for me through every aspect of my time as a postgraduate, from science discussions to organising a social culture around the department, and also in dreaming about the future. I would finally like to acknowledge all the others in the MLS building who made it such a conducive place to do research, from the teaching staff such as Lynn, Tony and Garry, to those such as Serge working in the store and TSU, and to those working in the other 3rd floor labs who not only helped with my research by most importantly contributed to the warm and social atmosphere that made coming to the lab every day something to look forward to.

Abbreviations

bHLH	Basic Helix-Loop-Helix
bp	Base pairs
cDNA	Complementary DNA (from mRNA)
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP, with detection by deep sequencing
dATP	Deoxyadenosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMOG	Dimethyloxallyl glycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	2,2'-dipyridyl
DTT	Dithiothreitol
ES	Embryonic stem (cells)
FCS	Fetal calf serum
HRE	Hypoxia response element
HUVEC	Human umbilical vein endothelial cell
MEM	Minimal essential medium
mES	Mouse embryonic stem (cells)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcriptase
qRT-PCR	Reverse transcription of mRNA to cDNA, with subsequent qPCR
siRNA	Small interfering RNA
SDS	Sodium dodecyl sulfate
TSS	Transcription start site
UV	Ultraviolet light

1. Introduction

1.1.1. Hypoxia and the Hypoxia Inducible Factors

Oxygen is critical for the survival of metazoan cells due to its central role in metabolism. The mitochondria of these cells use diatomic oxygen as the final electron acceptor of the electron transfer chain, where oxidative phosphorylation produces ATP from the proton gradient across the inner mitochondrial membrane which is generated by oxidation of NADH from the Krebs cycle. If cellular oxygen demand outweighs supply (hypoxia), the efficient conversion of carbon energy sources to ATP will be disrupted. In this situation cells are well-adapted to counter with a variety of hypoxic response pathways, which are utilised in both adaptation to hypoxia and organism development, as well as dysregulated in some disease states.

Cells can respond to hypoxia by modulating metabolism, in order to conserve and make more efficient use of the oxygen that is available. Furthermore, the efficiency of oxygen delivery can be increased, such as through the activation of angiogenic and erythropoietic pathways. Hypoxia also serves as an important developmental signal, such that disruption of hypoxia signalling pathways causes severe developmental phenotypes (Iyer *et al.*, 1998; Tian *et al.*, 1998). In addition, acute and chronic hypoxia are sensed and responded to in differing ways. For instance, the carotid body acts as an acute hypoxia sensor through use of O_2-regulated K^+ channels (reviewed by Lopez-Barneo *et al.*, 2009). These channels signal for a hyperventilatory response, which improves the delivery of oxygen to all tissues. However, almost all mammalian cells respond to chronic hypoxia through activation of the Hypoxia Inducible Factor (HIF) pathway. HIF is a heterodimeric transcription factor which binds at over 100 genomic sites to activate transcription of specific genes which allow cellular response and adaptation to hypoxia (Wenger *et al.*, 2005). This pathway has been the focus of much research due to its involvement in adaptation, development and pathogenesis, in particular oncogenesis.

HIF is a heterodimer comprised of two basic-Helix-Loop-Helix/Per-Arnt-Sim domain (bHLH/PAS) proteins, known as HIF-α and HIF-β (Wang and Semenza, 1995), which

dimerise in the nucleus to activate transcription (Konietzny *et al.*, 2009). The N-terminal bHLH domains of HIF-α and -β allow for DNA binding and primary dimerisation, while the PAS domains provide secondary dimerisation. HIF-α also contains two transactivation domains, known as the N-terminal Transactivation Domain (N-TAD) and the C-Terminal Transactivation Domain (C-TAD), the latter of which binds coactivators CBP or p300 to promote target gene transcription (Arany *et al.*, 1996; Ebert and Bunn, 1998). HIF-β, alternatively known as the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), is constitutively expressed and unresponsive to hypoxia. HIF-β also acts as a general partner factor for other dimeric bHLH/PAS transcription factors such as NPAS4 (Ooe *et al.*, 2004), SIM-1, SIM-2 and the Aryl Hydrocarbon Receptor (AhR) (Swanson *et al.*, 1995). These heterodimers respond to different stimuli, such as membrane depolarisation for NPAS4, but bind very similar genomic DNA sequences, all with a core CGTG consensus. In this way, crosstalk between some members of the bHLH/PAS family at genomic binding sites has been demonstrated previously (Farrall and Whitelaw, 2009; Ooe *et al.*, 2004). The HIF-α subunits are the only members of the bHLH/PAS family of proteins to be regulated at the level of stability and activity by oxygen concentration.

There are three genes encoding HIF-α subunits: *Hif1a*, which encodes HIF-1α protein; *Epas1*, which encodes HIF-2α protein, also known as HIF-Like Factor (HLF) or Endothelial Per-ARNT-Sim protein-1 (EPAS1); and *Hif3a*, which encodes various splice variants of HIF-3α protein. HIF-1α and -2α bind to HIF-β in order to form active HIF transcription factors HIF-1 or HIF-2, respectively. When compared to HIF-1α and HIF-2α, HIF-3α variants lack transactivation domains, and therefore are thought to have only an inhibitory or dominant-negative role on the transcriptional role of HIF (Hara *et al.*, 2001; Makino *et al.*, 2001; Makino *et al.*, 2002). There are also two genes encoding HIF-β subunits, *Arnt1* and *Arnt2*, both of which encode proteins capable of partnering HIF-1α (Drutel *et al.*, 2000; Stolze *et al.*, 2002). Many of the features of HIF-1α and HIF-2α regulation and function are similar, and therefore the term HIF-α is used here to refer to these two subunits collectively.

1.1.2. Post-Translational HIF Regulation

Both HIF-α proteins are sensitive to oxygen availability as a result of hydroxylation reactions in two of their domains. Normal oxygen levels, known as normoxia, enable

proline residues (P402 and P564 in human HIF-1α) to be hydroxylated by the HIF Prolyl Hydroxylase Domain proteins (PHD1, PHD2 and PHD3) (Jaakkola *et al.*, 2001). These hydroxylated prolines are located in a domain named the Oxygen Dependent Degradation domain (ODD), which overlaps with the N-TAD (Huang *et al.*, 1998). Hydroxylation of these prolines leads to recognition by an E3 ubiquitin ligase (Von-Hippel Lindau protein, pVHL) (Jaakkola *et al.*, 2001), ubiquitylation of HIF-α, and subsequent rapid turnover of the protein via the ubiquitin-proteasome degradation pathway (Huang *et al.*, 1998; Salceda and Caro, 1997). Generally, this results in no detectable HIF-α protein in well-oxygenated mammalian cells. In addition, interaction between HIF-α and transcriptional coactivators p300 or CBP is inhibited during normoxia by hydroxylation of an asparagine residue (N803 in human HIF-1α) in the HIF-α C-TAD by Factor Inhibiting HIF (FIH) (Hewitson *et al.*, 2002; Lando *et al.*, 2002a; Lando *et al.*, 2002b). Consequently, hydroxylation at this residue prevents transcriptional activation from the HIF-α C-TAD. In contrast, hypoxic conditions prevent hydroxylation and lead to HIF-α stabilisation and subsequent dimerisation with HIF-β to constitute the HIF transcription factor, which is then transcriptionally active (*Figure 1.1*).

The hydroxylation activity of the PHD and FIH enzymes requires diatomic oxygen, 2-oxoglutarate and an appropriate peptide substrate, producing a hydroxylation modification on the peptide substrate, and CO_2 and succinate as byproducts. Fe-II is also required by these enzymes for catalysis (Aravind and Koonin, 2001; Epstein *et al.*, 2001). The best characterised substrates of these enzymes are HIF-1α and HIF-2α, but other substrates are known. For example, the Notch-1, -2 and -3 Intracellular Domains (ICD) can be hydroxylated by FIH (Coleman *et al.*, 2007; Zheng *et al.*, 2008). As this hydroxylation reaction directly requires O_2, and hypoxia therefore inhibits hydroxylation of HIF, the PHD and FIH enzymes are thought to be the primary oxygen sensors of this the HIF pathway (Epstein *et al.*, 2001).

Post-translational modifications other than the hydroxylation pathway can also affect HIF activity. Increased levels of HIF-1α protein and activity have been observed following insulin, IGFI, IGFII, EGF (Feldser *et al.*, 1999; Jiang *et al.*, 2001), angiotensin-II, thrombin, FGF2 and PDGF (Richard *et al.*, 1999), lipopolysaccharide (Blouin *et al.*, 2004)

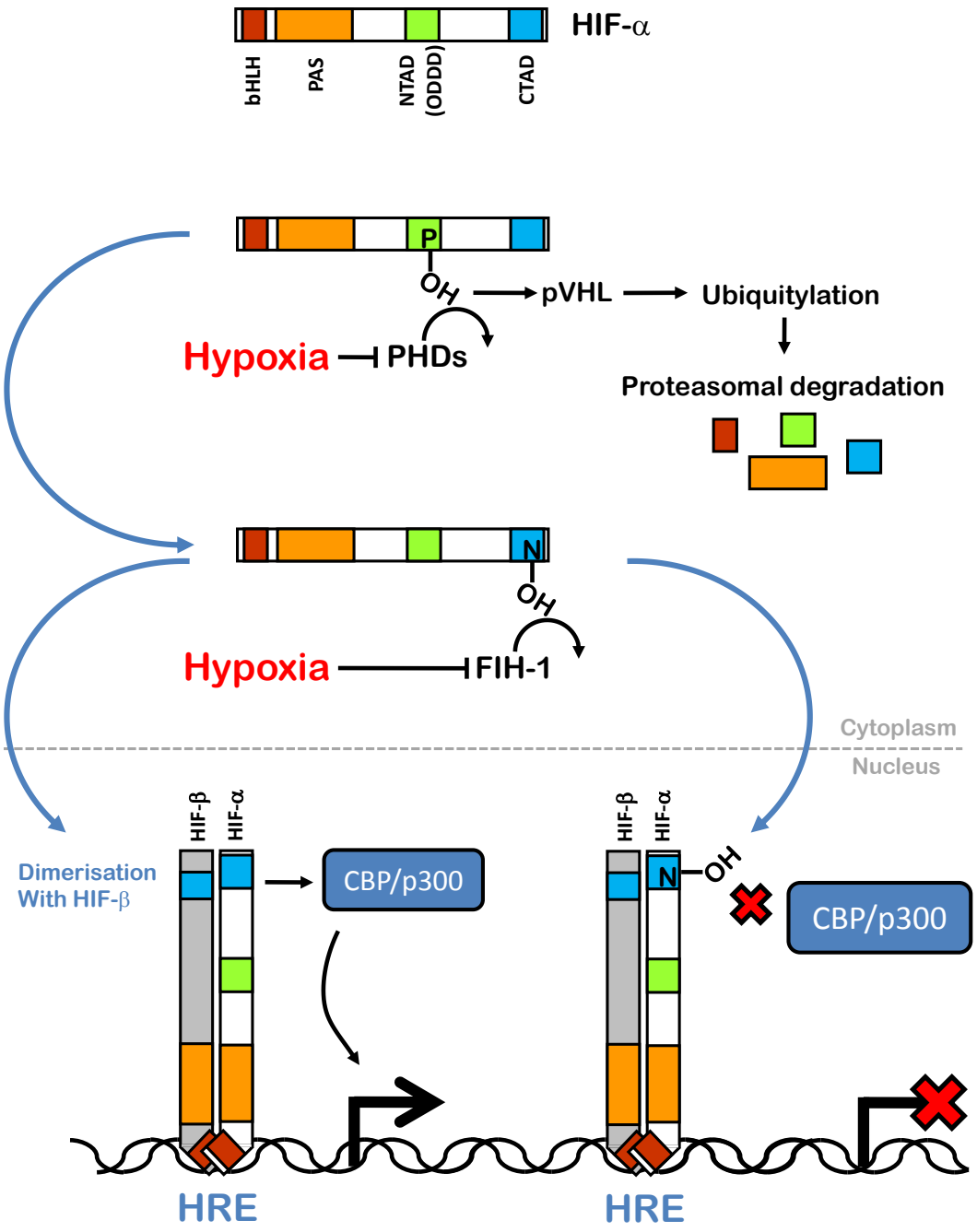


Figure 1.1. The domain structure of HIF-α (either HIF-1α or HIF-2α, shown at top) and canonical post-translational regulatory pathway. Hypoxic conditions (left) prevent HIF-α degradation and allow interaction with coactivators to promote target gene transcription. Normoxic conditions (right) allow PHD and FIH hydroxylation of HIF-α, promoting degradation and inhibiting interaction with transcriptional coactivators.

or TNFα (Sandau *et al.*, 2001) treatment of certain cell culture lines. Many of these signalling pathways result in phosphorylation of HIF-1α by kinases such as the p42 and p44 MAPKs (Richard *et al.*, 2000) and PI3K (Jiang *et al.*, 2001), resulting in increased HIF activity. However, it should be noted that some of these treatments also affect transcription of the HIF-α encoding genes. There is also evidence that reactive oxygen species (ROS) (Chandel *et al.*, 2000) and nitric oxide (Sandau *et al.*, 2001) can signal to activate the HIF pathway. Furthermore, the activity of HIF-1α can be modified by post-translational modifications other than those mentioned above, such as acetylation, sumoylation and S-nitrosation (reviewed by Brahimi-Horn *et al.*, 2005).

1.1.3. Targets of HIF Transactivation

HIF binds directly to a DNA sequence known as the Hypoxia Response Element (HRE), with a consensus sequence of RCGTG (Pescador *et al.*, 2005; Wang and Semenza, 1995; Wenger *et al.*, 2005), where R can be an adenine or guanine. There is also some evidence for a secondary cis-acting element named the HIF Ancillary Sequence (HAS), which is separated from the HRE core sequence by 8bp and improves transactivation by HIF (Kimura *et al.*, 2001). As mentioned previously, there are over 100 known genomic HIF binding sites (Wenger *et al.*, 2005), with novel sites still being discovered. As DNA binding specificity is apparently indistinguishable between HIF-1 and HIF-2 at a consensus HRE, many genes under the control of a HRE are responsive to both forms of HIF. One example of a non-specific HRE is located upstream of *Vegf* (Vascular Endothelial Growth Factor) (Levy *et al.*, 1995; Liu *et al.*, 1995), a gene which was originally identified as a target of HIF-1 transactivation (Forsythe *et al.*, 1996), but was later found to activatable by HIF-2 (Flamme *et al.*, 1997; Flamme *et al.*, 1998; Wiesener *et al.*, 1998). This HRE is located about 975bp upstream of the human *Vegf* transcription start site. Similarly, hypoxic response of *Dec1* in human neuroblastoma cells was found to be mediated through HIF-1 binding at a HRE 145bp upstream of its transcription start site (Miyazaki *et al.*, 2002), yet further analysis using chromatin immunoprecipitation (ChIP) and siRNA techniques has shown that both HIF-1 and HIF-2 are capable of binding at this HRE and activating *Dec1* transcription (Holmquist-Mengelbier *et al.*, 2006). *Phd3* (encoding the HIF Proline Hydroxylase Domain protein 3) is another example of a gene which is sensitive to both HIF forms, although it is more sensitive to ectopic expression of HIF-2 than HIF-1 (Aprelikova *et al.*, 2004; Lau *et al.*, 2007).

There are also examples of target gene specificity between HIF-1 and HIF-2. Hypoxic response of genes such as *Ca9* (encoding Carbonic Anhydrase IX, a pH regulator) and *Slc2a1* (also known as *Glut-1*, encoding a glucose transporter) occurs only through HIF-1, as knockdown of HIF-1 alone in breast cancer cells results in loss of the response of these genes to hypoxia, despite the presence of HIF-2 (Sowter *et al.*, 2003). Glycolytic enzyme genes such as *Ldha* (encoding Lactate Dehydrogenase) or *Pgk1* (encoding Phosphoglycerate Kinase 1) respond to HIF-1 but not HIF-2 (Hu *et al.*, 2003; Semenza *et al.*, 1994). The predominance of HIF-1 target genes compared to HIF-2 was initially not surprising, given that most of the initial studies were specifically designed to identify HIF-1 responsive genes, with relatively few studies aimed at identifying target genes of HIF-2. However, subsequent microarray experiments comparing hypoxic activation of genes in breast cancer cells to HIF-α siRNA-treated cells have since shown that the majority of hypoxia responsive genes are dependent on the presence of HIF-1 alone (Aprelikova *et al.*, 2006; Elvidge *et al.*, 2006). When compared with the essentially ubiquitous expression pattern of HIF-1α, this implies that HIF-1 is the most important isoform for the general hypoxic response, as the response of few genes is affected by loss of HIF-2α.

As fewer genes which display specificity towards HIF-2 transactivation are known, HIF-2 activity is thought to provide a more specific set of cellular responses. The *Epo* gene (which encodes Erythropoietin) is currently considered to be the best characterised *in vivo* HIF-2 specific target gene. Knockout or knockdown of HIF-1α in mouse astrocytes results in a significant reduction in the hypoxic response of *Vegf* and *Ldha*, while no change in *Epo* response is observed at all (Chavez *et al.*, 2006). Conversely, the same authors observed significant decrease in *Epo* response after infection with lentivirus encoding HIF-2α-directed siRNA, and that HIF-2α rather than HIF-1α was predominantly detectable bound at the *Epo* HRE by ChIP. Importantly, similar results have subsequently been observed for the hepatic *Epo* response to hypoxia or anaemia in mice (Rankin *et al.*, 2007). Similarly, *Flk1*, *Cited2* (Aprelikova *et al.*, 2006; Elvert *et al.*, 2003) and *Col10a1* (Saito *et al.*, 2010) are regulated by HIF-2 alone in specific environments. The exact mechanism for this specificity is unclear, but may relate to the profile of other transcription factors bound in the vicinity.

Selection between HIF forms at enhancers regulating *Flk1* and *Cited2* appear to be related to specific interaction between HIF-2 and ETS family transcription factors. ELK1 (ETS-LiKe protein 1) binds DNA proximal to the HRE at *Cited2* and is required for its hypoxic

activation (Aprelikova *et al.*, 2006), while ETS1 performs a similar role at the *Flk1* HRE (Elvert *et al.*, 2003). Although bioinformatic analysis suggests a role for ETS factor binding at other HIF-2 preferred sites, these have not been experimentally verified and so the extent to which ETS factors affect HIF binding specificity at other loci or in various cell types is not known.

Experiments using HIF-1α/HIF-2α over-expressed chimeric protein also imply that selection of target genes by either HIF-1 or HIF-2 is not dependent on the DNA-binding domain of the HIF-α protein, using *Ca9* as an example of a HIF-1 specific target gene, and *Phd3* as an example of a gene which is more sensitive to HIF-2 than HIF-1 overexpression. Hypoxic activation of *Ca9* transcription requires the presence of HIF-1α PAS, ODDD and N-TAD domains in a chimeric HIF-α protein, but these can be ligated to the DNA binding domain of either HIF-1α or HIF-2α for equal effect. Meanwhile, strong activation of *Phd3*, achieved by over-expression of HIF-2, requires only the presence of the HIF-2α C-TAD ligated to other domains from either HIF-α subunit (Lau *et al.*, 2007). Extrapolated, these experiments suggest that a protein consisting of the HIF-1α bHLH, PAS, ODDD and N-TAD domains ligated to the HIF-2α C-TAD domain would be capable of activating all known HIF targets, unless target specificity at genes other than *Ca9* and *Phd3* is regulated in a different way. Another group has published similar data indicating that the N-TAD of HIF-1α or HIF-2α is required for target specificity, while the C-TAD confers activation of shared transcriptional targets (Hu *et al.*, 2007). Furthermore, there are sites known where both HIF-1 and HIF-2 binding can be detected by ChIP, but one or the other HIF form cannot activate gene transcription (Mole *et al.*, 2009). This evidence taken together suggests that target genes specificity is due primarily to post-DNA-binding events and genomic context, rather than primary DNA sequence recognition alone.

HIF-1 is also involved in activation of Notch-1 pathway gene targets. Hypoxia was first noted to decrease expression of differentiation markers in neuroblastoma cells, resulting in a more undifferentiated gene expression profile (Jogi *et al.*, 2002). Notably, the expression of Notch-1 was increased upon hypoxic exposure. Further experiments show that hypoxia increases Notch-1 activation of its target genes, and that HIF-1α binds directly to the Notch-1 Intracellular Domain (ICD), rather than the DNA, to produce this effect (Gustafsson *et al.*, 2005). Therefore, alternative arrangements of HIF-1 at responsive genes are possible, and HIF-responsive genes may not require a RCGTG motif for HIF

recruitment. The Notch-1 ICD is also hydroxylated at two asparagines by FIH (Coleman *et al.*, 2007; Zheng *et al.*, 2008), and although functional effects of this modification are yet to be found, this does further connect the HIF and Notch transcriptional regulation pathways.

1.1.4. Complex Patterns of HIF Hypoxic Regulation

The differing physiological functions of HIF-1 and HIF-2 are due to more than simple differences in target gene selection. Although the response of the HIFs to hypoxia is broadly similar due to regulation of both by the oxygen sensing HIF hydroxylases outlined previously (*Chapter 1.1.2*), there is additional modulation using this pathway and others for each HIF-1α and HIF-2α. These intricacies indicate that the response to hypoxia is not equivalent between different cell types, in terms of both regulation of the HIF pathway and the downstream effects. Research has shown that exposure to different concentrations of oxygen and different periods of hypoxia can invoke different levels and types of HIF activation between cell types. One example is observed during cycles of hypoxia and normoxia, known as intermittent hypoxia. Rat pheochromocytoma PC12 cells show no change in HIF signalling after 10 cycles of 30 seconds hypoxia followed by 4 minutes of normoxia, but HIF-1α protein levels are increased after 30 cycles and HIF-dependent transcription is observed after 60 cycles (Yuan *et al.*, 2005). This demonstrates that the response of a cell to hypoxia is not only affected by its current conditions, but also the preconditioning of the cell. Intermittent hypoxia is commonly a result of sleep apnea, therefore HIF signalling in response to this stimulus is likely to be medically relevant.

The atmospheric oxygen concentration which results in stabilisation and activation of HIF-α differs between cell types, and in some cell types one subunit can be activated while the other is not. *Figure 1.2a* describes the difference in HIF-1α stabilisation in moderate hypoxia between CACO2 and 293T cells, where an oxygen concentration of between 2% and 5% gives different levels of detectable HIF-1α protein between the two cell lines (Bracken *et al.*, 2006). Another example has been described in the human neuroblastoma SK-N-BE(2)C cell line, which stabilise only HIF-2α at moderate hypoxia (5% O_2), while both HIF-1α and HIF-2α are stable and active after exposure of these cells to 1% oxygen (Holmquist-Mengelbier *et al.*, 2006).

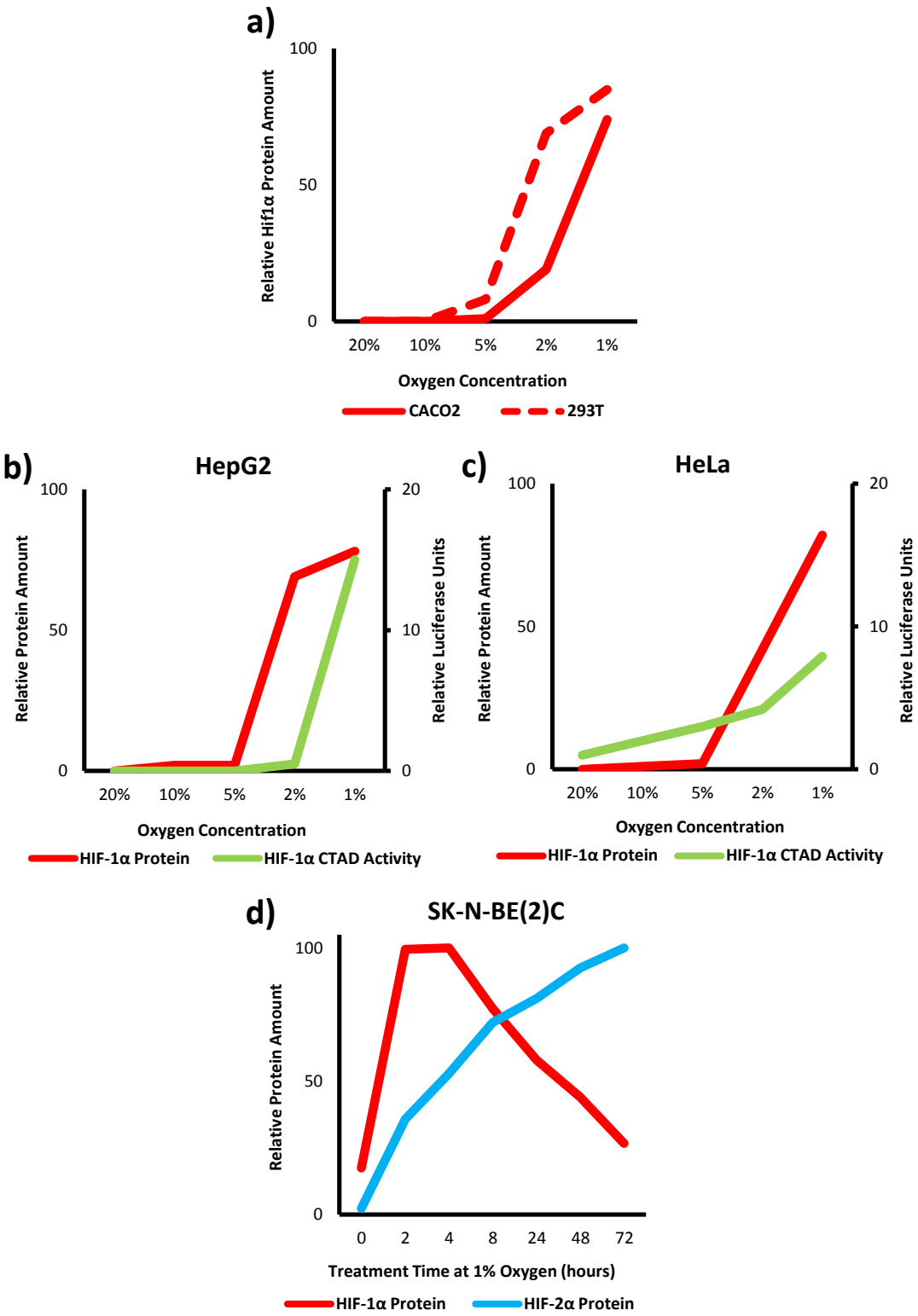


Figure 1.2. Examples of differences in HIF-α regulation between cell types. (*a*) HIF-1α protein is stabilised at higher oxygen concentrations in 293T cells than CACO2 cells (adapted from Bracken *et al.*, 2006). PHD activity compared to FIH activity (measured by HIF-1α protein level and C-TAD activity respectively, see *Figure 1.1*) is affected by oxygen concentration differently between HepG2 (*b*) and HeLa (*c*) cells (adapted from Bracken *et al.*, 2006). (*d*) SK-N-BE(2)C cells demonstrate an alternative HIF-1α and HIF-2α stabilisation pattern over extended hypoxia (adapted from Holmquist-Mengelbier *et al.*, 2006).

Differences in PHD (as indicated by HIF-1α protein level) and FIH (by HIF-1α C-TAD transactivation) activity in graded hypoxia are observed between hepatocellular carcinoma HepG2 and cervical adenocarcinoma HeLa cells, as an example (*Figure 1.2b,c*). This shows that in moderate hypoxia some cell lines have stable yet C-TAD-inactive HIF-α at moderate hypoxia (Bracken *et al.*, 2006), suggesting that some HIF protein may be able to activate transcription of genes using only its N-TAD. This form of HIF may only be able to activate a subset of HIF targets compared to HIF with both active TADs. This idea is further supported by the work of Dayan *et al.* (2006), who show that some HIF target genes can be classified as FIH sensitive or insensitive. As cell types are adapted to survive in different oxygen concentrations depending on the quality of their vascularisation and required metabolic rate, it is sensible that there are differences in the conditions required for HIF activation between tissues.

However, despite these differences, in most cell types studied induction of both HIF-α subunits is consistent over time. HIF-1α and HIF-2α protein are detectable after 30-60 minutes of hypoxia in HeLa cells, reaching a maximum induction after 2 hours. After this point, both HIF-α proteins are present and continue to be readily detectable after 48 hours of hypoxia (Wiesener *et al.*, 1998). This is consistent with observations from other cell types such as the CACO2, monkey kidney fibroblast COS-1 and human kidney 293T lines (Bracken *et al.*, 2006). In contrast, some cell types also display differential stabilisation of HIF-1 compared to HIF-2 in prolonged hypoxia (*Figure 1.2d*). Early transient stabilisation of HIF-1α followed by HIF-2α stabilisation in prolonged hypoxia has been described for the PC12, SK-N-BE(2)C and A549 cell lines (Bracken *et al.*, 2006; Holmquist-Mengelbier *et al.*, 2006; Li *et al.*, 2006; Uchida *et al.*, 2004). Therefore, this represents an alternative stabilisation pattern in a subset of cell types for HIF-α under extended hypoxia. Regulation of *Hif1a* or *Epas1* mRNA transcription is likely to play a role, as Holmquist-Mengelbier and colleagues (2006) describe increased *Epas1* message in prolonged neuroblastoma hypoxia. Alternatively, message stability or efficiency of translation may play some role, as described in the next section, or other unknown post-translational modifications on HIF-α stability.

Transcriptional activity of HIF-1 and HIF-2 may also be regulated in extended hypoxia by products of the *Hif3a* gene. *Hif3a* produces many alternatively spliced isoforms, one of which is termed Inhibitory PAS domain protein (IPAS, also known as HIF-3α2) (Makino *et al.*, 2002; Maynard *et al.*, 2003). IPAS acts in a dominant-negative fashion, binding to

HIF-α in place of HIF-β and preventing DNA binding, which affects vascularisation of the cornea in mice (Makino *et al.*, 2001). Another HIF-3α isoform, HIF-3α4, has been observed to work in a similar way (Maynard *et al.*, 2005). Some HIF-3α variants contain an ODD domain, and are therefore responsive to hypoxia through proline hydroxylation and VHL-mediated degradation by the same pathway as the other HIF-α proteins (Maynard *et al.*, 2005). The *Hif3a* gene is also under the direct transcriptional control of HIF through a HRE enhancer (Makino *et al.*, 2007; Tanaka *et al.*, 2009). Therefore, this pathway provides a mechanism for negative feedback in the HIF pathway.

1.1.5. Regulation of HIF-α mRNA Transcription by Hypoxia

Considering that the post-translational regulation of HIF-α by hydroxylation has been well-characterised, there has been relatively little research into the regulation of *Hif1a* or *Epas1* transcription. Indeed, the cellular response to hypoxia is primarily driven by HIF in coordination with the oxygen sensing activity of the PHD and FIH hydroxylases (Elvidge *et al.*, 2006). Wenger and colleagues reported no change in levels of *Hif1a* transcript in mouse tissues upon treatment with carbon monoxide (Wenger *et al.*, 1996), nor in mouse hepatoma Hepa1 or human astroglioma LN229 cell lines after 4 hours hypoxic treatment, although results were inconclusive in HeLa cells (Wenger *et al.*, 1997a). Anoxic treatment of cultured rat cardiomyocytes for 2 or 4 hours also fails to alter *Hif1a* mRNA expression (Ladoux and Frelin, 1997). Similarly, no change has been observed for *Epas1* mRNA levels in human colorectal adenocarcinoma HT-29, human kidney 293, human hepatocellular carcinoma Hep3B or HeLa cell lines (Wiesener *et al.*, 1998).

However, there have been reports of altered expression of HIF transcripts in hypoxia treated cells. Wang and colleagues observed hypoxic induction of *Hif1a* and *Arnt* mRNA in Hep3B cells (Wang *et al.*, 1995), while increased *Hif1a* message has been detected in rat and mouse organs such as the brain and lungs upon systemic hypoxic treatment (Wiener *et al.*, 1996). Although the primary mouse and human *Hif1a* promoters are insensitive to hypoxia (Luo *et al.*, 1997; Wenger *et al.*, 1997b; Wenger *et al.*, 1998), there is some evidence to suggest that there is an alternate *Hif1a* promoter for the mouse gene which is constitutive in mouse fibroblast L929 cells, but hypoxia-responsive in the Hepa1 mouse hepatoma cell line (Wenger *et al.*, 1997b; Wenger *et al.*, 1998).

1.1.6. Post-transcriptional Control of HIF-α Message by Hypoxia

More recently, experiments with different cell types have shown a negative regulation of *Hif1a* message in response to hypoxia treatment. First noted in immortalised human lymphocytes, *Hif1a* message is also reduced in the A549, PC12, rat neuroblastoma NB104 and mouse colon adenocarcinoma CT-26 cell lines upon exposure to 4 hours or longer of continuous hypoxia (Li *et al.*, 2006; Rossignol *et al.*, 2004; Thrash-Bingham and Tartof, 1999; Uchida *et al.*, 2004). This response is dependent on protein synthesis and the HIF pathway, and appears to act through antisense *Hif1a* transcription (*aHIF*), resulting in an antisense transcript which overlaps the 3'UTR of the *Hif1a* message (Rossignol *et al.*, 2002; Thrash-Bingham and Tartof, 1999; Uchida *et al.*, 2004). Transcription of *aHIF* is controlled by HIF-1 itself at HREs downstream of the *Hif1a* gene (Uchida *et al.*, 2004). The *aHIF* RNA is thought to bind the *Hif1a* mRNA 3'UTR and disrupt a hairpin structure, revealing an AU rich element which promotes degradation of the message (Rossignol *et al.*, 2002). This is supported by evidence the *Hif1a* message has a considerably shorter half-life in hypoxia-mimetic (cobalt chloride) treated A549 cells compared to controls (Uchida *et al.*, 2004). *aHIF* is expressed in many human, mouse and rat tissues, but is not hypoxia responsive in all cell types (Rossignol *et al.*, 2004; Rossignol *et al.*, 2002). This mechanism of destabilising *Hif1a* mRNA is likely to be connected to the lowered expression of HIF-1α protein described previously (*Chapter 1.1.4*) during extended hypoxia in PC12, SK-N-BE(2)C and A549 cells. However, this does not account for the increase in HIF-2α protein levels observed in these cell types after similar periods of hypoxic treatment, the cause of which is currently unknown.

The HIF pathway can also be affected by conditions other than hypoxia. In addition to regulation by antisense HIF RNA, the HIF pathway is responsive to many microRNA (miRNA) transcripts, which are short RNA duplexes that promote degradation and inhibit translation of target mRNAs. The first example of this was described for the miR-17-92 cluster, expression of which results in lowered HIF-1α and HIF-2α protein levels in immortalised lung epithelial cells (Taguchi *et al.*, 2008). In addition, mouse *Hif1a* is regulated by miR-199a in cardiac myocytes (Rane *et al.*, 2009), while miR-20b can inhibit its translation (Cascio *et al.*, 2010; Lei *et al.*, 2009). Both of these miRNAs act using a site in the *Hif1a* 3'UTR. Other messages encoding proteins of the HIF pathway are also under

the control of miRNAs, as VHL is targeted by miR-92-1 (Ghosh *et al.*, 2009), while FIH-1 levels are affected by miR-31 (Liu *et al.*, 2010). However, the effects of these miRNAs on the HIF pathway appear to be restricted to a fine-tuning role, as expression of any one of the *Hif1a* targeted miRNAs reduces hypoxic HIF-1α protein detection, but does not completely repress it.

Epas1 mRNA has been shown to be under the control of the RNA-binding Iron Regulatory Proteins IRP1 and IRP2 (Sanchez *et al.*, 2007). In low cellular iron conditions, the IRPs bind to hairpin structures in target mRNA sequences known as Iron Responsive Elements (IRE), and in the case of *Epas1*, inhibit translation of the message into protein. As iron levels are crucial in erythropoiesis, which in turn has an effect on oxygen delivery and occurs in response to hypoxia, regulation of HIF-2α translation directly adds another level of control to this system.

Hypoxia has been shown to downregulate translation of most mRNAs, as the cell apparently attempts to conserve ATP by avoiding translation of unnecessary proteins (Liu and Simon, 2004), through regulation of the mTOR pathway (Brugarolas *et al.*, 2004; Connolly *et al.*, 2006; Liu *et al.*, 2006) in a HIF-independent fashion (Arsham *et al.*, 2003). However, HIF-α escapes this regulation, along with some but not all HIF-responsive genes (Thomas and Johannes, 2007). This may be related to the RNA-binding proteins HuR and PTB, which bind and promote translation of *Hif1a* message in response to the hypoxia mimetic cobalt chloride (Galban *et al.*, 2008).

Between the effect of RNA-binding proteins, miRNAs, antisense HIF transcripts and transcriptional regulation, it is clear that there are several levels of regulation of HIF-α message working to fine tune the response to cellular hypoxia (see *Figure 1.3*), in addition to hydroxylation-dependent regulation of HIF-α described earlier (*Chapter 1.1.2*). The stabilisation and activation of HIF-α through activity of the PHD and FIH hydroxylases is the most powerful activator of the HIF pathway, yet different cell types in various situations could feasibly call on additional regulators to produce more subtle changes, in order to effect a more cell-appropriate response. Although not discussed in detail here, there are also some mechanisms where HIF can be activated by non-hypoxic means. As an example, signalling from growth factors such as IGF-1 has been shown to promote translation of *Hif1a* transcript in normoxia through MAPK and AKT kinase pathways (reviewed by Bilton and Booker, 2003).

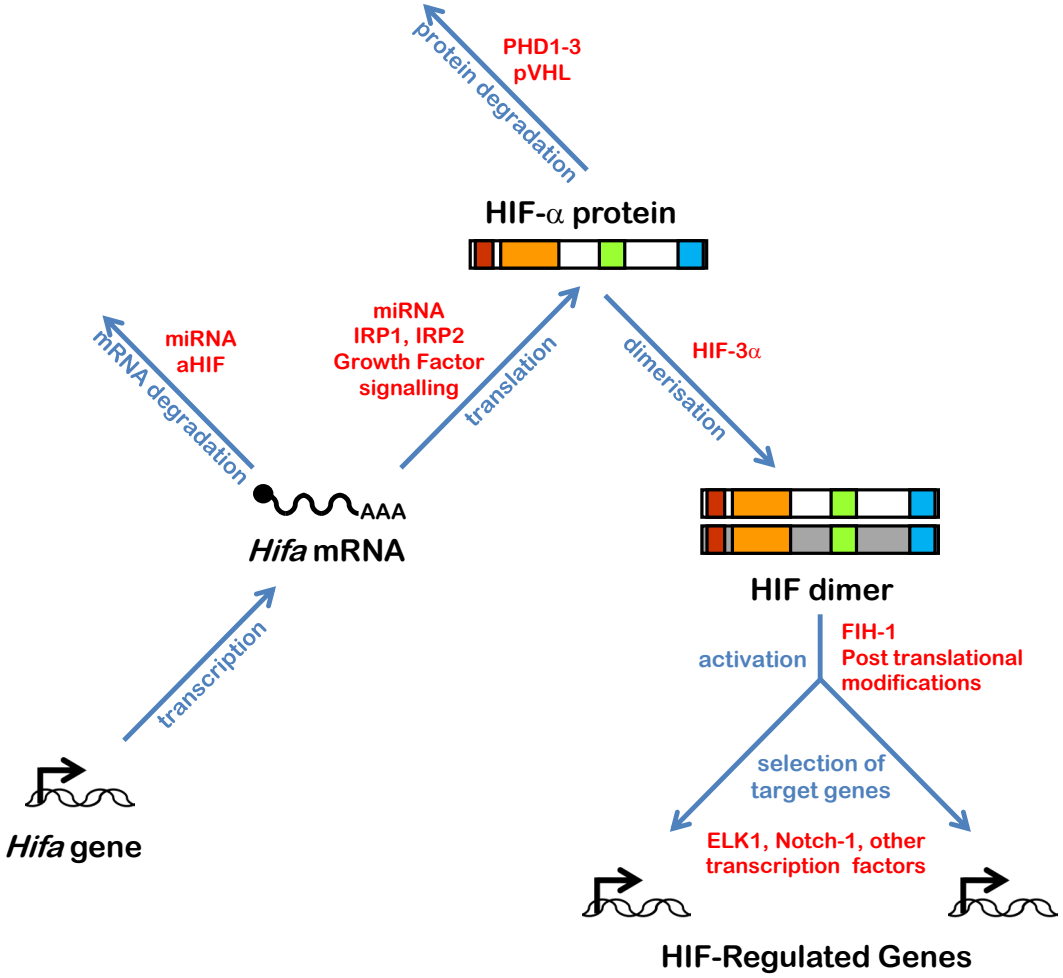


Figure 1.3. Examples of various mechanisms of regulation imposed on the HIF-α subunits, with processes affected shown in blue and the molecular effector of regulation shown in red. A selection of processes affecting either HIF-1α or HIF-2α (or both) are all shown on one diagram for simplicity, and not all regulatory mechanisms may be active at the same time or in the same type of cell. See text for details.

1.1.7. HIF-α Physiological Roles in Mice and Humans

The extent of redundancy between the two HIF-α subunits has been examined through generation of knockout mice. *Arnt1*$^{-/-}$ embryos do not survive past 10.5 days after fertilisation, due to several defects in angiogenesis which appear to be related to impaired HIF pathway function, rather than the other bHLH/PAS transcription factors which dimerise with ARNT (Kozak *et al.*, 1997; Maltepe *et al.*, 1997). *Arnt1*$^{-/-}$, *Hif1a*$^{-/-}$ and *Epas1*$^{-/-}$ embryos all display impaired placenta vascularisation and development (Cowden Dahl *et al.*, 2005), which would also be expected to impact negatively on the overall health of the embryo. In order to elucidate the functions of each HIF-α not compensated for by the other, mice with homozygous deletions of *Hif1a* and *Epas1* have been studied in further detail.

In wild-type animals *Hif1a* mRNA is detectable in all organs and is thought to be essentially ubiquitous (Wiener *et al.*, 1996). As the majority of currently known HIF target genes are activated by HIF-1 in preference to HIF-2 in cell culture models, it is thought that HIF-1 is responsible for the general cellular response to hypoxia, while HIF-2 performs more specific roles. *Hif1a*$^{-/-}$ stem cells exhibit poor induction of glycolytic pathway encoding genes such as *Glut1*, lower expression of *Vegf* and less growth under hypoxic conditions (Iyer *et al.*, 1998; Ryan *et al.*, 1998). Generation of mice from these stem cells showed that *Hif1a*$^{-/-}$ embryos were morphologically abnormal after 8.5-9.5 days of development, and no longer viable after 10.5 days due to failed neural tube closure and impaired vascularisation, causing reduced oxygenation of tissues (Iyer *et al.*, 1998; Ryan *et al.*, 1998). In order to investigate roles of HIF-1α other than embryonic development, conditional *Hif1a* knockout mice have been generated, implicating HIF-1 function in brain development (Milosevic *et al.*, 2007; Tomita *et al.*, 2003), normal heart function (Huang *et al.*, 2004), active skeletal muscle (Mason *et al.*, 2004), inflammation, efficiency of bacteria killing by macrophages (Cramer *et al.*, 2003; Peyssonnaux *et al.*, 2005), chondrogenesis in joints (Provot *et al.*, 2007), skin (Boutin *et al.*, 2008) and haematopoietic stem cell maintenance (Takubo *et al.*, 2010). Therefore, while HIF-1 regulates the general response of cells to hypoxia, it also has functions in many specific contexts, in roles not limited to systemic or cellular hypoxic response.

Epas1, which encodes for HIF-2α, has been the subject of repeated study through use of knockout mouse technology. Tian and colleagues (1998) generated *Epas1*$^{-/-}$ embryos and observed homozygous mutant death from 12.5 days post fertilisation, with a more subtle morphological disruption than had been observed for *Hif1a*$^{-/-}$ mice. The cause of death was attributed to bradycardia arising from insufficient catecholamine synthesis and release, as supplementing water with catecholamine precursor DOPS rescued some of the embryos to birth (Tian *et al.*, 1998). Curiously, heterozygous transgenic mice originating from this stock were able to survive to adulthood after backcrossing heterozygotes with another strain of mouse (Scortegagna *et al.*, 2003a; Scortegagna *et al.*, 2003b), allowing some study of more subtle adult phenotypes. Of particular note were hematopoietic deficiencies (Scortegagna *et al.*, 2003b), reactive oxygen species stress, low body weight, hypocellular bone marrow and testes, hypertrophic heart and steatotic liver (Scortegagna *et al.*, 2003a), reduced hypoxic erythropoietin production (Scortegagna *et al.*, 2005) and retinopathy resulting in blindness (Ding *et al.*, 2005). A second set of *Epas1*$^{-/-}$ mice have been independently generated by Peng and colleagues (2000), who observed some embryo death from 13.5 days post fertilisation, yet survival of some embryos to birth without DOPS supplements. The homozygous mutants in these experiments appeared to die from vascular remodelling defects, with reduced catecholamine release playing only a small role. Generation of a third *Epas1*$^{-/-}$ mouse line resulted in embryos which again mostly died 13.5 days post fertilisation, while in this case those which survived to birth displayed respiratory distress due to lowered lung surfactant production (Compernolle *et al.*, 2002).

Epas1 mRNA was first detected in vascular endothelial and highly vascularised tissues, has a more restricted expression profile compared to HIF-1α, and is often only expressed within subsets of cells in a tissue (Wiesener *et al.*, 2003). *Epas1* is expressed at a high level in the developing heart, placenta, organ of Zuckerkandl, adrenal glands, carotid body, regions of the brain and neonatal lungs (Ema *et al.*, 1997; Tian *et al.*, 1998; Tian *et al.*, 1997; Trollmann *et al.*, 2008). As the organ of Zuckerkandl, adrenal glands and carotid body all produce catecholamines, expression of *Epas1* in these areas may relate to the originally discovered catecholamine-deficiency phenotype of *Epas1*$^{-/-}$ mice, while the expression of *Epas1* in the heart, placenta and lungs is matched by developmental phenotypes in these organs in the subsequent knockout mouse studies as described above. The differences between the survival and phenotypes of *Epas1*$^{-/-}$ mice between different experiments are confounding, but appears to be related to differences in genetic backgrounds of the mice. Nonetheless, the presence of HIF-2α clearly has several

functions during development which align with its normal expression pattern, and HIF-2α is at least partially non-redundant with respect to the more ubiquitous HIF-1α.

As with *Hif1a*, Conditional *Epas1* knockout mice have been generated to examine roles for HIF-2 later in development. Deletion of *Epas1* only in VE-Cadherin-expressing endothelial cells results in blood vessels with increased permeability (Skuli *et al.*, 2009), which may be related to the vascular remodelling defects observed previously in *Epas1*$^{-/-}$ mice (Peng *et al.*, 2000). Gruber and colleagues generated mice with *Epas1* deletion in all cells after birth by tamoxifen-inducible Cre recombinase, which caused anaemia and loss of stress-induced erythropoiesis in the immature and adult mice (Gruber *et al.*, 2007). Interestingly, the previously mentioned HIF target gene *Epo* was found to be unresponsive to erythropoietic signals resulting from haemolysis in the *Epas1*$^{-/-}$ mice, whereas *Epo* response was not adversely affected by loss of *Hif1a*. This, along with further studies with *Epas1* deletion in either mouse hepatocytes (Rankin *et al.*, 2007) or astrocytes (Weidemann *et al.*, 2009), indicates that adaptive erythropoiesis due to induction of the *Epo* gene is predominantly regulated by HIF-2 *in vivo*, despite responding to either HIF homolog *in vitro*. In addition, deletion of *Epas1* in the duodenum indicates that HIF-2 but not HIF-1 is involved in regulation of iron absorption through activation of genes such as *DMT1-1A* (Mastrogiannaki *et al.*, 2009). In support, cases of familial erythrocytosis in humans have been genetically linked to dysfunction of the HIF pathway, and in particular HIF-2α. Two separate loss-of-function mutations at the gene encoding HIF-α-regulator PHD2 have been detected which are linked to this disease (Al-Sheikh *et al.*, 2008; Percy *et al.*, 2006), while in other families with the disease at least five different polymorphisms have been found within the *Epas1* gene (Percy, 2008; Percy *et al.*, 2008a; Percy *et al.*, 2008b; van Wijk *et al.*, 2010). These mutations have either been shown to or are expected to give rise to gain-of-function HIF-2α protein by disrupting interaction with negative regulators such as PHD2. This provides supporting evidence to the controlled transgenic mouse experiments in describing the central role of HIF-2α in regulating erythropoiesis, as well as the supply of nutrients involved in erythropoiesis.

Transgenic mouse models have recently connected aberrant HIF-2 function to the onset of osteoarthritis. Mice which overexpress *Epas1* in specifically in chondrocytes exhibit spontaneous cartilage destruction, while *Epas1*$^{+/-}$ mice were resistant to cartilage insult from collagenase treatment or destabilisation of the medial meniscus (Yang *et al.*, 2010).

In support, proinflammatory cytokines potently upregulate HIF-2α at both the protein and message level in mouse articular chondrocytes (Yang *et al.*, 2010).

Finally, transgenic mice have been generated in order to address the issue of redundancy between HIF-1α and HIF-2α. Since HIF-1α is expressed nearly ubiquitously while HIF-2α has a more restricted expression pattern, it is likely that the phenotypes observed in *Hif1a*$^{-/-}$ mice are not compensated for by HIF-2 due in part to the lack of expression of *Epas1* in some cell types. An allele of *Hif1a* was constructed with the *Epas1* gene inserted in place of the *Hif1a* coding sequence (*Hif1a*^{Epas1KI}) (Covello *et al.*, 2006). A mouse heterozygous for this allele expresses HIF-2α in place of HIF-1α, in an effective *Hif1a*$^{-/-}$ background. This means that any compensation for the loss of HIF-1α by HIF-2α will result in a *Hif1a*$^{Epas1KI/Epas1KI}$ mouse phenotype which is less severe than the *Hif1a*$^{-/-}$ phenotype. However, *Hif1a*$^{Epas1KI/Epas1KI}$ embryos were detected in a significantly smaller numbers than expected 7 days post-fertilisation (Covello *et al.*, 2006), compared to *Hif1a*$^{-/-}$ embryos which show normal percentages of homozygotes until 9 days post-fertilisation (Iyer *et al.*, 1998). Further experiments on this knock-in line show that ectopic HIF-2α expression has a dominant effect which results in a more severe phenotype than the *Hif1a*$^{-/-}$ background alone would be expected to produce. Therefore, HIF-2α must have functions which are not shared by HIF-1α. The converse experiment of a *Hif1a* knock-in at the *Epas1* locus has not been published, as HIF-1α is ubiquitous. This means that phenotypes observed in *Epas1*$^{-/-}$ mice already indicate functions of HIF-2α which are not compensated for by HIF-1α.

1.1.8. Effects of the HIF Pathway on Cancer

As potent regulators of angiogenic, metabolic and apoptotic pathways, HIF-1 and HIF-2 are increasingly being studied with relation to their oncogenic properties. Dysregulation of the HIF pathway can drive cancer progression as a result of accumulated inherited or somatic mutations, or from the environmental hypoxia found within a solid tumour.

The von Hippel-Lindau (VHL) disease is a well characterised familial cancer caused by various mutations of the *Vhl* gene, which encodes the E3 ubiquitin ligase pVHL. Increased susceptibility to kidney and other forms of cancer are dominantly transmitted in families with *Vhl* loss of function mutations, resulting in highly vascularised solid tumours (Kaelin and Maher, 1998). The previously described model of HIF-α normoxic degradation

through the pVHL mediated ubiquitin-proteasome pathway was originally discovered when *Vhl*$^{-/-}$ renal carcinoma cells were observed to have constitutive HIF activity (Cockman *et al.*, 2000; Krieg *et al.*, 2000; Maxwell *et al.*, 1999). VHL patients are heterozygous for functional *Vhl* and are therefore susceptible to somatic genetic loss of VHL protein. This results in constitutive activation of the HIF pathway and rapid development of tumours such as renal cell carcinoma (Kondo *et al.*, 2002; Maranchie *et al.*, 2002). Interestingly, it appears that HIF-2α is a more potent oncogene than HIF-1α when constitutively activated in cultured cells of this type (Kondo *et al.*, 2002; Maranchie *et al.*, 2002). However, it is interesting that patients heterozygous for *Vhl* are only susceptible to specific forms of cancer, rather than all types of cancer.

The oncogenic properties of the HIF pathway have been tested in other broader contexts, and show that these effects of aberrant HIF activity are not confined to the VHL disease state. Maxwell and colleagues employed a technique using xenografts of hepatoma cells, including experiments where the cells contained mutant HIF-1β. Using this model, tumour angiogenesis and overall growth of solid tumours were observed to be dependent on HIF *in vivo* (Maxwell *et al.*, 1997). In support of this role for HIF in cancer, increased presence of nuclear HIF-1α and HIF-2α has been detected by immunohistochemistry of primary and metastatic tumour sections of widely varying origins, relative to normal tissue (Talks *et al.*, 2000; Zhong *et al.*, 1999).

Further insights into the specific functions of HIF-1α and HIF-2α in tumour development can be gained from transgenic embryonic stem (ES) cell models, which form solid teratocarcinoma tumours when injected into nude mice. *Hif1a*$^{-/-}$ ES cells form significantly smaller teratocarcinomas with fewer blood vessels compared to wildtype ES tumours (Ryan *et al.*, 1998). Similarly, injecting *Hif1a*$^{-/-}$ mouse embryonic fibroblast (mEF) cells results in the formation of smaller fibrosarcomas than wildtype mEFs (Ryan *et al.*, 2000). The fibrosarcoma *Hif1a*$^{-/-}$ model exhibits lowered expression of *Vegf*, yet unlike the teratocarcinoma model no difference in blood vessel density was observed. In another set of experiments designed to study breast cancer development and metastasis, Liao and colleagues generated mice with a mammary epithelial conditional knockout *Hif1a* locus in a breast-cancer susceptible genetic background (Liao *et al.*, 2007). In accordance with the other models, tumour growth was slowed by the lack of HIF-1, and HIF target genes were expressed at lower levels than for the control. In this case, however, metastatic growths in the lungs were also quantified, showing significantly fewer resulting from the *Hif1a*$^{-/-}$

tumours, demonstrating that HIF-1 can also promote metastasis. These experiments imply that HIF-1 dependent progression of cancer occurs through multiple downstream pathways, and the effect of each is context dependent.

However, HIF-2 is increasingly thought to provide an even more potent stimulus than HIF-1 for advancement of cancer. Teratocarcinomas formed from mouse ES cells with replacement of the *Hif1a* gene with *Epas1* (*Hif1a*$^{Epas1KI/Epas1KI}$) form faster growing, more vascularised tumours with higher expression of target genes such as *Vegf*, when compared to teratocarcinomas formed from wildtype ES cells (Covello *et al.*, 2005). High expression of HIF-2α in clinical neuroblastoma cases correlates with a poor prognosis (Holmquist-Mengelbier *et al.*, 2006), while aberrant expression of HIF-2α results in high expression of progenitor markers and low expression of markers of differentiation (Pietras *et al.*, 2008; Pietras *et al.*, 2009). As HIF-2 has separately been found to bind and activate transcription of *Pou5f1*, which encodes the pluripotency marker Oct-4 (Covello *et al.*, 2006), it seems that activity of HIF-2 is linked to increased pluripotency, a trait which allows for increased tumour adaptivity. Curiously, in the context of neuroblastomas, HIF-1α expression is correlated with a positive outcome (Noguera *et al.*, 2009). In addition, forced expression of stabilised HIF-2α in a mouse strain susceptible to lung tumours results in larger, more invasive tumours than in control mice (Kim *et al.*, 2009). Furthermore, conditional endothelial *Epas1* knockout mice display reduced vascularisation of lung carcinoma xenografts compared to wildtype mice (Skuli *et al.*, 2009), highlighting an important HIF-2 function in not only the tumour itself, but also the supporting endothelial cells.

1.1.9. Summary and Preliminary Approach

Clearly, the HIF pathway is more than a simple hypoxia response pathway, as it is under the control of a complex regulation system which allows for differential activation of different HIF-α subunits or even different domains within each HIF-α protein, depending on the type of activating stimulus and cellular context. In particular, many facets of the regulation of HIF are highly dependent on the experimental cell type used, as described in *Chapters 1.1.4*, *1.1.5* and *1.1.6*. Therefore, we reasoned that selection of an appropriate set of cell lines and conditions was critical for continuing research in the HIF field, given that the contrasting functions of this pathway are most pronounced when comparing different cell types.

There are aspects of the HIF transactivational pathway which are not explained by its currently known components, in particular when comparing HIF-1 function to HIF-2. In transgenic mouse models HIF-1α and HIF-2α are non-redundant, and while the two proteins are regulated in a similar way, differences in regulation and target gene specificity have been observed. Most of the research published in the field to date has focussed on HIF-1, while the specifics of HIF-2 function are still poorly understood. Therefore, in order to further study the differences between the two HIF-α subunits, two approaches are possible. On one hand, different regulation and activation conditions can be studied, with the aim of elucidating differences in pathways upstream of HIF activation. Alternatively, the different downstream effects of HIF-1 and HIF-2 activity can be examined directly by testing for changes in target genes. We decided to use the second approach, as at the time this project commenced HIF-2α had been poorly studied and consequently relatively few target genes were known for HIF-2, and none of these displayed preference for HIF-2 over HIF-1. Therefore, we postulated that there were more HIF-2 responsive genes which had not yet been discovered, which may be preferred or even specific for HIF-2 activity with respect to HIF-1. The identification and characterisation of unknown HIF-2 target genes would help to explain with more clarity the physiological function of HIF-2, with particular reference to the multiple phenotypes of *Epas1*$^{-/-}$ mice. Novel target genes may also help to shed light on the mechanisms of HIF binding site selection, or be used as a readout when studying regulation of the two HIF forms. As such, the long term aim of this research was to identify novel HIF-2 target genes, and this Ph.D project was a continuation of that research.

Preliminary Experiments

1.2.1. Novel HIF Target Genes

For the reasons outlined in the previous chapter, a microarray experiment was designed and performed by Anthony Fedele as part of his Ph.D project to search for novel HIF target genes (Fedele, 2004). At the time, few microarray experiments had been published concerning the HIF pathway, but since this time a number of microarray experiments have been used to search for HIF responsive genes. These published microarrays used conditions of hypoxia alone (Aravindan *et al.*, 2005; Burke *et al.*, 2003; Elvidge *et al.*, 2006; Greijer *et al.*, 2005; Jin *et al.*, 2002; Jogi *et al.*, 2004; Leonard *et al.*, 2003; Mense *et*

al., 2006; Robins *et al.*, 2005; Warabi *et al.*, 2004; Zhang and Hill, 2004), overexpression of HIF-1α or HIF-2α (Takeda *et al.*, 2004; Wang *et al.*, 2005), and knock-down of HIF-1α or HIF-2α (Aprelikova *et al.*, 2006; Hu *et al.*, 2003), and used cell types derived from the kidney, endothelium, breast and cervical cancers. However, the function of the HIF pathway can be altered by cellular context, therefore these multiple microarray experiments are not redundant, and alternative target genes of HIF may still be found which are silenced in certain cell types. Given the known defects of the *Epas1*$^{-/-}$ mouse models in catecholamine synthesis (Tian *et al.*, 1998), we decided to use a cell line which can synthesise and release catecholamines. This was hoped to make the microarray data relate to possible HIF-2 functions, such that HIF targets could be found which may not be hypoxia-regulated in other cell types. At this time, erythropoiesis was thought to be regulated by either HIF form, rather than HIF-2 preferentially (as discussed in *Chapter 1.1.7*), so erythrocyte-related cell lines were not considered. Our group used the PC-12 rat pheochromocytoma cell line, as it has a well characterised ability to produce catecholamines in response to hypoxia and expresses relatively high, inducible levels of HIF-2α (Kumar *et al.*, 1998; Taylor and Peers, 1998).

1.2.2. The PC12/TetON System

To identify the genes regulated by HIF-1 or HIF-2 individually, a Tet-ON doxycycline-inducible expression system was incorporated into the PC12 cells by sequential stable transfections. The Tet-ON system consists of one expression cassette which provides constitutive expression of the Reverse Tetracycline Transactivator (rtTA), while a second provides the coding sequence for protein to be overexpressed downstream of a Tetracycline Responsive Element (TRE). In the presence of tetracycline, or its derivative doxycycline, rtTA is able to bind at the TRE and activate transcription. Three monoclonal cell lines were generated using this system, differing only in the coding sequence under the control of the TRE: one encoding mouse HIF-2α; one encoding human HIF-1α with an activating N803A mutation; and one control PC12 TetON cell line with an empty expression cassette. Transcription of the HIF-α cDNA leads to stabilised protein at normoxia by overloading the regulatory hydroxylases, while the N803A mutation was required to achieve robust HIF-1 activity by preventing FIH-mediated asparagine hydroxylation of the HIF-1α, leading to activation in normoxia. The differences in species and incorporated mutations of HIF-1α and HIF-2α in the Tet-ON cell lines are a result of

optimisation for maximum doxycycline-responsive HIF activity. Microarrays were then performed comparing cDNA from doxycycline-treated TetON-HIF-2α and TetON-HIF-1α^{N803A} cells (Fedele, 2004). Cell lines were treated with doxycycline for 16 hours before lysis in order to match previous data indicating that PC-12 cells express both endogenous HIF-1α and HIF-2α this timepoint (Bracken *et al.*, 2006). The data from the first microarray were not reproducible by replicate semi-quantitative PCR experiments.

1.2.3. Verification of Microarray Data

After the inconsistent results of the first experiment, a subsequent microarray was performed to identify HIF-2 targets alone, comparing cDNA from doxycycline-treated TetON-HIF-2α cells to the TetON-Control cell line. Verification of the results of this second microarray was performed as part of my research towards my Honours thesis. The twelve genes most consistently upregulated by HIF-2α overexpression were selected for further analysis (*Table 1.1*). Two of these genes were previously characterised HIF targets: *Vegf* and *Bnip3*. These twelve genes were retested by northern blot for response to HIF-α in the TetON overexpression PC12 cell lines, and also for response to hypoxia in unmodified PC12 cells. *Vegf* and *Bnip3* were consistently upregulated by HIF-1α, HIF-2α and hypoxia, although the effect of HIF-1α was greater than that of HIF-2α. Other genes such as *INrf2*, *Spr*, *Scn3b* and *Cacna2d1* were not reproducibly regulated by either HIF-1α or HIF-2α overexpression, and were therefore likely false positives. However, two genes not previously known as HIF targets were consistently detected by northern blot as a higher level in both hypoxic and HIF-α overexpressing PC12 cells: *Slc16a1* and *Rgs4* (Olechnowicz, 2005).

Gene ID	Gene Name	Fold Change		P.Value	B
AF304364	**INrf2**; cytosolic inhibitor of Nrf2	4.30	Up	6.98E-05	8.42
AJ277828	**HIF-2a**; Hypoxia Inducible Factor 2a, Rattus norvegicus	8.04	Up	8.33E-04	6.74
U44845	**Vitronectin**; S-protein; epibolin; adhesion protein.	3.23	Up	8.33E-04	6.64
NM_017214	**Rgs4**; Regulator of G-protein Signaling 4	2.12	Up	2.60E-03	5.36
M36410	**Sepiapterin Reductase**	1.80	Up	6.01E-03	4.61
L03556	**Hox1.3** protein 3' end (clone RAHB2 8/10)	4.04	Up	1.09E-02	3.72
AF243515	**Bnip3**; BCL2/adenovirus E1B 19 kDa-interacting protein 3	1.69	Up	1.10E-02	3.64
M31178	**Calbindin D28**	1.51	Up	1.80E-02	3.04
AF215726	**VEGF**; vascular endothelial growth factor-A120 (alternatively spliced)	1.97	Up	2.07E-02	2.77
NM_012716	**Slc16a1**; Solute carrier 16 member 1; MCT1	1.61	Up	2.51E-02	2.42
U04933	**NACA7**; Sprague-Dawley (CD-1) Na-Ca exchanger isoform NACA7	1.59	Up	2.55E-02	2.21
AJ243395	**Na+b3**; Voltage-gated sodium channel b3 subunit	1.62	Up	4.08E-02	1.18
AF286488	**Ca+a2d1**; Voltage-gated calcium channel a2/d-1 subunit	1.55	Up	4.08E-02	1.15

Table 1.1. The twelve most consistently upregulated genes in the doxycycline treated PC12-TetON-HIF2α cell line, compared to the TetON control line (Fedele, 2004). Genes are ranked by *B* value, which depends on both the fold change and *P* value. Note the presence of *HIF-2a* is an artefact of the cDNA-mediated HIF-2α overexpression used in this cell line, and *Bnip3* and *Vegf* are previously characterised HIF target genes. The response of these genes to doxycycline and hypoxia was verified by northern blot (Adapted from Olechnowicz, 2005).

Slc16a1 encodes a solute carrier which transports monocarboxylic acids across the plasma membrane. Over three independent northern blot experiments, *Slc16a1* mRNA was detected at 3.1 ±0.7 fold higher levels in 16-hour hypoxia treated PC12 cells than in normoxia (Standard Error of the Mean given for *n*=2). The response to doxycycline treatment of the TetON-HIF-1α, TetON-HIF-2α and TetON-Control cell lines was consistent with HIF dependent regulation, but less dramatic changes of 2.5 ±1.7, 1.6 ±0.4 and 0.7 ±0.5 respectively (with Standard Deviation for *n*=3) were observed. Therefore, while *Slc16a1* may represent a novel hypoxia regulated gene, the response to overexpression was only subtle, and stronger for HIF-1α than HIF-2α. Many HIF-1α target genes are already well characterised, while novel HIF-2α target genes are more rare, so this gene was deemed to be a low priority for further testing.

Rgs4 encodes Regulator of G-Protein Signalling 4 (RGS4), one of a family of proteins which contain an RGS domain. *Rgs4* was upregulated by hypoxia (2.8 ±1.3 fold, SEM for *n*=2) and HIF-2α overexpression (2.8 ±1.3 fold, SD for *n*=3), but was unresponsive to HIF-1α overexpression (1.2 ±0.1, SD for *n*=3) or the TetON control (1.2 ±0.2, SD for *n*=3) (*Figure 1.4*). Although the regulation of *Rgs4* observed was moderate, the apparent preference for HIF-2α over HIF-1α in regulation made this putative target gene worthy of further investigation. Furthermore, the known role of the *Rgs4* gene product in modulating G-protein function provided a putative link of considerable interest between hypoxic signalling and G-Protein coupled receptor signalling. Therefore, this research for this thesis focuses on the hypothesis that transcription of *Rgs4* is regulated by hypoxia through the HIF pathway.

Regulator of G-Protein Signalling 4

1.3.1. G-Protein Coupled Receptors and the RGS Family of Proteins

G-Protein Coupled Receptors (GPCRs) are seven transmembrane domain proteins which are found at the plasma membrane in eukaryotes, with an extracellular ligand receptor domain and a cytosolic G-protein interaction domain. In its inactive state, a GDP-bound $G_{\alpha\beta\gamma}$ (G-protein) trimer binds at the cytosolic domains of the GPCR. Once activated by

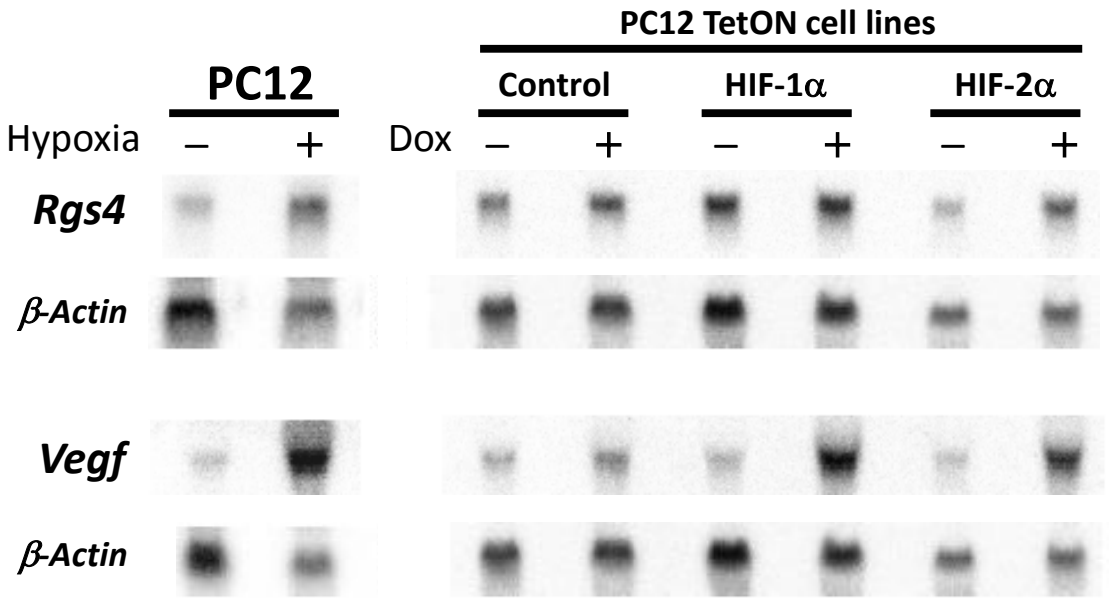


Figure 1.4. Representative northern blots of unmodified PC12 cells with or without 16 hours of hypoxic treatment, and the TetON-Control, TetON-HIF1α and TetON-HIF-2α PC12 cell lines with or without 16 hours of doxycycline treatment (adapted from Olechnowicz, 2005). Total RNA was extracted and separated by 1% agarose/formamide/MOPS gel electrophoresis, northern blotted, then probed with ^{32}P-labelled *Rgs4*, *Vegf* (positive control) or *β–actin* (loading control) coding sequence clones. Blots are representative of either three (*Rgs4* with doxycycline treatments), two (*Rgs4* with hypoxia, *Vegf* with doxycycline) or one (*Vegf* with hypoxia) independent experiments (Adapted from Olechnowicz, 2005).

ligand binding, the G_α subunit exchanges GDP for GTP, and the G-protein trimer dissociates to a GTP-bound G_α monomer and a $G_{\beta\gamma}$ dimer. These subunits then move away from the receptor to signal to secondary effectors. G_α has intrinsic GTPase activity, and will eventually hydrolyse its bound GTP to GDP. At this point, the G_α subunit will recondense with the $G_{\beta\gamma}$ dimer at the GPCR to await reactivation by the receptor. RGS family proteins contain a RGS domain, which binds to GTP-bound G_α and increases the rate of GTP hydrolysis, resulting in reduced signal transduction (Berman *et al.*, 1996b; Watson *et al.*, 1996). This interaction occurs at the switch regions of G_α, rather than the GTPase catalysis site (Tesmer *et al.*, 1997). A bound RGS domain therefore lowers the transition state energy of GTP hydrolysis allosterically, rather than contributing directly to the reaction. This role of the RGS domain can itself be allosterically inhibited by phosphatidylinositol 3,4,5-trisphosphate (PIP_3), an effect which is relieved by Ca^{2+}-bound-Calmodulin binding (reviewed by Ishii and Kurachi, 2003). RGS4 is also subject to N-terminal palmitoylation, which inhibits its effect as a GTPase activator (Tu *et al.*, 1999).

The family of proteins which contain Regulator of G-Protein Signalling (RGS) domains includes the products from at least 37 different genes (Siderovski and Willard, 2005). These proteins are classed by homology of RGS domains and presence of other domains into 8 subfamilies: RZ, R4, R7, R12, RA, GRK, GEF and SNX. While many of these families include proteins which also contain other recognisable domains, the R4 sub-family comprises relatively short proteins with no known domains other than the RGS domain (reviewed by Willars, 2006). *Rgs4* encodes RGS4, which was amongst the first of the RGS proteins to be discovered (Druey *et al.*, 1996) and encodes the prototypical member of the R4 subfamily. There are numerous forms of RGS4 (see *Chapter 1.3.2*), but the most prominent form of RGS4 has 205 amino acids, of which 120 form the RGS domain (Popov *et al.*, 1997). Other R4 subfamily members such as RGS5 and RGS16 are of a similar size and composition.

1.3.2. Expression Pattern of RGS4

Rgs4 mRNA is detected at high levels in adult brain and heart in rats and mice, with lower expression detectable in lung, liver and skeletal muscle (Nomoto *et al.*, 1997; Zhang *et al.*, 1998). However, the specific regions of *Rgs4* expression within the brain are less clear, as *in-situ* hybridisation and qPCR studies of the developing or adult brain often give conflicting results (Erdely *et al.*, 2004; Gold *et al.*, 1997; Grillet *et al.*, 2003; Grillet *et al.*,

2005; Ingi and Aoki, 2002; Larminie *et al.*, 2004). The cause of these differences is unclear, but nonetheless it appears that *Rgs4* expression within the brain is very specific between regions and possibly dynamic. Notably, expression of *Rgs4* overlaps to some extent with expression of other R4 subfamily members. *Rgs5* is strongly expressed in the heart, vasculature and skeletal muscle (Seki *et al.*, 1998), but is restricted in the brain to regions such as the amygdala and hypothalamus (Gold *et al.*, 1997). *Rgs16* also has a specific expression pattern within the brain, highest in the thalamus (Grafstein-Dunn *et al.*, 2001).

Human *Rgs4* produces at least 5 different transcripts through either alternative splicing or alternative promoters, of which two encode the previously mentioned 205 amino acid (aa) protein, one encodes a 302aa protein (extended at the N-terminus) and two produce truncated RGS4 forms (187aa and 93aa) (Ding *et al.*, 2007). The relative expression of each of these variants *in vivo* is not clear, although the transcript designated as *Rgs4-1* by Ding *et al.* (2007) is identical to the originally discovered 205aa form. Different variants of mouse *Rgs4* transcript all give rise to the same open reading frame, which is equivalent to the coding sequence of human splice variant *Rgs4-1* (Ding *et al.*, 2007), implying that this is the prototypical *Rgs4* form.

1.3.3. Regulation of RGS Genes and Proteins by Hypoxia

Regulation of *Rgs4* and its protein product by hypoxia is not unprecedented in the literature. *Rgs4* has appeared in the results of one published hypoxia-related microarray. Treatment of SK-N-BE(2)C human neuroblastoma cells with hypoxia for 72 hours led to increased hybridisation to a transcript listed as *FLJ38885* by between 5 and 8.2 fold (Jogi *et al.*, 2004). Upon further investigation, this sequence aligns as a partially-processed form of *Rgs4* mRNA, which is likely to also detect fully processed *Rgs4* transcript. *Rgs5* was also consistently detected at 2.2 to 2.6 fold increased levels. However, the authors did not verify these positive responses with further experiments (Jogi *et al.*, 2004). The other published microarray experiments described earlier (*Chapter 1.2.1*) used either arrays which did not feature an *Rgs4* probe, or reported no significant change in *Rgs4* levels to either hypoxia or change in HIF-α expression. As these studies used cell types which are not known to express *Rgs4*, it is likely that the hypoxic response of *Rgs4* is restricted to

cell types which express basal *Rgs4* before treatment, such as neuroblastoma or pheochromocytoma cells. It is interesting to note that other bHLH/PAS family members have also been linked to regulation of *Rgs4* expression levels. *Rgs4* transcript levels are decreased by 3 fold in the anterior hypothalamus of *Sim1*$^{-/-}$ embryonic mice (Caqueret *et al.*, 2006), and also decreased in cultured mouse hippocampal neurons after RNAi-mediated knockdown of *Npas4* (Lin *et al.*, 2008). As the DNA binding specificities of HIF, SIM-1 and NPAS4 are similar, it is possible that these is some crosstalk between these factors at an enhancer which regulates *Rgs4* expression.

It has been reported that exposing Human Umbilical Vein Endothelial Cells (HUVECs) to hypoxia causes a rapid yet modest induction of *Rgs5* message, which encodes an R4 subfamily member closely related to *Rgs4* (Jin *et al.*, 2009). Levels remain increased for at least 12 hours of treatment before returning to control levels by 24 hours. Curiously, while this report showed western blot data indicating similarly increased levels of RGS5 protein, no change in RGS4 protein level was detected (Jin *et al.*, 2009). Although no experimental test for *Rgs4* message levels was presented, this indicates that the response of *Rgs4* to hypoxia may be replaced in some contexts by other RGS family members. It is interesting to note that *Rgs4* and *Rgs5* are adjacent in the human genome, separated by about 50kb of intervening sequence, and as such may feasibly share some transcriptional enhancers.

At the posttranslational level of control, protein stability of RGS4, RGS5 and RGS16 is regulated by the N-end rule pathway (Davydov and Varshavsky, 2000; Lee *et al.*, 2005), where the N-terminal residue of a protein affects its degradation. N-terminal residues other than Met, Gly, Val, Ile or Pro signal for protein degradation through the ubiquitin-proteasome pathway (Gonda *et al.*, 1989). These residues can be exposed by proteolysis or activity of methionine aminopeptidases (MetAPs), which remove the N-terminal methionine of a target protein (Davydov and Varshavsky, 2000). MetAP acts on RGS4 to reveal its second amino acid, Cys. This residue is oxidised, leading to recognition by ATE1, an enzyme which ligates an arginine to the N-terminus of RGS4 (Lee *et al.*, 2005). This arginine is then recognised directly by E3 ubiquitin ligases UBR1 and UBR2 resulting in ubiquitylation of RGS4 and subsequent proteasomal degradation (Lee *et al.*, 2005). Curiously, the cysteine oxidation step is sensitive to hypoxia, such that RGS4 (Hu *et al.*, 2005) and RGS5 (Lee *et al.*, 2005) exhibit hypoxic protein stabilisation and accumulation. It is unknown whether there is a hypoxia-sensitive enzyme responsible for this oxidation step, in a role similar to that of FIH and the PHD enzymes in the HIF

pathway, or if cysteine oxidation occurs spontaneously in the presence of oxygen. The phenotypes of mice defective for ATE1, UBR1 and UBR2 imply that the N-end rule pathway has physiological roles in neural development (An *et al.*, 2006), cardiovascular development and remodelling (An *et al.*, 2006; Kwon *et al.*, 2002), pancreas function (Zenker *et al.*, 2005) and meiosis in spermatocytes (Kwon *et al.*, 2003). It is unclear whether the pathway is in effect throughout the body or only in a subset of tissues as there are other poorly characterised components (Tasaki *et al.*, 2005). However, while the effect of hypoxia on RGS4 has been characterised in terms of the N-end rule pathway, no experiments were presented in these papers addressing *Rgs4* mRNA levels. Altered *Rgs4* mRNA may augment the effect of N-end rule mediated protein stability, or may produce a similar hypoxia-responsive outcome in cell types which lack the N-end rule pathway components. Furthermore, as we theorise that the N-end rule pathway and the now the HIF pathway are both involved in a hypoxic increase of RGS4, it is interesting to note that the impaired vascular remodelling phenotype of *Ate1*$^{-/-}$ (Kwon *et al.*, 2002) and *Ubr1*$^{-/-}$*Ubr2*$^{-/-}$ (An *et al.*, 2006) mice overlaps with the phenotype of *Epas1*$^{-/-}$ mice (Peng *et al.*, 2000).

1.3.4. G_α-selectivity of RGS4 Function

There are at least sixteen known genes which encode G_α subunits, which can be divided into four subfamilies by sequence and function: α_s; α_{12}; α_i; and α_q (reviewed by Hildebrandt, 1997; Neves *et al.*, 2002; and Simon *et al.*, 1991). There are also multiple different G_β and G_γ subunits, and alternative splicing occurs on some subunits, so defining roles for a specific G_α is complicated. RGS4 exhibits some specificity in G_α selection, determined by both biochemical interaction and functional assays. Signalling through α_i subfamily members α_{i1}, α_{i2}, α_{i3}, α_o and α_z (Berman *et al.*, 1996b), as well as α_t and α_q (Berman *et al.*, 1996a; Hepler *et al.*, 1997) is dampened by RGS4 presence, while RGS4 can be immunoprecipitated with members of the α_i subfamily (Watson *et al.*, 1996). However, RGS4 exerts no effect on α_s (Berman *et al.*, 1996b) or α_{12} (Berman *et al.*, 1996a). Other RGS proteins also display similar selectivity between subfamilies of G_α (reviewed by De Vries *et al.*, 2000). The secondary effectors for members of different G_α subfamilies are diverse: α_s subunits activate adenylyl cyclase, while α_i subunits generally inhibit adenylyl cyclase. Signalling by α_q triggers production of inositol triphosphate by Phospholipase C, while effectors for the α_{12} are still unclear. Also, some specific ion channels can be regulated through the effect of the partner $G_{\beta\gamma}$ dimer (reviewed by Neves

et al., 2002). Novel secondary effectors are still being discovered for each of these G proteins, and each subfamily of G-proteins can be used by a variety of different GPCRs, so the scope for effects of RGS4 function is wide. In this way, the molecular basis for RGS4-regulated physiological roles is poorly understood.

1.3.5. RGS4 Function in the Brain

In keeping with its observed expression pattern, many proposed functions of RGS4 relate to the brain, as described on the right-hand side of *Figure 1.5*. In particular, alterations at the *Rgs4* gene locus are thought to be a contributing factor for schizophrenia, a psychosis which has a heritability component of up to 80% (Merikangas and Risch, 2003). This link was first established by microarray analysis of matched schizophrenic and control postmortem prefrontal cortices (Mirnics *et al.*, 2001), an area which becomes dysfunctional in affected individuals (Goldman-Rakic and Selemon, 1997). Mirnics and colleagues (2001) report that out of 6 matched microarray studies, expression of only one gene was found to be significantly altered in all experiments: *Rgs4*, which exhibited increased expression in affected individuals. In support, the human *Rgs4* locus (at 1q23) is located near to a previously discovered genomic region with strong linkage to familial schizophrenia at 1q21-22 (Brzustowicz *et al.*, 2000). Since then, multiple studies have included *Rgs4*-linked single nucleotide polymorphisms to determine whether this locus is associated with schizophrenia susceptibility. The Schizophrenia Research Forum collate these results, showing that of 31 studies, 15 present data supporting a link between the *Rgs4* locus and schizophrenia, while 18 present negative results (Heimer, 2010). This indicates that while polymorphisms of the *Rgs4* locus may be related to heritable schizophrenia in some cases, the disease is also likely to involve other loci as well as environmental factors, such that *Rgs4* represents one of many possible susceptibility loci. Indeed, there are other susceptibility genes known, such as *Nrg1*, *Comt* and *Notch4* (reviewed by Schmidt-Kastner *et al.*, 2006).

One of the several theories for the molecular cause of schizophrenia is the dopamine hypothesis, which states that excess dopamine signalling in the central nervous system is a major factor in the disease (reviewed by Murray *et al.*, 2008). This theory originates from the eventual discovery that antipsychotic drugs such as haloperidol used in the treatment of schizophrenia target the D_2 dopamine receptor (D_2R), one of a family of dopamine receptors which either signal for activation (D_1-like) or inhibition (D_2-like) of adenylyl

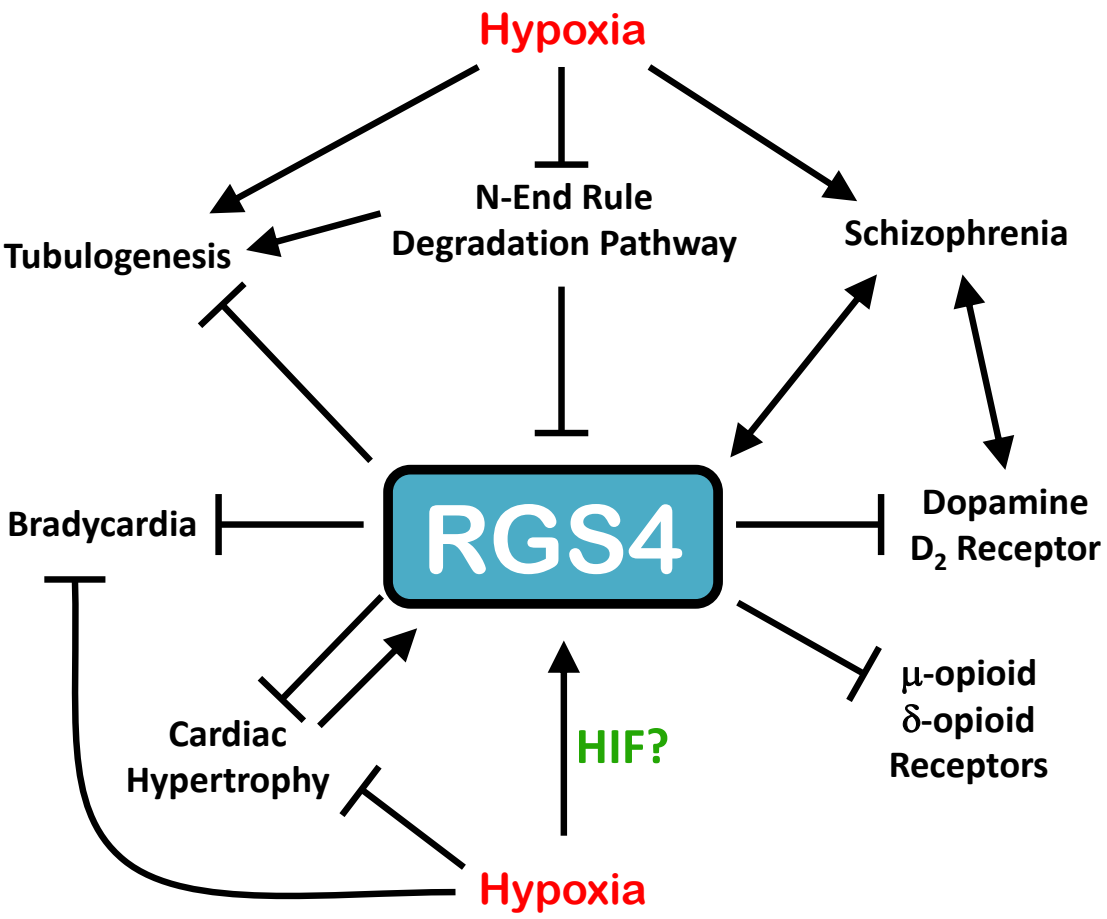


Figure 1.5. Flowchart of possible functions of RGS4 in the cardiovascular (left) and nervous (right) systems. Also pictured are the influences of hypoxia on these systems, and on the regulation of RGS4, in many cases through the HIF transcriptional pathway. The connector labelled "HIF?" question mark denotes the novel mechanism of RGS4 regulation by the HIF pathway under investigation in this thesis, which may describe the molecular mechanism behind some of the effects of hypoxia shown.

cyclase (reviewed by Seeman, 2006). Interestingly, as the D_2R is a $G_{\alpha i}$-linked GPCR, RGS4 can inhibit its downstream signalling. RGS4 has been shown to directly promote the GTPase activity of $G_{\alpha i}$ after activation by the D_2R, causing a decrease in not only $G_{\alpha i}$ but also $G_{\beta\gamma}$ signalling (Ho *et al.*, 2007; Yan *et al.*, 1997). In this way, RGS4 regulates a signalling system thought to be central to the molecular pathogenesis of schizophrenia. Furthermore, there is some suggestion of a link between obstetric complications involving hypoxia and schizophrenic risk later in life (Cannon *et al.*, 2000).

RGS4 can negatively regulate $G_{\alpha i}$-mediated signalling from the μ-opioid (morphine) and/or δ-opioid receptors depending on context (Garnier *et al.*, 2003; Leontiadis *et al.*, 2009; Wang *et al.*, 2009). As opiates such as morphine are used widely in the treatment of pain, it is intriguing that *Rgs4* mRNA is upregulated in rat spinal cord following neuropathic pain (Garnier *et al.*, 2003), possibly to act in a negative feedback loop on the opioid receptors. *Rgs4* message also increases in the locus coeruleus during morphine withdrawal (Gold *et al.*, 2003). *Rgs4*$^{-/-}$ mice have been generated which display only subtle phenotypes, as they develop without gross morphological abnormalities, possibly due to compensation by other RGS family members. Knockout mice are smaller than wildtype, fall from a rotating rod more quickly, and are more resistant to some pain treatments (Grillet *et al.*, 2005), while later studies indicate these mice have some disruption to metabolic homeostasis pathways (Iankova *et al.*, 2008). They display mostly normal responses to morphine withdrawal, the sole difference being the lack of a characteristic sniffing response (Grillet *et al.*, 2005). However, Grillet and colleagues note that this relates to the phenotype of *D$_2$R*$^{-/-}$ mice, where withdrawal symptoms are also identical to wildtype save for an enhanced sniffing response (Maldonado *et al.*, 1997). Furthermore, more detailed studies using conditional and total *Rgs4* knockout and knock-in mice indicate significant roles of *Rgs4* in different regions of the brain in dampening the effect of opiate signalling (Han *et al.*, 2010).

1.3.6. RGS4 Function in the Cardiovascular and Other Systems

Although expressed at a relatively lower level than in the brain, *Rgs4* is thought to also have roles in the heart and vascular system, as described on the left-hand side of *Figure 1.5*. Both *in vitro* and *in vivo* models of heart hypertrophy result in increased levels of

Rgs4 mRNA (Zhang *et al.*, 1998), while conversely, overexpression of *Rgs4* inhibits the hypertrophic response in cell culture models (Tamirisa *et al.*, 1999) and in vivo (Rogers *et al.*, 1999). Therefore, regulation of *Rgs4* transcription appears to give rise to a negative feedback loop. In support, increased expression of *Rgs4* has been shown to inhibit GPCR signalling from ligands such as endothelin and phenylephrine, which normally invoke hypertrophy in cultured cardiomyocytes (Schaub *et al.*, 1997; Snabaitis *et al.*, 2005; Tamirisa *et al.*, 1999). Additionally, endothelin signalling has also been shown to activate HIF through downregulation of *Phd2* expression in melanoma, so HIF may be included in this negative feedback loop (Spinella *et al.*, 2010). Heart hypertrophy has been described in *Epas1*$^{-/-}$ mice (Scortegagna *et al.*, 2003a), which may therefore be related to the proposed transcriptional activation of *Rgs4* by HIF, as lack of HIF-2 may allow hypertrophy to occur in an unregulated manner.

While no heart disorders were originally noted in *Rgs4*$^{-/-}$ mice (Grillet *et al.*, 2005), more detailed studies have since noted that knockout mice display an increased sensitivity to the muscarinic M_2 acetylcholine receptor (M_2R) agonist carbachol (Cifelli *et al.*, 2008). Activation of M_2R signalling in cardiomyocytes results in bradycardia, while RGS4 modulates M_2R signalling *in vitro* by inhibiting $G_{\beta\gamma}$-mediated activation of G-Protein-regulated Inward Rectifying K^+ channels (GIRK) (Doupnik *et al.*, 1997; Fujita *et al.*, 2000; Inanobe *et al.*, 2001). Once again, this may relate to the bradycardic phenotype observed in *Epas1*$^{-/-}$ mice (Tian *et al.*, 1998), as lack of HIF-2 may prevent upregulation of RGS4 resulting in excess bradycardic signalling.

There may also be a role for *Rgs4* in the development of tubules in the vascular and respiratory system. *In vitro* assays show increased *Rgs4* in MB114 endothelial and Mv1Lu lung epithelial cells during tubulogenesis, yet viral expression of *Rgs4* in the same cells inhibits tubulogenesis (Albig and Schiemann, 2005). This implies another regulatory role for RGS4 in a negative feedback loop. However, similar effects are observed for *Rgs5*, indicating that there may be some degree of redundancy between these RGS proteins in the regulation of vascular development (Albig and Schiemann, 2005; Manzur and Ganss, 2009). This would explain the lack of major cardiovascular developmental defects in *Rgs4*$^{-/-}$ mice (Grillet *et al.*, 2005). Overexpression of *Rgs4* inhibits signalling by VEGF in endothelial cells, despite this signal occurring through a receptor tyrosine kinase (Albig and Schiemann, 2005), while the HIF pathway is well characterised in its activation of *Vegf* transcription, so a connection between these pathways would be of interest.

Rgs4 may also have a role in cancer progression, as it is a negative regulator of tumour size, lamellipodia formation and subsequent cell motility in breast cancer (Xie *et al.*, 2009). This may be a cell type specific effect, as previous work has instead correlated *Rgs4* overexpression with a decrease in glioma cell motility (Tatenhorst *et al.*, 2004). As described previously (*Chapter 1.1.8*), the HIF pathway is also known to be highly involved in oncogenesis.

Summary and Approach

1.4.1. Summary

Mammalian cellular hypoxia sensing and response is a vital function for survival and adaptation of cells individually, as well as for development and adaptation of the entire organism, as the HIF pathway is strongly conserved through mammalian evolution, while disruption of this pathway causes severe deleterious phenotypes. Although previous research has identified numerous hypoxia responsive genes, it appears that the hypoxic response is not uniform between different cell types and contexts. Furthermore, current knowledge of HIF-responsive genes does not fully explain the range of phenotypes observed in HIF knockout mice. As such, we reasoned that there were more hypoxia responsive genes yet to be found, and that the careful selection of cell types used may be critical in their identification. In this way, novel genes may be found which are responsive to hypoxia in a subset of cell types, but do not respond in others and have therefore been overlooked in previous studies. Preliminary studies in our lab using microarray technology indicated that *Rgs4*, encoding RGS4 protein, may in fact be a novel HIF-responsive gene in the PC12 rat pheochromocytoma cell line.

Curiously, RGS4 has proposed roles in development and regulation of signalling in both the nervous and cardiovascular systems, as well as pathophysiological roles in psychological disorders, heart disease and cancer. Several of these processes have links to hypoxia, but molecular mechanisms have not yet been found. **The hypothesis investigated in this thesis is that hypoxic regulation of *Rgs4* is mediated by HIF. My aim is to elucidate the molecular mechanism of transcriptional regulation of *Rgs4* in response to hypoxia, and to define the contexts in which it occurs.** This interaction may then provide explanations for some of the uncharacterised effects of HIF pathway

disruption, and provide a more complete understanding of the physiological mammalian response to hypoxia.

1.4.2. Approach

To address this aim, I designed experiments around two related approaches. The first of these aimed to clarify the types of cells in which *Rgs4* is responsive to hypoxia, by testing for increased relative *Rgs4* mRNA levels in neural and endothelial related cell lines using qRT-PCR and northern blotting. In these experiments, hypoxia, hypoxia mimetics and siRNA knockdown were all used to implicate the HIF transcription factors in this regulation.

Secondly, I aimed to test whether *Rgs4* is regulated in a similar fashion to other known HIF-responsive genes. If this is the case, it would be expected that the HIF transcription factors bind at a genomic site proximal to the *Rgs4* transcription start site, known as a HRE. To eliminate other possibilities, the effect of transcription-independent regulation, 3'UTR mRNA elements and the hypoxia-response time were tested. Finally, enhancer reporter assays were used alongside bioinformatics to search for a putative direct HIF binding site at the human and rat genomic *Rgs4* loci.

2. Methods

2.1.1. Commercially Sourced Reagents

Standard solutions supplied by Technical Services Unit (TSU) unless otherwise described, and used at 1x concentration unless otherwise specified.

All water used is Milli-Q purified (Millipore).

All chemicals from Sigma-Aldrich, unless otherwise stated.

All restriction enzymes, related buffers and BSA from New England Biosciences.

1kb+ DNA marker: Invitrogen

^{32}P-dATP: Perkin Elmer

AnaeroGen sachets: Oxoid

Betaine: Sigma

Big Dye Terminator Ver.3: Amersham

DMEM, MEM and RPMI1640: Gibco

dNTPs: Finnzymes

Dual Luciferase Reporter Assay Kit: Promega

FCS: JRH

Gel Purification Kit: Qiagen

Horse Serum: Sigma

Klenow fragment: NEB

Lipofectamine2000: Invitrogen

Nytran Membrane: Schleicher and Schuell

Oligo(15)-dTs: Geneworks

pGEM-T Easy Kit: Promega

PfuTurbo: Stratagene

ProbeQuant G-50 Microcolumns: Amersham

Proteinase K: Roche

qPCR Fast SYBR Green Mastermix: Applied Biosystems

Random dNTP hexamer: Geneworks

RNaseIN: Ambion

Superscript III: Invitrogen

Taq Polymerase: NEB

TRI Reagent: Sigma

X-gal: Biovectra

2.1.2. PCR Primer Sequences

Oligonucleotides for use as PCR primers were designed with the aid of UCSC Genome Browser (Kent *et al.*, 2002) and Primer3 (Rozen and Skaletsky, 2000), and synthesised by Geneworks (Thebarton, South Australia) at 40nmole synthesis scale and sequencing/PCR purity grade. Primers were resuspended in H_2O to 100μM, diluted to a 10μM working stock in H_2O, and stored at -20ºC.

The following primers were used to clone probes for use in northern blot probing:

rat Rgs4 - 618 bp PCR product

Rgs4 upper (110)	ATG TGC AAA GGA CTC GCT GGT
Rgs4 lower (727)	TTA GGC ACA CTG AGG GAC TAG

rat Vegf - 477 bp PCR product

hVEGF upper (37)	GCC TTG CTG CTC TAC CTC CAC
hVEGF lower (513)	CAA ATG CTT TCT CCG CTC TGA

The following primers were used to sequence plasmids as indicated:

pGEM-T Easy

BS M13-20	GTA AAA CGA CGG CCA GT
BS Reverse	AAC AGC TAT GAC CAT G

pGL3basic, pGL3promoter

RVPrimer3	CTA GCA AAA TAG GCT GTC CC
GLPrimer2	CTT TAT GTT TTT GGC GTC TTC CA
RVPrimer4	GAC GAT AGT CAT GCC CCG CGC

The following primers were used for amplification of target gene cDNA in qRT-PCR. Primers were designed using Primer3 (Rozen and Skaletsky, 2000), set to anneal at a predicted temperature of 60ºC and amplify a target sequence of 75-150bp in length, crossing at least one intron boundary to minimise amplification from genomic DNA contamination.

human Rgs4 – 106bp PCR product from cDNA

hRgs4_qPCR_F	TTGCGAATTCCAAGCTGTTA
hRgs4_qPCR_R	AGCAGGAAACCTAGCCGATG

human Vegf – 119bp PCR product from cDNA

VEGF_qPCR_F	CCTTGCTGCTCTACCTCCAC
VEGF_qPCR1_R	GCAGTAGCTGCGCTGATAGA

human Polr2a – 67bp PCR product from cDNA

Polr2a_qPCR1_F	ACCCTCCGTCACAGACATTC
Polr2a_qPCR1_R	CCATCAAGAGAGTCCAGTTCG

human Actb – 75bp PCR product from cDNA

hActb_qPCR_F	ACAGAGCCTCGCCTTTGC
hActb_qPCR_R	GCGGCGATATCATCATCC

mouse/rat Polr2a – 85bp PCR product from cDNA

mrPolr2a_qPCR1_F	GCA CCA TCA AGA GAG TGC AG
mrPolr2a_qPCR1_R	GGG TAT TTG ATA CCA CCC TCT G

mouse Rgs4 – 100bp PCR product from cDNA

mRgs4_qPCR1_F	TCC TCG CTA AGA ATC CCT CAG
mRgs4_qPCR1_R	CCA GCC GAT GTT TGA TGT C

mouse Vegf – 101bp PCR product from cDNA

mVegfa_qPCR1_F CTG CTG TAC CTC CAC CAT GC

mVegfa_qPCR1_R CGC TGG TAG ACA TCC ATG AAC

rat Rgs4 – 74bp PCR product from cDNA

rRgs4_qPCR1_F CAA GAT GTG CAA AGG ACT CG

rRgs4_qPCR1_R CCA GCC GAT GTT TCA TAT CC

rat Vegf – 116bp PCR product from cDNA

rVegfa_qPCR1_F GGC TTT ACT GCT GTA CCT CCA C

rVegfa_qPCR1_R AAT AGC TGC GCT GGT AGA CG

human Hif1a – 89bp PCR product from cDNA

hHIF1A_qPCR2_F CATGTGACCATGAGGAAATGAG

hHIF1A_qPCR2_R AAAGCTTCGCTGTGTGTTTTG

human Epas1 – 98bp PCR product from cDNA

hEPAS_qPCR2_F CTGCGACCATGAGGAGATTC

hEPAS_qPCR2_R TGAAGAAGTCCCGCTCTGTG

mouse/rat Hif1a – 125bp PCR product from cDNA

mrHif1a_qPCR_F CGG CGA GAA CGA GA GA

mrHif1a_qPCR_R GAA GTG GCA ACT GAT GAG CA

mouse/rat Epas1 – 104bp PCR product from cDNA

mrHif2a_qPCR_F GGT TAA GGA ACC CAG GTG

mrHif2a_qPCR_R GGG ATT TCT CCT TCC TCA GC

human Dec1 – 97bp PCR product from cDNA

hDec1_qPCR1_F GAC CGG ATT AAC GAG TGC AT

hDec1_qPCR1_R CAA GAA CCA CTG CTT TTT CCA

human/mouse/rat Rgs5 – 181bp PCR product from cDNA

hmrRgs5_qPCR1_F TCAGTGAGGAAAACCTTGAGTTC

hmrRgs5_qPCR1_R AGGTTCCACCAGGTTCTTCAT

human Mlh1 – 88bp PCR product from cDNA

hMLH1_qPCR1_F AGC CTA TTT GCC CAA AAA CA

hMLH1_qPCR2_R TGT GGG GTG CAC ATT AAC AT

The following primers were used to amplify parts of the human *Rgs4* genomic locus as described in *Figure 4.6a* for ligation to pGEM-T Easy and subcloning to pGL3 or pCI_FL. Note that some primers incorporate 5' restriction enzyme sites, and therefore may not align perfectly to genomic sequence. Some primer sequences are duplicated.

Sequence A

hRgs4_-15kb_F ACCTGGAAAATCCTGCCTCT

hRgs4_-11.3kb_R_XhoI CTCGAGCTCCATCCTTCACTGCCCTA

Sequence B

hRgs4_-11.5kb_F GGAATGGAAAACTCCCCTTG

hRgs4_-8.4kb_R_XhoI CTCGAGTGGTGCTCTAAAATGTTCTATCCA

Sequence C

hRgs4_-8.6kb_F_MluI- AAGAAAACGCGTAAGCCAGAAGTGATGGAGGA

hRgs4_-6.7kb_R_XhoI- GCTAATCTCGAGAATCAAGGGGCATTCTACCC

Sequence D

hRgs4_-2.2kb_R_XhoI CTCGAGTAACAGAGCTGGGTCCAGATACA

hRgs4_5'FS_-6.5kb_F	GGCACAGAACAGGGGAAATA

Sequence E

hRgs4_5'FS_-6.5kb_F	GGCACAGAACAGGGGAAATA
hRgs4_TSSsv_R	TCCAAGATCTGATCCTCACGA

Sequence F

hRgs4_5'FS_-2.2kb_F	GATGGGATAGCATGGTGGAC
hRgs4_TSSsv_R	TCCAAGATCTGATCCTCACGA

Sequence G

hRgs4_5'FS_-2.2kb_F	GATGGGATAGCATGGTGGAC
hRgs4_5'FS_0.05kb_R	CTTCGGCTTTGAGCGTACTT

Sequence H

hRgs4_5'FS_-2.2kb_F	GATGGGATAGCATGGTGGAC
2kbRgs4_NheI	GCTAGCGAAAGCAGTAGCGGCAAAAG

Sequence I

hRgs4_qPCR_F	TTGCGAATTCCAAGCTGTTA
2kbRgs4_NheI	GCTAGCGAAAGCAGTAGCGGCAAAAG

Sequence J

hRgs4_3.3kb_F_KpnI	GGTACCCGAGGTGCTTCTACAGTT
hRgs4_6kb_R_XhoI	CTCGAGTATTTGGCCTCAGGTTTTCC

Sequence K

hRgs43UTRpA_XbaI_F	TCTAGATTCTCACCTGAAGGCAGAGG
hRgs43UTRpA_XhoXba_R	CTCGAGTCTAGACACTGGCACAGGAGGGATTA

Sequence L

hRgs4_8.2kb_F_MluI	ACGCGTAAACATCAGGGTGTAGGGTGA
hRgs4_12.9kb_R_XhoI	CTCGAGTTCCTTCCTTGTGTCATATTTCC

Sequence M

hRgs4_8.2kb_F_MluI	ACGCGTAAACATCAGGGTGTAGGGTGA
hRgs4_17.9kb_R_XhoI	CTCGAGTTGCCAGTGGGGAAACTAAC

Sequence N

hRgs4_12.8kb_F_MluI	ACGCGTCTGCTTAGGCCATTAAACCA
hRgs4_17.9kb_R_XhoI	CTCGAGTTGCCAGTGGGGAAACTAAC

2.1.3. siRNA Duplexes

siHIF1A_1541	Sense	r(CUG AUG ACC AGC AAC UUG A) dTdT
	Antisense	r(UCA AGU UGC UGG UCA UCA G) dTdT
siHIF2A_1599	Sense	r(CAG CAU CUU UGA UAG CAG U) dTdT
	Antisense	r(ACU GCU AUC AAA GAU GCU G) dTdT
siHIF1A_1530	Sense	r(CGA CAC AGA AAC UGA UGA C) dTdT
	Antisense	r(GUC AUC AGU UUC UGU GUC G) dTdT
siHIF2A_668	Sense	r(UCA GCU UCC UGC GAA CAC A) dTdT
	Antisense	r(UGU GUU CGC AGG AAG CUG A) dTdT

siRNA sequences target the sites of *Hif1a* and *Epas1* mRNA as described originally by Sowter *et al.* (2003), and were synthesised by Qiagen. RNA/DNA oligonucleotides were resuspended in siRNA Suspension Buffer at 20μM concentration, heated at 90ºC for 1 minute then 37ºC for 60 minutes to anneal duplexes, then stored at -20ºC.

2.1.4. Plasmids

pGEM-T Easy: Promega

pGL3basic: Promega

pGL3promoter: Promega

pHRE4 ($pHRE_4GL3$, containing 4 repeats of the *Epo* HRE in pGL3promoter): Ema *et al.* (1997)

pCI_FL: Peter McCarthy (Adelaide University)

pRLTK: Promega

phRLCMV: Promega

Experimental Procedures

2.2.1. Tissue Culture

PC12 rat pheochromocytoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Horse Serum and 5% Fetal Calf Serum (FCS). Neuro-2A mouse neuroblastoma and SK-N-SH human neuroblastoma cells were grown in Minimal Essential Medium (MEM) supplemented with 10% FCS. SK-N-BE(2)C human neuroblastoma cells were grown in RPMI-1640 supplemented with 10% FCS. Mouse embryonic fibroblasts (mEFs) were grown in DMEM supplemented with 10% FCS. Human Umbilical Vein Endothelial Cells (HUVECs) were a kind gift from Dr Claudine Bonder (Institute of Medical and Veterinary Science, South Australia), while mouse embryonic stem cell maintenance and differentiation in N2B27 medium was performed by Tom Klarić (Adelaide University), following previously established protocols for neural differentiation (Ying *et al.*, 2003). All media contained both L-glutamine and phenol red, and cells were grown on standard tissue culture plasticware.

Cells were maintained at 37ºC and 5% CO_2, and passaged with trypsinisation when near-confluent. Stocks of each cell-line were frozen in cryotubes in FCS supplemented with 10% DMSO, using a "Mr. Frosty" (Nalgene) container to provide slow cooling to -80ºC.

Cryotubes were then stored at -80ºC for a period of 6-12 months, or archived in liquid nitrogen storage.

Hypoxic conditions were achieved by sealing the culture vessel inside an airtight container along with an AnaeroGen sachet (Oxoid). Testing of this procedure with an oxygen meter indicated that oxygen levels are depleted over the course of the first hour to less than 0.1% oxygen. Treatment times are given as the time from sealing the container. Dimethyloxalylglycine (DMOG) stocks were prepared at 1M in DMSO, while 2,2'-Dipyridyl (DP) stocks were prepared at 100mM in DMSO, such that both chemicals were used at a dilution factor of 1000x in tissue culture medium. An equivalent amount of DMSO (0.1%) was used as a negative (vehicle) control.

2.2.2. Total RNA Extraction and Northern Transfer

Subconfluent PC12 and Neuro-2A cells were treated with normoxia, hypoxia, DMSO, DMOG or DP for 16 hours as described above. Total RNA for this and other methods was harvested by lysing cells in 1mL TriReagent (Sigma) as per the Sigma protocol (substituting 200μL chloroform for 100μL BCP at the biphase formation step), or by lysis in RLT/β-mercaptoethanol (Qiagen) buffer and extraction using the Qiagen RNeasy Mini column kit. All manipulation of RNA or preparation of materials which come into contact with RNA was performed with either designated RNase-free reagents or cleaned with a solution consisting of 200 mM NaOH and 1% SDS. Extracted total RNA was dissolved in 30-50μL H_2O, 1μl of which was diluted in 99μl H_2O for concentration quantification by spectrophotometry at 260nm using an Eppendorf BioPhotometer. RNA was also tested for purity by the measured A260/A280 ratio, and integrity of RNA was assessed by running 1μL on a 1% agarose/TBE gel and visualising rRNA bands by ethidium bromide staining.

10x MOPS buffer was prepared in H_2O: 200 mM MOPS, 50 mM NH_4Ac, 10 mM EDTA, to pH 7 with NaOH. To set a formamide/MOPS/1% agarose gel, 1.5 g of agarose was dissolved by boiling in 108 mL H_2O, then cooled to around 65°C. At this point, 15 mL of 10x MOPS buffer and 27 mL of formaldehyde was added and mixed in. The gel was then poured into a moulding apparatus and allowed to cool in a fumehood. 20 μg of each total RNA sample was diluted to a final concentration of 10 μL in H_2O. 3μL MOPS buffer

(10x), 10 μL formamide and 3.5 μL formaldehyde was added to each sample, mixed and heated at 65°C for 10 minutes to denature RNA. The samples were then transferred to ice, and 0.5 μL ethidium bromide and 7 μL loading buffer added to each sample. The samples were loaded to the gel immediately after mixing, then the gel was run at 70V for 2 hours in 1x MOPS buffer, or until the loading dye had migrated to ¾ the length of the gel. After running, the gel was soaked twice for 15 minutes in H_2O, then photographed under 70% UV-light.

A transfer stack was constructed as follows, in order from the base to the top: dish containing 20x SSC buffer; gel support (upturned moulding apparatus); long piece of Whatmann paper as a wick, covering the support and extending into the 20x SSC; the gel containing the electrophoresed RNA (face down); slices of parafilm around the edge of the gel to prevent contact between the above paper and the wick; the Nytran membrane; three wet pieces of Whatmann paper, cut to membrane size; a 5-10 cm stack of paper towel, cut to the size of the membrane; a weight. Transfer occurred for around 24 hours at room temperature, after which the stack was disassembled and the membrane rinsed briefly in H_2O. The RNA was then auto-UV-crosslinked (Stratagene Stratalinker) to the membrane and sealed in plastic for storage at 4°C until use.

2.2.3. Generation of Radiolabelled Probes

Due to high homology between sequences, cDNA probes for rat target sequences were used for probing both mouse and rat cell line extracts. Probes were excised from their corresponding pGEM-T Easy plasmid by digestion with EcoRI, then separated by 1% agarose gel electrophoresis. Bands of expected length were recovered by gel purification (Qiagen), and recovered DNA concentration was estimated by running a small amount on a second 1% agarose gel in comparison to the 1kb+ DNA ladder standard.

200ng of DNA probe was used per 20mL of pre-hybridisation buffer in hybridisation flask. This DNA was added to 1μL of random dNTP hexamer (100 μM), and made up to 14 μL with H_2O. The DNA was denatured at 95ºC for 5 minutes, then cooled on ice. Next, 2.5 μL of dNTPs(-dATP) (5 mM) and 2.5 μL of TM buffer (10x), then 5 μL of ^{32}P-dATP (10 μCi/μL) and 1μL of Klenow fragment (10U/μL) were added, mixed and incubated at 37ºC for 1 hour. 25 μL of TE was added, and the labelled DNA fragments retrieved using ProbeQuant G-50 microcolumns. The reaction product was applied to the top of a prepared G-50 column, and centrifuged for 2 minutes at 3 kRPM into a fresh

microcentrifuge tube. The eluant was diluted by addition of 150-350 µL 1xTE and stored at -20ºC until use.

2.2.4. Hybridisation of Northern Transfer Membrane

The membranes were blocked in 20mL of prehybridisation buffer, or 40mL if multiple membranes were being prehybridised simultaneously, overnight at 42ºC with slow rotation. Prehybridisation buffer: 50% formamide, 5x SSC, 50 mM $NaPO_4$ (pH 6.5), 5x Denhardt's solution, 0.1% SDS, 0.3 mg/mL sheared salmon sperm DNA in H_2O. Labelled probes were heated at 95ºC for 5 minutes to denature the DNA, then cooled on ice for 5 minutes before application to the membranes. All 400 µL of the labelled probe was transferred to the membrane in prehybridisation buffer, and was allowed to hybridise overnight at 42ºC with slow rotation.

After hybridisation, the hybridisation buffer was replaced with 2x SSPE/0.1% SDS and washed for 15 minutes at 42ºC with rotation. This was repeated once with 2x SSPE/0.1% SDS at the same conditions, then finally the wash buffer was replaced with 0.1x SSPE/0.1% SDS and washed for 45 minutes at 65ºC. The membranes were then removed from the hybridisation flasks and sealed in plastic, then exposed to a phosphorimager screen for 48-72 hours. The phosphorimager screen was scanned to computer using a Typhoon Trio (Amersham Biosciences), and images were manipulated using QuantityOne (Bio-Rad) and Adobe Photoshop software.

Probes were stripped from membranes with stripping buffer (10 mM Tris pH 7.5/0.1% SDS) and boiled in a microwave for at least 5 minutes. The membrane was then rinsed three times in RO water and sealed in plastic for storage at 4ºC until needed.

2.2.5. Reverse Transcription of mRNA

2µg of total RNA (generated as described in *Chapter 2.2.2*) was mixed with 1µL of 300ng/µL random oligonucleotide hexamer and 1µL of 500ng/µL oligo(15)dT in a volume made up to 19µL with H_2O, and annealed by heating to 70ºC for 10 minutes in a theromocycler, followed by 5 minutes on ice. These tubes were set up in duplicate for

each RNA sample. Mastermixes are then prepared, resulting in each tube receiving 6μL 5x First Strand Buffer (Invitrogen), 2μL 0.1M DTT, 1μL 10mM dNTPs and 1μL SUPERase-In RNase inhibitor (Ambion). To one tube ("cDNA") 1μL Superscript III Reverse Transcriptase (RT) (Invitrogen) was added, while the other received 1μL H_2O ("noRT"). Reactions progressed in a thermocycler set at 50ºC for 150 minutes, then 70ºC for 15 minutes to inactivate the reverse transcriptase. cDNA was stored at -20ºC until use.

2.2.6. Quantitative PCR

Quantitative PCR was performed in triplicate on cDNA samples (qRT-PCR), while one reaction per no RT sample was also set up as a negative control. Reactions were set up on ice using 2x Fast SYBR Green Mastermix (Applied Biosystems) according to the manufacturer's protocol: per reaction, 10μL 2x Fast SYBR Green Mastermix, 1.0μL cDNA or noRT sample (template), 0.4μL of left primer and 0.4μL of right primer (each at 10μM), and 8.2μL H_2O. Mastermixes of SYBR Green Mastermix and template were set up, mixed and added to wells of a 96well (BIOPlastics Cat#AB19809) tray first, followed by a mastermix of primer pairs and water. Trays were sealed with an Opti-Seal (BIOPlastics), then vortexed at low power to mix contents gently. Thermal cycling and detection was performed using the StepOne Plus (Applied Biosystems), using the following cycling parameters: 95ºC for 10 minutes, 95ºC for 15 seconds, 60ºC for 1 minute and read tray, cycle to step two 39 additional times, then for melting curve analysis 95ºC for 15 seconds, 60ºC for 1 minute, then ramping temperature by 0.3ºC increments, reading tray at each temperature. Primary amplification curves were analysed using the StepOne v2.1 (Applied Biosystems) software. Briefly, thresholds were set at the same point for an entire tray, at a level which aligned to the most reproducible detection of amplification yet below the end of the logarithmic amplification period. Resulting target Cycle(threshold) values (C(*t*)) were converted to arbitrary relative quantities with *Polr2a* or *Actb* as a reference gene using either StepOne or Q-Gene software (Muller *et al.*, 2002), both of which use modified versions of the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001). These arbitrary values were then normalised to a control sample set at 1, to allow for comparison of fold change as a mean with standard deviation across *n* independent experiments and statistical analysis. For a new primer set, qRT-PCR products were run on a 3% agarose/TBE gel to confirm product size compared to expected size, then melt curve analysis was used in subsequent experiments to ensure specificity of amplification.

To determine statistical significance of target gene fold change in expression, the mean and standard deviation of the independent normalised relative expression values were transferred to Excel (Microsoft). As the control to which comparison was being made is set at 1 (with no resulting standard deviation between independent experiments), Student's *t*-test cannot be used, as this calculation can only compare two values each with error values. Therefore, 99% confidence intervals (p=0.01) for the experimental value were calculated using standard deviation and n. Significance was assigned (indicated by an asterisk) when the value 1 (control value) was outside of the 99% confidence interval of the experimental value.

PCR efficiency of each primer pair was calculated by performing qPCR in triplicate on a set of serial dilutions of species-appropriate cDNA. This data was plotted to a log10/linear graph, and a line of best fit was calculated by Excel. The slope of this line was converted to an efficiency value (where 1 equals perfect amplification) using the equations below, with the aid of Q-Gene software. Primer pairs were accepted if E was greater than 0.9 and less than 1.1.

$$N_c = N_o\,(E+1)^{\,C}$$

Equation 2.1. Amplification of target DNA by PCR. N_c: Template amount after cycle C; N_o: Original template amount; E: PCR efficiency, between 0 and 1 (Adapted from Rutledge and Cote, 2003).

$$E = 10^{-(Slope)^{-1}} - 1$$

Equation 2.2. PCR efficiency calculation from $\log_{10}$[cDNA] vs C(t) standard curve, as depicted in *Figure 6.1* (Adapted from Rutledge and Cote, 2003).

2.2.7. Transfection of siRNA Duplexes

SK-N-BE(2)C cells were plated at a confluency of around 40% in 6 well trays, in normal growth media. siRNA duplex sequences targeted towards *Hif1a* or *Epas1* mRNA were ordered, as described by Sowter *et al.* (2003) and *Chapter 2.1.3*. For *Figure 3.5a*, cells were transfected the day after plating with 50nM of each siRNA and 5µL Lipofectamine2000 (Invitrogen), diluted in RPMI 1640 without serum as directed by the Invitrogen protocol. 12-24 hours later, media was replaced with normal growth media, before a repeat transfection using the same method. 12-24 hours later, media was once

again replaced with normal growth media, supplemented with either 100μM DP or 0.1% DMSO (vehicle). After 8 hours treatment, media was removed and cells were lysed for total RNA extraction or western blot as described in other chapters. Cells for *Figure 3.5b* were treated in the same way, but volumes were scaled up to 6cm^2 dish scale.

For *Figure 3.6*, a similar protocol was followed, with the following exceptions. Cells were transfected with 50nM siRNA and 5μL siLentFect (Bio-Rad) per well instead of Lipofectamine2000. Growth media was replaced 24 hours after the second transfection, and cells were grown for an additional 12-24 hours before treatment. Cells were treated with normoxia or hypoxia for 8 hours as described in *Chapter 2.2.1*, followed by lysis for total RNA extraction or western blot.

2.2.8. Western Transfer

Growth media was aspirated from cells, then cells were rinsed with cold 1x PBS and lysed in 80μL 1x Laemmli buffer with Protease Inhibitor Cocktail (2μg/mL aprotinin, 4μg/mL bestatin, 5μg/mL leupeptin and 1μg/mL pepstatin at final concentration) and 40mM DTT. 2x Laemmli buffer consists of: 125mM tris-HCl pH 6.8, 20% Glycerol, and 5% SDS in H_2O. 40μL of this whole cell extract mixed with was loaded with 13.3μL 4x SDS Gel Running Buffer: 50% glycerol, 0.05% bromophenol blue, 0.1mM EDTA pH 8 in H_2O. This mixture was heated for 5 minutes at 95ºC, briefly centrifuged, then loaded to a 7.5% SDS/polyacrylamide gel. 5-10mL of Precision Plus Protein Dual Color Standard (Bio-Rad) was also loaded in a separate lane. 7.5% Separating (lower) gel: 2.5mL Lower buffer, 1.875mL 40% 29:1 Bis-Acrylamide, 5.625mL H_2O, 8μL TEMED and 80μL 10% ammonium persulphate; 4.5% Stacking (upper) gel: 2.5mL 4x Upper buffer, 1.13mL 40% 29:1 Bis-Acrylamide, 6.37mL H_2O, 8μL TEMED and 80μL 10% ammonium persulphate. Lower buffer: 181.1g tris and 40mL 10% SDS, made up to 1L with H_2O, with pH adjusted to 8.8 with HCl. Upper buffer: 60.5g tris and 40mL 10%SDS, made up to 1L with H_2O, with pH adjusted to 6.8 with HCl. The gel was run at 140V in 1x GTS until adequate separation between high molecular weight bands could be observed. 10x GTS solution: 30.3g Tris base, 144g Glycine, 10g SDS, made up to 1L with H_2O.

Protein was transferred to nitrocellulose membrane (Pall) by wet transfer. Transfer apparatus was set up in transfer buffer from negative to positive as follows: mesh sponge, Whatmann paper, polyacrylamide gel (facing the negative side), nitrocellulose membrane,

Whatmann paper, mesh sponge. 1x Transfer buffer: 10mL 10% SDS, 100mL methanol, 3g tris and 14.4g glycine, made up to 1L with H_2O. Western transfer then progressed at 250mA for 1½ hours in transfer buffer at 4ºC. After transfer, nitrocellulose membrane was stained briefly with 0.1% Ponceau in 5% acetic acid for visualisation of transfer effectiveness, and to facilitate cutting the membrane between the 75 and 50kDa size markers. Membranes were then blocked in 7.5% skim milk in 1x PBS for at least 1 hour.

The high molecular weight portion of the membrane was probed for HIF-2α with anti-HIF-2α rabbit antibody Cat# NB100-122 (Novus) diluted at 1:1000 in 1x PBS with 1% skim milk. Following detection, membrane was rinsed in 1x PBS and HIF-1α was detected with anti-HIF-1α mouse antibody Cat# 610959 (BD Biosciences) at 1:500 dilution in 1x PBS with 1% skim milk. The low molecular weight portion of the membrane was probed for α-Tubulin with anti-α-Tubulin rat antibody (Novus), diluted at 1:10000 in 1x PBS with 1% skim milk. Primary antibody binding occurred overnight with rocking at 4ºC. Antibody solutions were poured off and stored at 4ºC with 0.02% sodium azide, while membranes were washed 3 times with rocking for 5 minutes in 1x PBS at room temperature. Horseradish Peroxidase (HRP)-conjugated secondary antibodies against rabbit, mouse or rat primary antibodies were then applied at 1:10000 dilution in 1x PBS for 1 hour at room temperature with rocking. Following this, antibody solutions were aspirated and membranes were washed 3 times in 1x PBS as previously. Membranes were then dried by blotting, and enhanced chemiluminescence reagents (Pierce and Millipore) were used to detect HRP by 1-10 minute exposure of X-ray film to membranes.

2.2.9. Actinomycin D Treatment and mRNA Decay Calculation

SK-N-SH or SK-N-BE(2)C cells were grown subconfluency in 8x 10cm^2 dishes containing 10mL growth media. Cells were treated by adding either 20μL of 2mg/mL actinomycin D (in DMSO, resulting in 4μg/mL final concentration), or an equivalent amount of DMSO as a negative control. Media was gently mixed in the dish by swirling, left for 10 minutes, then cells were treated with normoxia or hypoxia as described in *Chapter 2.2.1* for 4, 8 or 12 hours, while a negative control sample was lysed immediately to determine starting relative expression levels. After treatment, cells were lysed and total RNA was extracted and expression levels quantified with qRT-PCR as described above.

qRT-PCR results were analysed as described above to give Rgs4 mRNA levels relative to Polr2a for each time point, presented as a representative experiment in *Figure 4.1a* and *d*. As cDNA samples are normalised for total RNA amounts used in reverse transcription reaction, C(*t*) values without normalisation to reference gene levels were used to compute target gene mRNA half-life, to remove the effect of reference gene degradation. C(*t*) values were converted to values relative to basal expression levels using *Equation 2.1* by setting $N_0 = 1$ for normoxic control expression, giving an arbitrary common threshold value N_c. Values from two independent experiments were entered into Prism (GraphPad) and graphed as an *x*,*y* scatter plot with one phase decay line fitted, constraining the plateau to 0 as mRNA was assumed to become eventually undetectable as *time* approaches ∞. Calculated half-life ranges at 99% confidence were then computed by Prism software.

2.2.10. Mammalian Luciferase Reporter Assays

PC12 or SK-N-BE(2)C cells were plated in 24 well trays at 20-30% confluency in 2mL normal growth media per well. Cells were transfected twice (at 24 and 48 hours), each time by adding 1μL Lipofectamine2000 (Invitrogen), 100ng of pGL3 or 25ng of pHRE4 firefly luciferase reporter and 25ng of phRLCMV renilla luciferase reporter in 0.5mL serum-free media per well. Reporter plasmids are described in *Chapter 2.1.3*. 100ng of pGFP was transfected in separate wells to assess transfection efficiency. Growth media was replaced before the second transfection, and before treatment. On the third day, cells were treated with 16 hours of normoxia or hypoxia as described in *Chapter 2.2.1*, following which media was aspirated and cells were lysed in 100μL 1x Passive Lysis Buffer (Promega) per well, shaking for at least 20 minutes at room temperature. 10μL of lysate from each well was then measured using the Dual Luciferase System (Promega) reagents and Glomax luminometer (Promega). Luminometer plate read settings: Injection 1 (Luciferase Assay Reagent II) 75μL, wait 3 seconds, integrate 10 seconds, injection 2 (Stop and Glo reagent) 75μL, wait 3 seconds, integrate 10 seconds. Data was then processed with Excel (Microsoft) and Prism (GraphPad), and presented as a representative of three independent experiments.

2.2.11. Published ChIP-seq Data Alignment and Analysis

Chromatin immunoprecipitation with deep sequencing DNA detection (ChIP-seq) data for NPAS4, CBP, and PolII in mouse cortical neurons treated with either 2 hours KCl-induced membrane depolarisation or control culture conditions was retrieved from bigWig files provided in the supplementary figures of reference (Kim *et al.*, 2010). These files were applied to the *mm9* mouse genome assembly using the Genomes tab of the UCSC Genome Browser (Kent *et al.*, 2002), and regions covering genes *Drebrin* (Chromosome 13, bases 55450000-55700000) and *Rgs4* (Chromosome 1, bases 171580000-171830000) were viewed (*Figure 4.7*). HRE-like sequences were searched for using the MBCS plug-in for Microsoft Word (Muller *et al.*, 2001), and located in the UCSC Genome Browser using the each site's flanking 20 bases.

3. *Rgs4* is Responsive to Hypoxia and the HIF Pathway

3.1.1. Preamble

The preliminary data described in *Chapter 1.2* and background literature described in *Chapter 1.3* suggest that the *Rgs4* gene and its product are under the control of hypoxia-sensitive pathways. In particular, the data described in *Figure 1.4* show that exogenous expression of HIF-2α or hypoxic treatment of the rat pheochromocytoma PC12 cell line both result in increased expression of *Rgs4* mRNA. However, it is not clear whether these responses are conserved in other species or even different cell types. It is also unclear whether this hypoxic response is dependent on the endogenous HIF pathway, as ectopic expression of HIF-α factors could feasibly lead to activation of genes not normally affected by HIF. Finally, if the hypoxic response of *Rgs4* is controlled by HIF, it is unclear whether this response is solely due to activity of HIF-2 or whether both HIF forms are involved, as overexpression of these proteins may distort their natural specificity between DNA binding target sequences.

Therefore, the work described in this chapter aims to further explore the conditions in which *Rgs4* mRNA is regulated by hypoxia, and to investigate whether the regulation of *Rgs4* levels by hypoxia utilises the HIF pathway.

3.1.2. Northern Blot Analysis of *Rgs4* Response to Hypoxia and Mimetics

The preliminary experiments of *Figure 1.4* were initially extended by testing for a response of *Rgs4* message to hypoxia in other cell lines, taken from different species and cell type origins. In the first instance, this selection included human embryonal kidney 293T cells, mouse neuroblastoma Neuro-2A cells, and mouse P19 embryonal carcinoma cells in either undifferentiated, neural or muscle differentiation states. These were tested alongside the PC12 cell line that was originally used, as a positive control. These cells were treated with or without hypoxia for 16 hours before total RNA harvest and analysis by northern blotting. *Rgs4* mRNA was not detectable in normoxic or hypoxic 293T cells, nor in any of the P19 cell treatments (data not shown). *Rgs4* transcript was readily

detectable at a higher level in hypoxic PC12 cells than in normoxic control cells as observed previously, but was also weakly detected in hypoxic but not normoxic Neuro-2A cells (*Figure 3.1*). Housekeeping gene *γ-Actin* was probed for as a loading and transfer control. Average fold change for *Rgs4* in PC12 cells relative to *γ-Actin* was similar to the measurements in the preliminary data (*Figure 1.4*), while in the Neuro-2A cells fold change in message was not able to be calculated due to the lack of detection at normoxia. *Vegf* and *Ldha* were also probed for as a positive control for hypoxic gene activation in each cell line tested, as they are well characterised target genes of HIF, and were detected in all cases at elevated levels in response to hypoxia regardless of the detection of *Rgs4*. Weaker secondary bands were commonly seen after probing, most notably with *γ-Actin* and *Ldha* probes, and may relate to lowly expressed splice variants or non-specific hybridisation. In the case of the loading control, alignment of the coding sequences of actin family members *γ-actin* and *β-actin* in rat result in over 80% identity, so multiple similar members from the actin family may be detected with one probe.

The experiment was also extended to include treatment with dimethyloxalylglycine (DMOG) and 2,2'-dipyridyl (DP) for 16 hours, compared to vehicle (DMSO) controls. DMOG is an analogue of 2-oxoglutarate (2-OG), while DP chelates Fe-II. As the PHD and FIH regulatory enzymes require 2-OG and Fe-II to hydroxylate HIF-α, treatment with these chemicals is known to stabilise and activate both HIF-α subunits (Jaakkola *et al.*, 2001). Therefore, these hypoxia mimetic chemicals may be expected to provide a stimulus for activating the HIF pathway without activation of other hypoxia-responsive pathways. PC12 cells increased *Rgs4* message in response to 16 hour treatment with either DMOG or DP relative to vehicle treatment, while *Rgs4* was responsive to DMOG but not DP in Neuro-2A cells (*Figure 3.1*). Positive control *Vegf* and *Ldha* were upregulated in this cell line by both treatments, as expected. In all cases the fold change of *Rgs4* mRNA in hypoxia-mimetic treated cells was slightly greater than that observed for hypoxia treatment, with the curious exception of DP treatment of Neuro-2A cells.

In order to link these findings to human disease and other genetic information, and given the consistent hypoxic induction of *Rgs4* in the neuroblastoma cell line, human neuroblastoma cell lines were selected for use in subsequent experiments. Initial experiments were performed using northern blotting (data not shown), but quantitative RT-PCR (qRT-PCR) used for subsequent analyses presented in the next chapter.

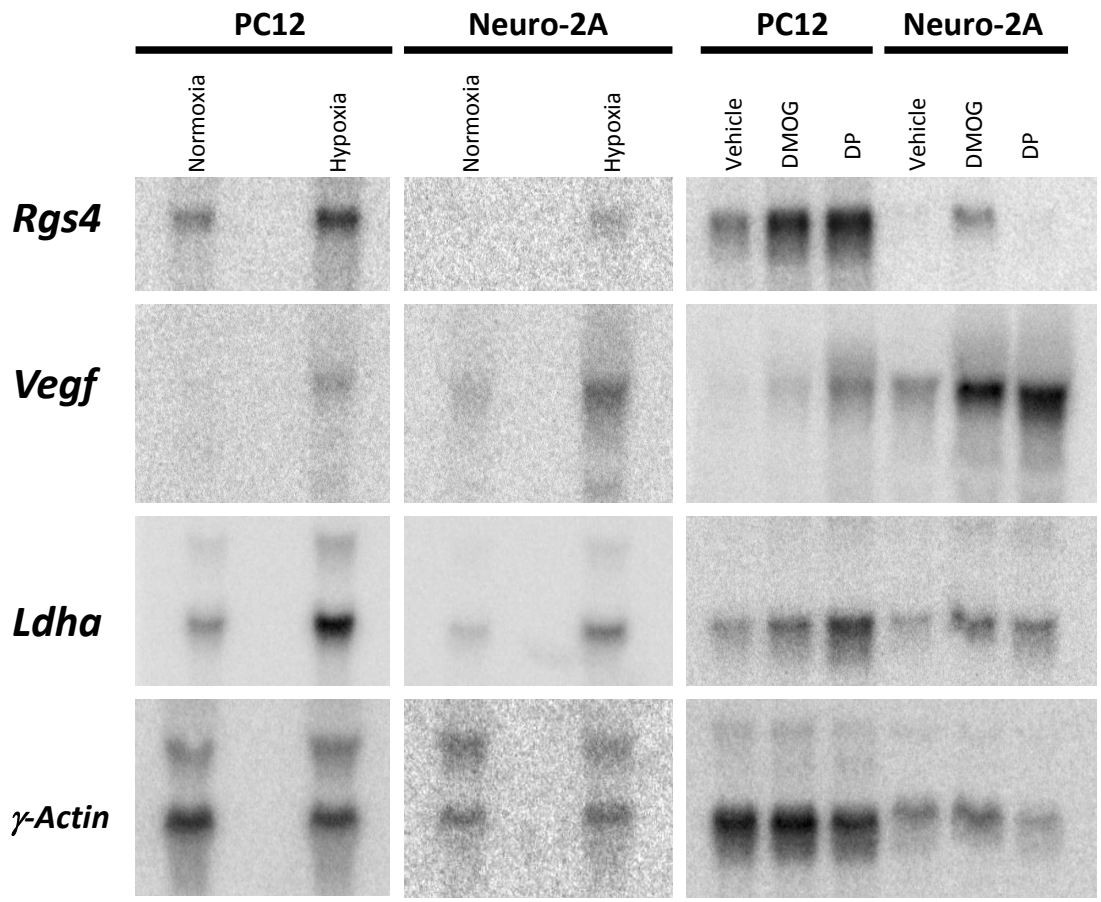


Figure 3.1. Cultured rat pheochromocytoma PC-12 and mouse neuroblastoma Neuro-2A cells were treated for 16 hours with normoxia, hypoxia or hypoxia mimetics. 1mM dimethyloxalylglycine (DMOG) or 100μM 2,2'-dipyridyl (DP) was added to normal growth medium, while vehicle (0.1% DMSO) was used as a negative control. Following treatment, total RNA was analysed by northern transfer and detection of the indicated target mRNAs by ^{32}P-labelled cDNA probes and exposure to a phosphorimaging screen. Representative scans of at least two independent experiments are shown.

3.1.3. Response of *Rgs4* in Human Neuroblastoma to Hypoxia and Mimetics

The response of *Rgs4* to hypoxia was next examined in human neuroblastoma cell lines. Quantitative RT-PCR was optimised in order to enable accurate quantification of changes in expression levels, increased sensitivity and a higher throughput of experiments. The results of optimisation and verification of this technique are presented in the appendix (*Chapter 6.1.1*). The SK-N-SH and SK-N-BE(2)C are independent human cell lines derived from neuroblastoma biopsies, and therefore were expected to display a similar response to that observed in the mouse neuroblastoma cell line (*Chapter 3.1.2*). Neuroblastoma and pheochromocytoma are both cancers of the sympathetic nervous system, with pheochromocytoma arising from the chromaffin cells of the adrenal medulla, and neuroblastoma from primitive neuroblast cells of the developing sympathetic nervous system (reviewed by Fung *et al.*, 2008). These human neuroblastoma lines were tested for *Rgs4* response to a 16 hour treatment with hypoxia, DMOG or DP by qRT-PCR (*Figure 3.2*). Neuro-2A (data not shown) and PC12 samples were retested by this method to compare the qRT-PCR detection method with the previously presented northern blots (*Figure 1.4* and *Figure 3.1*). For PC12 cells, quantification by qRT-PCR gave a 4-fold response of *Rgs4* to hypoxia, and a 3-fold response to either DMOG or DP treatment, which is consistent with the data obtained by northern blot.

Three independent experiments were performed with the SK-N-SH cell line, with relative *Rgs4* levels showing a statistically significant mean change of 4.8 fold in hypoxia, 4.3 fold in DMOG and 3.3 fold in DP, when comparing each treatment to appropriate negative controls (*Figure 3.2a*). Statistical analyses across the three independent experiments were performed using 99% confidence intervals for the treated samples compared to untreated samples as described in *Chapter 2.2.6*. Known HIF target gene also displayed significant responses to the treatments, albeit at a higher fold change than observed for *Rgs4*. Similar results were observed for the SK-N-BE(2)C (*Figure 3.2b*) and previously tested PC12 (*Figure 3.2c*) cell lines, which are presented as representative qRT-PCR experiments of n=2 relative to *Polr2a* prior to normalisation to calibrator (negative control) sample. Therefore, the two human neuroblastoma lines are good models for investigating the hypoxic response of *Rgs4*, and show that this response is conserved over evolution between rat, mouse and human cells.

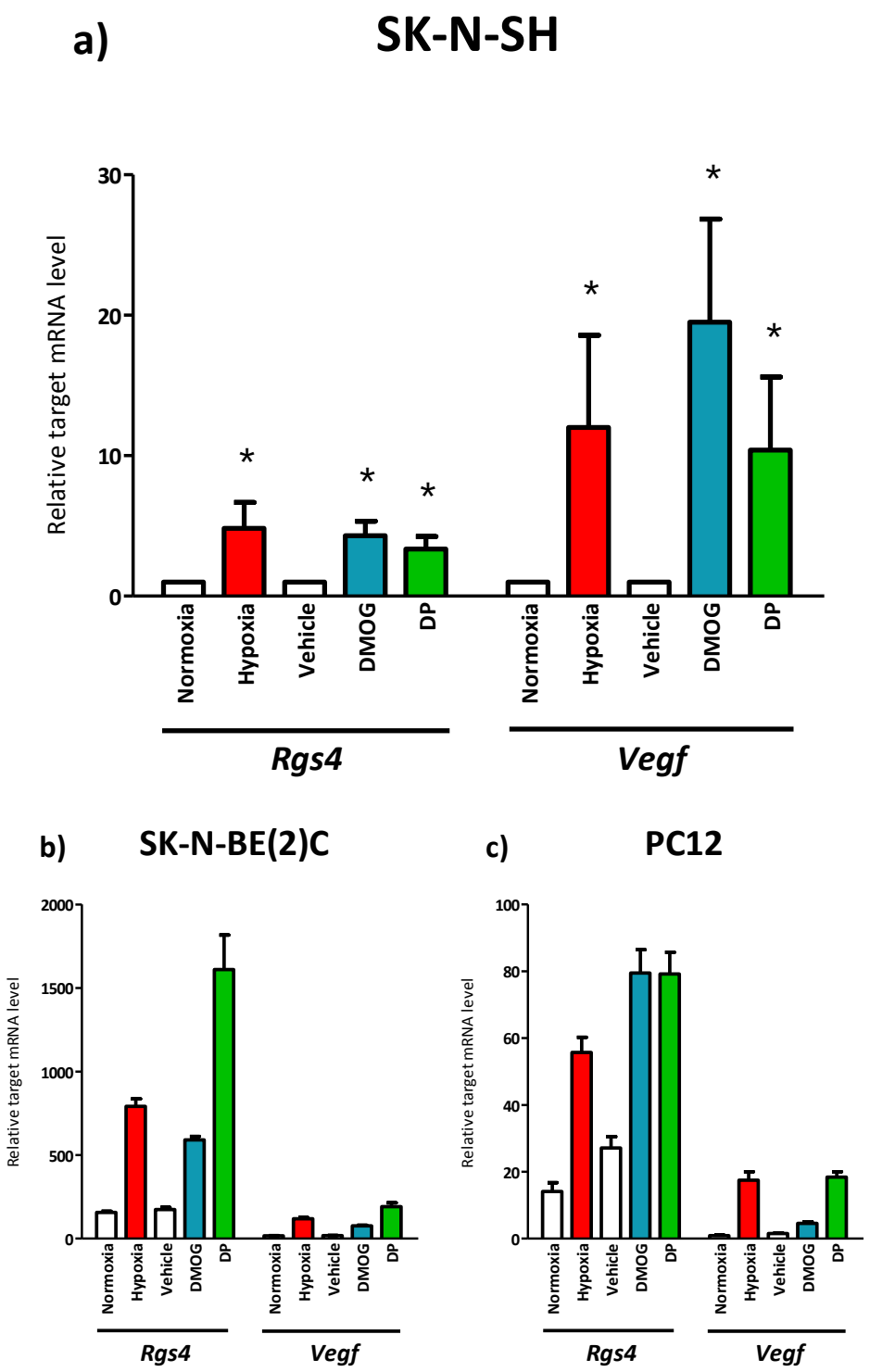


Figure 3.2. (**a**) SK-N-SH cells were treated with normoxia, hypoxia, 0.1% DMSO (vehicle), 1mM DMOG or 100μM DP for 16 hours before target gene analysis by qRT-PCR. Mean fold change with standard deviation between three independent experiments is shown, with asterisks indicating that normoxic control lies outside of the 99% confidence interval of relative target gene expression. (**b**) SK-N-BE(2)C and (**c**) PC12 cell lines were tested in two independent experiments in the same manner, representative experiments are shown with standard deviation between three qRT-PCR replicate reactions.

Using the qRT-PCR technique, we also tested other cell lines for *Rgs4* expression. cDNA samples of mouse embryonic stem cells undergoing neural differentiation generated by Tom Klarić were tested, (*Figure 3.3a*) and although *Rgs4* could not be detected by qRT-PCR in cultured undifferentiated mouse embryonic stem cells, after 3 days of neural differentiation triggered by growth in N2B27 medium *Rgs4* was readily detectable. *Rgs4* was then further upregulated in continued neural differentiation until a maximum expression relative to *Polr2a* after 10 days of treatment. However, as only one set of differentiated cells were tested, and reference gene *Polr2a* expression was not stable across these samples, these results can be taken as preliminary only. Differentiated stem cells may therefore provide a non-cancerous model for testing the hypoxic regulation of *Rgs4*, however this was not attempted due to time constraints. Mouse embryonic fibroblasts (mEFs) were also tested by 16 hour normoxic or hypoxic treatment, showing strong upregulation of *Vegf*, while *Rgs4* was detectable but not positively regulated by hypoxia (*Figure 3.3b*).

3.1.4. Hypoxic Response is Specific for *Rgs4* in Neural-like Cells

As outlined in *Chapter 1.3.3*, it has previously been suggested that *Rgs5* but not *Rgs4* message levels are responsive to hypoxia in Human Umbilical Vein Endothelial Cells (HUVECs) (Jin *et al.*, 2009). *Rgs4* and *Rgs5* both encode members of the R4 subfamily of RGS domain proteins, and are both located in the human genome on Chromosome 1 q23.3, separated by 50kb of intervening sequence. Experiments were designed to test for *Rgs4* and *Rgs5* hypoxic response in the SK-N-BE(2)C cell line in comparison to primary HUVECs. As Jin and colleagues report a maximum increase in *Rgs5* after 3 hours hypoxic treatment, but a return to control levels after 24 hours of treatment, we treated cells for 0, 3 or 16 hours with hypoxia. We then tested by qRT-PCR for *Rgs4*, *Rgs5* and positive control *Vegf* expression relative to *Polr2a* (*Figure 3.4*). Across three independent experiments, *Rgs4* and *Vegf* were significantly upregulated in SK-N-BE(2)C cells after hypoxic treatment, yet *Rgs5* was not significantly responsive. Interestingly, no increase in *Rgs4* or *Rgs5* levels could be detected in hypoxic HUVECs, despite significant upregulation of positive control *Vegf*. Furthermore, small yet significant decreases in *Rgs4* and *Rgs5* (0.51 and 0.69 fold change respectively) could be detected after the longer treatment with hypoxia. These results oppose the findings of Jin *et al.* (2009), as no hypoxic *Rgs5*

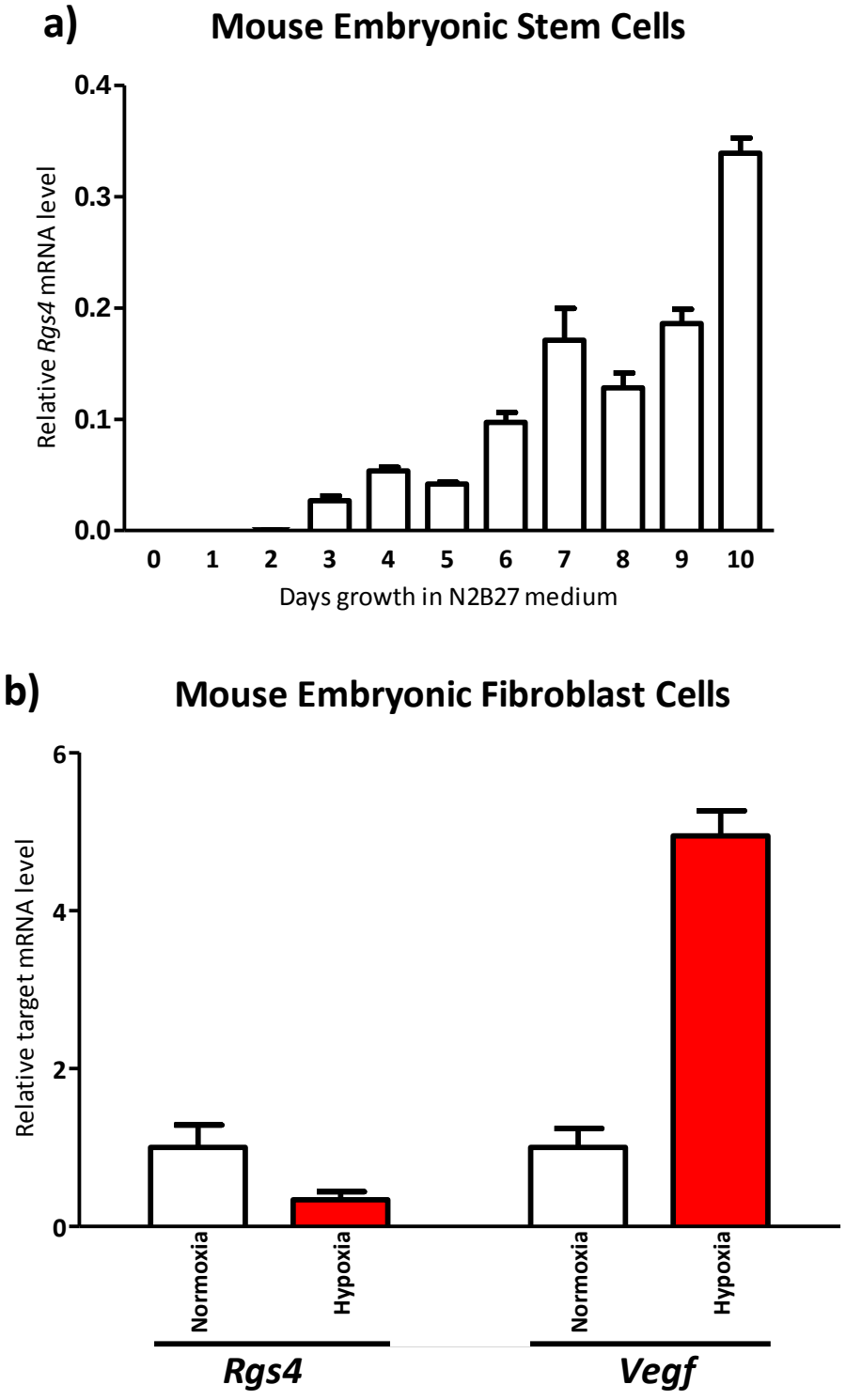


Figure 3.3. (**a**) cDNA samples from undifferentiated (day 0) and neural differentiated (N2B27 medium treated) mouse embryonic stem cells were analysed for *Rgs4* relative to *Polr2a*. (**b**) Mouse embryonic fibroblasts (mEF) were exposed to normoxia or hypoxia for 16 hours before cDNA preparation. In both figures, target genes were quantified relative to *Polr2a* in three replicate qRT-PCR reactions, on one set of experimental cDNA.

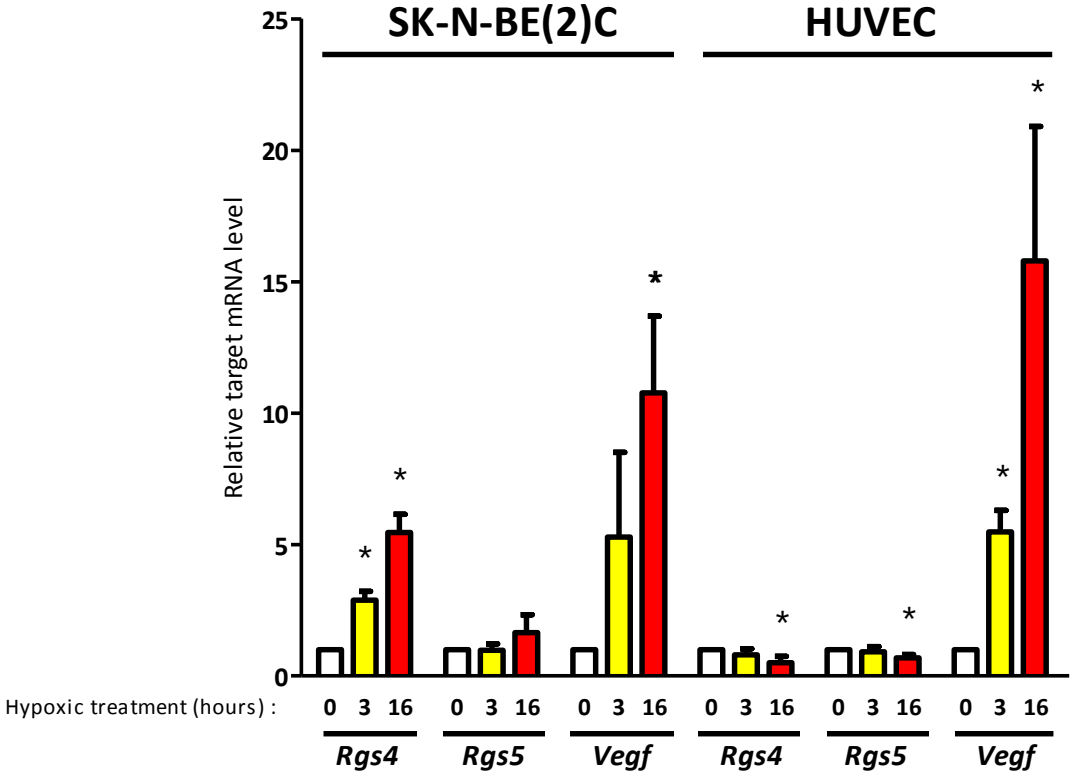


Figure 3.4. SK-N-BE(2)C and HUVEC cell cultures were treated with 0 (normoxia), 3 and 16 hours of hypoxia before lysis and RNA extraction. Quantification of *Rgs4*, *Rgs5* and *Vegf* mRNA levels relative to *Polr2a* was performed using qRT-PCR, then normalised to normoxic levels to give fold change. Mean fold change with standard deviation between three independent experiments is shown, with asterisks indicating that normoxic control lies outside of the 99% confidence interval of relative target gene expression.

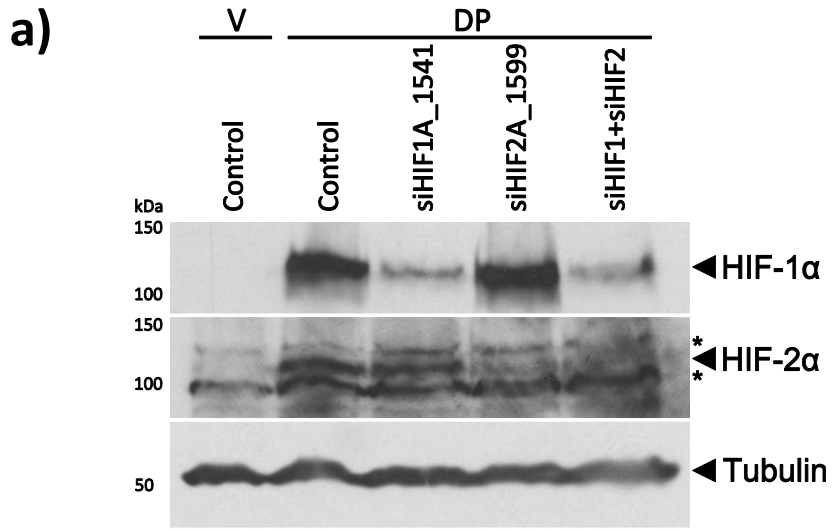
response is detected, and further indicate that the hypoxic responses of *Rgs4* in neuroblastoma and pheochromocytoma cells are observed in neural-like cell types only.

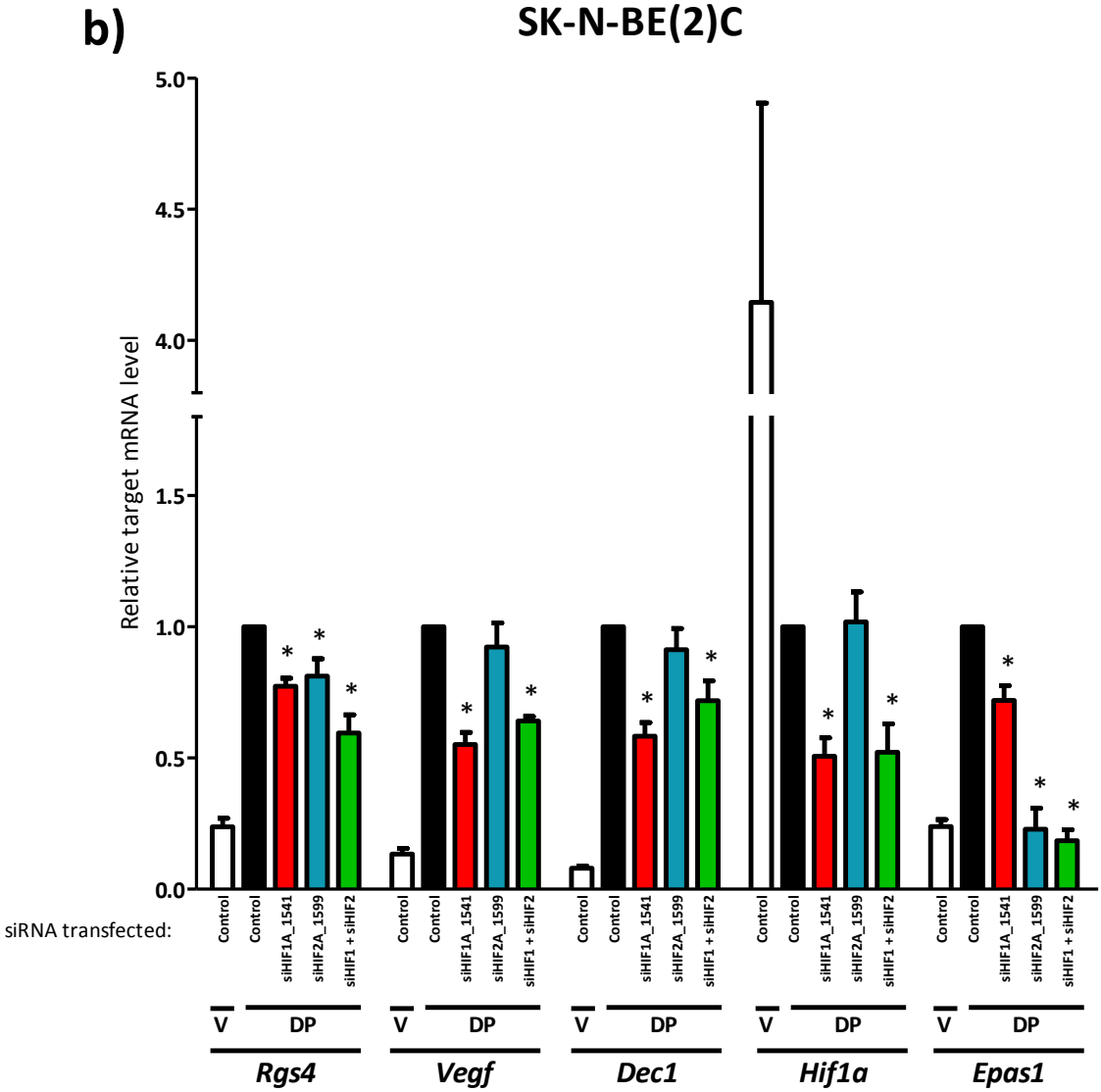
3.1.5. Loss of HIF-1 or HIF-2 Impairs the Response of *Rgs4* to 2,2'Dipyridyl

The response of *Rgs4* to hypoxia, the hypoxia-mimetic chemicals DMOG and DP, and to overexpressed HIF-2α in the PC-12 cell line strongly suggests that the HIF pathway is responsible for regulation of *Rgs4*. However, in order to directly test the involvement of HIF in this regulation, and the contribution of endogenous HIF-1α compared to HIF-2α, experiments involving knockdown of *Hif1a* (encoding HIF-1α) or *Epas1* (encoding HIF-2α) were designed. RNA interference (RNAi) has been successfully used previously to knockdown *Hif1a* and *Epas1* by transfection of small interfering RNA (siRNA) molecules to human cell lines (Sowter *et al.*, 2003), so we applied the same technique with optimisation to the SK-N-BE(2)C cell line. A standard negative control siRNA tagged with AlexaFluor-546 was also used to control for non-specific RNAi and transfection effects, as well as allowing optimisation of transfection efficiency by microscopy (data not shown).

After optimisation, siRNA-mediated knockdown of HIF-1α and HIF-2α in SK-N-BE(2)C cells was found to be most efficient after two rounds of transfection, and 8 hours of hypoxia or DP treatment. Both HIF-1α and HIF-2α are induced after 8 hours of treatment in these cells (Holmquist-Mengelbier *et al.*, 2006), so it would be expected that at this timepoint both are functional and knockdown would cause a negative effect on target gene response. As a larger target gene response was observed in SK-N-BE(2)C cells following DP treatment compared to hypoxia (*Figure 3.2c*), we reasoned that this diminished response of HIF target genes due to siRNA-mediated knockdown would be most detectable following DP treatment.

Sub-confluent SK-N-BE(2)C were plated, then transfected twice with 50nM of negative non-specific control siRNA, siHIF1A_1541 (targeted to *Hif1a*), siHIF2A_1599 (targeted to *Epas1*) or a combination of siHIF1A_1541 and siHIF2A_1599 (siHIF1 + siHIF2) before treatment with 100μM DP (or 0.1% DMSO vehicle control). Three independently transfected and treated cell cultures were tested by western blot for HIF-1α, HIF-2α and


a)
V
DP
Control
Control
siHIF1A_1541
siHIF2A_1599
siHIF1+siHIF2
kDa
150
100
150
100
50
HIF-1α
HIF-2α
Tubulin


b)
SK-N-BE(2)C
Relative target mRNA level
5.0
4.5
4.0
1.5
1.0
0.5
0.0
siRNA transfected:
Control
Control
siHIF1A_1541
siHIF2A_1599
siHIF1 + siHIF2
V
DP
Rgs4
Vegf
Dec1
Hif1a
Epas1

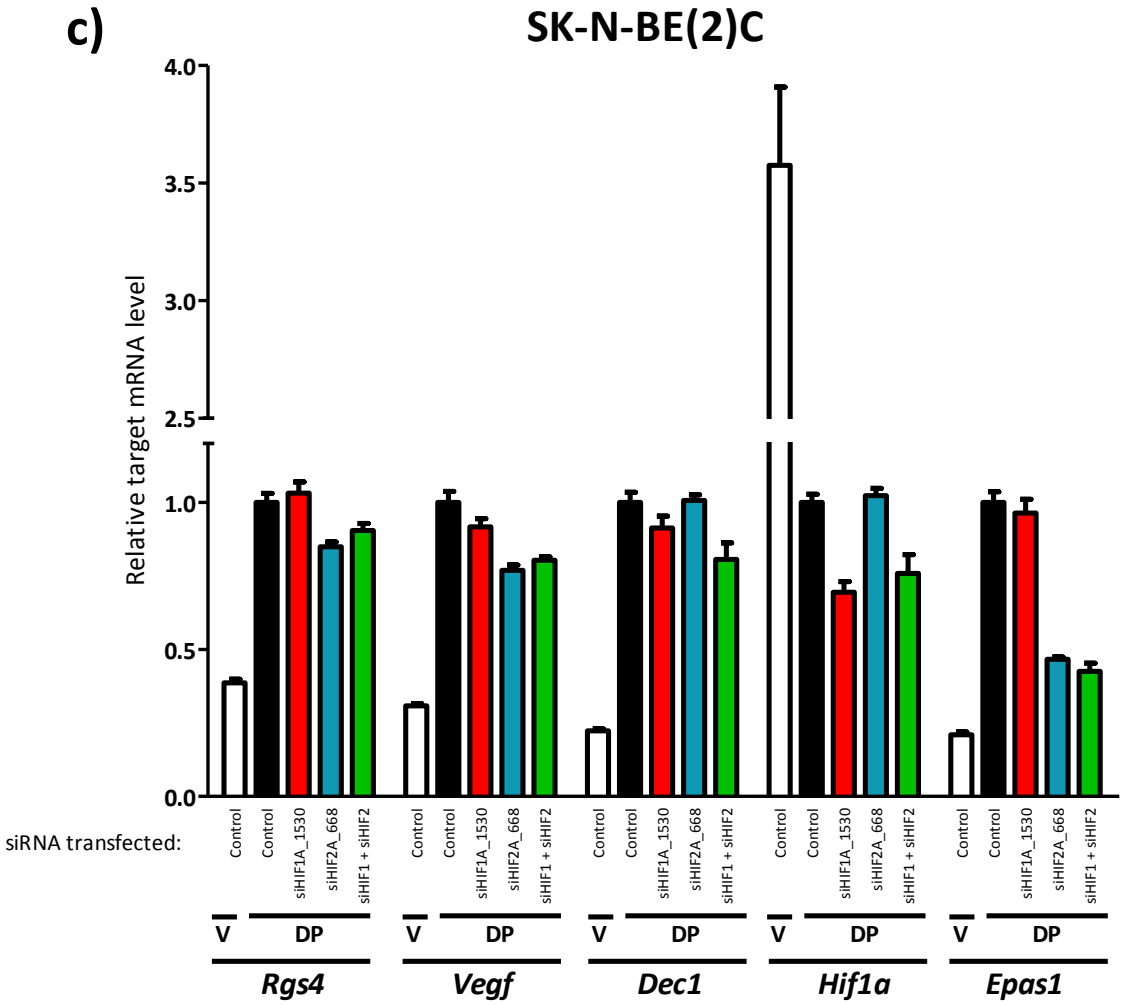


Figure 3.5. (**a**) SK-N-BE(2)C cells were transfected twice with 50nM siRNA using Lipofectamine2000 reagent, before 8 hours 0.1% DMSO vehicle (V) or 100μM DP treatment. Western blot was then performed for HIF-1α, HIF-2α and Tubulin as indicated, with asterisks to indicate non-specific bands. (**b**) Following identical treatment, SK-N-BE(2)C cells were analysed for *Rgs4*, *Vegf*, *Dec1*, *Hif1a* and *Epas1* expression levels relative to *Polr2a* by qRT-PCR. Mean fold change with standard deviation between three independent experiments, each normalised to the DP-treated level of expression of each gene after negative control siRNA transfection, is shown with asterisks indicating that normoxic control lies outside of the 99% confidence interval of relative target gene expression. (**c**) Representative experiment set up identically to (b), but using the different siRNA sequences.

Tubulin protein levels (*Figure 3.5a*), and by qRT-PCR for *Rgs4, Vegf, Dec1, Hif1a* and *Epas1* transcript expression relative to *Polr2a* (*Figure 3.5b*). Expression was normalised to the DP-treated Control siRNA sample for each replicate, then mean and standard deviation was calculated across the three independent experiments for statistical analysis.

Western blot analysis of identically-treated SK-N-BE(2)C cell cultures detected HIF-1α and HIF-2α at decreased levels in the relevant siRNA transfected cultures, although reliable detection of HIF-2α was problematic despite repeated optimisation (*Figure 3.5a*). Similarly, partial knockdown of the HIF-α subunits was observed by qRT-PCR, as *Hif1a* and *Epas1* mRNA levels are decreased but still detectable upon relevant siRNA transfection. Unexpectedly, levels of both HIF encoding mRNAs were also found to change upon DP treatment alone, with *Hif1a* decreasing while *Epas1* levels increased. Regulation of HIF-α encoding mRNAs by hypoxia or hypoxia mimetics is not commonly observed in other cell types, therefore this result was investigated further in later experiments (*Chapter 4.1.5*).

Induction by DP compared to vehicle was observed for known HIF target genes *Vegf* and *Dec1*, as well as *Rgs4*, in accordance with previous results (*Figure 3.2b*). Knockdown of HIF-1α but not HIF-2α significantly repressed induction of *Vegf* and *Dec1* by DP treatment, yet knockdown of HIF-2a gave no significant effect on these target genes. *Rgs4* induction was significantly repressed by knockdown of either HIF-1α or HIF-2α, but maximum repression of response required a combination of both HIF-1α and HIF-2α knockdown.

Unfortunately, the effectiveness of siRNA-mediated knockdown of HIF-1α and HIF-2α was not consistent with repeated experiments. An identical protocol was used to transfect SK-N-BE(2)C cells with a secondary set of siRNA sequences targeted to the HIF-α encoding transcripts: siHIF1A_1530 and siHIF2A_668. However, as shown by a representative experiment (*Figure 3.5c*), knockdown efficiency of HIF-1α was poor, as indicated by both *Hif1a* transcript knockdown and knock target gene (*Vegf, Dec1*) knockdown. Nonetheless, a small reduction in *Rgs4* response can be observed with siRNA treatment against HIF-2α.

3.1.6. HIF-α Knockdown has a Small Effect on *Rgs4* Hypoxic Response

To further test the requirement of the HIF pathway in *Rgs4* regulation, siRNA-treated SK-N-BE(2)C cells were also tested under hypoxic conditions, rather than with a hypoxia mimetic. SK-N-BE(2)C cells were transfected with the secondary set of duplex siRNAs in a similar fashion to the experiments of *Chapter 3.1.5*, following which cells were exposed to hypoxia for 8 hours before lysis for analysis by qRT-PCR (*Figure 3.6*). Due to ongoing optimisation, siLentFect transfection reagent was used, which resulted in more effective knockdown than previous experiments which used Lipofectamine2000.

Hif1a and *Epas1* message levels were significantly decreased in the appropriate siRNA-transfected cells, and *Hif1a* and *Epas1* responses to hypoxia were similar to the trends observed following DP treatment (*Figure 3.5*), as treatment results in a relative decrease in *Hif1a* levels and an increase in *Epas1*. Knockdown of either HIF-α subunit caused significant decreases in the hypoxic response of known HIF target genes *Vegf* and *Dec1*, although knockdown of both HIF-α subunits did not improve repression of the hypoxic responses of either target gene. Notably, knockdown of HIF-2α alone inhibited *Vegf* and *Dec1* hypoxic response, where HIF-2α knockdown previously had little effect on the response of either of these genes to DP (*Figure 3.5*).

In this experiment, knockdown of the HIF-α subunits had a subtle negative effect on *Rgs4* upregulation. However, this effect was not consistent between the four independent experiments, resulting in a large standard deviation when taken together as displayed in *Figure 3.6*. As a result the effects of these siRNAs on *Rgs4* hypoxic regulation were not statistically significant. This implies that the DP-induced activation of *Rgs4* is somewhat different to hypoxia, and that *Rgs4* is different in its response to both stimuli than *Vegf* and *Dec1*.

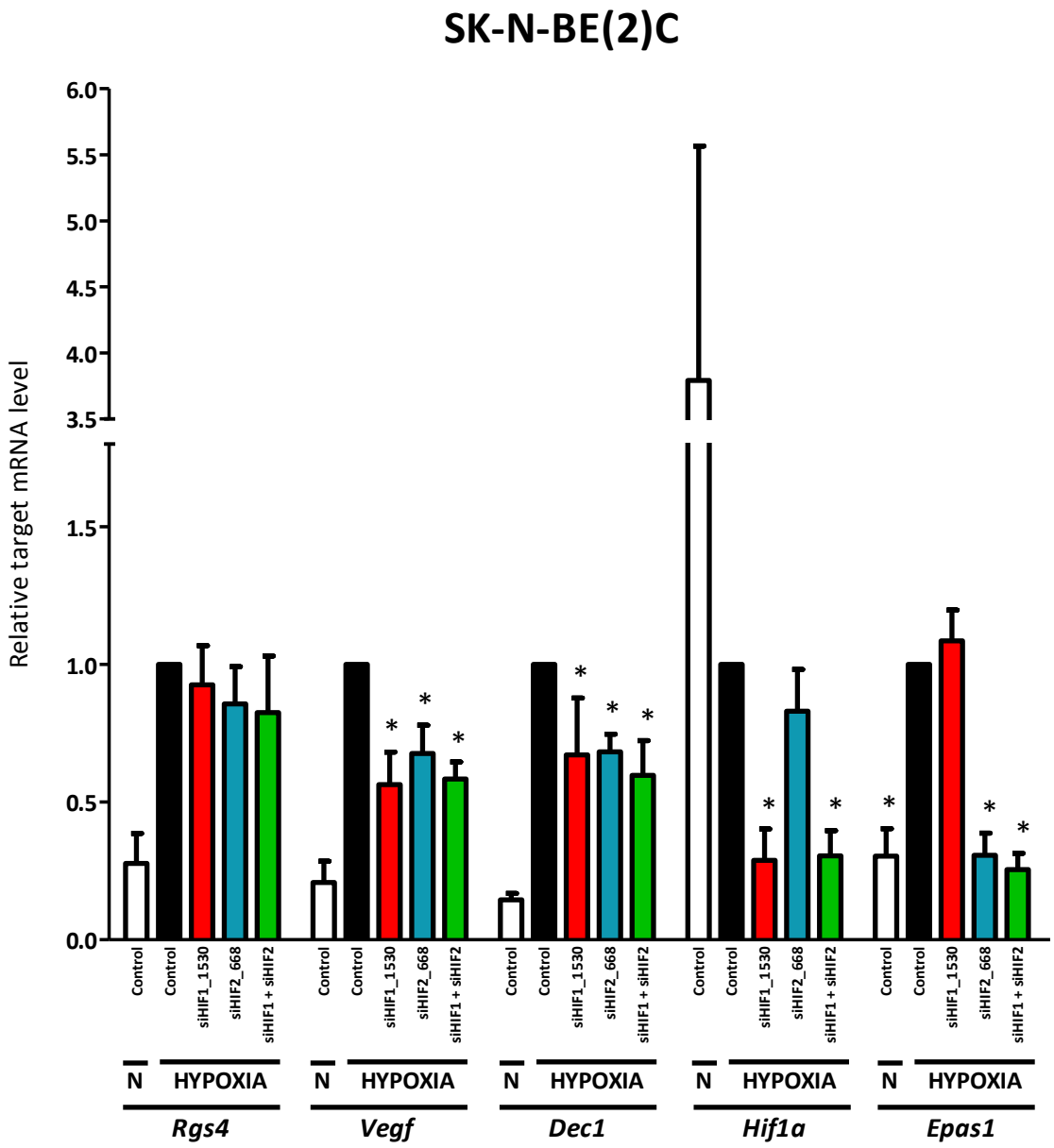


Figure 3.6. SK-N-BE(2)C cells were transfected twice sequentially with 50nM siRNA using siLentFect reagent, before 8 hours normoxic (N) or hypoxic treatment. Total RNA was then prepared for analysis of *Rgs4*, *Vegf*, *Dec1*, *Hif1a* and *Epas1* levels relative to *Polr2a* by qRT-PCR. Mean fold change with standard deviation between four independent experiments, normalised to the hypoxia-treated level of expression of each gene after negative control siRNA transfection, is shown with asterisks indicating that normoxic control lies outside of the 99% confidence interval of relative target gene expression.

3.2.1. Hypoxic Response of *Rgs4* is Conserved, but Cell Type Specific

The work presented in the previous chapters shows that Rgs4 is regulated by hypoxia in a cell type specific manner, and that the HIF pathway plays a role in this regulation. *Rgs4* mRNA is detected at increased levels after 16 hour hypoxic treatment of rat pheochromocytoma PC-12 and mouse neuroblastoma Neuro-2A cells by northern blot (*Chapter 3.1.2*), and subsequently in these cell lines along with human neuroblastoma SK-N-SH and SK-N-BE(2)C cells by qRT-PCR (*Chapter 3.1.3*). The PC-12 samples also demonstrate the accuracy of the qRT-PCR method in comparison to northern blot, as a similar fold induction was obtained for quantification from both detection methods. The detection of a hypoxic response of *Rgs4* between cells of rat, mouse and human species indicates that this response predates the divergence of humans and rodents in the *Euarchontoglires* clade of mammals. Conservation of such a response implies that upregulation of *Rgs4* mRNA by hypoxia has an important physiological role.

Rgs4, like other HIF targets such as *Vegf*, is upregulated by hypoxia-mimetic chemicals DMOG and DP, which are potent inducers of the HIF pathway. These chemicals inhibit the activity of 2-OG dependent hydroxylases, which includes the enzymes which hydroxylate key residues of HIF-α in normoxia to inhibit HIF function (*Chapter 1.1.2*). Some off-target cellular effects would be expected from treatment with each of these chemicals or hypoxic treatment, yet the response of *Rgs4* to both of these chemicals and hypoxia strongly implicates HIF in the regulation of this gene. One exception was observed, in which *Rgs4* is responsive to hypoxia and DMOG treatment, but not DP in the mouse Neuro-2A cell line (*Figure 3.1*). This result is perplexing, as other HIF responsive genes *Ldha* and *Vegf* are upregulated by DP treatment in the same samples tested, therefore the treatment is activating the HIF pathway as expected. This observation was confirmed by qRT-PCR detection on the same RNA samples (n=3, data not shown). Therefore, the regulation of *Rgs4* or the HIF pathway may be more complex in the Neuro-2A cell line than in the PC12, SK-N-SH or SK-N-BE(2)C cell lines, so for simplicity Neuro-2A cells were not used for further analysis (*Chapter 4*).

Rgs4 is known to be expressed in the cardiovascular system (*Chapter 1.3.6*), and Jin *et al.* (2009) describe detection of both RGS4 and RGS5 protein in Human Umbilical Vein Endothelial Cells (HUVECs). Furthermore, experiments presented in that publication show increased *Rgs5* mRNA and protein levels in hypoxia-treated HUVECs, yet RGS4 protein levels did not change and *Rgs4* mRNA levels were not tested. In the experiments presented in this thesis, HUVECs were compared to a neuroblastoma cell line, using both 3 and 16 hour hypoxia treatments, as the published *Rgs5* response in HUVECs was observed to be maximal at the 3 hour timepoint (Jin *et al.*, 2009). The results in this thesis disagree with the published data for *Rgs5*, as *Figure 3.4* shows a hypoxic response at both timepoints for *Vegf* in HUVECs, but no positive change in either *Rgs4* or *Rgs5* mRNA, and actually a modest but statistically significant decrease in levels of both after 16 hours of treatment relative to *Pol2a*. However, the lack of hypoxic regulation of RGS4 in HUVECs presented by Jin *et al.* (2009) is supported by these results. Additionally, *Rgs4* and *Vegf* respond to hypoxia in SK-N-BE(2)C neuroblastoma cells, but *Rgs5* levels are not significantly responsive at either treatment timepoint. This shows that the response of *Rgs4* to hypoxia is not a general property of RGS encoding genes, and also that *Rgs4* responds to hypoxia only in a subset of cell types in which it is expressed.

The differences between the results presented in this thesis and those published by Jin *et al.* (2009) are difficult to reconcile, but the published results show only a modest 2-fold change in *Rgs5* mRNA, normalised to 28S RNA levels detected before northern transfer, which do not account for slight errors in transfer or probing. Jin *et al.* (2009) also present *Rgs5* promoter reporter studies showing a maximum 2 fold change in relative luciferase activity, so even if this transcriptional response is real it may be difficult to reproducibly detect. The published experiments use an oxygen-regulated chamber to achieve 1% atmospheric oxygen, whereas the experiments presented in this thesis use AnaeroGen sachets in sealed containers, which achieve a final oxygen concentration of below 0.1%, so different hypoxic treatment conditions may also explain the differences in results. Furthermore, Jin et al. (2009) do not present positive controls for hypoxic gene response, so the efficiency of hypoxic treatment and HIF pathway induction in these published experiments is not clear. Nonetheless, our experiments show that the hypoxic response of *Rgs4* in neuroblastoma is of a greater magnitude than that of *Rgs5*, which is not upregulated in either cell type at 0.1% oxygen.

Rgs4 was not detected in many of the other cell types tested in either normoxia or hypoxia, suggesting that the *Rgs4* gene is silenced in these cell types. However, as *Rgs4* transcript is readily detectable in both neuroblastoma and HUVEC samples, but responsive to hypoxia in neuroblastoma and not the endothelial cells, HIF activity on the *Rgs4* gene does not appear to be the only requirement for hypoxic induction for the gene. *Vegf* is responsive to HIF in both cell types, so *Rgs4* may have a more complex mechanism of regulation than other HIF target genes. As *Rgs4* is not silenced in HUVECs, there may be factors which prevent HIF activity on *Rgs4* transcription in a HUVEC context, or alternatively factors which allow HIF activity on *Rgs4* in neuroblastoma cells.

These experiments suggest that the hypoxic response of *Rgs4* is limited to certain cell types. Although a thorough set of *Rgs4*-expressing cells has not yet been tested, the only cell types discovered with a positive *Rgs4* hypoxic response have been derived from neuroblastoma or pheochromocytoma. These are both cancers of the sympathetic nervous system (SNS), and these derived cells are both able to be differentiated to neural cells with Nerve Growth Factor (NGF) treatment, so *Rgs4* hypoxic response may be limited to neural-like cells or even restricted to only cells of the SNS. Alternatively, there may be other types of cells which respond to hypoxia with upregulation of *Rgs4* transcript that have not yet been tested. To test these alternatives, a more comprehensive approach would involve *in situ* hybridisation for *Rgs4* using mice treated with systemic hypoxia, to test for tissues which exhibit *Rgs4* hypoxic response. *In situ* hybridisation could also be used to compare *Epas1*$^{-/-}$ and wildtype mice, as *Epas1*$^{-/-}$ mice can develop to birth in certain genetic backgrounds (Scortegagna *et al.*, 2003a). In this case, impaired HIF signalling may affect normal *Rgs4* expression in some tissues.

To test whether the *Rgs4* response is specific to cancerous cells, the sympathetic nervous system or a general phenomenon for neural-like cells, mouse embryonic stem (mES) cells in different states of differentiation can also be tested. *Figure 3.3a* shows that *Rgs4* is readily detectable in mES cells after 3 days of neural differentiation in N2B27 medium, so cells at representative stages of this process could be treated with normoxia or hypoxia and tested for *Rgs4* expression. Finally, elucidation of the molecular mechanisms behind HIF activation of *Rgs4* in neuroblastoma may determine whether additional factors are required for this response, which may in turn aid in prediction of cell types express increased levels of *Rgs4* in hypoxia. Experiments on this approach are described in *Chapter 4.*

3.2.2. *Rgs4* is Regulated by Both HIF Forms

To directly test the role of endogenous HIF in the regulation of *Rgs4*, siRNA duplexes antisense to HIF-1α or HIF-2α were transfected to human neuroblastoma cells. Two siRNA sequences against *Hif1a* and *Epas1* were used to control for off-target effects, but as these sequences have been used before in several publications (Chen *et al.*, 2009; Lau *et al.*, 2007; Raval *et al.*, 2005; originally by Sowter *et al.*, 2003), specificity to the respective HIF-α transcripts was not expected to be problematic. Despite problems visualising HIF-2α knockdown by western blot due to poor antibody specificity, knockdown of both proteins after DP treatment was detected and transcripts of both HIF-α encoding genes were observed to be significantly lower when targeted with either primary siRNA sequences (*Figure 3.5a,b*). Only partial knockdown of *Hif1a* or *Epas1* transcripts were observed by qRT-PCR, although some additional knockdown effect may be expected through inhibition of message translation, as protein knockdown of HIF-1α appears to be greater than that observed at the message level.

Known HIF target genes *Vegf* and *Dec1* both exhibit a smaller response to hypoxia when siRNA against both HIF-α subunits is employed, showing that the HIF-α knockdown is functional but still incomplete. However, considerable variation between experiments occurred in terms of knockdown efficiency, despite repeated attempts at optimisation. In the case of experiments using the secondary siRNA sequences and DP treatment (*Figure 3.5c*), inconsistent knockdown was observed which could possibly be attributed to varying transfection efficiencies resulting from differences between frozen stocks of the same cell lines. The same sequences acted at a greater efficiency on cells treated with hypoxia in a later experiment, although a modified protocol using a different transfection reagent was used. These problems may be remedied by viral transfer of short hairpin-encoding genes, or possibly use of conditional *Hif1a* or *Epas1* mouse ES cells differentiated to neural precursor cells, if such cells behave in a similar fashion to these neuroblastoma cell lines in terms of hypoxic *Rgs4* regulation

Cells transfected with siRNA were exposed to 8 hours of treatment with either DP (*Figure 3.5*) or hypoxia (*Figure 3.6*), a treatment period at which protein levels of both HIF-1α and HIF-2α are expected to be strong (Holmquist-Mengelbier *et al.*, 2006). At this timepoint, knockdown of either HIF-1α or HIF-2α during DP treatment resulted in a modest yet significant reduction in the response to either treatment of *Rgs4*, while

knockdown of both HIF-α subunits resulted in a larger reduction. Positive controls *Vegf* and *Dec1* were affected only by siRNA directed against HIF-1α. Therefore, in terms of DP response, *Rgs4* is regulated by both HIF-1 and HIF-2, such that only loss of both forms effectively prevents hypoxic gene induction, while *Vegf* and *Dec1* act as HIF-1 targets alone.

This result is somewhat contrary to the preliminary results observed in the inducible HIF-α TetON PC-12 cell lines, where overexpression of HIF-2α resulted in increased *Rgs4* mRNA, while overexpressed HIF-1α did not (*Figure 1.4*). However, knockdown of endogenous proteins is a more biologically relevant experiment to determine the effect of each subunit than overexpression of a cDNA, as overexpressed proteins can overload other endogenous regulatory pathways or bind DNA with lowered specificity and therefore act in a non-physiological manner. Therefore, the most logical conclusion is that both HIF forms are capable of transactivating *Rgs4* expression in neuroblastoma. There is, however, a possibility that there are differences in HIF selection between cell types or even species, as these siRNA experiments have only been performed in human neuroblastoma and not the rat pheochromocytoma cells.

However, inconsistent results were observed when the HIF-α subunits are targeted by siRNA in hypoxic rather than DP treatment. Knockdown of either HIF-α subunit successfully inhibited the hypoxic regulation of *Vegf* and *Dec1*, yet only a small and inconsistent decrease in *Rgs4* regulation was observed. The reason for this difference between hypoxic and DP treatments is not clear. Different HIF target genes may not have the same sensitivity to a partial loss of HIF-α protein by siRNA, such that a more efficient knockdown of HIF is required to significantly inhibit the response of *Rgs4*. This would be supported by the variability observed, as in some independent experiments good inhibition of *Rgs4* hypoxic response was observed, while in other cases no change could be detected at all. Alternatively, there may be other hypoxia-responsive but not DP-responsive pathways that can compensate for the loss of HIF in regulation of *Rgs4* in hypoxia only.

One of the few transcription factors to have been shown to directly upregulate *Rgs4* is NF-κβ. Treatment of rabbit smooth muscle cells with interleukin-1β (IL-1β) causes phosphorylation of IKK2 and degradation of Iκβα, two events which form part of the canonical NF-κβ activation pathway, resulting in modestly increased *Rgs4* mRNA and protein levels (Hu *et al.*, 2008). An NF-κβ binding site was discovered in the rabbit *Rgs4*

5’ proximal promoter 179bp upstream of the transcription start site, where NF-κβ subunit p65 binding could be detected by chromatin immunoprecipitation (Li *et al.*, 2010b). Although this site is not completely conserved between rabbit and human sequences, luciferase reporter assays indicate that IL-1β treatment is able to activate transcription from both the rabbit and human *Rgs4* promoters (Li *et al.*, 2010b). NF-κβ signalling is also known to be activated by hypoxic treatment in some cellular contexts, apparently through a tyrosine kinase pathway (Koong *et al.*, 1994a; Koong *et al.*, 1994b). However, the reporter assays described in *Chapter 4.1.6* do not detect any hypoxic response in an area of the *Rgs4* promoter which would cover the equivalent NF-κβ binding site from the rabbit genome (Li *et al.*, 2010b). Therefore, hypoxic response compensation by NF-κβ in this context is unlikely.

HIF-1α and HIF-2α are known to be stabilised differentially in these neuroblastoma cell lines. HIF-1α is the predominant form in acute hypoxia, rapidly reaching maximal protein levels within 2 hours of exposure to hypoxia, then gradually decreasing after 8 hours, while HIF-2α accumulates to a maximum after 24 or more hours of hypoxic treatment (Holmquist-Mengelbier *et al.*, 2006). Therefore, genes under preferential control of either HIF form may be expected to show a related pattern of induction across a hypoxia timecourse. Experiments performed in the next chapter give results which imply that *Rgs4*, *Vegf* and *Dec1* are all target genes of both HIF-1 and HIF-2, as these target genes are upregulated in both acute (1-4 hours) and chronic (8-16 hours) hypoxic treatments (*Figure 4.3*).

The hypoxic regulation of HIF-α encoding mRNAs *Hif1a* and *Epas1* is an interesting result, as it may shed some light on the mechanisms behind the aforementioned differences in HIF-1α and HIF-2α protein levels over hypoxic treatment time. *Hif1a* message was detected at a decreased level after 8 hours treatment with hypoxia (*Figure 3.6*) or DP (*Figure 3.5*), while *Epas1* message was increased in the same treatments. As inhibition of the HIF-regulatory PHD and FIH enzymes is the intended cellular effect of DP treatment, the response of *Hif1a* and *Epas1* mRNA to DP implies that this regulation may be downstream of post-translational HIF activation by the PHD and FIH enzymes. In this way, a negative (*Hif1a*) or positive (*Epas1*) feedback loop may be affecting the changes in HIF-a protein observed in chronic hypoxia. Such regulation of the HIF-α encoding genes is not commonly reported for most cell lines, however Holmquist-Mengelbier *et al.* (2006) report similar positive changes for *Epas1* mRNA in SK-N-

BE(2)C cells at 5% and 1% hypoxia, yet more modest negative changes in *Hif1a*. The exact nature of this regulation is expanded upon in *Figure 4.4a* and discussed in greater detail in *Chapter 4.2.3*.

Chromatin immunoprecipitation could help to further define the role of HIF-1 and HIF-2 in *Rgs4* regulation, however it has been difficult to determine a HIF binding site at the *Rgs4* locus (see *Chapter 4.2.2*), and more importantly, presence of HIF at a locus is not sufficient for activation of transcription, as target gene selection appears to act after DNA binding (Hu *et al.*, 2007; Lau *et al.*, 2007; Mole *et al.*, 2009). As previously described, mouse knockout models could be used to further pursue the *in vivo* specificity between HIF-1 and HIF-2 in transactivation of *Rgs4*.

Taken together, the data presented within this Chapter implicate both HIF-1α and HIF-2α in the regulation of *Rgs4* by hypoxia. Furthermore, the hypoxic regulation of *Rgs4* is cell type specific, unlike other known HIF target genes such as *Vegf*. HIF is likely to play a major role in hypoxic regulation of *Rgs4*, however the contribution of other unknown hypoxia-responsive pathways cannot be determined without a more complete experimental protein knockdown system.

4. The Molecular Mechanism of *Rgs4* Response to Hypoxia

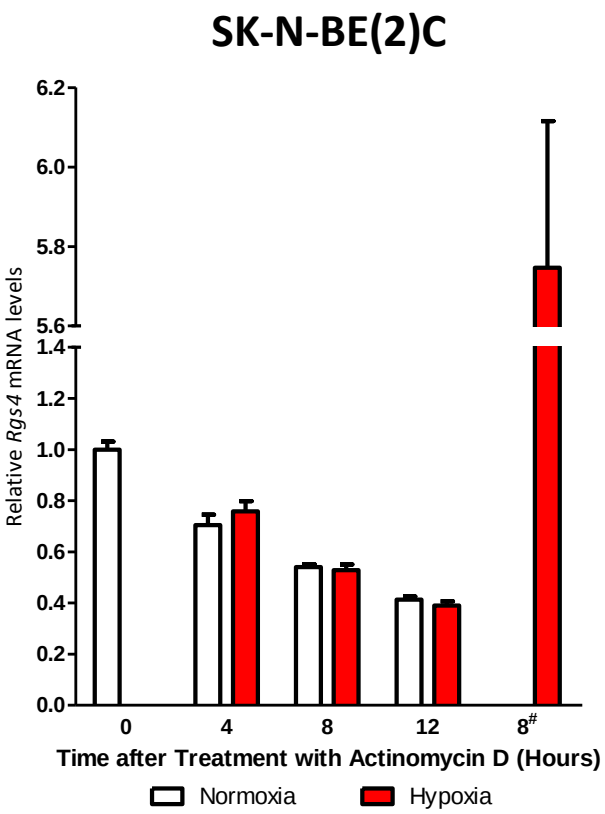
4.1.1. Preamble

As *Rgs4* is regulated in some cell types by hypoxia and the HIF pathway, and the HIF transcription factors can directly bind and activate transcription of promoters, the simplest hypothesis is that HIF directly activates transcription of *Rgs4* in hypoxia. However, HIF is also known to activate transcription of other transcription factors such as DEC1, and specific micro RNA (miRNAs) transcripts, and could feasibly activate expression of RNA stabilising proteins, all of which could in turn regulate *Rgs4* mRNA levels as secondary effectors. There are also examples of RNA-binding proteins which can alter stability or translation of target mRNAs in response to hypoxia independent of the HIFs, such as HuR (reviewed by Masuda *et al.*, 2009).Therefore, in order to implicate direct activity of HIF or other transcription factors on *Rgs4* transcription, experiments were designed to test whether *Rgs4* is regulated in hypoxia by transcriptional or post-transcriptional mechanisms, whether the response of *Rgs4* occurs in a similar timeframe to other direct HIF targets, and whether a hypoxia responsive enhancer element could be found at the *Rgs4* locus.

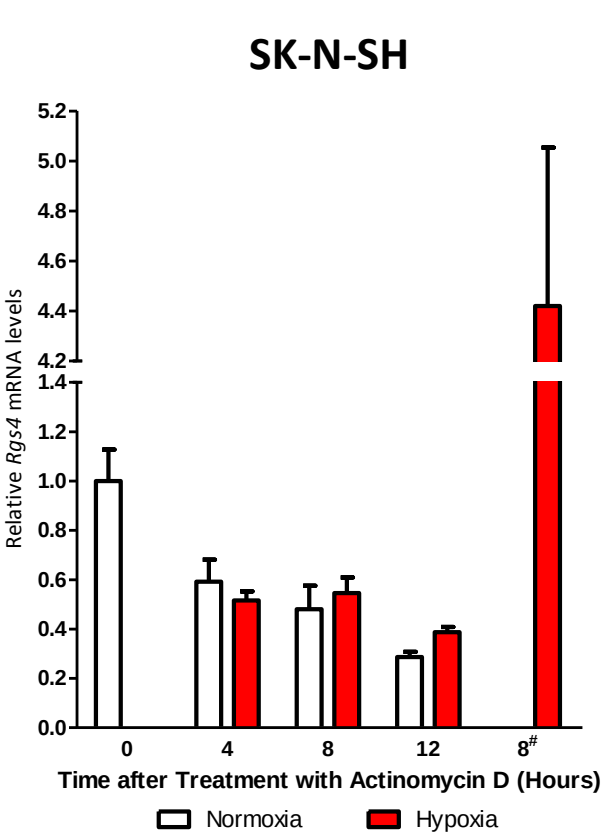
4.1.2. *Rgs4* Hypoxic Response is Dependent on Transcription

To test whether hypoxic *Rgs4* mRNA accumulation was dependent on transcription, RNA degradation assays were performed in both SK-N-BE(2)C and SK-N-SH cells. Cells were plated and treated with 4μg/ml of actinomycin D to block transcription, then subjected to a timecourse of hypoxic or normoxic treatment. *Rgs4* mRNA was quantified by qRT-PCR relative to an untreated normoxic control. Untreated cells were also included in the 8 hour hypoxia treatment as a positive control for normal *Rgs4* regulation. No difference in *Rgs4* levels was observed between the actinomycin D treated or untreated cells at any of the normoxic or hypoxic timepoints in SK-N-BE(2)C (n=2) or SK-N-SH (n=2) cells (*Figure 4.1a,b*).

a)


SK-N-BE(2)C
Relative Rgs4 mRNA levels
6.2
6.0
5.8
5.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
8#
Time after Treatment with Actinomycin D (Hours)
Normoxia
Hypoxia

b)


SK-N-SH
Relative Rgs4 mRNA levels
5.2
5.0
4.8
4.6
4.4
4.2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
8#
Time after Treatment with Actinomycin D (Hours)
Normoxia
Hypoxia

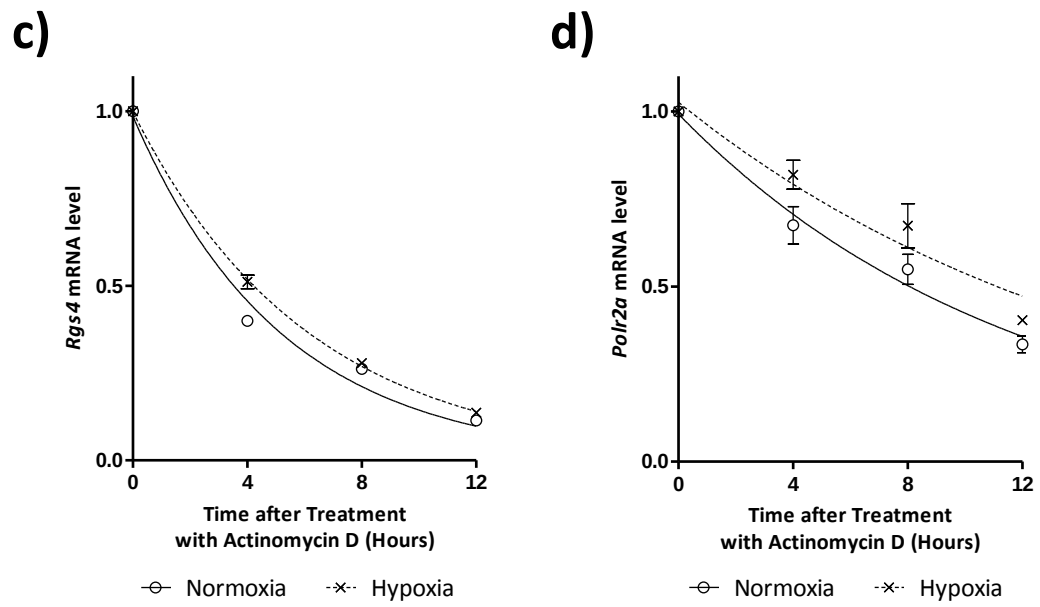


Figure 4.1. (**a**) SK-N-BE(2)C cells were treated with 4μg/ml actinomycin D prior to treatment for 4, 8 or 12 hours with normoxia or hypoxia. Actinomycin D-untreated cells were also used to determine control normoxic (*0 hours*) and hypoxic (*8# hours*) *Rgs4* levels. *Rgs4* mRNA levels were quantified using qRT-PCR relative to *Polr2a*, then normalised to normoxic levels. One representative of *n*=2 is shown. (**b**) Experiments from (a) were replicated using SK-N-SH cells, a representative of *n*=2 is shown. For SK-N-BE(2)C data from (a), *Rgs4* (**c**) and *Polr2a* (**d**) mRNA levels relative to total RNA were also calculated from C(t) values without normalisation to reference gene relative to normoxia. The mean detection with standard error (*n*=2) is plotted to give a one-phase decay using GraphPad Prism 5. Estimated mRNA half-lives are described in the text.

The mean detected levels of *Rgs4* and *Polr2a* without normalisation to reference gene for SK-N-BE(2)C cells were also plotted against time for normoxic and hypoxic treatments, in order to calculate the rate of mRNA degradation by fitting the these points to a one phase exponential decay curve (*Figure 4.1c,d*). For this graph, raw C(*t*) values for *Rgs4* and *Polr2a* were converted to relative amounts by setting the normoxic level of transcript in each experiment to 1 (such that for normoxic samples, $N_0 = 1$ giving an aribitrary threshold value N_c, see *Equation 2.1, Chapter 2.2.6*). Given that the same amount of total RNA was used in cDNA generation, and the same amount of cDNA was used in each qRT-PCR reaction, the half-life of *Rgs4* and *Polr2a* mRNA can be estimated for each curve. *Polr2a* is unresponsive to hypoxia, so no difference is expected in its degradation in either condition, while any differences in *Rgs4* half-life would be due to transcription-independent regulation of *Rgs4* message stability. The one-phase decay curve for *Rgs4* (*Figure 4.1c*) gave 99% confidence intervals for message half-life of 3.53 - 4.03 hours in normoxia, and 3.96 – 4.55 hours in hypoxia. For comparison, the calculated half-lives for *Polr2a* message were 6.57 – 10.72 in normoxia, and 8.22 – 15.39 hours in hypoxia (*Figure 4.1d*). Similar half-lives were determined by applying the same calculations to the SK-N-SH cell line data (not shown).

These data demonstrate that there is no substantial change in *Rgs4* mRNA stability in response to hypoxia, and the small change that is observed is similar to that observed with *Polr2a*. Given the previously demonstrated robust induction of *Rgs4* (*Figure 3.2*), it is clear from the results presented here that the hypoxic accumulation of *Rgs4* mRNA requires active transcription.

4.1.3. The *Rgs4* 3'UTR does not Confer Hypoxic Regulation

The 3' Untranslated Region (3'UTR) of *Rgs4* has regions of high conservation through mammalian evolution according to the UCSC genome alignment browser (Kent *et al.*, 2002). This indicates that there may be functional elements within the 3'UTR which could regulate the stability of *Rgs4* mRNA. Although the previous experiments show transcription is required for hypoxic induction, there is a possibility that hypoxia activates transcription of a miRNA or RNA-binding protein.

In order to test for hypoxia-responsive RNA elements in the human *Rgs4* 3'UTR, the entire 3'UTR was cloned to the *XbaI* site downstream of the firefly luciferase (luc^+) coding sequence in the pCI_FL plasmid, upstream of the SV40 late polyadenylation signal. This plasmid has a CMV promoter driving expression of luciferase, as well as a chimeric intron in the 5'UTR of luc^+ in order to ensure processing by post-transcriptional machinery. The pCI_FL(hRgs4-3'UTR) construct was transfected to SK-N-BE(2)C cells and tested for hypoxic luciferase response in comparison to pCI_FL which contains no 3'UTR, relative to a constitutive renilla luciferase expressing plasmid using the Dual Luciferase system (*Figure 4.2*). Over three independent experiments a small change in relative luciferase levels was observed between normoxia and hypoxia treatments, but this was observed with or without the presence of the *Rgs4* 3'UTR. Furthermore, the change observed is too small to account for the level of *Rgs4* mRNA induction observed in SK-N-BE(2)C cells by qRT-PCR (*Figure 3.2*), indicating that the *Rgs4* 3'UTR is unlikely to make a substantial contribution to the hypoxic induction of *Rgs4* mRNA or RGS4 protein. Notably, presence of the *Rgs4* 3'UTR did lower relative luciferase readings by around 2-fold in both conditions compared to the control plasmid, which suggests the presence of an inhibitory element within this 3'UTR.

4.1.4. *Rgs4* is Co-regulated with Other Known HIF Targets

The above experiments show that hypoxic regulation of *Rgs4* mRNA is dependent on transcription (*Chapter 4.1.2*) and not dependent on the *Rgs4* 3'UTR (*Chapter 4.1.3*), and that *Rgs4* is not only responsive to hypoxia but also hypoxia mimetics that activate the HIF pathway (*Chapter 3.2.2*). Therefore, the simplest and most likely hypothesis is that *Rgs4* is regulated at the transcriptional level as either a direct HIF target, or as a secondary response through other transcription factors under the direct transcriptional control of HIF. To ascertain which of these mechanisms is more likely to be occurring, a series of timecourse experiments were performed.

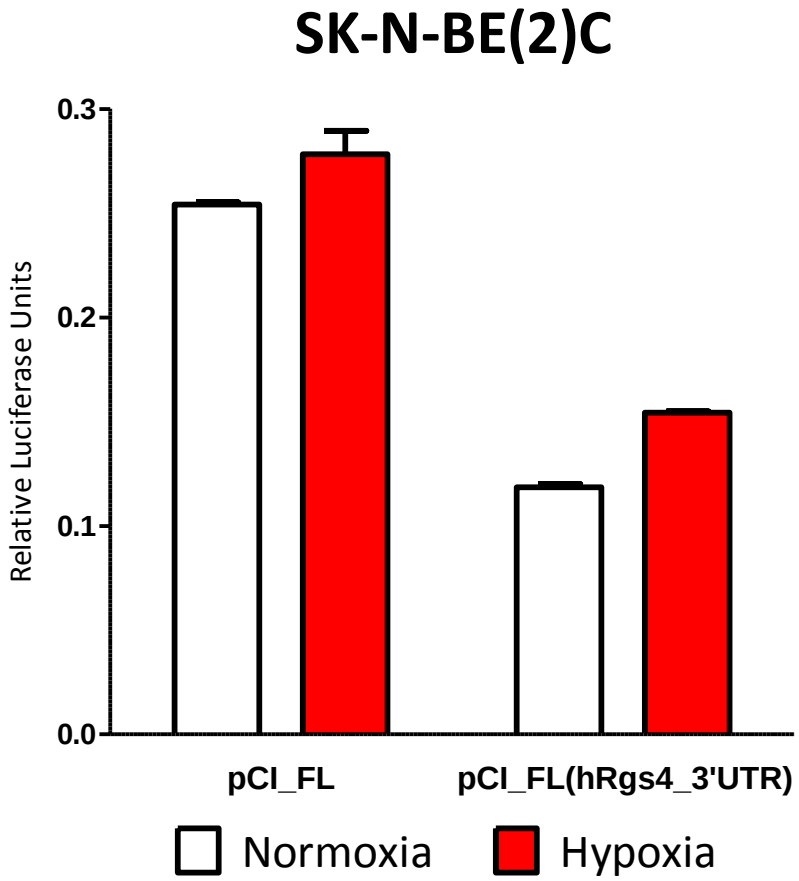


Figure 4.2. SK-N-BE(2)C cells were plated in triplicate in 24-well trays and transfected twice sequentially with 100ng of pCI_FL or pCI_FL incorporating the 3'UTR of human *Rgs4* between the *luc*$^{+}$ coding sequence and polyadenylation sequence (pCI_FL(hRgs4_3'UTR)), along with 25ng of phRLCMV as control for transfection efficiency and survival. Separate wells were also transfected with pHRE4 as a positive control for hypoxia treatment (see *Figure 4.6d*, as both experiments were performed concurrently). Cells were treated with hypoxia for 16 hours, then lysed in Passive Lysis Buffer for analysis using the Dual Luciferase System (Promega). Mean Relative Luficerase Units with standard deviation from 3 replicate wells of one experiment are shown as a representative of n=3.

Other direct HIF target genes such as *Vegf* and *Dec1* are well characterised in terms of their response to HIF, as both have defined HREs in their promoters which have been verified by chromatin immunoprecipitation (Gray *et al.*, 2005; Holmquist-Mengelbier *et al.*, 2006), reporter assays and electrophoretic mobility shift assays (Liu *et al.*, 1995; Miyazaki *et al.*, 2002). *Dec1* encodes DEC1 (also known as BHLHB2), a direct negative regulator of *Mlh1* transcription (Nakamura *et al.*, 2008), making *Mlh1* repression an example of a secondary HIF responsive gene. Therefore, if *Rgs4* is a direct HIF target gene, the time between hypoxic treatment and response should be similar to that of *Vegf* and *Dec1*, whereas if *Rgs4* is regulated by a secondary HIF-responsive transcription factor, it will respond in a similar time to *Mlh1*.

Three independent timecourse experiments were performed, where SK-N-BE(2)C cells were exposed to increasing periods of DP treatment before RNA harvest and qRT-PCR (*Figure 4.3*). *Rgs4* mRNA was detected at significantly increased levels after only 1 hour of DP treatment, as were *Vegf* and *Dec1*. The levels of these transcripts continued to increase until a maximum at 4 hours, after which they remained effectively constant. However, levels of secondary response gene *Mlh1* (indirectly repressed by HIF) were not significantly altered until 4 hours of hypoxic treatment, when levels were significantly lowered, reaching a minimum after 8 hours. Therefore, *Rgs4* response time to DP is consistent with it being a direct HIF target gene.

4.1.5. *Hif1a* and *Epas1* are Co-regulated with Secondary HIF Targets

SK-N-BE(2)C cells are known to display an alternative pattern of HIF-α stabilisation to most cell types during prolonged hypoxia. As described previously (*Chapter 1.1.4, Figure 1.2d*), in short-term hypoxia these cells stabilise HIF-1α rapidly, yet HIF-2α protein stabilisation is delayed. After 8 hours of continuous hypoxia, HIF-1α protein levels gradually decrease, while HIF-2α becomes more prominent (Holmquist-Mengelbier *et al.*, 2006). Altered *Epas1* mRNA levels in hypoxia have previously been described for this cell line, although no change in *Hif1a* levels has been reported (Holmquist-Mengelbier *et al.*, 2006). To test whether this effect may be related to altered mRNA levels, we subjected the three independent SK-N-BE(2)C samples generated for *Figure 4.3* to qRT-PCR for *Hif1a* and *Epas1* transcripts (*Figure 4.4a*). *Epas1* message was significantly upregulated

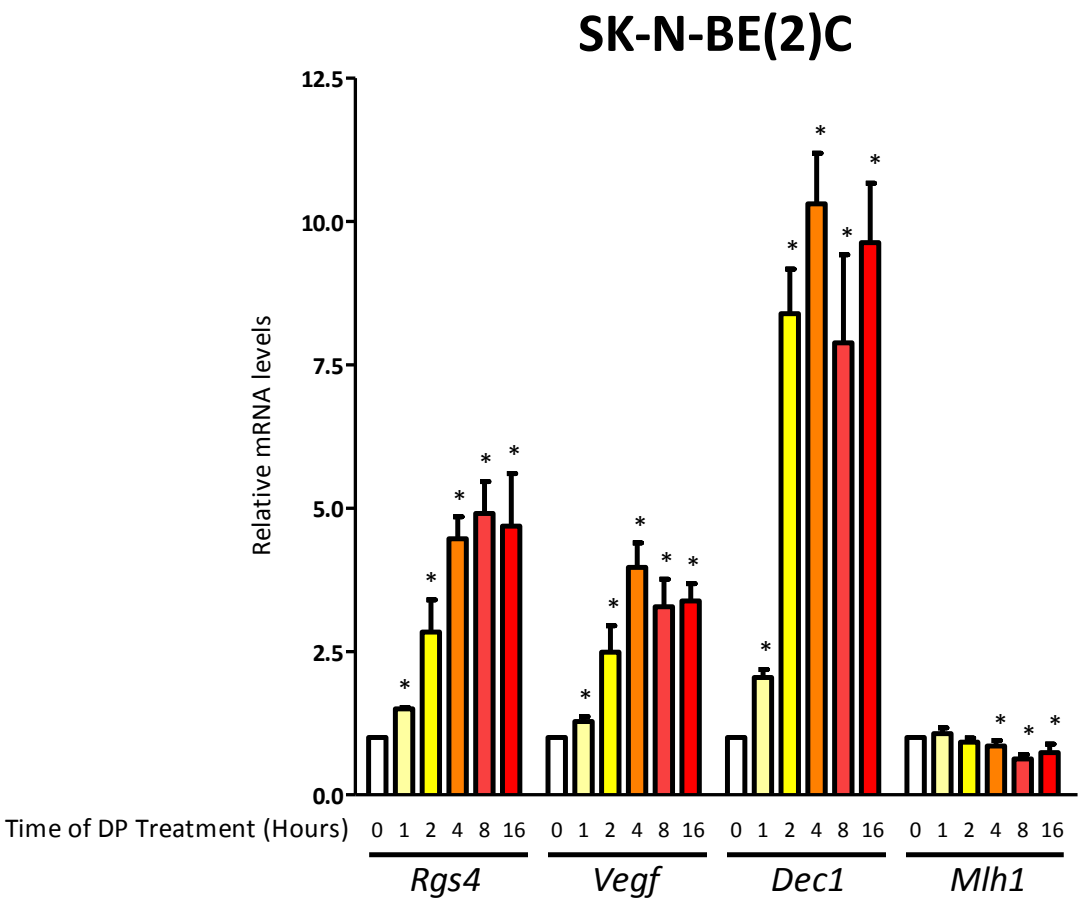


Figure 4.3. SK-N-BE(2)C cells were treated with 100μM DP for 1, 2, 4, 8 or 16 hours in comparison to an untreated (0 hours) control. *Rgs4*, *Vegf*, *Dec1* and *Mlh1* message levels were quantified by qRT-PCR relative to reference gene *Polr2a*, then normalised to normoxic levels. Data is presented as the mean and standard deviation from three independent experiments. Asterisks indicate 99% confidence in difference from normoxic levels.

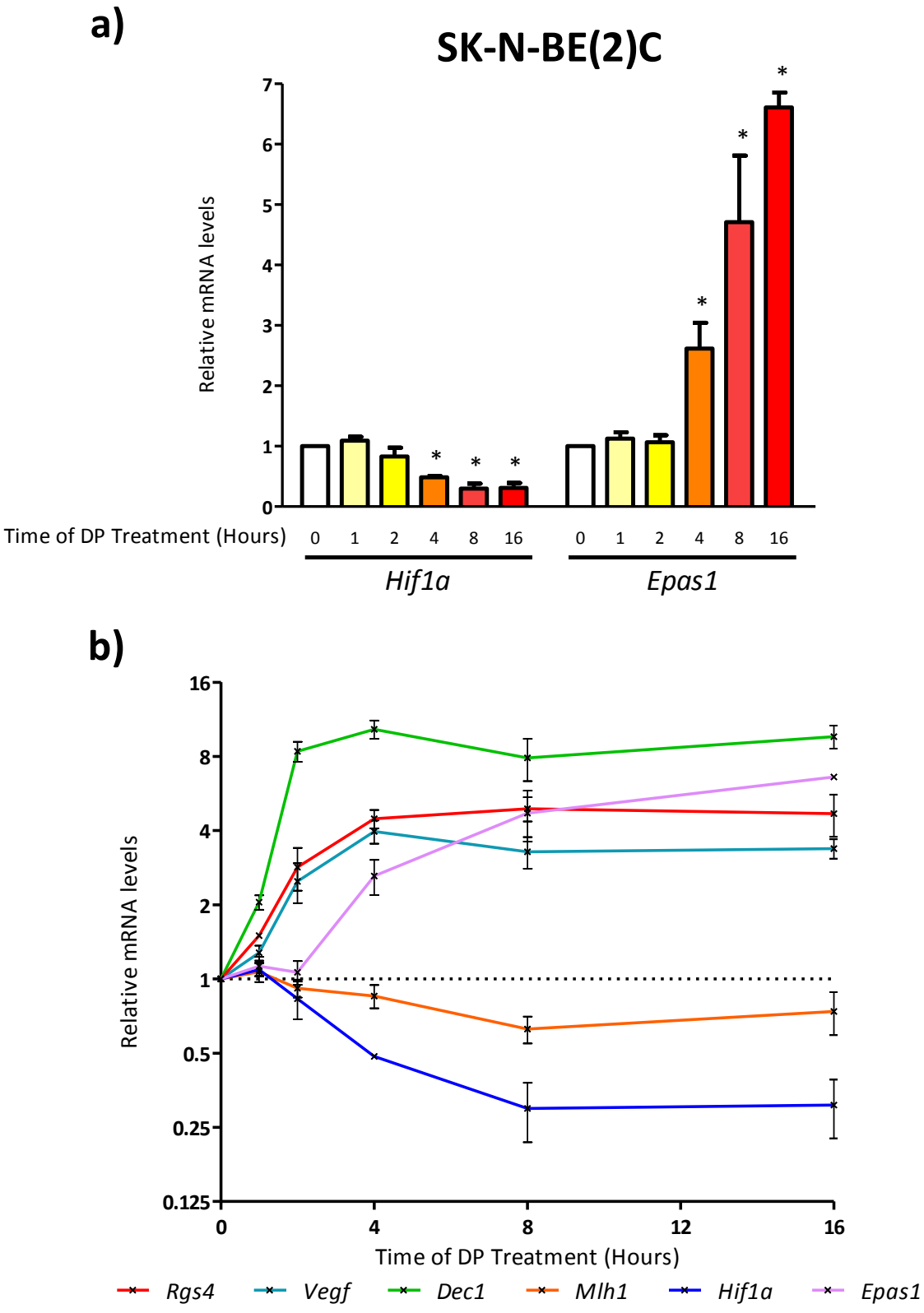


Figure 4.4. (**a**) SK-N-BE(2)C cDNA samples tested in *Figure 4.3* were also analysed for *Hif1a* and *Epas1* message levels, relative to *Polr2a* and negative control levels. Asterisks indicate 99% confidence in difference from control levels, *n*=3. (**b**) Data from (a) and *Figure 4.3* plotted together on a proportional *x*-axis and $\log_2$ *y*-axis.

only after 4 hours of hypoxic treatment, to a maximum induction of approximately 6.5 fold at or after 16 hours. Similarly, *Hif1a* message did not change until 4 hours of hypoxic treatment, where upon levels were significantly decreased to a minimum 3-fold reduction at and after 8 hours. *Figure 4.4b* depicts this data on a proportional *x*-axis, with a $\log_2$ y-axis to emphasise similarities between message level increases and decreases. Both *Hif1a* and *Epas1* mRNA levels respond to hypoxia at a similar time to *Mlh1*, a gene regulated indirectly by HIF, indicating that this regulation is likely to be a secondary response to hypoxia.

4.1.6. Reporter Assays do not Detect an *Rgs4* Proximal Hypoxia Responsive Enhancer

The evidence presented in *Chapters 4.1.2, 4.1.3* and *4.1.4* strongly suggests that *Rgs4* mRNA hypoxic regulation is mediated by direct binding of HIF and activation of transcription at the *Rgs4* locus, rather than through secondary effectors. As this response is conserved between rat, mouse and human cell lines (*Chapter 3.1.2 and 3.1.3*), a bioinformatics search was performed for HIF binding sites (HREs) at the *Rgs4* locus in both the rat and human genome. HIF is able to bind and induce transcription from cloned HREs in transient transfection reporter gene assays, and there are numerous experiments in the literature which use this method to determine HRE positions (for examples, see Firth *et al.*, 1995; Liu *et al.*, 1995; Miyazaki *et al.*, 2002), many of which have been later verified by ChIP experiments. Therefore, we cloned overlapping regions of the rat and human *Rgs4* loci and tested them for hypoxic response in luciferase reporter assays, in an attempt to keep the search as unbiased as possible.

The minimal HRE consensus sequence has been previously determined from known genomic HIF binding sites as RCGTG (Wenger *et al.*, 2005), which can occur in either forwards or reverse orientation relative to a Transcription Start Site (TSS). However, due to their short length RCGTG sequences occur frequently in the genome, and most of these sites do not act as functional HREs. The other factors determining the whether a RCGTG sequence can bind HIF functionally are mostly unknown, although other nearby sequences and binding factors do have some role (see *Chapter 1.1.3*). The majority of HREs are found within 2kb of the TSS of a regulated gene (Mole *et al.*, 2009), but there are

examples of more distant functional HREs, such as that of the *Phd3* gene which is found over 10kb from its TSS (Pescador *et al.*, 2005).

Various fragments of the rat *Rgs4* proximal promoter were amplified and cloned from PC-12 genomic DNA by PCR of sequences 5kb upstream of the TSS (*Figure 4.5a*). Ten RCGTG sequences of either orientation were identified in the 5kb rat *Rgs4* promoter region. Amplified fragments were cloned to pGem-T Easy for sequencing and comparison to expected sequences from the UCSC Genome Browser, then subcloned to the pGL3-basic luciferase reporter construct. Fragments were cloned into pGL3-basic such that the TSS of *Rgs4* and a small sequence of the 5'UTR were ligated to the *NcoI* site at the start of the *luc*$^+$ (firefly luciferase) coding sequence, in order to replace the entire pGL3 5' gene flanking sequence and provide the most native promoter context possible.

These pGL3-*Rgs4* plasmids were transfected into PC-12 cells in tandem with a renilla luciferase constitutive expression vector (pRLTK) and tested for response to 16 hours of hypoxic treatment by the Dual Luciferase system (*Figure 4.5b*). No region of the 5kb sequence tested was detectably responsive to hypoxia in two independent experiments, yet the shortest *A* fragment was clearly a stronger promoter for *luc*$^+$ than the longer variants. A previously constructed plasmid containing 4 tandem copies of the *Epo* HRE upstream of *luc*$^+$ was used as a positive control for hypoxic treatment and endogenous HIF activity, and displayed strong hypoxic response, while unmodified pGL3-basic served as a negative control.

Considering that no functional hypoxia responsive element could be detected in the 5kb promoter of the rat *Rgs4* locus, it was decided to widen the search when testing the human *Rgs4* locus. PCR was used to amplify overlapping genomic fragments from SK-N-BE(2)C derived genomic DNA covering 32.9kb of the locus, starting from 15.3kb upstream of the *Rgs4-1* TSS through to 9.7kb downstream of the transcript poly-adenylation site (*Figure 4.6a*). This region was scanned for the minimal HIF-binding RCGTG sequence in either orientation, resulting in 27 hits (4 of which relate to 2 palindromic CACGTG sequences). Conservation between mammalian species was also analysed using the UCSC genome browser, showing some short conserved sequences in the *B*, *D* and *E* regions. However, as known HREs for other HIF responsive genes were not necessarily depicted by the Genome Browser as being conserved, we decided not to discriminate between genomic sequences based on predicted features.

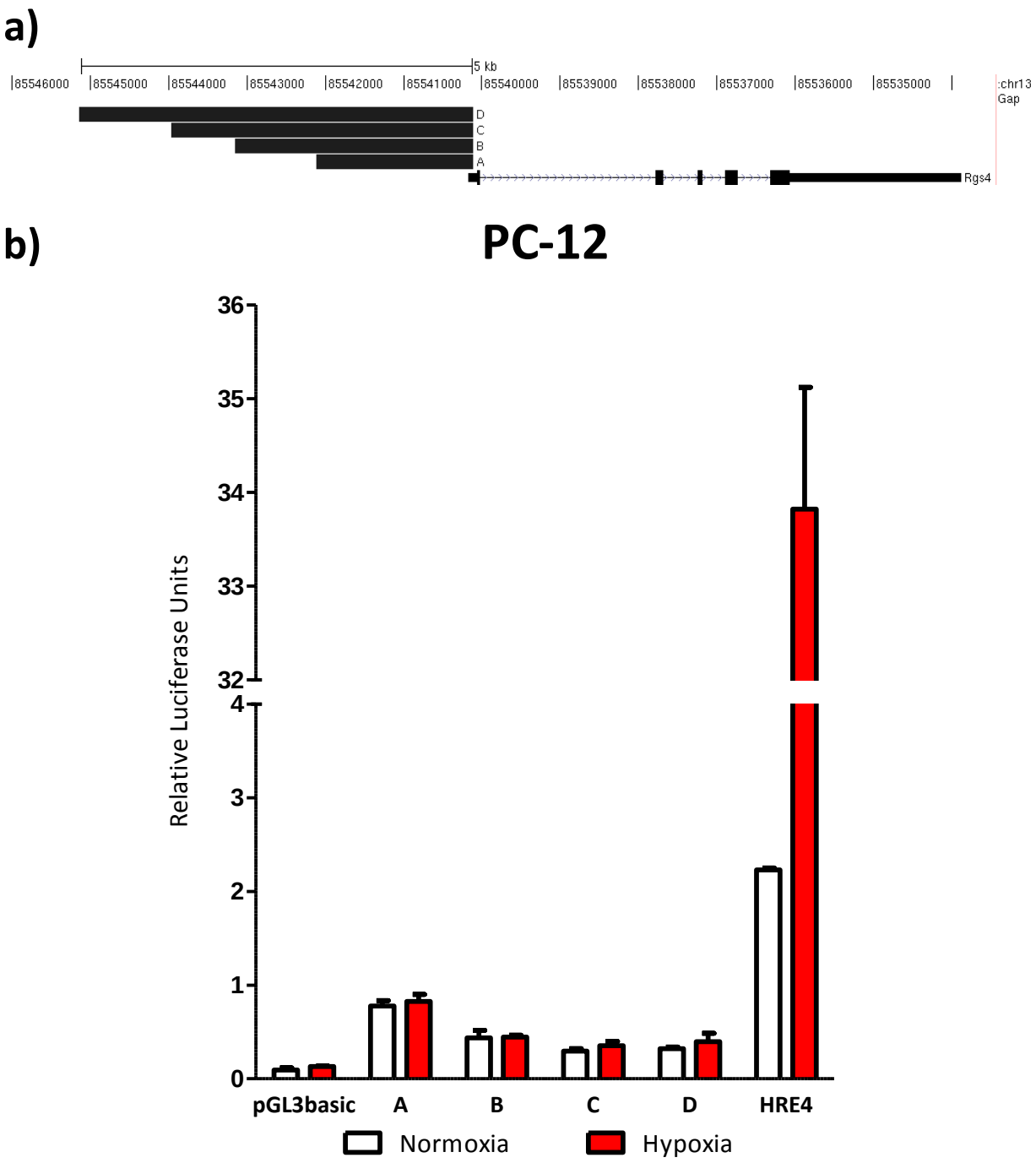
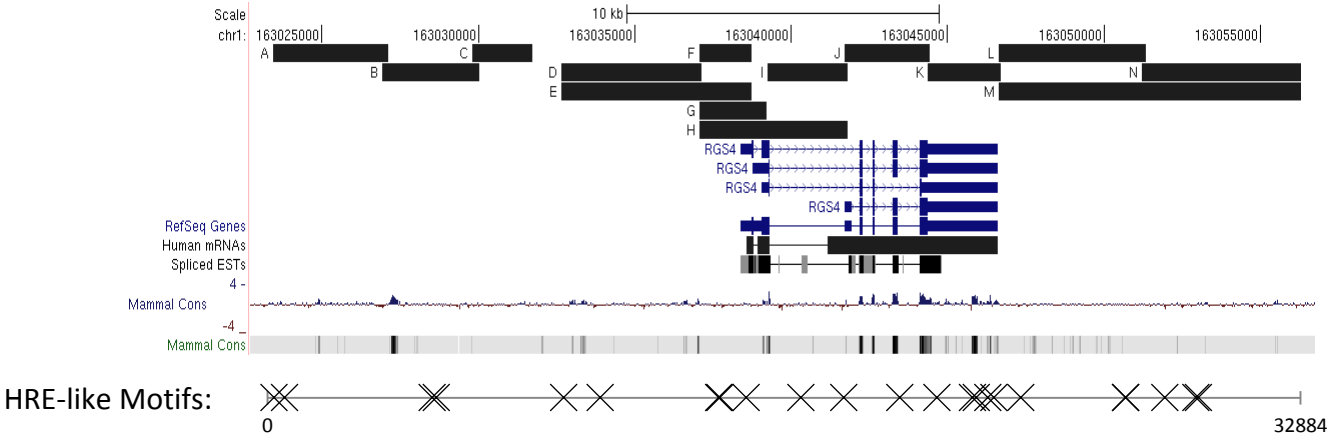


Figure 4.5. (**a**) Diagram of the rat *Rgs4* locus, with promoter regions selected for analysis shown. Regions corresponding to A-D were amplified by genomic PCR and cloned into pGL3basic. (**b**) Reporter gene assay of pGL3-*Rgs4* constructs. PC-12 cells were plated in triplicate in 24-well trays and transfected with 100ng of pGL3basic, or pGL3basic incorporating the sequences A-D depicted in (**a**), while pHRE4 was transfected separately as a positive control for hypoxic induction. 100ng of pRLTK was included in all transfections as control for transfection efficiency and survival. Cells were treated with hypoxia for 16 hours, then analysed using the Dual Luciferase System (Promega). Mean Relative Luciferase Units for a representative of two independent experiments with standard deviation between replicate wells is depicted.

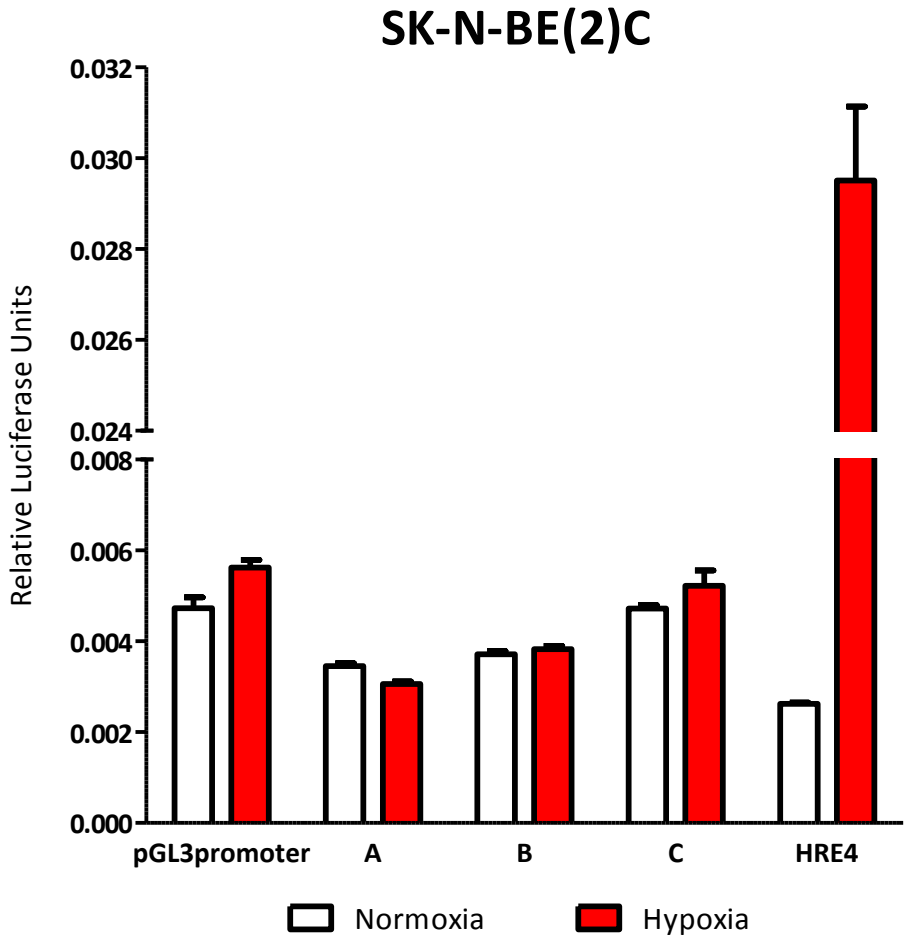
Sequences *E*, *F* and *G* contain TSSs, and were therefore subcloned to the *NcoI* site of pGL3-basic, directly upstream of the *luc*$^+$ coding sequence, to test for promoter and enhancer activity. Sequence *G* contains three TSS according to the UCSC genome browser, which give rise to three different transcripts, one of which results in an extended coding sequence. Therefore, transcription from this most distal TSS would result in a spurious coding sequence before the luciferase gene coding sequence, while transcription for either of the other two TSSs would result in normal luciferase coding sequences. To control for this, constructs *E* and *F* were generated to include only the most distal TSS, and therefore test for transcription at that promoter only. All other fragments do not include a TSS, and were therefore subcloned upstream of the SV40 promoter of the pGL3-promoter plasmid to determine whether they contained enhancers. Sequence *H* also includes the three TSSs described for sequences *E*, *F* and *G*, but places them in the enhancer context. As there is a TAA stop codon in frame at the start of the first shared intron, the effect of transcription on *luc*$^+$ expression from the TSS within sequence *H* should be minimal, leaving only enhancer effects on transcription from the SV40 promoter.

Unfortunately, one region between sequences *C* and *D* could not be cloned or tested. Despite repeated attempts to clone this region, either in isolation or together with sequences *B* or *C*, ligation of this sequence produced plasmids which inhibited bacterial growth, making subcloning and preparation of the plasmid impossible. The 1.16kb unclonable sequence between sequences C and D (*Figure 4.6a*) contains no RCGTG sites, and displays no notable sequence homology between mammalian species according to the UCSC genome browser. Therefore, it seems unlikely that a HRE or other hypoxia responsive enhancer is located within this region. 42% of this sequence is comprised of repeats, in particular a TcMar-Tigger element as detected by RepeatMasker software (Smit, 1996). These elements may have contributed to the problems in subcloning this sequence of DNA.

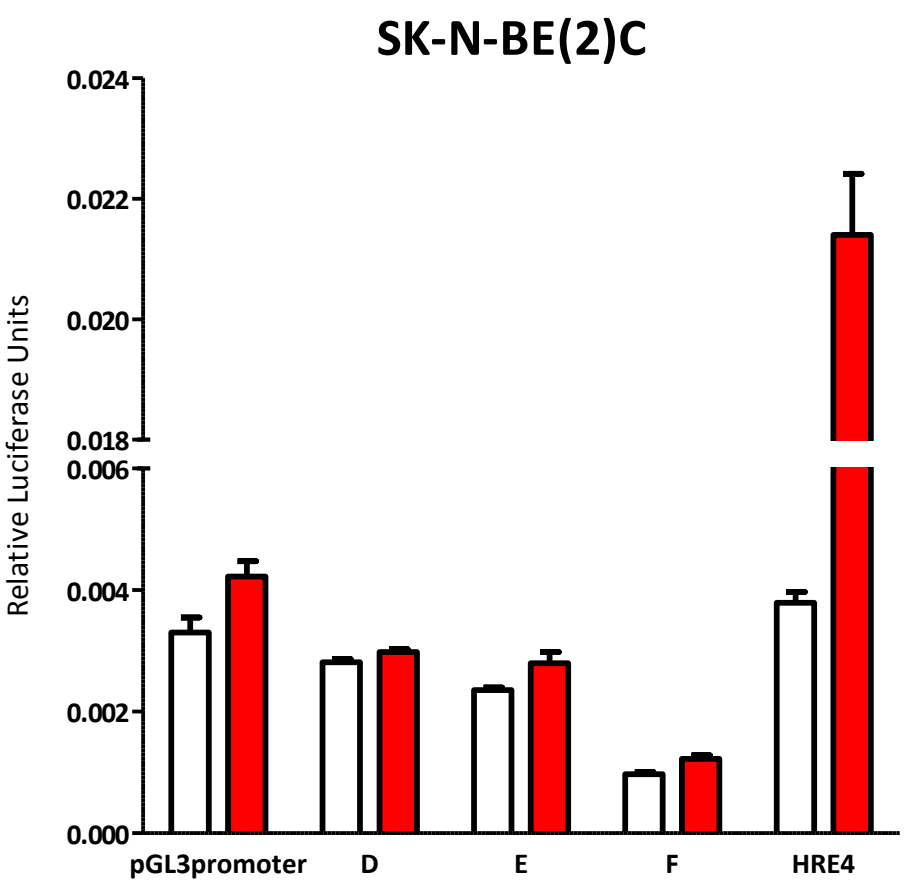
a)


Scale
chr1:
163025000
163030000
163035000
163040000
163045000
163050000
163055000
10 kb
A
B
C
D
E
F
G
H
I
J
K
L
M
N
RGS4
RefSeq Genes
Human mRNAs
Spliced ESTs
Mammal Cons
Mammal Cons
HRE-like Motifs:
0
32884

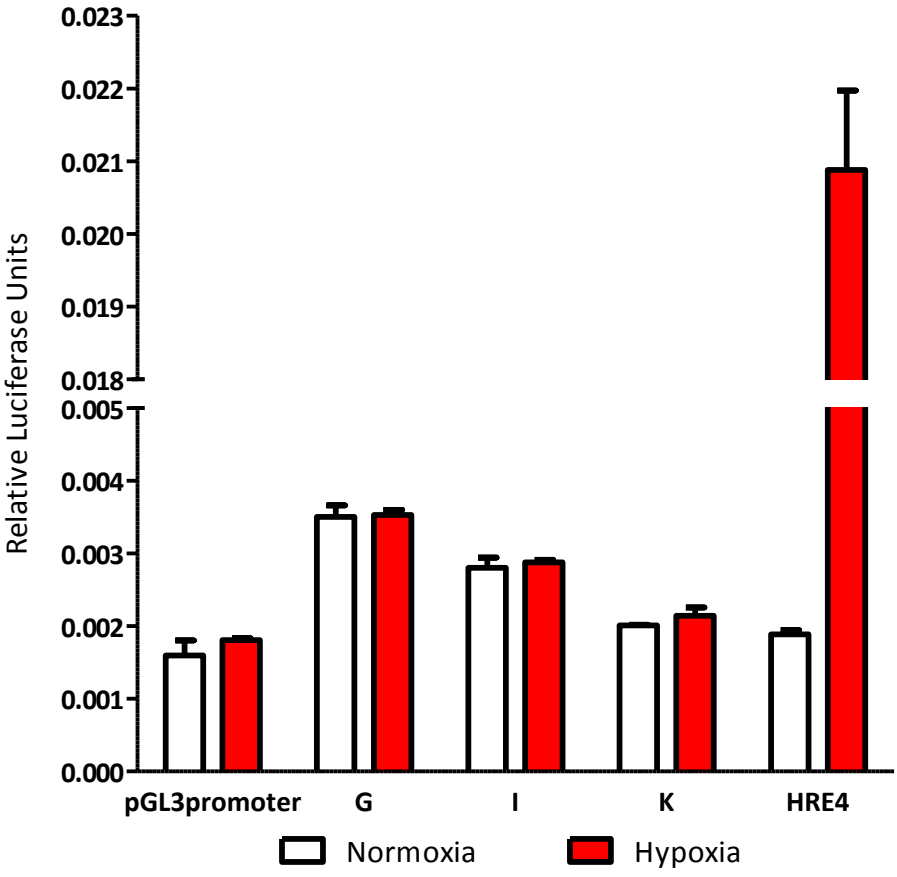
b)


SK-N-BE(2)C
Relative Luciferase Units
0.032
0.030
0.028
0.026
0.024
0.008
0.006
0.004
0.002
0.000
pGL3promoter
A
B
C
HRE4
Normoxia
Hypoxia

c)


SK-N-BE(2)C
Relative Luciferase Units
0.024
0.022
0.020
0.018
0.006
0.004
0.002
0.000
pGL3promoter
D
E
F
HRE4

d)


Relative Luciferase Units
0.023
0.022
0.021
0.020
0.019
0.018
0.005
0.004
0.003
0.002
0.001
0.000
pGL3promoter
G
I
K
HRE4
Normoxia
Hypoxia

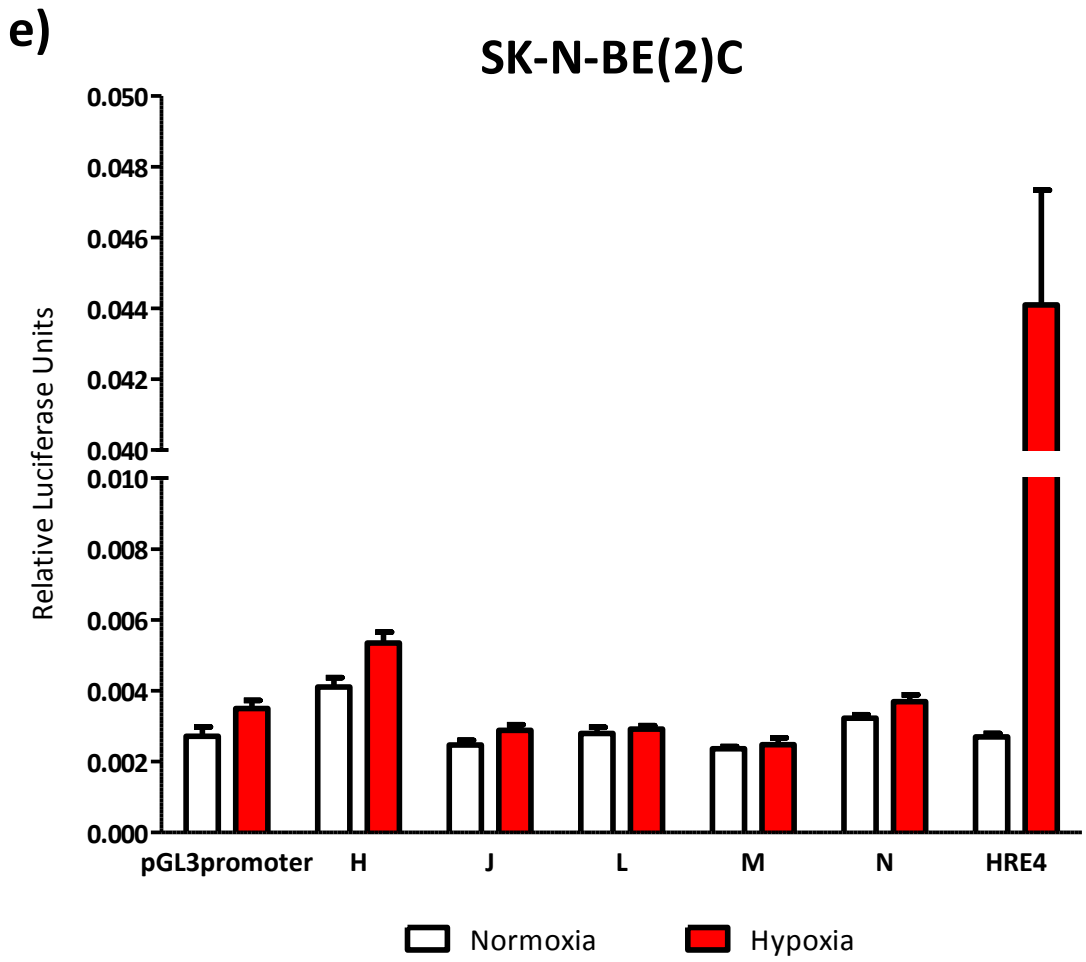


Figure 4.6. (**a**) Diagram of the human *Rgs4* locus, with regions selected for analysis shown. Regions corresponding to fragments A-N were amplified by genomic PCR and cloned into pGL3basic or pGL3promoter upstream of the *luc*$^{+}$ coding sequence, as described in the text. *hRgs4* splice variants and regions of high conservation within mammals as shown as generated by UCSC Genome Browser. HRE-like motifs (RCGTG) locations are also depicted, as detected by MBCS software (Muller *et al.*, 2001). (**b**) SK-N-BE(2)C cells were plated in triplicate in 24-well trays and transfected twice sequentially with 100ng of pGL3promoter or pGL3-based reporter plasmids incorporating the sequences depicted in (**a**), while 25ng pHRE4 was transfected separately as a positive control for hypoxia treatment. Each transfection included 25ng of phRLCMV as control for transfection efficiency and survival. Cells were treated with hypoxia for 16 hours, then analysed using the Dual Luciferase System (Promega). Mean Relative Luficerase Units for a representative of three independent experiments with standard deviation between replicate wells is depicted.

Constructs were transfected into SK-N-BE(2)C cells together with the constitutive Renilla Luciferase expressing contructs, and treated for 16 hours with hypoxia before analysis by the Dual Luciferase system. Results are presented as one representative of three independent experiments, and display no change in relative luciferase upon hypoxic treatment compared to normoxia (*Figure 4.6b, 4.6c and 4.6d*). As the constructs were not all tested at the same time, positive (pHRE4) and negative (pGL3-promoter) controls were performed alongside each set of tested constructs to ensure HIF activation and test for non-specific regulation, respectively. As the pHRE4 plasmid contains 4 copies of the *Epo* HRE, a second positive control with only one copy of the *Vegf* HRE in pGL3 was used to confirm that the assay was sensitive enough to detect a single HRE. This reporter was activated by hypoxia to a similar extent to the pHRE4 plasmid (data not shown).

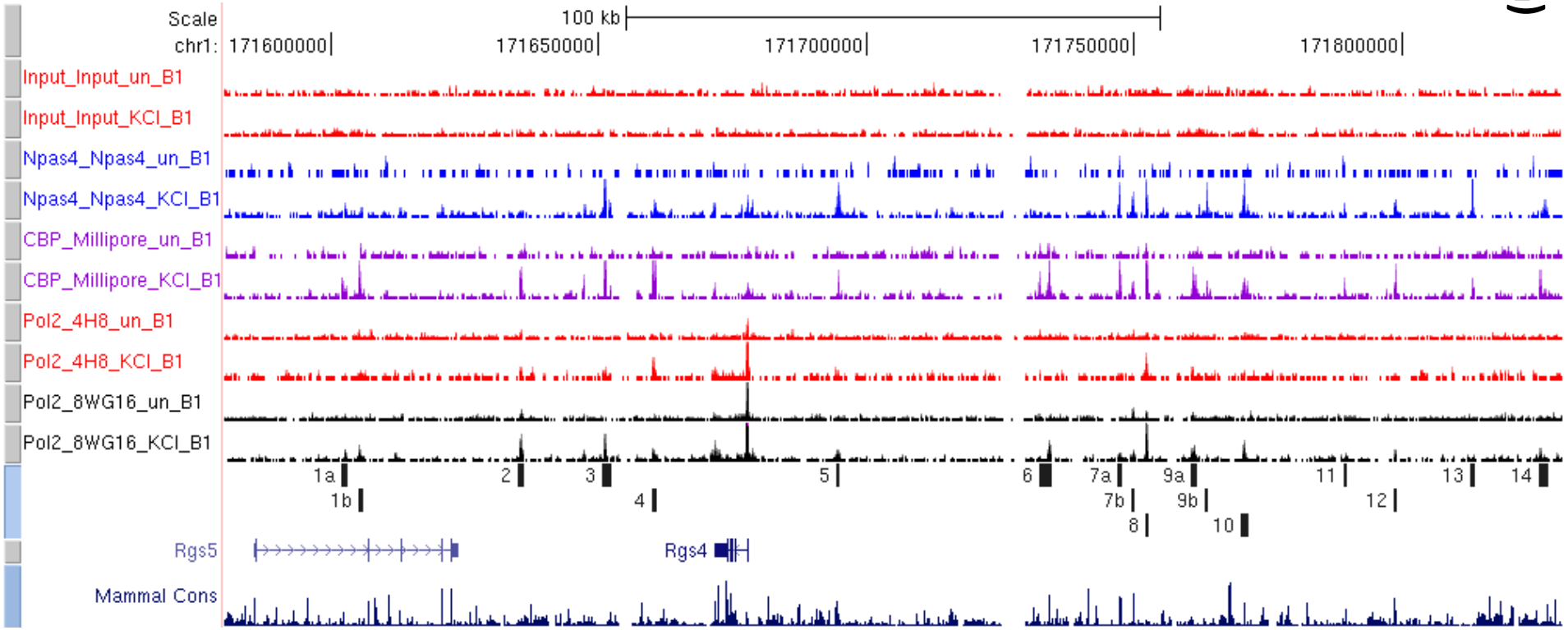
4.1.7. Further Bioinformatic Analyses

Recently, technological advances have allowed a combination of chromatin immunoprecipitation (ChIP) and deep sequencing detection of resulting DNA fragments, termed ChIP-seq. This technique is currently being applied to the HIF transcription factors in SK-N-BE(2)C cells by a collaborating group, but data is not yet available. There is, however, a published set of genomic DNA binding data for RNA PolII, coactivator CBP and the transcription factor NPAS4 in cultured mouse cortical neurons after membrane depolarisation, an activating condition for NPAS4 (Kim *et al.*, 2010). NPAS4 is a member of the bHLH/PAS transcription factor family, heterodimerises with ARNT and binds a NCGTG core DNA sequence (Ooe *et al.*, 2004), all of which are traits shared by HIF. Nonetheless, NPAS4 is not known to be activated or regulated by hypoxia, and is expected to have distinct target genes. However, it is possible that NPAS4 and HIF heterodimers bind some of the same genomic sites, given the similarity between their consensus DNA binding sequences.

Rgs4 has previously been detected at decreased levels following *Npas4* RNAi-mediated knockdown in mouse hippocampal neurons, and as such it is considered to be a candidate target gene for NPAS4 (Lin *et al.*, 2008). The ChIP-seq was used to detect not only NPAS4, but also coactivator CBP, as it is often found bound at enhancer regions. This coactivator also interacts with the C-TAD of HIF-α in hypoxia, so the enhancer sites of NPAS4 and CBP binding in the *Rgs4* locus may also provide some hints as to the locations of hypoxia-regulated enhancers.

The ChIP-seq data from the experiments described above are available in a *bigWig* format in the supplementary section of the Kim *et al.* (2010) publication, which can be applied to the "NCBI37/mm9" assembly of the mouse genome using the UCSC genome browser (Kent *et al.*, 2002). *Figure 4.7a* depicts 250kb of the genomic region surrounding *Rgs4* with this ChIP-seq data aligned, while *Figure 4.7b* shows the 250kb locus surrounding a previously characterised direct NPAS4 target gene, *Drebrin* (Ooe *et al.*, 2004). While *Drebrin* is located within a relatively gene-rich region, *Rgs4* has fewer neighbouring genes, the closest of which (*Rgs5*) is 50kb away. Increased NPAS4, CBP and PolII levels were detected in stimulated cells at the promoter and transcription end site for both *Rgs4* and *Drebrin*. The *Drebrin* 250kb locus contains several sites which display stimulus-enhanced binding of these factors, yet these sites localise almost exclusively to the promoters or transcription end sites of other known genes. Therefore, there appear to be few enhancers present in intervening sequences between genes at the *Drebrin* locus. The 250kb *Rgs4* locus is quite different however, and displays multiple NPAS4, CBP and PolII binding sites in regions where no gene is annotated and no ESTs have been recorded.

We retrieved the genomic sequences of multiple inducible transcription factor binding sites in the 250kb *Rgs4* locus (*Figure 4.7a*, indicated by black bars "1a"–"14") and searched for HRE-like sequences (RCGTG) in either orientation. Of these seventeen sequences, eight contained HRE-like motifs, two of which contained multiple motifs. These enhancer regions for regulation of *Rgs4* by NPAS4 may provide some clues to finding enhancers for the regulation by HIF.


(a)
Scale
100 kb
chr1:
171600000
171650000
171700000
171750000
171800000
Input_Input_un_B1
Input_Input_KCl_B1
Npas4_Npas4_un_B1
Npas4_Npas4_KCl_B1
CBP_Millipore_un_B1
CBP_Millipore_KCl_B1
Pol2_4H8_un_B1
Pol2_4H8_KCl_B1
Pol2_8WG16_un_B1
Pol2_8WG16_KCl_B1
1a
1b
2
3
4
5
6
7a
7b
8
9a
9b
10
11
12
13
14
Rgs5
Rgs4
Mammal Cons

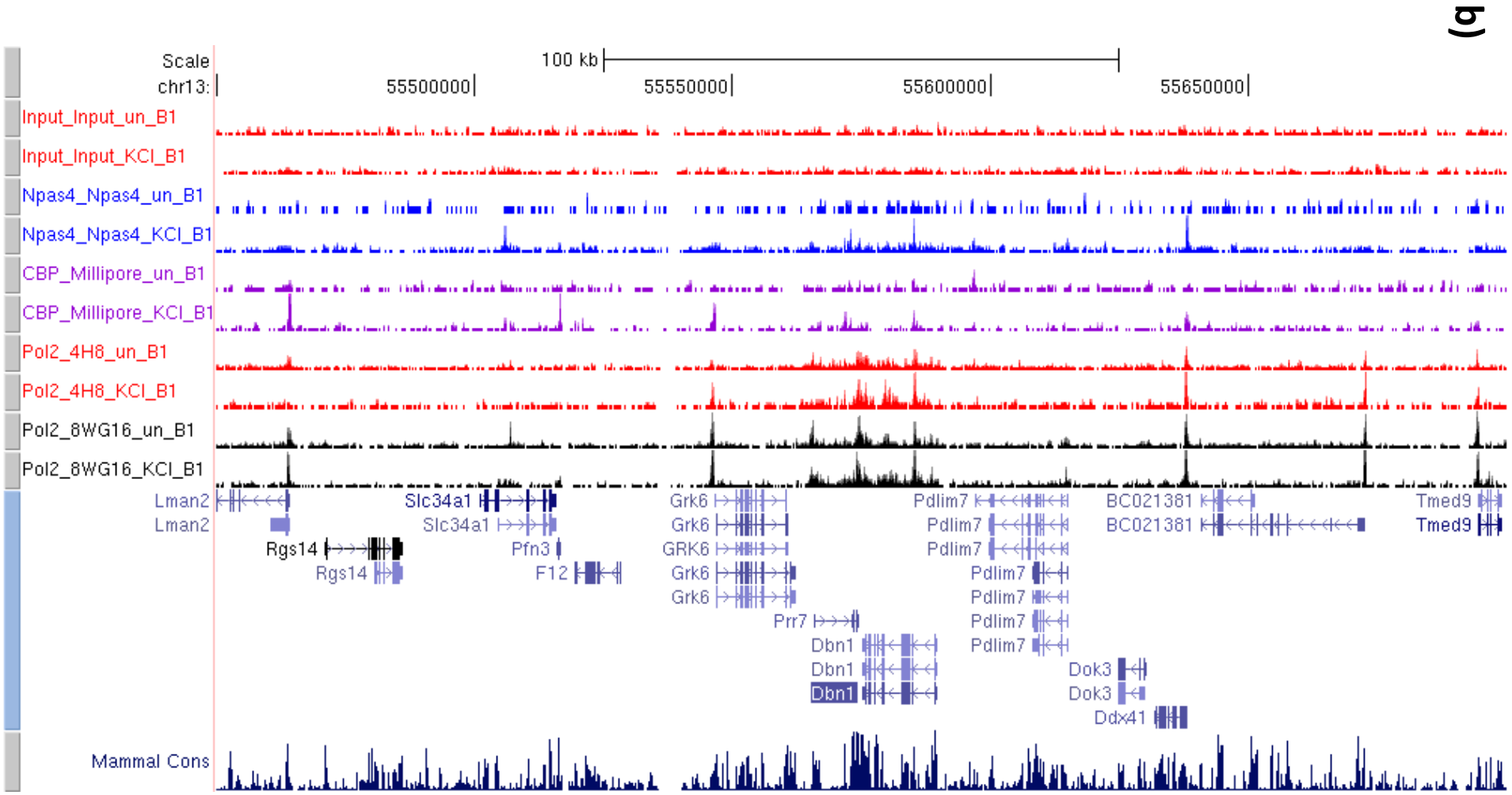

(g
Scale
chr13:
100 kb
55500000
55550000
55600000
55650000
Input_Input_un_B1
Input_Input_KCl_B1
Npas4_Npas4_un_B1
Npas4_Npas4_KCl_B1
CBP_Millipore_un_B1
CBP_Millipore_KCl_B1
Pol2_4H8_un_B1
Pol2_4H8_KCl_B1
Pol2_8WG16_un_B1
Pol2_8WG16_KCl_B1
Lman2
Lman2
Rgs14
Rgs14
Slc34a1
Slc34a1
Pfn3
F12
Grk6
Grk6
GRK6
Grk6
Grk6
Prr7
Dbn1
Dbn1
Dbn1
Pdlim7
Pdlim7
Pdlim7
Pdlim7
Pdlim7
Pdlim7
Pdlim7
Dok3
Dok3
Ddx41
BC021381
BC021381
Tmed9
Tmed9
Mammal Cons

Figure 4.7. Alignment of NPAS4, CBP and PolII Chromatin IP Deep Sequencing (ChIP-Seq) data (Kim *et al.*, 2010) to the UCSC Genome Browser for 250kb-spanning regions centred on the mouse *Rgs4* gene (**a**) and mouse *Drebrin* gene (**b**). "Un" refers to untreated cells, whereas "KCl" are KCl-depolarised cells, a stimulus which activates NPAS4-mediated transcription. "Pol2_4H8" antibody detects PolII with either phosphorylated or unphosphorylated C-Terminal Domains (CTDs), while "Pol2_8WG16" detects the unphosphorylated form preferentially. Black bars in (**a**) labelled "1a" to "14" indicate regions of interest which appear to act as enhancers. UCSC curated genes are depicted in the lower portion of each figure, with arrows indicating direction of transcription (and introns), while wide bars indicate exons. Mammalian conservation according to UCSC Genome Browser alignment is also depicted at the bottom of each image.

4.2.1. The *Rgs4* Locus is Likely to be a Direct HIF Binding Target

The data presented in *Chapter* 3 provides evidence for the regulation of *Rgs4* levels by the HIF transcription factor pathway in mouse, rat and human pheochromocytoma and neuroblastoma cells. In this chapter, the possibility of RNA-binding protein involvement in *Rgs4* hypoxic response was addressed by RNA degradation assays using SK-N-BE(2)C and SK-N-SH cells (*Figure 4.1*). *Rgs4* mRNA was shown to not be differentially stabilised by normoxic or hypoxic conditions in the absence of transcription, relative to *Polr2a* or relative to total RNA levels. This is evidence against regulation of *Rgs4* at the level of mRNA stability, and shows *Rgs4* mRNA accumulation involves a transcriptional response. However, this result in isolation does not exclude the possibility that there is hypoxia responsive transcription of RNA-binding proteins or antisense RNAs, which in turn affect *Rgs4* at the post-transcriptional level.

Calculations for the half-life of *Rgs4* or *Polr2a* mRNA in these cells were made difficult by their high stability, as it becomes difficult to take long timepoints of cells treated with actinomycin D as cells become decreasingly viable, introducing variability in detection. Also, as total RNA amounts were normalised before cDNA generation, *Rgs4* and *Polr2a* detection may be influenced by the stability of ribosomal RNA, which forms the bulk of total RNA samples. This variability was observed when testing *Rgs4* or *Polr2a* degradation without normalisation to a reference gene (*Figure 4.1c,d*), but was much less notable when *Rgs4* was normalised to *Polr2a* levels (*Figure 4.1a,b*), reinforcing the importance of internal qRT-PCR reference gene normalisation. However, in the calculation of the half-life of *Rgs4* mRNA, this normalisation step would skew the calculations. Nonetheless, it was clear that any differences in the half-life of *Rgs4* mRNA between normoxia and hypoxia were minimal, and not responsible for the large induction of mRNA observed in response to hypoxia in these cells in the absence of actinomycin D.

Much post-translational regulation occurs through the 3'UTR of target mRNAs, and the *Rgs4* 3'UTR contains several sites of strong conservation between species. To test for hypoxic response conferred through this sequence, we ligated the human *Rgs4* 3'UTR between the firefly luciferase encoding gene (luc^+) and polyadenylation sequence of pCI_FL, and tested it in normoxia and hypoxia by luciferase reporter assay (*Figure 4.2*). A small increase in relative luciferase levels was observed in hypoxia, but this was not dependent on or increased by the presence of the *Rgs4* 3'UTR, indicating that this RNA sequences is not involved in this regulation.

However, inclusion of the *Rgs4* 3'UTR did generally decrease relative luciferase detection compared to the control without additional 3'UTR. This indicates that there may be some destabilising elements in the human *Rgs4* 3'UTR which are not regulated by hypoxia. This observation is likely to be conferred through one or more of the highly-conserved 3'UTR elements as depicted in *Figure 4.6a*. For example, the largest of the conserved regions appears to contain an AU-rich element, as it contains 42.5% uridine and 29.5% adenine bases, and two AUUUA core motifs within 200bp, of which only 24 are mismatched or inserted bases compared to the mouse sequence. AU-rich elements similar to this cause destabilisation of mRNA (reviewed by Chen and Shyu, 1995). Alternatively, the conserved elements of the *hRgs4* 3'UTR may control mRNA translation, or sub-cellular localisation of transcript. These sequences could provide *Rgs4* response to presently unknown signals other than hypoxia, or may simply increase the overall rate of *Rgs4* mRNA turnover to promote a return to normal levels after removal of stimuli, for example reoxygenation. In support of this result, AU-rich elements have recently been found within the rabbit *Rgs4* 3'UTR, which negatively regulate *Rgs4* expression through association with RNA-binding protein HuR (Li *et al.*, 2010a).

A significant response of *Rgs4* was detectable within 1 hour of DP treatment, in a similar pattern to that observed for other direct HIF targets *Vegf* and *Dec1* (*Figure 4.3*). *Dec1* encodes a transcription factor, DEC1, which is known to negatively regulate transcription of the *Mlh1* gene. Levels of the *Mlh1* transcript were not significantly altered until 4 hours or more of treatment. Therefore, the timing of *Rgs4* response to DP is consistent with the hypothesis that it is a direct target of HIF, and the role of intermediary transcription events in its regulation is unlikely.

4.2.2. *Rgs4* Transcription May be Regulated by a Distant Enhancer

It seems likely from the discussion above that the HIF pathway directly binds and activates transcription of *Rgs4*. Therefore, a search was undertaken for DNA sequences at the rat and human *Rgs4* loci which could mediate this response. Functional HIF-1 or HIF-2 binding sites (HREs) are commonly detected by cloning and testing in mammalian reporter assays, although selection between the two HIF forms can become distorted in a plasmid context (Lau *et al.*, 2007). However, no functional HRE was detectable in either the 5kb upstream sequence of rat *Rgs4*, nor in any sequence of the 32.9kb human *Rgs4* locus. Fragment H displays a small change upon hypoxic treatment, yet as this fragment is overlapped by Fragments E, F, G and I, all of which do not display a similar response, this is not likely to represent a real hypoxia-responsive sequence. This is a surprising result, as most HIF binding sites in the genome are found by chromatin-IP within 2kb of a TSS (Mole *et al.*, 2009).

Different human *Rgs4* promoters were also tested, as this gene has multiple TSSs. The originally discovered human *Rgs4* promoter (Fragment G) resulted in stronger luciferase expression than the alternative promoter (Fragment F), indicating that the former is more likely to be the predominant start site of transcription in the SK-N-BE(2)C cell line. The different mRNA forms resulting from the alternative transcription start sites are not discriminated by the qRT-PCR primers used, nor the northern blot probe used, so previous quantification would not indicate the relative presence of these subtly different transcript forms.

Given the unlikelihood of post-transcriptional regulation as discussed in the previous chapter, there are two possible explanations for these results: that there is a hypoxia responsive element within the regions cloned, but it is not detectable by the conditions used in our luciferase reporter assay; or that there is a hypoxia-responsive element outside of the regions tested in our assay. In relation to the first alternative, it is possible that the *Rgs4* HRE is only functional when it is located in the context of native chromatin (as distinct from a transfected plasmid), due to interaction between transcription factors, other chromatin-bound factors or histone marks. There may also be long-range interaction between transcription factors across the locus, all of which are required to allow hypoxia-

responsive transcription, which would not be recapitulated by testing each fragment individually. However, minimal HIF target sequences discovered to date have all been found to be responsive in reporter assays such as those performed for this thesis (for examples, see Firth *et al.*, 1995; Liu *et al.*, 1995; Miyazaki *et al.*, 2002), so there is no precedence for this explanation.

The alternative explanation is that there is a HRE located outside of the regions of the human *Rgs4* locus tested here, either more than 15.3kb upstream of the *Rgs4* TSS, or more than 9.7kb downstream of its poly-adenylation site. This would make the *Rgs4* HRE atypical, but as mentioned, not without precedence. Although most HREs are found proximal to a TSS, there are exceptions to this observation, such as the HRE for *Phd3* which is found 12.59kb downstream of the TSS (Pescador *et al.*, 2005). In the absence of HIF ChIP-seq data, to determine whether *Rgs4* transcription could be under the control of distant enhancers we analysed ChIP-Seq data for NPAS4 binding, which is a related bHLH-PAS family member, as well as for coactivator CBP and RNA PolII (*Figure 4.7*). Both *Drebrin* and *Rgs4* mRNA levels are responsive to NPAS4 (Lin *et al.*, 2008; Ooe *et al.*, 2004), and NPAS4, coactivator CBP and RNA PolII are found at the *Drebrin* locus bound to only gene proximal sites. However, at the *Rgs4* locus these transcription factors are found at several specific loci in distant non-coding regions. Aside from the sites found at the *Rgs4* proximal promoter and coding sequence, seventeen regions were determined where at least two of either NPAS4, CBP and RNA PolII were detected binding in response to depolarisation stimulus, in the 250kb flanking the *Rgs4* locus (*Figure 4.7*). These binding sites do not coincide with characterised genes, nor do any known expressed sequenced tags (ESTs) align to these regions. Ten such sites could be found within the same span of the *Drebrin* locus, all of which are localised to the TSSs of *Drebrin* or other previously characterised genes.

Therefore, *Drebrin* appears to be regulated by NPAS4 through enhancers found proximal to its promoter, while *Rgs4* is appears to be under the control of several enhancers located up to 150kb away from its TSS, one or more of which bind NPAS4. These may access the *Rgs4* promoter through DNA looping, as indicated by the detection of RNA PolII at these distal sites. This also indicates that the detection of NPAS4 and CBP at some of these distal sites may be due to indirect interactions, such as crosslinking of factors which are in proximity to but not bound directly to the DNA at that location. Futhermore, as NPAS4 is a bHLH-PAS transcription factor like HIF-1α and HIF-2α, and also heterodimerises with

ARNT (HIF-β), there may be crosstalk between NPAS4 and HIF pathways in *Rgs4* regulation. Crosstalk between NPAS4 and another bHLH/PAS member (SIM-2) has already been described (Ooe *et al.*, 2004). The consensus binding sequence for NPAS4 is NCGTG, which is similar to that of the HIFs (Ooe *et al.*, 2004), so binding sites for NPAS4 could also be binding sites for HIF. Alternatively, at the very least this NPAS4 enhancer information could be taken as an analogy for the regulation of *Rgs4* by HIF, which would indicate that *Rgs4* is under the control of several distant enhancers, one of which may be hypoxia-responsive.

The sequences of these seventeen *Rgs4* locus peaks bound by NPAS4, CBP and/or RNA PolII were retrieved and RCGTG motifs (which could theoretically bind both HIF and NPAS4) were found in eight of these regions. HRE-like motifs in sequences 3, 5 and 7b correlated to peaks of NPAS4 binding from the ChIP-Seq data, while motifs in 6 and 9a correlated to CBP binding only (*Figure 4.7a*). Some of these sites were also found in regions of high overall mammalian sequence conservation, yet few of these central RCGTG elements were conserved in the corresponding human or rat sequences. If NPAS4 interacts directly with one of these sites, it may be that the site has moved over mammalian evolution. Evolutionary movement of enhancers, either through translocation of a locus or generation of a novel superceding enhancer while maintaining the regulation of a gene, has been noted as being common for other transcription factors by use of ChIP combined with custom microarrays (Odom *et al.*, 2007) and ChIP-Seq technology (Schmidt *et al.*, 2010). Hence, the lack of conservation at a candidate binding site should not preclude it from analysis as a likely enhancer element.

Since we do not know whether NPAS4 and HIF commonly bind at the same genomic sites, a definitive experiment would need to include ChIP-Seq against HIF-1α and HIF-2α in normoxic and hypoxic cells. This experiment is currently being performed by collaborators using SK-N-BE(2)C cells, and although this data would then align to a different species genome than this NPAS4 data, general comparison would still be possible. Alternatively, as HIF has been shown to in some cases bind to DNA indirectly through the Notch intracellular domain (Gustafsson *et al.*, 2005), consensus HIF binding sites may not be found at HIF controlled *Rgs4* enhancers. Both direct and indirect HIF binding sites may be confirmed by reporter gene assay.

If the regulation of *Rgs4* transcription by hypoxia occurs though distal enhancers interacting by DNA looping, it is feasible that this more complex mode of transactivation

explains the previous observation that *Rgs4* hypoxic response is limited to a narrow range of cell types. Considering that active *Rgs4* transcription and HIF activity is not necessarily sufficient for its hypoxic regulation, whereas *Vegf* appears to be regulated by HIF in all cell types tested, the alternative enhancer set-up at the *Rgs4* locus may allow this restriction. In this case, there may be additional neural or neural-precursor specific transcription factors which HIF must interact with at this locus in order to promote *Rgs4* transcription.

4.2.3. Regulation of *Hif1a* and *Epas1* Message in Prolonged Hypoxia

One of the unexpected results of the siRNA knockdown experiment control (*Figure 3.5*) was that *Hif1a* and *Epas1* mRNA levels are regulated during prolonged DP treatment of SK-N-BE(2)C cells. This effect is also present during hypoxic treatment, as shown by analysis of the timecourse hypoxia samples (*Figure 4.4*), which were primarily intended to quantify the time delay in *Rgs4* response to hypoxia (*Figure 4.3*). Therefore, *Hif1a* and *Epas1* message levels are responsive to both hypoxia and a HIF-activating hypoxia mimetic chemical, and are likely to be regulated through HIF. Interestingly, while *Rgs4* responds in a similar time to known HIF target genes, *Hif1a* and *Epas1* are only detected at altered levels after 4 hours of hypoxia, akin to *Mlh1*, a secondary responder to the HIF pathway. By the 16 hour timepoint, *Hif1a* mRNA has decreased to close to one-third of its normoxic levels (relative to *Polr2a*), while *Epas1* levels have increased by almost 7 fold. It is unclear whether these changes are maintained in longer hypoxia treatments, in part due to the decreasing health of the cell culture in prolonged hypoxia. However, these results do relate to previous observations of HIF-α protein levels in SK-N-BE(2)C cells, amongst others, during prolonged hypoxic treatment in certain cell types (see *Chapter 1.1.4*).

Holmquist-Mengelbier *et al.* (2006) present data demonstrating increased HIF-2α and decreased HIF-1α protein levels in prolonged severe hypoxia, using the SK-N-BE(2)C and KCN-69n human neuroblastoma cell lines, and similar results have also described for A549 lung epithelial cells (Uchida *et al.*, 2004). Interestingly, published qRT-PCR experiments in neuroblastoma indicate an increase in SK-N-BE(2)C *Epas1* mRNA levels within 2 hours of hypoxia, yet the maximum induction was smaller than the data presented

here in *Figure 4.4* (Holmquist-Mengelbier *et al.*, 2006). Furthermore, no reduction in *Hif1a* message was described for this cell line at any timepoint, disagreeing with the results presented in this thesis. Previously published data for the KCN-69n cell line, however, fit more closely with the results presented in this thesis, as *Epas1* is increased while *Hif1a* levels are decreased by severe hypoxia, after a brief delay of around 4 hours (Holmquist-Mengelbier *et al.*, 2006). Subtle changes in reference gene levels are unlikely to cause the differences observed in our data, since significant regulation is observed in both the positive and negative directions.

Therefore, the differences between these experiments are likely to result from different cell culturing conditions, or different hypoxic conditions. In particular, Holmquist-Mengelbier and colleagues use an oxygen-regulated chamber to achieve a specific environmental oxygen percentage of 1%, whereas our experiments use a less controlled system, employing AnaeroGen sachets to absorb oxygen in a sealed container. Although the specific oxygen level was not tested concurrently with the experiments in *Figure 4.3* and *Figure 4.4*, later use of an oxygen meter in the same sealed containers with AnaeroGen sachets produces a final oxygen concentration at or below 0.1%. Therefore, it is likely that the hypoxia employed in the experiments here is more severe than those described by Holmquist-Mengelbier *et al.* (2006), such that the regulation of *Hif1a* and *Epas1* message levels is more pronounced in prolonged conditions of extreme hypoxia, or anoxia. HIF-1α and HIF-2α have also been previously shown to be differentially regulated by moderate hypoxia (Holmquist-Mengelbier *et al.*, 2006), so it would appear that the hypoxic response is significantly affected by the specific oxygen concentration available. However, this has not been directly tested here, so future experiments on this path would require comparison of hypoxic treatments at 1% and 0.1% oxygen.

Due to the timing of these responses, regulation of these transcripts by hypoxia is unlikely to be due to direct activity of HIF itself on *Hif1a* or *Epas1* transcription, but is likely to use an indirect mechanism involving regulation of an intermediate gene. For example, negative regulation of *Hif1a* levels can occur following transcription of *aHIF*, which is under the control of HIF. RNA from *aHIF* then causes *Hif1a* mRNA destabilisation (see *Chapter 1.1.6*), so the time taken to transcribe and accumulate *aHIF* RNA as an intermediate may result in the 4 hour delay before response observed here. Alternatively, there may be other as-yet unknown miRNA genes which could act in a similar fashion to *aHIF*. Another explanation involves activation by HIF of genes encoding transcription

factors such as *Dec1*, which in turn may bind at the *Hif1a* gene and repress its transcription. For example, the negative response of *Hif1a* mRNA occurs in a similar timeframe to that of another DEC1 target gene, *Mlh1*. The effect of either of these pathways could be tested by *Hif1a* 3'UTR reporter assays, promoter reporter assays, or ChIP assays to determine whether altered *Hif1a* levels in this case are due to transcriptional or post-transcriptional regulation. This could then be extended to test whether this pathway is active at 0.1% oxygen, but not 1% oxygen.

Similar explanations are possible for the positive regulation of *Epas1* message levels. There may be an as-yet unknown post-transcriptional pathway regulating *Epas1* levels, or *Epas1* may be regulated by a secondary HIF-responsive transcription factor. Notably, the increase in *Epas1* to hypoxia was detected in previous studies during prolonged 1% oxygen exposure (Holmquist-Mengelbier *et al.*, 2006), whereas the *Hif1a* response was only detected in this thesis which utilised more severe hypoxic conditions, indicating that dissimilar oxygen sensing pathways may be in effect for each of these responses.

This modulation of the two HIF isoforms may enable cell survival and adaptation during extended severe hypoxia or anoxia. Increased HIF-2α protein levels have been correlated to increased dedifferentiation of clinical neuroblastoma samples and poor prognosis (Holmquist-Mengelbier *et al.*, 2006; Pietras *et al.*, 2008), while *Epas1* RNAi knockdown promotes neuroblastoma differentiation (Pietras *et al.*, 2009). Therefore, the *Epas1*-mediated dedifferentiation in non-cancerous cells may enable survival, an effect which manifests as an aggressive phenotype in the context of neuroblastoma. It is then likely that in some cell types, HIF-1α provides adaptation during short hypoxic insults, but is not beneficial during long-term anoxia, while later HIF-2α activates a distinct set of genes to adapt the cell for survival in prolonged hypoxia.

5. Final Discussion

5.1.1. Cell Type Specific Transactivation of *Rgs4* by HIF

The experiments presented in this thesis indicate that *Rgs4* is responsive to hypoxia through the HIF pathway, but only in a subset of all *Rgs4* expressing cells. Although the specific genomic site from which HIF activates transcription of *Rgs4* is not clear, it is likely that long-range interactions are involved in the transcriptional activation of this gene, through DNA-looping. This is in contrast to many, but not all of the known HRE-containing enhancers that are found proximal to transcription start sites of other HIF target genes such as *Vegf*. Therefore, *Rgs4* may have a more complex hypoxia-responsive enhancer than most other characterised HIF target genes, raising the question as to why the *Rgs4* gene would need to be regulated in this fashion.

One possibility involves the specificity of the *Rgs4* hypoxia response to neural-related cells. Expression of *Rgs4* is not silenced in endothelial cells, as *Rgs4* transcript is readily detectable, however its expression is not altered by hypoxic treatment, in contrast to results using neuroblastoma cells. As a general observation, transcription of other HIF target genes such as *Vegf* is responsive to hypoxia in all cell types, excepting cell types in which the gene is silenced. In these cases, presumably the silencing elements outweigh the activating properties of HIF, or HIF is not permitted access to the HRE. However, as the *Rgs4* locus is not silenced in endothelial cells, there must be other elements which specifically prevent HIF transactivation of *Rgs4* in these cells, or specifically permit HIF activation of *Rgs4* in neural-like cells, while still allowing the usual HIF transactivation of other target genes such as *Vegf*. This unknown mechanism may be related to the long-range and complex style of NPAS4-bound enhancer observed at the *Rgs4* locus, such that if HIF acts in a similar fashion at the *Rgs4* locus, other cell-type specific factors or conditions may also be required for transactivation. Notably, if HIF transactivates *Rgs4* expression from a distant enhancer, the results presented here show this enhancer interacts specifically with the *Rgs4* gene and does not act on the nearby *Rgs5* gene.

The idea of a complex enhancer at *Rgs4* is supported in part by previous publications showing that differential transactivation of target genes by HIF-1 and HIF-2 is controlled by post-DNA-binding events, rather than differences in DNA binding (Hu *et al.*, 2007; Lau *et al.*, 2007; Mole *et al.*, 2009), indicating generally that the process of transactivation

by the HIFs involves regulatory mechanisms that are not yet fully understood. In other contexts, HIF is known to functionally interact with transcription factors such as ETS1 (Elvert *et al.*, 2003), ELK1 (Aprelikova *et al.*, 2006) and Notch-1 (Gustafsson *et al.*, 2005). Therefore, HIF regulation of *Rgs4* may require interaction with these or other as-yet unknown transcription factors, resulting in the cell-type specific hypoxic response observed in this thesis. There are many examples of other transcription factors which have distinct roles in different cell types, such as the RE1-Silencing Transcription factor (REST), which binds at distinct genomic sites in embryonic and neural stem cells (Johnson *et al.*, 2008), and the T-cell Acute Leukaemia protein-1 (TAL1), which binds DNA directly using its bHLH domain during erythrocyte maturation, but binds distinct genomic sites during specification of haematopoietic stem cells (Kassouf *et al.*, 2008; Kassouf *et al.*, 2010).

The *Rgs4* transcriptional response to hypoxia is affected by loss of either HIF-1α or HIF-2α, but is more sensitive to loss of HIF-2α than some other classical HIF-1 target genes (*Figure 3.5* and *Figure 3.6*), indicating that while *Rgs4* does not appear to be a target gene of HIF-2 alone, regulation of *Rgs4* is not identical to these other HIF target genes. A recent study using the ChIP-Seq method (Schodel *et al.*, 2011) indicates that long-range HIF interactions are more likely to be preferred by HIF-2, while HIF-1 often acts from a site proximal to the regulated gene. However, it is unclear whether HIF-2 regulates transcription from these enhancers using a different mechanism to HIF-1, and whether the difference in enhancer distance from the regulated gene has a functional effect on regulation. The use of deep sequencing with ChIP for other transcription factors such as p53 has revealed transcription factor binding sites up to 100kb from a regulated gene, and other binding site with no detectable direct effect on nearby gene transcription (Wei *et al.*, 2006). Nonetheless, this finding correlates well with the theory of distant enhancers controlling *Rgs4* transcription, as results presented here show that HIF-2 has more influence on *Rgs4* transactivation than it does on target genes such as *Vegf* or *Dec-1*, at least in the neuroblastoma cell line tested.

The transcriptional regulation of *Rgs4* expression described in this thesis is also interesting in the context of previously discovered regulation of RGS4 and RGS5 protein degradation by hypoxia through the N-end rule degradation pathway (*Chapter 1.3.3*). However, while HIF transactivation of *Rgs4* appears to be restricted to neural-like cells, the N-end rule pathway is most likely to be active in cardiovascular cell types (Kwon *et al.*, 2002).

Furthermore, hypoxia regulates *Rgs4* transcription but not that of *Rgs5*, while the N-end rule pathway affects both RGS4 and RGS5 proteins. Therefore, the two hypoxia response pathways may result in different modes of RGS4 response in vascular and non-vascular cell types, in terms of response time or other characteristics. Alternatively, the two hypoxia responsive pathways may provide a means of allowing RGS4 response in both vascular and neural cell types, while RGS5 is only regulated by hypoxia in vascular cells.

It is worth making note of the lack of obvious phenotype observed for *Rgs4*$^{-/-}$ mice, which is curious given the two distinct, conserved levels of hypoxic regulation of RGS4 levels. Presumably, the function of RGS4 is favourable in some response to hypoxia, as two distinct hypoxia response pathways have been conserved in regulation of RGS4. However, although both the HIF and N-end rule pathways are known to have influential roles in development, *Rgs4*$^{-/-}$ mice have no gross morphological defects (Grillet *et al.*, 2005), therefore RGS4 either has little effect in development, or is made redundant by the other RGS family proteins. In terms of the HIF-based hypoxic response, *Rgs5* does not compensate for *Rgs4* in neural-like cells, but other members of the family which have not yet been tested may be HIF responsive, and therefore compensate for loss of *Rgs4*, considering that many of the RGS family proteins appear to have overlapping specificities for G_{α} target proteins. Taken together, this might suggest that the physiological role of RGS4 hypoxic response is not developmental, but rather used in adaptation to hypoxia. Such a role may be too subtle to be observed in *Hif1a* or *Epas1* deficient mice, which have severe developmental problems due to disruption of transactivation of other HIF target genes. Therefore, it would be of interest to treat *Rgs4*$^{-/-}$ mice with systemic hypoxia and determine whether these mice have impaired survival or adaptation. Given that the experiments in this thesis show specific hypoxic regulation of *Rgs4* in cells which express catecholamines, and the original aims to find new HIF target genes using catecholamine expressing cell types due to the catecholamine-deficient phenotype of *Epas1*$^{-/-}$ mice, it would be of particular interest to test hypoxic and control *Rgs4*$^{-/-}$ mice for perturbations in circulating catecholamine levels, or catecholamine signalling within neural cell types. In this way, novel physiological roles of *Rgs4* may be elucidated using hypoxic conditions which could not be found under normal oxygen conditions.

The observations presented in this thesis can be further related to the putative physiological functions of RGS4 protein discussed in *Chapter 1.3.5* and *1.3.6*. In particular, as the HIF transcription factors regulate expression of *Rgs4* in neural-like cell

types, hypoxia and HIF may influence the regulation of opiate and/or dopamine signalling through $G_{\alpha i}$ proteins, by RGS4 protein activation of $G_{\alpha i}$-GTP hydrolysis. As mentioned previously, foetal hypoxia has been previously linked to risk of schizophrenia (Cannon *et al.*, 2000), a mechanism that could possibly involve HIF regulation of *Rgs4*. Therefore, the results of this thesis provide a possible link between several conditions correlated with schizophrenia susceptibility. Further experiments would involve testing schizophrenia-linked *Rgs4* SNPs in terms of disruption of gene transcription, which would then act as guide to locating novel enhancers of *Rgs4*. Alternatively, *Rgs4, Hif1a or Epas1* mutant alleles could be crossed into existing mouse models of schizophrenia to test for amplification or reduction of phenotypes. A mouse model for this disease has been developed (Hikida *et al.*, 2007), while excessive *Rgs4* expression (Mirnics *et al.*, 2001) and hypoxia (Cannon *et al.*, 2000) have been linked to the disease state, such that null alleles of *Rgs4*, *Hif1a* or *Epas1* might be expected to weaken the schizophrenic phenotype, while overexpression of these genes may cause an increase in deleterious phenotypes.

As the *Rgs4* gene is found to be unresponsive to hypoxia in endothelial cells (*Chapter 3.1.4*), it seems less likely that the regulation of *Rgs4* by HIF impacts on processes such as tubulogenesis, heart hypertrophy or bradycardia. However, the possibility of a role for *Rgs4* in these processes should not be entirely discounted as *Rgs4* may be regulated by hypoxia in cardiovascular cell types other than the umbilical endothelial cell type tested here. Furthermore, RGS4 protein is regulated by hypoxia in the cardiovascular system, so RGS4 may have roles in these processes independent of the HIF pathway. Experiments to further test for an *Rgs4* hypoxic response would include transcript expression analysis of a wider range of primary mouse cell types, and *in situ* hybridisation towards *Rgs4* and other relevant genes, using normoxic and hypoxic heart, brain and other tissue samples. In this way, the extent to which the transcriptional response of *Rgs4* to hypoxia is restricted in cardiovascular cell types can be determined.

Despite numerous published microarray experiments examining the transcriptional response to hypoxia (*Chapter 1.3.3*), *Rgs4* has only appeared in one other set of results other than those presented in this project. This is clearly due to the restriction of *Rgs4* hypoxic regulation to specific cell types that are not commonly used as models. However, these results now imply that there may be other hypoxia and HIF responsive genes which have gone hitherto undetected due to experimental cell type selection. While use of cell types such as breast cancer has been useful in determining the majority of general HIF

target genes, and in turn the binding profile of HIF throughout the genome (Schodel *et al.*, 2011), there are likely to be other functional HIF binding sites and responsive genes in cell types that have not yet been tested, whether in neural-like cells as tested in this thesis, or other unrelated cell types.

Performing microarrays or ChIP-seq experiments across an exhaustive set of cell types would be impractical, so more directed approaches would be required to find these elusive target genes. These atypical target genes of HIF would only be expected to represent a small subset of total hypoxia and HIF responsive genes, the majority of which have already been discovered and have less restricted responsiveness than *Rgs4*. To discover other such novel target genes, physiological functions of HIF which have not yet been described by known target genes could direct researchers to use of a specific model cell type in microarray or ChIP-seq studies, as the catecholamine-deficient phenotype of *Epas1*$^{-/-}$ mice did for the experiments presented in this project. However, due to the critical role of both HIF-1 and HIF-2 in development, carefully designed conditional knockout mouse models would be required to expose the more subtle physiological roles of HIF. Various conditional *Hif1a*$^{-/-}$ and *Epas1*$^{-/-}$ mice have been generated and tested, as described in *Chapter 1.1.7*. Alternatively, atypical HIF target genes such as *Rgs4* could be used as a model for discovery of other HIF-associated transcription factors, and the domains responsible for interaction within HIF-α. This would then aid in predicting cell types where a restricted hypoxic response might be found, and also would provide a method for screening for the genes regulated in this way, either through knockdown of the associated transcription factors alongside microarray technology, or through sequential ChIP (ReChIP) experiments. Considering that these putative novel protein interactions mediate a subset of HIF transactivation targets, some of which like *Rgs4* may be involved in pathogenesis, an attractive direction of research would be involve small molecule inhibitors. Such molecules could be designed to inhibit the formation of transactivation complexes involving HIF at specific atypical target genes, while allowing normal HIF-mediated regulation of classical target genes such as *Epo* and *Vegf*, thus minimising deleterious side effects.

In conclusion, the novel discovery presented here that the *Rgs4* gene is transcriptionally responsive to hypoxia and the HIF pathway in neural-like but not endothelial cells poses new questions about the complexity of regulation of HIF activity towards genes such as *Rgs4*, as well as elucidating a novel link between the HIF and G-protein signalling

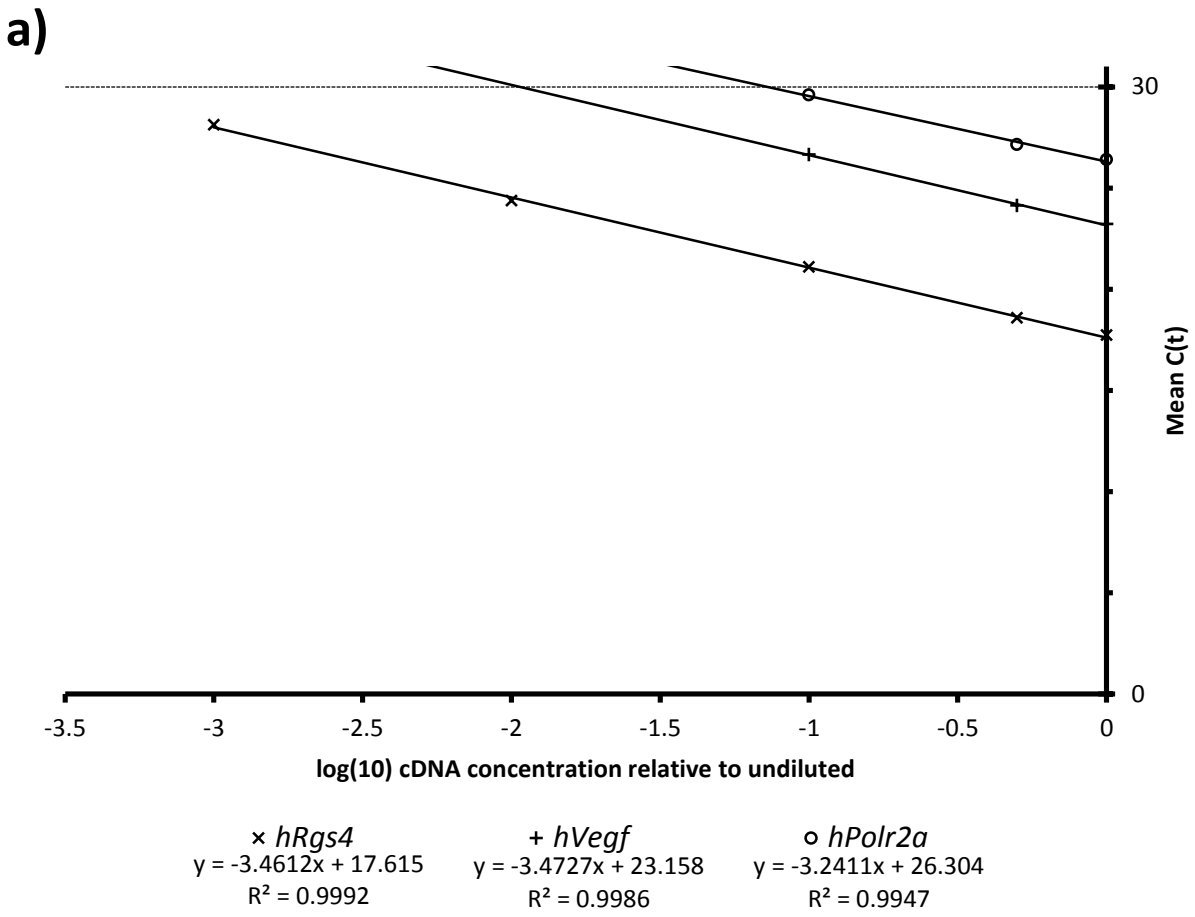
pathways which could lead to a better understanding of diseases such as schizophrenia. These questions can be addressed by further research into *Rgs4* transactivation using techniques such as ChIP-Seq and model systems such as transgenic mice. The direct binding site of HIF at the *Rgs4* locus remains elusive, yet its mechanism of HIF action at this locus is distinct from that previously discovered at other target genes, and may even describe a new subset of as-yet undiscovered HIF target genes.

6. Appendix

6.1.1. Quantitative PCR Optimisation

Initial experiments were designed to establish the quantitative PCR (qPCR) technique prior to the experiments of *Chapter 3.1.3,* as this had not been performed previously in our laboratory. Primers were designed across intron boundaries for human, mouse and rat target gene cDNA sequences retrieved from the UCSC Genome Browser (Kent *et al.*, 2002) using Primer3 software (Rozen and Skaletsky, 2000). Primer sets were designed for *Rgs4*, positive (HIF-regulated) controls *Vegf* and *Dec1*, reference (negative) controls *Polr2a* and *Actb*, as well as *Hif1a*, *Epas1* and *Mlh1* in later experiments. cDNA was generated from identical amounts of template total RNA, and as total RNA is predominantly composed of ribosomal RNA (rRNA), qRT-PCR reactions are already normalised to some extent relative to rRNA levels. Although some variability in mRNA detection is expected despite this step, *Actb* (*β-Actin*) was consistently detected in hypoxic samples about one PCR cycle later than the normoxic control. Therefore, *Actb* levels appear to be slightly responsive to hypoxia and not suitable for use as a reference. Another popular reference gene, *Gapdh*, is under the direct transcriptional control of HIF, making it also unsuitable (Graven *et al.*, 2003). *Polr2a* (encoding the major subunit of RNA Polymerase II), has appeared in several experiments as a more stable reference gene than *Actb* or *Gapdh* (Brattelid *et al.*, 2010; Radonic *et al.*, 2004; Saviozzi *et al.*, 2006), and was also less variable between normoxia and hypoxia that *Actb* in cDNA samples tested here (data not shown). Thus, *Polr2a* was selected as the most appropriate reference control.

The quality of each primer set was verified by qRT-PCR using serial dilutions of cDNA from an appropriate species. The equation for the progression of PCR is expressed in *Equation 2.1* (see *Chapter 2.2.6*), giving *E* as the efficiency of PCR, or in other words the average number of new product molecules produced per cycle per template molecule. *E* can be calculated from the slope of a $\log_{10}$[*template*] vs C(*t*) plot (*Figure 6.1, Equation 2.2*), where C(*t*) is the cycle of PCR where the product reaches a threshold amount (Rutledge and Cote, 2003). In the case of *Figure 6.1*, the template concentration used is relative to neat cDNA, which is set at 1. This PCR efficiency analysis was performed on all primer sets and was determined to be above 90% (0.9) and below 110% (1.1) for C(*t*) less than 30, after which PCR efficiency was less reliable due to the low amount of template being detected, impacting on efficiency. Examples for human *Rgs4*, *Vegf* and *Polr2a* are provided in *Figure 6.1a*, with calculations resulting in $E_{hRgs4} = 0.945$, $E_{hVegf} = 0.941$ and $E_{hPolr2a} = 1.035$. PCR efficiency over 100% can result from formation of



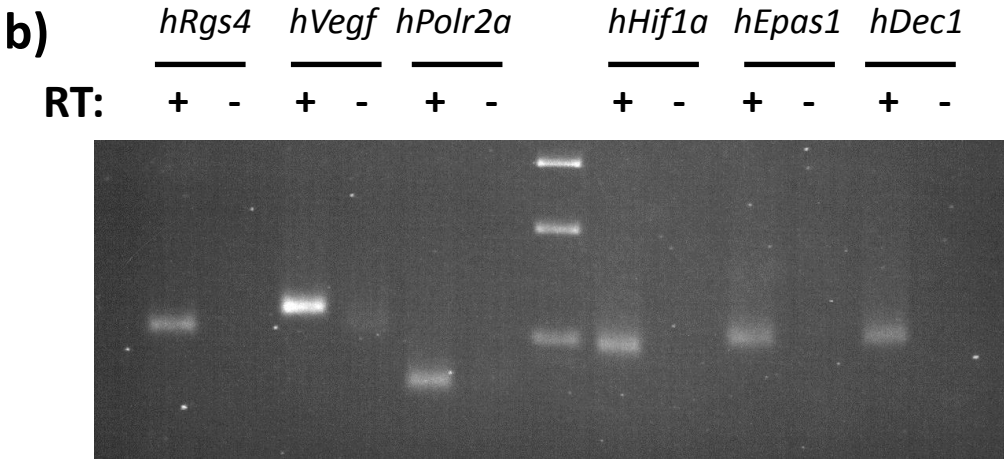


Figure 6.1. (**a**) Representative quantitative PCR efficiency test for *hRgs4*, *hVegf* and *hPolr2a* primer sets on serial dilutions of human (SK-N-SH) cDNA. Mean C(*t*) detection between three replicate qPCR reactions is plotted, for $\log_{10}$ concentrations of cDNA that result in detection of target at C(*t*) < 30. Standard deviations of points are too small to accurately plot, and line slope is used to calculate PCR efficiency calculation using *Equation 2.2*, giving *E* (*hRgs4*) = 0.945, *E* (*hVegf*) = 0.941, and *E* (*hPolr2a*) = 2.035. (**b**) Entire qRT-PCR products from *Figure 3.5c* were run on a 3% agarose/TBE gel, with the DNA size ladder (central lane, from top: 300, 200 and 100bp markers) to estimate PCR specificity. RT refers to Reverse Transcriptase.

non-specific DNA duplexes such as primer-dimers, or from the presence of PCR inhibitors. For the purposes of the following experiments, detection of amplified target sequences later than 30 cycles was considered to represent levels of target gene expression which are at the limit of reliable detection, while C(t) > 35 or no detectable amplification within 40 cycles was considered to be effectively 'undetectable' target message levels. Using more cDNA template per reaction could result in improved detection sensitivity, but this was not required for the experiments presented in this thesis.

Relative amount of target gene mRNA was determined using StepOne (Applied Biosystems) and Q-Gene software (Muller *et al.*, 2002), both of which use modified versions of the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001). This method uses the cycle number (C(t)) at which the amount of PCR product detection reaches a threshold for both target and reference genes, and gives a target expression value in arbitrary units relative to the reference gene. To perform statistical analysis, expression was further normalised to the calibrator (negative control) cDNA, then the mean and standard deviation across at least three independent experiments was taken. After verifying PCR primer set efficiencies to be 90% < E < 110%, the programs were set to assume PCR efficiency of 1, as even a 10% difference in calculated efficiency produces only a small effect. For example, when comparing a hypothetical qPCR result of a 2 cycle difference in C(t) of target between two samples, which equates to a 4-fold relative difference in target amount, altering the theoretical PCR efficiency E from 0.9 to 1.1 gives a 10% range around the expected result, 4. The effects of imperfect PCR efficiency are more pronounced with higher differences in C(t) between samples, and effectively non-existent between samples which give the same C(t), so small errors in calculated efficiency are not likely to give false positive results, but may slightly affect the fold change observed. In addition, the specific PCR efficiency of each subsequent reaction is likely to be slightly variable, so attempting to correct for this using a predetermined efficiency value may introduce more error into the calculation than it removes. *Chapter 3.1.3* describes qRT-PCR experiments using the same samples tested previously by northern blot (*Figure 3.1*), which further demonstrate the comparability of the qRT-PCR technique to northern blots.

In all experiments, qRT-PCR runs were subjected to melting curve analysis to provide data on the specificity of amplification (data not shown). This was compared to the apparent size of amplified DNA product by electrophoresis in a 3% agarose/TBE gel, with reference to the expected size of amplicon (see *Chapter 2.1.2*). In all cases, single

amplicons were detected, and strong amplification was dependent on reverse transcriptase (RT) presence in cDNA generation (*Figure 6.1b*).

Replicate cDNA generation using a combination of poly-d(T) and random hexamer primers with Superscript III reverse transcriptase was performed to determine the variation inherent in reverse transcription (RT), resulting in slightly greater but acceptable standard deviation in relative *Rgs4* detection between cDNA from triplicate independent RT reactions (*Figure 6.2b*) when compared to triplicate qPCR experiments on the same cDNA template (*Figure 6.2a*). This indicates that bias is not likely to incorporated by generation of only one cDNA sample from each RNA sample, followed by triplicate qPCR reactions, provided that biological replicates are also performed. Other qRT-PCR parameters such as amount of template, PCR reagents, cycling conditions, trays and thermocyclers were also optimised to provide the most sensitive and reproducible data possible.

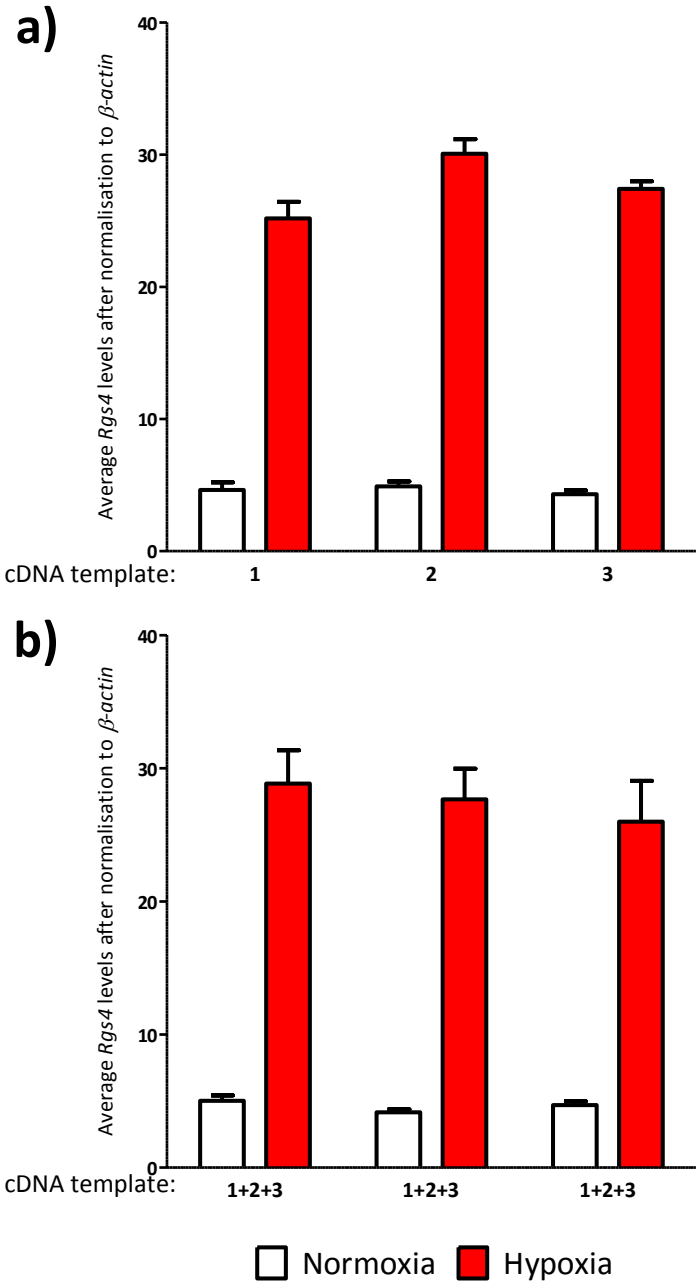


Figure 6.2. Quantitative PCR analysis of cDNA generated from three Reverse Transcriptase (RT) reactions, using the same RNA templates. SK-N-SH cells were treated with 16 hours of normoxia or hypoxia, after which total RNA was extracted. Three identical RT reactions were set up using this RNA as template, and qPCR was performed in triplicate on each resulting cDNA sample using *Rgs4* and *Actb* primers. (**a**) *Rgs4* levels normalised to *Actb* for each independent cDNA triplicate, where each column represents the relative mean of three qPCR reactions on the same cDNA template. Error bars indicate standard error of the mean as determined by *qGene*. (**b**) *Rgs4* levels normalised to *Actb*, where each column represents the relative mean of three qPCR reactions, each using a different cDNA template. Error bars indicate standard error of the mean as determined by *qGene*.

7. References

Al-Sheikh, M., Moradkhani, K., Lopez, M., Wajcman, H., and Prehu, C. (2008). Disturbance in the HIF-1alpha pathway associated with erythrocytosis: further evidences brought by frameshift and nonsense mutations in the prolyl hydroxylase domain protein 2 (PHD2) gene. Blood Cells Mol Dis *40*, 160-165.

Albig, A.R., and Schiemann, W.P. (2005). Identification and characterization of regulator of G protein signaling 4 (RGS4) as a novel inhibitor of tubulogenesis: RGS4 inhibits mitogen-activated protein kinases and vascular endothelial growth factor signaling. Mol Biol Cell *16*, 609-625.

An, J.Y., Seo, J.W., Tasaki, T., Lee, M.J., Varshavsky, A., and Kwon, Y.T. (2006). Impaired neurogenesis and cardiovascular development in mice lacking the E3 ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. Proc Natl Acad Sci U S A *103*, 6212-6217.

Aprelikova, O., Chandramouli, G.V., Wood, M., Vasselli, J.R., Riss, J., Maranchie, J.K., Linehan, W.M., and Barrett, J.C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem *92*, 491-501.

Aprelikova, O., Wood, M., Tackett, S., Chandramouli, G.V., and Barrett, J.C. (2006). Role of ETS transcription factors in the hypoxia-inducible factor-2 target gene selection. Cancer Res *66*, 5641-5647.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F., and Livingston, D.M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A *93*, 12969-12973.

Aravind, L., and Koonin, E.V. (2001). The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol *2*, RESEARCH0007.

Aravindan, N., Williams, M.T., Riedel, B.J., and Shaw, A.D. (2005). Transcriptional responses of rat skeletal muscle following hypoxia-reoxygenation and near ischaemia-reperfusion. Acta Physiol Scand *183*, 367-377.

Arsham, A.M., Howell, J.J., and Simon, M.C. (2003). A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. J Biol Chem *278*, 29655-29660.

Berman, D.M., Kozasa, T., and Gilman, A.G. (1996a). The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. J Biol Chem *271*, 27209-27212.

Berman, D.M., Wilkie, T.M., and Gilman, A.G. (1996b). GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. Cell *86*, 445-452.

Bilton, R.L., and Booker, G.W. (2003). The subtle side to hypoxia inducible factor (HIFalpha) regulation. Eur J Biochem *270*, 791-798.

Blouin, C.C., Page, E.L., Soucy, G.M., and Richard, D.E. (2004). Hypoxic gene activation by lipopolysaccharide in macrophages: Implication of hypoxia-inducible factor 1 alpha. Blood *103*, 1124-1130.

Boutin, A.T., Weidemann, A., Fu, Z., Mesropian, L., Gradin, K., Jamora, C., Wiesener, M., Eckardt, K.U., Koch, C.J., Ellies, L.G., Haddad, G., Haase, V.H., Simon, M.C., Poellinger, L., Powell, F.L., and Johnson, R.S. (2008). Epidermal sensing of oxygen is essential for systemic hypoxic response. Cell *133*, 223-234.

Bracken, C.P., Fedele, A.O., Linke, S., Balrak, W., Lisy, K., Whitelaw, M.L., and Peet, D.J. (2006). Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. J Biol Chem *281*, 22575-22585.

Brahimi-Horn, C., Mazure, N., and Pouyssegur, J. (2005). Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. Cell Signal *17*, 1-9.

Brattelid, T., Winer, L.H., Levy, F.O., Liestol, K., Sejersted, O.M., and Andersson, K.B. (2010). Reference gene alternatives to Gapdh in rodent and human heart failure gene expression studies. BMC Mol Biol *11*, 22.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., and Kaelin, W.G., Jr. (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev *18*, 2893-2904.

Brzustowicz, L.M., Hodgkinson, K.A., Chow, E.W., Honer, W.G., and Bassett, A.S. (2000). Location of a major susceptibility locus for familial schizophrenia on chromosome 1q21-q22. Science *288*, 678-682.

Burke, B., Giannoudis, A., Corke, K.P., Gill, D., Wells, M., Ziegler-Heitbrock, L., and Lewis, C.E. (2003). Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy. Am J Pathol *163*, 1233-1243.

Cannon, T.D., Rosso, I.M., Hollister, J.M., Bearden, C.E., Sanchez, L.E., and Hadley, T. (2000). A prospective cohort study of genetic and perinatal influences in the etiology of schizophrenia. Schizophr Bull *26*, 351-366.

Caqueret, A., Boucher, F., and Michaud, J.L. (2006). Laminar organization of the early developing anterior hypothalamus. Dev Biol *298*, 95-106.

Cascio, S., D'Andrea, A., Ferla, R., Surmacz, E., Gulotta, E., Amodeo, V., Bazan, V., Gebbia, N., and Russo, A. (2010). miR-20b modulates VEGF expression by targeting HIF-1alpha and STAT3 in MCF-7 breast cancer cells. J Cell Physiol.

Chandel, N.S., McClintock, D.S., Feliciano, C.E., Wood, T.M., Melendez, J.A., Rodriguez, A.M., and Schumacker, P.T. (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem *275*, 25130-25138.

Chavez, J.C., Baranova, O., Lin, J., and Pichiule, P. (2006). The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes. J Neurosci *26*, 9471-9481.

Chen, C.Y., and Shyu, A.B. (1995). AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci *20*, 465-470.

Chen, Y.Q., Zhao, C.L., and Li, W. (2009). Effect of hypoxia-inducible factor-1alpha on transcription of survivin in non-small cell lung cancer. J Exp Clin Cancer Res *28*, 29.

Cifelli, C., Rose, R.A., Zhang, H., Voigtlaender-Bolz, J., Bolz, S.S., Backx, P.H., and Heximer, S.P. (2008). RGS4 regulates parasympathetic signaling and heart rate control in the sinoatrial node. Circ Res *103*, 527-535.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem *275*, 25733-25741.

Coleman, M.L., McDonough, M.A., Hewitson, K.S., Coles, C., Mecinovic, J., Edelmann, M., Cook, K.M., Cockman, M.E., Lancaster, D.E., Kessler, B.M., Oldham, N.J., Ratcliffe, P.J., and Schofield, C.J. (2007). Asparaginyl

hydroxylation of the Notch ankyrin repeat domain by factor inhibiting hypoxia-inducible factor. J Biol Chem *282*, 24027-24038.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D., and Carmeliet, P. (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med *8*, 702-710.

Connolly, E., Braunstein, S., Formenti, S., and Schneider, R.J. (2006). Hypoxia inhibits protein synthesis through a 4E-BP1 and elongation factor 2 kinase pathway controlled by mTOR and uncoupled in breast cancer cells. Mol Cell Biol *26*, 3955-3965.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C., and Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev *20*, 557-570.

Covello, K.L., Simon, M.C., and Keith, B. (2005). Targeted replacement of hypoxia-inducible factor-1alpha by a hypoxia-inducible factor-2alpha knock-in allele promotes tumor growth. Cancer Res *65*, 2277-2286.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P., and Simon, M.C. (2005). Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol *25*, 10479-10491.

Cramer, T., Yamanishi, Y., Clausen, B.E., Forster, I., Pawlinski, R., Mackman, N., Haase, V.H., Jaenisch, R., Corr, M., Nizet, V., Firestein, G.S., Gerber, H.P., Ferrara, N., and Johnson, R.S. (2003). HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell *112*, 645-657.

Davydov, I.V., and Varshavsky, A. (2000). RGS4 is arginylated and degraded by the N-end rule pathway in vitro. J Biol Chem *275*, 22931-22941.

Dayan, F., Roux, D., Brahimi-Horn, M.C., Pouyssegur, J., and Mazure, N.M. (2006). The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. Cancer Res *66*, 3688-3698.

De Vries, L., Zheng, B., Fischer, T., Elenko, E., and Farquhar, M.G. (2000). The regulator of G protein signaling family. Annu Rev Pharmacol Toxicol *40*, 235-271.

Ding, K., Scortegagna, M., Seaman, R., Birch, D.G., and Garcia, J.A. (2005). Retinal disease in mice lacking hypoxia-inducible transcription factor-2alpha. Invest Ophthalmol Vis Sci *46*, 1010-1016.

Ding, L., Mychaleckyj, J.C., and Hegde, A.N. (2007). Full length cloning and expression analysis of splice variants of regulator of G-protein signaling RGS4 in human and murine brain. Gene *401*, 46-60.

Doupnik, C.A., Davidson, N., Lester, H.A., and Kofuji, P. (1997). RGS proteins reconstitute the rapid gating kinetics of gbetagamma-activated inwardly rectifying K+ channels. Proc Natl Acad Sci U S A *94*, 10461-10466.

Druey, K.M., Blumer, K.J., Kang, V.H., and Kehrl, J.H. (1996). Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. Nature *379*, 742-746.

Drutel, G., Kathmann, M., Heron, A., Gros, C., Mace, S., Schwartz, J.C., and Arrang, J.M. (2000). Two splice variants of the hypoxia-inducible factor HIF-1alpha as potential dimerization partners of ARNT2 in neurons. Eur J Neurosci *12*, 3701-3708.

Ebert, B.L., and Bunn, H.F. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol Cell Biol *18*, 4089-4096.

Elvert, G., Kappel, A., Heidenreich, R., Englmeier, U., Lanz, S., Acker, T., Rauter, M., Plate, K., Sieweke, M., Breier, G., and Flamme, I. (2003). Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem *278*, 7520-7530.

Elvidge, G.P., Glenny, L., Appelhoff, R.J., Ratcliffe, P.J., Ragoussis, J., and Gleadle, J.M. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate dependent dioxygenase inhibition; the role of HIF-1alpha , HIF-2alpha and other pathways. J Biol Chem.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and 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. PNAS *94*, 4273-4278.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J., and Ratcliffe, P.J. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Erdely, H.A., Lahti, R.A., Lopez, M.B., Myers, C.S., Roberts, R.C., Tamminga, C.A., and Vogel, M.W. (2004). Regional expression of RGS4 mRNA in human brain. Eur J Neurosci *19*, 3125-3128.

Farrall, A.L., and Whitelaw, M.L. (2009). The HIF1alpha-inducible pro-cell death gene BNIP3 is a novel target of SIM2s repression through cross-talk on the hypoxia response element. Oncogene *28*, 3671-3680.

Fedele, A. (2004). Differential function and regulation of the Hypoxia Inducible Factors in the rat pheochromocytoma cell line PC12. In School of Molecular and Biomedical Sciences (Biochemistry) (Adelaide, Australia, The University of Adelaide).

Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G., and Semenza, G.L. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res *59*, 3915-3918.

Firth, J.D., Ebert, B.L., and Ratcliffe, P.J. (1995). Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem *270*, 21021-21027.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and 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. Mech Dev *63*, 51-60.

Flamme, I., Krieg, M., and Plate, K.H. (1998). Up-regulation of vascular endothelial growth factor in stromal cells of hemangioblastomas is correlated with up-regulation of the transcription factor HRF/HIF-2alpha. Am J Pathol *153*, 25-29.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol *16*, 4604-4613.

Fujita, S., Inanobe, A., Chachin, M., Aizawa, Y., and Kurachi, Y. (2000). A regulator of G protein signalling (RGS) protein confers agonist-dependent relaxation gating to a G protein-gated K+ channel. J Physiol *526 Pt 2*, 341-347.

Fung, M.M., Viveros, O.H., and O'Connor, D.T. (2008). Diseases of the adrenal medulla. Acta Physiol (Oxf) *192*, 325-335.

Galban, S., Kuwano, Y., Pullmann, R., Jr., Martindale, J.L., Kim, H.H., Lal, A., Abdelmohsen, K., Yang, X., Dang, Y., Liu, J.O., Lewis, S.M., Holcik, M., and Gorospe, M. (2008). RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1alpha. Mol Cell Biol *28*, 93-107.

Garnier, M., Zaratin, P.F., Ficalora, G., Valente, M., Fontanella, L., Rhee, M.H., Blumer, K.J., and Scheideler, M.A. (2003). Up-regulation of regulator of G protein signaling 4 expression in a model of neuropathic pain and insensitivity to morphine. J Pharmacol Exp Ther *304*, 1299-1306.

Ghosh, A.K., Shanafelt, T.D., Cimmino, A., Taccioli, C., Volinia, S., Liu, C.G., Calin, G.A., Croce, C.M., Chan, D.A., Giaccia, A.J., Secreto, C., Wellik, L.E., Lee, Y.K., Mukhopadhyay, D., and Kay, N.E. (2009). Aberrant regulation of pVHL levels by microRNA promotes the HIF/VEGF axis in CLL B cells. Blood *113*, 5568-5574.

Gold, S.J., Han, M.H., Herman, A.E., Ni, Y.G., Pudiak, C.M., Aghajanian, G.K., Liu, R.J., Potts, B.W., Mumby, S.M., and Nestler, E.J. (2003). Regulation of RGS proteins by chronic morphine in rat locus coeruleus. Eur J Neurosci *17*, 971-980.

Gold, S.J., Ni, Y.G., Dohlman, H.G., and Nestler, E.J. (1997). Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. J Neurosci *17*, 8024-8037.

Goldman-Rakic, P.S., and Selemon, L.D. (1997). Functional and anatomical aspects of prefrontal pathology in schizophrenia. Schizophr Bull *23*, 437-458.

Gonda, D.K., Bachmair, A., Wunning, I., Tobias, J.W., Lane, W.S., and Varshavsky, A. (1989). Universality and structure of the N-end rule. J Biol Chem *264*, 16700-16712.

Grafstein-Dunn, E., Young, K.H., Cockett, M.I., and Khawaja, X.Z. (2001). Regional distribution of regulators of G-protein signaling (RGS) 1, 2, 13, 14, 16, and GAIP messenger ribonucleic acids by in situ hybridization in rat brain. Brain Res Mol Brain Res *88*, 113-123.

Graven, K.K., Bellur, D., Klahn, B.D., Lowrey, S.L., and Amberger, E. (2003). HIF-2alpha regulates glyceraldehyde-3-phosphate dehydrogenase expression in endothelial cells. Biochim Biophys Acta *1626*, 10-18.

Gray, M.J., Zhang, J., Ellis, L.M., Semenza, G.L., Evans, D.B., Watowich, S.S., and Gallick, G.E. (2005). HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene *24*, 3110-3120.

Greijer, A.E., van der Groep, P., Kemming, D., Shvarts, A., Semenza, G.L., Meijer, G.A., van de Wiel, M.A., Belien, J.A., van Diest, P.J., and van der Wall, E. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol *206*, 291-304.

Grillet, N., Dubreuil, V., Dufour, H.D., and Brunet, J.F. (2003). Dynamic expression of RGS4 in the developing nervous system and regulation by the neural type-specific transcription factor Phox2b. J Neurosci *23*, 10613-10621.

Grillet, N., Pattyn, A., Contet, C., Kieffer, B.L., Goridis, C., and Brunet, J.F. (2005). Generation and characterization of Rgs4 mutant mice. Mol Cell Biol *25*, 4221-4228.

Gruber, M., Hu, C.J., Johnson, R.S., Brown, E.J., Keith, B., and Simon, M.C. (2007). Acute postnatal ablation of Hif-2alpha results in anemia. Proc Natl Acad Sci U S A *104*, 2301-2306.

Gustafsson, M.V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J.L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell *9*, 617-628.

Han, M.H., Renthal, W., Ring, R.H., Rahman, Z., Psifogeorgou, K., Howland, D., Birnbaum, S., Young, K., Neve, R., Nestler, E.J., and Zachariou, V. (2010). Brain region specific actions of regulator of G protein signaling 4 oppose morphine reward and dependence but promote analgesia. Biol Psychiatry *67*, 761-769.

Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., and Imura, N. (2001). Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha. Biochem Biophys Res Commun *287*, 808-813.

Heimer, H. (2010). Schizophrenia Research Forum (http://www.schizophreniaforum.org/res/sczgene/default.asp), pp. The SZGene database provides a comprehensive, unbiased and regularly updated field synopsis of genetic association studies performed in schizophrenia. In addition, hundreds of up-to-date meta-analyses are available for all eligible polymorphisms with sufficient data.

Hepler, J.R., Berman, D.M., Gilman, A.G., and Kozasa, T. (1997). RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. Proc Natl Acad Sci U S A *94*, 428-432.

Hewitson, K.S., McNeill, L.A., Riordan, M.V., Tian, Y.M., Bullock, A.N., Welford, R.W., Elkins, J.M., Oldham, N.J., Bhattacharya, S., Gleadle, J.M., Ratcliffe, P.J., Pugh, C.W., and Schofield, C.J. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem *277*, 26351-26355. Epub 22002 May 26331.

Hikida, T., Jaaro-Peled, H., Seshadri, S., Oishi, K., Hookway, C., Kong, S., Wu, D., Xue, R., Andrade, M., Tankou, S., Mori, S., Gallagher, M., Ishizuka, K., Pletnikov, M., Kida, S., and Sawa, A. (2007). Dominant-negative DISC1 transgenic mice display schizophrenia-associated phenotypes detected by measures translatable to humans. Proc Natl Acad Sci U S A *104*, 14501-14506.

Hildebrandt, J.D. (1997). Role of subunit diversity in signaling by heterotrimeric G proteins. Biochem Pharmacol *54*, 325-339.

Ho, G., Wang, Y., Jones, P.G., and Young, K.H. (2007). Activation of serum response element by D2 dopamine receptor is governed by Gbetagamma-mediated MAPK and Rho pathways and regulated by RGS proteins. Pharmacology *79*, 114-121.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K., Poellinger, L., and Pahlman, S. (2006). Recruitment of HIF-1alpha and HIF-

2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell *10*, 413-423.

Hu, C.J., Sataur, A., Wang, L., Chen, H., and Simon, M.C. (2007). The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Mol Biol Cell *18*, 4528-4542.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., and Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol *23*, 9361-9374.

Hu, R.G., Sheng, J., Qi, X., Xu, Z., Takahashi, T.T., and Varshavsky, A. (2005). The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. Nature *437*, 981-986.

Hu, W., Li, F., Mahavadi, S., and Murthy, K.S. (2008). Interleukin-1beta up-regulates RGS4 through the canonical IKK2/IkappaBalpha/NF-kappaB pathway in rabbit colonic smooth muscle. Biochem J *412*, 35-43.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A *95*, 7987-7992.

Huang, Y., Hickey, R.P., Yeh, J.L., Liu, D., Dadak, A., Young, L.H., Johnson, R.S., and Giordano, F.J. (2004). Cardiac myocyte-specific HIF-1alpha deletion alters vascularization, energy availability, calcium flux, and contractility in the normoxic heart. FASEB J *18*, 1138-1140.

Iankova, I., Chavey, C., Clape, C., Colomer, C., Guerineau, N.C., Grillet, N., Brunet, J.F., Annicotte, J.S., and Fajas, L. (2008). Regulator of G protein signaling-4 controls fatty acid and glucose homeostasis. Endocrinology *149*, 5706-5712.

Inanobe, A., Fujita, S., Makino, Y., Matsushita, K., Ishii, M., Chachin, M., and Kurachi, Y. (2001). Interaction between the RGS domain of RGS4 with G protein alpha subunits mediates the voltage-dependent relaxation of the G protein-gated potassium channel. J Physiol *535*, 133-143.

Ingi, T., and Aoki, Y. (2002). Expression of RGS2, RGS4 and RGS7 in the developing postnatal brain. Eur J Neurosci *15*, 929-936.

Ishii, M., and Kurachi, Y. (2003). Physiological actions of regulators of G-protein signaling (RGS) proteins. Life Sci *74*, 163-171.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., and Semenza, G.L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1alpha. Genes Dev *12*, 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jiang, B.H., Jiang, G., Zheng, J.Z., Lu, Z., Hunter, T., and Vogt, P.K. (2001). Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth Differ *12*, 363-369.

Jin, K., Mao, X.O., Eshoo, M.W., del Rio, G., Rao, R., Chen, D., Simon, R.P., and Greenberg, D.A. (2002). cDNA microarray analysis of changes in gene expression induced by neuronal hypoxia in vitro. Neurochem Res *27*, 1105-1112.

Jin, Y., An, X., Ye, Z., Cully, B., Wu, J., and Li, J. (2009). RGS5, a hypoxia-inducible apoptotic stimulator in endothelial cells. J Biol Chem *284*, 23436-23443.

Jogi, A., Ora, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., Axelson, H., and Pahlman, S. (2002). Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A *99*, 7021-7026.

Jogi, A., Vallon-Christersson, J., Holmquist, L., Axelson, H., Borg, A., and Pahlman, S. (2004). Human neuroblastoma cells exposed to hypoxia: induction of genes associated with growth, survival, and aggressive behavior. Exp Cell Res *295*, 469-487.

Johnson, R., Teh, C.H., Kunarso, G., Wong, K.Y., Srinivasan, G., Cooper, M.L., Volta, M., Chan, S.S., Lipovich, L., Pollard, S.M., Karuturi, R.K., Wei, C.L., Buckley, N.J., and Stanton, L.W. (2008). REST regulates distinct transcriptional networks in embryonic and neural stem cells. PLoS Biol *6*, e256.

Kaelin, W.G., Jr., and Maher, E.R. (1998). The VHL tumour-suppressor gene paradigm. Trends Genet *14*, 423-426.

Kassouf, M.T., Chagraoui, H., Vyas, P., and Porcher, C. (2008). Differential use of SCL/TAL-1 DNA-binding domain in developmental hematopoiesis. Blood *112*, 1056-1067.

Kassouf, M.T., Hughes, J.R., Taylor, S., McGowan, S.J., Soneji, S., Green, A.L., Vyas, P., and Porcher, C. (2010). Genome-wide identification of TAL1's functional targets: insights into its mechanisms of action in primary erythroid cells. Genome Res *20*, 1064-1083.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, D. (2002). The human genome browser at UCSC. Genome Res *12*, 996-1006.

Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P.F., Kreiman, G., and Greenberg, M.E. (2010). Widespread transcription at neuronal activity-regulated enhancers. Nature *465*, 182-187.

Kim, W.Y., Perera, S., Zhou, B., Carretero, J., Yeh, J.J., Heathcote, S.A., Jackson, A.L., Nikolinakos, P., Ospina, B., Naumov, G., Brandstetter, K.A., Weigman, V.J., Zaghlul, S., Hayes, D.N., Padera, R.F., Heymach, J.V., Kung, A.L., Sharpless, N.E., Kaelin, W.G., Jr., and Wong, K.K. (2009). HIF2alpha cooperates with RAS to promote lung tumorigenesis in mice. J Clin Invest *119*, 2160-2170.

Kimura, H., Weisz, A., Ogura, T., Hitomi, Y., Kurashima, Y., Hashimoto, K., D'Acquisto, F., Makuuchi, M., and Esumi, H. (2001). Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J Biol Chem *276*, 2292-2298.

Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W.G., Jr. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell *1*, 237-246.

Konietzny, R., Konig, A., Wotzlaw, C., Bernadini, A., Berchner-Pfannschmidt, U., and Fandrey, J. (2009). Molecular imaging: into in vivo interaction of HIF-1alpha and HIF-2alpha with ARNT. Ann N Y Acad Sci *1177*, 74-81.

Koong, A.C., Chen, E.Y., and Giaccia, A.J. (1994a). Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. Cancer Res *54*, 1425-1430.

Koong, A.C., Chen, E.Y., Mivechi, N.F., Denko, N.C., Stambrook, P., and Giaccia, A.J. (1994b). Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). Cancer Res *54*, 5273-5279.

Kozak, K.R., Abbott, B., and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation. Dev Biol *191*, 297-305.

Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K.H. (2000). Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene *19*, 5435-5443.

Kumar, G.K., Overholt, J.L., Bright, G.R., Hui, K.Y., Lu, H., Gratzl, M., and Prabhakar, N.R. (1998). Release of dopamine and norepinephrine by hypoxia from PC-12 cells. Am J Physiol *274*, C1592-1600.

Kwon, Y.T., Kashina, A.S., Davydov, I.V., Hu, R.G., An, J.Y., Seo, J.W., Du, F., and Varshavsky, A. (2002). An essential role of N-terminal arginylation in cardiovascular development. Science *297*, 96-99.

Kwon, Y.T., Xia, Z., An, J.Y., Tasaki, T., Davydov, I.V., Seo, J.W., Sheng, J., Xie, Y., and Varshavsky, A. (2003). Female lethality and apoptosis of spermatocytes in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway. Mol Cell Biol *23*, 8255-8271.

Ladoux, A., and Frelin, C. (1997). Cardiac expressions of HIF-1 alpha and HLF/EPAS, two basic loop helix/PAS domain transcription factors involved in adaptative responses to hypoxic stresses. Biochem Biophys Res Commun *240*, 552-556.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev *16*, 1466-1471.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science *295*, 858-861.

Larminie, C., Murdock, P., Walhin, J.P., Duckworth, M., Blumer, K.J., Scheideler, M.A., and Garnier, M. (2004). Selective expression of regulators of G-protein signaling (RGS) in the human central nervous system. Brain Res Mol Brain Res *122*, 24-34.

Lau, K.W., Tian, Y.M., Raval, R.R., Ratcliffe, P.J., and Pugh, C.W. (2007). Target gene selectivity of hypoxia-inducible factor-alpha in renal cancer cells is conveyed by post-DNA-binding mechanisms. Br J Cancer *96*, 1284-1292.

Lee, M.J., Tasaki, T., Moroi, K., An, J.Y., Kimura, S., Davydov, I.V., and Kwon, Y.T. (2005). RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. Proc Natl Acad Sci U S A *102*, 15030-15035.

Lei, Z., Li, B., Yang, Z., Fang, H., Zhang, G.M., Feng, Z.H., and Huang, B. (2009). Regulation of HIF-1alpha and VEGF by miR-20b tunes tumor cells to adapt to the alteration of oxygen concentration. PLoS One *4*, e7629.

Leonard, M.O., Cottell, D.C., Godson, C., Brady, H.R., and Taylor, C.T. (2003). The role of HIF-1 alpha in transcriptional regulation of the proximal tubular epithelial cell response to hypoxia. J Biol Chem *278*, 40296-40304.

Leontiadis, L.J., Papakonstantinou, M.P., and Georgoussi, Z. (2009). Regulator of G protein signaling 4 confers selectivity to specific G proteins to modulate mu- and delta-opioid receptor signaling. Cell Signal *21*, 1218-1228.

Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. (1995). Transcriptional Regulation of the Rat Vascular Endothelial Growth Factor Gene by Hypoxia. J Biol Chem *270*, 13333-13340.

Li, F., Hu, D.Y., Liu, S., Mahavadi, S., Yen, W., Murthy, K.S., Khalili, K., and Hu, W. (2010a). RNA-binding protein HuR regulates RGS4 mRNA stability in rabbit colonic smooth muscle cells. Am J Physiol Cell Physiol *299*, C1418-1429.

Li, F., Murthy, K.S., Khalili, K., and Hu, W. (2010b). Cloning and characterization of rabbit Rgs4 promoter in gut smooth muscle. Gene *451*, 45-53.

Li, Q.F., Wang, X.R., Yang, Y.W., and Lin, H. (2006). Hypoxia upregulates hypoxia inducible factor (HIF)-3alpha expression in lung epithelial cells: characterization and comparison with HIF-1alpha. Cell Res *16*, 548-558.

Liao, D., Corle, C., Seagroves, T.N., and Johnson, R.S. (2007). Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res *67*, 563-572.

Lin, Y., Bloodgood, B.L., Hauser, J.L., Lapan, A.D., Koon, A.C., Kim, T.K., Hu, L.S., Malik, A.N., and Greenberg, M.E. (2008). Activity-dependent regulation of inhibitory synapse development by Npas4. Nature *455*, 1198-1204.

Liu, C.J., Tsai, M.M., Hung, P.S., Kao, S.Y., Liu, T.Y., Wu, K.J., Chiou, S.H., Lin, S.C., and Chang, K.W. (2010). miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma. Cancer Res *70*, 1635-1644.

Liu, L., Cash, T.P., Jones, R.G., Keith, B., Thompson, C.B., and Simon, M.C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol Cell *21*, 521-531.

Liu, L., and Simon, M.C. (2004). Regulation of transcription and translation by hypoxia. Cancer Biol Ther *3*, 492-497.

Liu, Y., Cox, S.R., Morita, T., and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res *77*, 638-643.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lopez-Barneo, J., Ortega-Saenz, P., Pardal, R., Pascual, A., Piruat, J.I., Duran, R., and Gomez-Diaz, R. (2009). Oxygen sensing in the carotid body. Ann N Y Acad Sci *1177*, 119-131.

Luo, G., Gu, Y.Z., Jain, S., Chan, W.K., Carr, K.M., Hogenesch, J.B., and Bradfield, C.A. (1997). Molecular characterization of the murine Hif-1 alpha locus. Gene Expr *6*, 287-299.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature *414*, 550-554.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem *277*, 32405-32408.

Makino, Y., Uenishi, R., Okamoto, K., Isoe, T., Hosono, O., Tanaka, H., Kanopka, A., Poellinger, L., Haneda, M., and Morimoto, C. (2007). Transcriptional up-regulation of inhibitory PAS domain protein gene expression by hypoxia-

inducible factor 1 (HIF-1): a negative feedback regulatory circuit in HIF-1-mediated signaling in hypoxic cells. J Biol Chem *282*, 14073-14082.

Maldonado, R., Saiardi, A., Valverde, O., Samad, T.A., Roques, B.P., and Borrelli, E. (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. Nature *388*, 586-589.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403-407.

Manzur, M., and Ganss, R. (2009). Regulator of G protein signaling 5: a new player in vascular remodeling. Trends Cardiovasc Med *19*, 26-30.

Maranchie, J.K., Vasselli, J.R., Riss, J., Bonifacino, J.S., Linehan, W.M., and Klausner, R.D. (2002). The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell *1*, 247-255.

Mason, S.D., Howlett, R.A., Kim, M.J., Olfert, I.M., Hogan, M.C., McNulty, W., Hickey, R.P., Wagner, P.D., Kahn, C.R., Giordano, F.J., and Johnson, R.S. (2004). Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. PLoS Biol *2*, e288.

Mastrogiannaki, M., Matak, P., Keith, B., Simon, M.C., Vaulont, S., and Peyssonnaux, C. (2009). HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. J Clin Invest *119*, 1159-1166.

Masuda, K., Abdelmohsen, K., and Gorospe, M. (2009). RNA-binding proteins implicated in the hypoxic response. J Cell Mol Med *13*, 2759-2769.

Maxwell, P.H., Dachs, G.U., Gleadle, J.M., Nicholls, L.G., Harris, A.L., Stratford, I.J., Hankinson, O., Pugh, C.W., and Ratcliffe, P.J. (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A *94*, 8104-8109.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Maynard, M.A., Evans, A.J., Hosomi, T., Hara, S., Jewett, M.A., and Ohh, M. (2005). Human HIF-3alpha4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. FASEB J *19*, 1396-1406.

Maynard, M.A., Qi, H., Chung, J., Lee, E.H.L., Kondo, Y., Hara, S., Conaway, R.C., Conaway, J.W., and Ohh, M. (2003). Multiple Splice Variants of the Human HIF-3alpha Locus Are Targets of the von Hippel-Lindau E3 Ubiquitin Ligase Complex. J Biol Chem *278*, 11032-11040.

Mense, S.M., Sengupta, A., Zhou, M., Lan, C., Bentsman, G., Volsky, D.J., and Zhang, L. (2006). Gene expression profiling reveals the profound upregulation of hypoxia-responsive genes in primary human astrocytes. Physiol Genomics *25*, 435-449.

Merikangas, K.R., and Risch, N. (2003). Will the genomics revolution revolutionize psychiatry? Am J Psychiatry *160*, 625-635.

Milosevic, J., Maisel, M., Wegner, F., Leuchtenberger, J., Wenger, R.H., Gerlach, M., Storch, A., and Schwarz, J. (2007). Lack of hypoxia-inducible factor-1 alpha impairs midbrain neural precursor cells involving vascular endothelial growth factor signaling. J Neurosci *27*, 412-421.

Mirnics, K., Middleton, F.A., Stanwood, G.D., Lewis, D.A., and Levitt, P. (2001). Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. Mol Psychiatry *6*, 293-301.

Miyazaki, K., Kawamoto, T., Tanimoto, K., Nishiyama, M., Honda, H., and Kato, Y. (2002). Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. J Biol Chem *277*, 47014-47021.

Mole, D.R., Blancher, C., Copley, R.R., Pollard, P.J., Gleadle, J.M., Ragoussis, J., and Ratcliffe, P.J. (2009). Genome-wide association of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha DNA binding with expression profiling of hypoxia-inducible transcripts. J Biol Chem *284*, 16767-16775.

Muller, P.Y., Janovjak, H., Miserez, A.R., and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques *32*, 1372-1374, 1376, 1378-1379.

Muller, P.Y., Studer, E., and Miserez, A.R. (2001). Molecular Biocomputing Suite: a word processor add-in for the analysis and manipulation of nucleic acid and protein sequence data. Biotechniques *31*, 1306, 1308, 1310-1303.

Murray, R.M., Lappin, J., and Di Forti, M. (2008). Schizophrenia: from developmental deviance to dopamine dysregulation. Eur Neuropsychopharmacol *18 Suppl 3*, S129-134.

Nakamura, H., Tanimoto, K., Hiyama, K., Yunokawa, M., Kawamoto, T., Kato, Y., Yoshiga, K., Poellinger, L., Hiyama, E., and Nishiyama, M. (2008). Human mismatch repair gene, MLH1, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2. Oncogene *27*, 4200-4209.

Neves, S.R., Ram, P.T., and Iyengar, R. (2002). G protein pathways. Science *296*, 1636-1639.

Noguera, R., Fredlund, E., Piqueras, M., Pietras, A., Beckman, S., Navarro, S., and Pahlman, S. (2009). HIF-1alpha and HIF-2alpha are differentially regulated in vivo in neuroblastoma: high HIF-1alpha correlates negatively to advanced clinical stage and tumor vascularization. Clin Cancer Res *15*, 7130-7136.

Nomoto, S., Adachi, K., Yang, L.X., Hirata, Y., Muraguchi, S., and Kiuchi, K. (1997). Distribution of RGS4 mRNA in mouse brain shown by in situ hybridization. Biochem Biophys Res Commun *241*, 281-287.

Odom, D.T., Dowell, R.D., Jacobsen, E.S., Gordon, W., Danford, T.W., MacIsaac, K.D., Rolfe, P.A., Conboy, C.M., Gifford, D.K., and Fraenkel, E. (2007). Tissue-specific transcriptional regulation has diverged significantly between human and mouse. Nat Genet *39*, 730-732.

Olechnowicz, S.W.Z. (2005). Characterisation of Hypoxia Inducible Factor-2α Target Genes in the PC12 Cell Line. In School of Molecular and Biomedical Sciences (Biochemistry) (Adelaide, Australia, The University of Adelaide), pp. 122.

Ooe, N., Saito, K., Mikami, N., Nakatuka, I., and Kaneko, H. (2004). Identification of a novel basic helix-loop-helix-PAS factor, NXF, reveals a Sim2 competitive, positive regulatory role in dendritic-cytoskeleton modulator drebrin gene expression. Mol Cell Biol *24*, 608-616.

Peng, J., Zhang, L., Drysdale, L., and Fong, G.H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A *97*, 8386-8391.

Percy, M.J. (2008). Familial erythrocytosis arising from a gain-of-function mutation in the HIF2A gene of the oxygen sensing pathway. Ulster Med J *77*, 86-88.

Percy, M.J., Beer, P.A., Campbell, G., Dekker, A.W., Green, A.R., Oscier, D., Rainey, M.G., van Wijk, R., Wood, M., Lappin, T.R., McMullin, M.F., and Lee, F.S. (2008a). Novel exon 12 mutations in the HIF2A gene associated with erythrocytosis. Blood *111*, 5400-5402.

Percy, M.J., Furlow, P.W., Lucas, G.S., Li, X., Lappin, T.R., McMullin, M.F., and Lee, F.S. (2008b). A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. N Engl J Med *358*, 162-168.

Percy, M.J., Zhao, Q., Flores, A., Harrison, C., Lappin, T.R., Maxwell, P.H., McMullin, M.F., and Lee, F.S. (2006). A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. Proc Natl Acad Sci U S A *103*, 654-659.

Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landazuri, M.O., and Del Peso, L. (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J *390*, 189-197.

Peyssonnaux, C., Datta, V., Cramer, T., Doedens, A., Theodorakis, E.A., Gallo, R.L., Hurtado-Ziola, N., Nizet, V., and Johnson, R.S. (2005). HIF-1alpha expression regulates the bactericidal capacity of phagocytes. J Clin Invest *115*, 1806-1815.

Pietras, A., Gisselsson, D., Ora, I., Noguera, R., Beckman, S., Navarro, S., and Pahlman, S. (2008). High levels of HIF-2alpha highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. J Pathol *214*, 482-488.

Pietras, A., Hansford, L.M., Johnsson, A.S., Bridges, E., Sjolund, J., Gisselsson, D., Rehn, M., Beckman, S., Noguera, R., Navarro, S., Cammenga, J., Fredlund, E., Kaplan, D.R., and Pahlman, S. (2009). HIF-2alpha maintains an undifferentiated state in neural crest-like human neuroblastoma tumor-initiating cells. Proc Natl Acad Sci U S A *106*, 16805-16810.

Popov, S., Yu, K., Kozasa, T., and Wilkie, T.M. (1997). The regulators of G protein signaling (RGS) domains of RGS4, RGS10, and GAIP retain GTPase activating protein activity in vitro. Proc Natl Acad Sci U S A *94*, 7216-7220.

Provot, S., Zinyk, D., Gunes, Y., Kathri, R., Le, Q., Kronenberg, H.M., Johnson, R.S., Longaker, M.T., Giaccia, A.J., and Schipani, E. (2007). Hif-1alpha regulates differentiation of limb bud mesenchyme and joint development. J Cell Biol *177*, 451-464.

Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., and Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun *313*, 856-862.

Rane, S., He, M., Sayed, D., Vashistha, H., Malhotra, A., Sadoshima, J., Vatner, D.E., Vatner, S.F., and Abdellatif, M. (2009). Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circ Res *104*, 879-886.

Rankin, E.B., Biju, M.P., Liu, Q., Unger, T.L., Rha, J., Johnson, R.S., Simon, M.C., Keith, B., and Haase, V.H. (2007). Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest *117*, 1068-1077.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol *25*, 5675-5686.

Richard, D.E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem *274*, 32631-32637.

Richard, D.E., Berra, E., and Pouyssegur, J. (2000). Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem *275*, 26765-26771.

Robins, J.C., Akeno, N., Mukherjee, A., Dalal, R.R., Aronow, B.J., Koopman, P., and Clemens, T.L. (2005). Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. Bone *37*, 313-322.

Rogers, J.H., Tamirisa, P., Kovacs, A., Weinheimer, C., Courtois, M., Blumer, K.J., Kelly, D.P., and Muslin, A.J. (1999). RGS4 causes increased mortality and reduced cardiac hypertrophy in response to pressure overload. J Clin Invest *104*, 567-576.

Rossignol, F., de Laplanche, E., Mounier, R., Bonnefont, J., Cayre, A., Godinot, C., Simonnet, H., and Clottes, E. (2004). Natural antisense transcripts of HIF-1alpha are conserved in rodents. Gene *339*, 121-130.

Rossignol, F., Vache, C., and Clottes, E. (2002). Natural antisense transcripts of hypoxia-inducible factor 1alpha are detected in different normal and tumour human tissues. Gene *299*, 135-140.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol *132*, 365-386.

Rutledge, R.G., and Cote, C. (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res *31*, e93.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo J *17*, 3005-3015.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res *60*, 4010-4015.

Saito, T., Fukai, A., Mabuchi, A., Ikeda, T., Yano, F., Ohba, S., Nishida, N., Akune, T., Yoshimura, N., Nakagawa, T., Nakamura, K., Tokunaga, K., Chung, U.I., and Kawaguchi, H. (2010). Transcriptional regulation of endochondral ossification by HIF-2alpha during skeletal growth and osteoarthritis development. Nat Med.

Salceda, S., and Caro, J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem *272*, 22642-22647.

Sanchez, M., Galy, B., Muckenthaler, M.U., and Hentze, M.W. (2007). Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. Nat Struct Mol Biol *14*, 420-426.

Sandau, K.B., Zhou, J., Kietzmann, T., and Brune, B. (2001). Regulation of the hypoxia-inducible factor 1alpha by the inflammatory mediators nitric oxide and tumor necrosis factor-alpha in contrast to desferroxamine and phenylarsine oxide. J Biol Chem *276*, 39805-39811.

Saviozzi, S., Cordero, F., Lo Iacono, M., Novello, S., Scagliotti, G.V., and Calogero, R.A. (2006). Selection of suitable reference genes for accurate normalization of gene expression profile studies in non-small cell lung cancer. BMC Cancer *6*, 200.

Schaub, M.C., Hefti, M.A., Harder, B.A., and Eppenberger, H.M. (1997). Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. J Mol Med *75*, 901-920.

Schmidt-Kastner, R., van Os, J., H, W.M.S., and Schmitz, C. (2006). Gene regulation by hypoxia and the neurodevelopmental origin of schizophrenia. Schizophr Res.

Schmidt, D., Wilson, M.D., Ballester, B., Schwalie, P.C., Brown, G.D., Marshall, A., Kutter, C., Watt, S., Martinez-Jimenez, C.P., Mackay, S., Talianidis, I., Flicek, P., and Odom, D.T. (2010). Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. Science *328*, 1036-1040.

Schodel, J., Oikonomopoulos, S., Ragoussis, J., Pugh, C.W., Ratcliffe, P.J., and Mole, D.R. (2011). High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A., Bennett, M.J., and Garcia, J.A. (2003a). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet *35*, 331-340. Epub 2003 Nov 2009.

Scortegagna, M., Ding, K., Zhang, Q., Oktay, Y., Bennett, M.J., Bennett, M., Shelton, J.M., Richardson, J.A., Moe, O., and Garcia, J.A. (2005). HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood *105*, 3133-3140.

Scortegagna, M., Morris, M.A., Oktay, Y., Bennett, M., and Garcia, J.A. (2003b). The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood *102*, 1634-1640.

Seeman, P. (2006). Targeting the dopamine D2 receptor in schizophrenia. Expert Opin Ther Targets *10*, 515-531.

Seki, N., Sugano, S., Suzuki, Y., Nakagawara, A., Ohira, M., Muramatsu, M., Saito, T., and Hori, T. (1998). Isolation, tissue expression, and chromosomal assignment of human RGS5, a novel G-protein signaling regulator gene. J Hum Genet *43*, 202-205.

Semenza, G.L., Roth, P.H., Fang, H.M., and Wang, G.L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem *269*, 23757-23763.

Siderovski, D.P., and Willard, F.S. (2005). The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci *1*, 51-66.

Simon, M.I., Strathmann, M.P., and Gautam, N. (1991). Diversity of G proteins in signal transduction. Science *252*, 802-808.

Skuli, N., Liu, L., Runge, A., Wang, T., Yuan, L., Patel, S., Iruela-Arispe, L., Simon, M.C., and Keith, B. (2009). Endothelial deletion of hypoxia-inducible factor-2alpha (HIF-2alpha) alters vascular function and tumor angiogenesis. Blood *114*, 469-477.

Smit, A., Hubley, R & Green, P (1996). RepeatMasker Open-3.0 (http://www.repeatmasker.org).

Snabaitis, A.K., Muntendorf, A., Wieland, T., and Avkiran, M. (2005). Regulation of the extracellular signal-regulated kinase pathway in adult myocardium: differential roles of G(q/11), Gi and G(12/13) proteins in signalling by alpha1-adrenergic, endothelin-1 and thrombin-sensitive protease-activated receptors. Cell Signal *17*, 655-664.

Sowter, H.M., Raval, R.R., Moore, J.W., Ratcliffe, P.J., and Harris, A.L. (2003). Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res *63*, 6130-6134.

Spinella, F., Rosano, L., Del Duca, M., Di Castro, V., Nicotra, M.R., Natali, P.G., and Bagnato, A. (2010). Endothelin-1 inhibits prolyl hydroxylase domain 2 to activate hypoxia-inducible factor-1alpha in melanoma cells. PLoS One *5*, e11241.

Stolze, I., Berchner-Pfannschmidt, U., Freitag, P., Wotzlaw, C., Rossler, J., Frede, S., Acker, H., and Fandrey, J. (2002). Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells. Blood *100*, 2623-2628.

Swanson, H.I., Chan, W.K., and Bradfield, C.A. (1995). DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. J Biol Chem *270*, 26292-26302.

Taguchi, A., Yanagisawa, K., Tanaka, M., Cao, K., Matsuyama, Y., Goto, H., and Takahashi, T. (2008). Identification of hypoxia-inducible factor-1 alpha as a novel target for miR-17-92 microRNA cluster. Cancer Res *68*, 5540-5545.

Takeda, N., Maemura, K., Imai, Y., Harada, T., Kawanami, D., Nojiri, T., Manabe, I., and Nagai, R. (2004). Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, Flt-1. Circ Res *95*, 146-153.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M., and Suda, T. (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell *7*, 391-402.

Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., and Harris, A.L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol *157*, 411-421.

Tamirisa, P., Blumer, K.J., and Muslin, A.J. (1999). RGS4 inhibits G-protein signaling in cardiomyocytes. Circulation *99*, 441-447.

Tanaka, T., Wiesener, M., Bernhardt, W., Eckardt, K.U., and Warnecke, C. (2009). The human HIF (hypoxia-inducible factor)-3alpha gene is a HIF-1 target gene and may modulate hypoxic gene induction. Biochem J *424*, 143-151.

Tasaki, T., Mulder, L.C., Iwamatsu, A., Lee, M.J., Davydov, I.V., Varshavsky, A., Muesing, M., and Kwon, Y.T. (2005). A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. Mol Cell Biol *25*, 7120-7136.

Tatenhorst, L., Senner, V., Puttmann, S., and Paulus, W. (2004). Regulators of G-protein signaling 3 and 4 (RGS3, RGS4) are associated with glioma cell motility. J Neuropathol Exp Neurol *63*, 210-222.

Taylor, S.C., and Peers, C. (1998). Hypoxia evokes catecholamine secretion from rat pheochromocytoma PC-12 cells. Biochem Biophys Res Commun *248*, 13-17.

Tesmer, J.J., Berman, D.M., Gilman, A.G., and Sprang, S.R. (1997). Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell *89*, 251-261.

Thomas, J.D., and Johannes, G.J. (2007). Identification of mRNAs that continue to associate with polysomes during hypoxia. RNA *13*, 1116-1131.

Thrash-Bingham, C.A., and Tartof, K.D. (1999). aHIF: a Natural Antisense Transcript Overexpressed in Human Renal Cancer and During Hypoxia. J Natl Cancer Inst *91*, 143a-151.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12*, 3320-3324.

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

Tomita, S., Ueno, M., Sakamoto, M., Kitahama, Y., Ueki, M., Maekawa, N., Sakamoto, H., Gassmann, M., Kageyama, R., Ueda, N., Gonzalez, F.J., and Takahama, Y. (2003). Defective brain development in mice lacking the Hif-1alpha gene in neural cells. Mol Cell Biol *23*, 6739-6749.

Trollmann, R., Strasser, K., Keller, S., Antoniou, X., Grenacher, B., Ogunshola, O.O., Dotsch, J., Rascher, W., and Gassmann, M. (2008). Placental HIFs as markers of cerebral hypoxic distress in fetal mice. Am J Physiol Regul Integr Comp Physiol *295*, R1973-1981.

Tu, Y., Popov, S., Slaughter, C., and Ross, E.M. (1999). Palmitoylation of a conserved cysteine in the regulator of G protein signaling (RGS) domain modulates the GTPase-activating activity of RGS4 and RGS10. J Biol Chem *274*, 38260-38267.

Uchida, T., Rossignol, F., Matthay, M.A., Mounier, R., Couette, S., Clottes, E., and Clerici, C. (2004). Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. J Biol Chem *279*, 14871-14878.

van Wijk, R., Sutherland, S., Van Wesel, A.C., Huizinga, E.G., Percy, M.J., Bierings, M., and Lee, F.S. (2010). Erythrocytosis associated with a novel missense mutation in the HIF2A gene. Haematologica *95*, 829-832.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wang, G.L., and Semenza, G.L. (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*, 1230-1237.

Wang, Q., Liu-Chen, L.Y., and Traynor, J.R. (2009). Differential modulation of mu- and delta-opioid receptor agonists by endogenous RGS4 protein in SH-SY5Y cells. J Biol Chem *284*, 18357-18367.

Wang, V., Davis, D.A., Haque, M., Huang, L.E., and Yarchoan, R. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. Cancer Res *65*, 3299-3306.

Warabi, E., Wada, Y., Kajiwara, H., Kobayashi, M., Koshiba, N., Hisada, T., Shibata, M., Ando, J., Tsuchiya, M., Kodama, T., and Noguchi, N. (2004). Effect on endothelial cell gene expression of shear stress, oxygen concentration, and low-density lipoprotein as studied by a novel flow cell culture system. Free Radic Biol Med *37*, 682-694.

Watson, N., Linder, M.E., Druey, K.M., Kehrl, J.H., and Blumer, K.J. (1996). RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature *383*, 172-175.

Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z., Liu, J., Zhao, X.D., Chew, J.L., Lee, Y.L., Kuznetsov, V.A., Sung, W.K., Miller, L.D., Lim, B., Liu, E.T., Yu, Q., Ng, H.H., and Ruan, Y. (2006). A global map of p53 transcription-factor binding sites in the human genome. Cell *124*, 207-219.

Weidemann, A., Kerdiles, Y.M., Knaup, K.X., Rafie, C.A., Boutin, A.T., Stockmann, C., Takeda, N., Scadeng, M., Shih, A.Y., Haase, V.H., Simon, M.C., Kleinfeld, D., and Johnson, R.S. (2009). The glial cell response is an essential component of hypoxia-induced erythropoiesis in mice. J Clin Invest *119*, 3373-3383.

Wenger, R.H., Kvietikova, I., Rolfs, A., Gassmann, M., and Marti, H.H. (1997a). Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level. Kidney Int *51*, 560-563.

Wenger, R.H., Rolfs, A., Kvietikova, I., Spielmann, P., Zimmermann, D.R., and Gassmann, M. (1997b). The mouse gene for hypoxia-inducible factor-1alpha--genomic organization, expression and characterization of an alternative first exon and 5' flanking sequence. Eur J Biochem *246*, 155-165.

Wenger, R.H., Rolfs, A., Marti, H.H., Guenet, J.L., and Gassmann, M. (1996). Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxia-inducible factor-1 alpha. Biochem Biophys Res Commun *223*, 54-59.

Wenger, R.H., Rolfs, A., Spielmann, P., Zimmermann, D.R., and Gassmann, M. (1998). Mouse hypoxia-inducible factor-1alpha is encoded by two different mRNA isoforms: expression from a tissue-specific and a housekeeping-type promoter. Blood *91*, 3471-3480.

Wenger, R.H., Stiehl, D.P., and Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE *2005*, re12.

Wiener, C.M., Booth, G., and Semenza, G.L. (1996). In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun *225*, 485-488.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W., Ratcliffe, P.J., Bachmann, S., Maxwell, P.H., and Eckardt, K.U. (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J *17*, 271-273.

Wiesener, M.S., Turley, H., Allen, W.E., Willam, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood *92*, 2260-2268.

Willars, G.B. (2006). Mammalian RGS proteins: multifunctional regulators of cellular signalling. Semin Cell Dev Biol *17*, 363-376.

Xie, Y., Wolff, D.W., Wei, T., Wang, B., Deng, C., Kirui, J.K., Jiang, H., Qin, J., Abel, P.W., and Tu, Y. (2009). Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. Cancer Res *69*, 5743-5751.

Yan, Y., Chi, P.P., and Bourne, H.R. (1997). RGS4 inhibits Gq-mediated activation of mitogen-activated protein kinase and phosphoinositide synthesis. J Biol Chem *272*, 11924-11927.

Yang, S., Kim, J., Ryu, J.H., Oh, H., Chun, C.H., Kim, B.J., Min, B.H., and Chun, J.S. (2010). Hypoxia-inducible factor-2alpha is a catabolic regulator of osteoarthritic cartilage destruction. Nat Med.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol *21*, 183-186.

Yuan, G., Nanduri, J., Bhasker, C.R., Semenza, G.L., and Prabhakar, N.R. (2005). Ca2+/calmodulin kinase-dependent activation of hypoxia inducible factor 1 transcriptional activity in cells subjected to intermittent hypoxia. J Biol Chem *280*, 4321-4328.

Zenker, M., Mayerle, J., Lerch, M.M., Tagariello, A., Zerres, K., Durie, P.R., Beier, M., Hulskamp, G., Guzman, C., Rehder, H., Beemer, F.A., Hamel, B., Vanlieferinghen, P., Gershoni-Baruch, R., Vieira, M.W., Dumic, M., Auslender, R., Gil-da-Silva-Lopes, V.L., Steinlicht, S., Rauh, M., Shalev, S.A., Thiel, C., Ekici, A.B., Winterpacht, A., Kwon, Y.T., Varshavsky, A., and Reis, A. (2005). Deficiency of UBR1, a ubiquitin ligase of the N-end rule pathway, causes pancreatic dysfunction, malformations and mental retardation (Johanson-Blizzard syndrome). Nat Genet *37*, 1345-1350.

Zhang, L., and Hill, R.P. (2004). Hypoxia enhances metastatic efficiency by up-regulating Mdm2 in KHT cells and increasing resistance to apoptosis. Cancer Res *64*, 4180-4189.

Zhang, S., Watson, N., Zahner, J., Rottman, J.N., Blumer, K.J., and Muslin, A.J. (1998). RGS3 and RGS4 are GTPase activating proteins in the heart. J Mol Cell Cardiol *30*, 269-276.

Zheng, X., Linke, S., Dias, J.M., Gradin, K., Wallis, T.P., Hamilton, B.R., Gustafsson, M., Ruas, J.L., Wilkins, S., Bilton, R.L., Brismar, K., Whitelaw, M.L., Pereira, T., Gorman, J.J., Ericson, J., Peet, D.J., Lendahl, U., and Poellinger, L. (2008). Interaction with factor inhibiting HIF-1 defines an additional mode of cross-coupling between the Notch and hypoxia signaling pathways. Proc Natl Acad Sci U S A *105*, 3368-3373.

Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L., and Simons, J.W. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res *59*, 5830-5835.

Addendum

The following corrections and additions are made in response to the thesis examiners' comments.

Page 14: The following paragraph should be inserted at the end of Chapter 1.1.2:

"Acetylation in particular has been shown to modify the activity and stabilisation of HIF-1α and HIF-2α. In mouse cells, acetylation of HIF-1α between the PAS A and PAS B domains by a splice variant of ARD1 promotes association with pVHL and proteasomal degradation (Jeong et al., 2002), however the relevance of this effect is questionable as similar effects are not observed in human cells (Bilton et al., 2005; Fisher et al., 2005; Kim et al., 2006). In human cells, acetylation by PCAF activates HIF-1α (Xenaki et al., 2008), while deacetylation of HIF-1α by Sirtuin1 or HDAC4 prevents HIF-1α transactivation of many target genes (Geng et al., 2011; Lim et al., 2010). Conversely, deacetylation of HIF-2α by Sirtuin1 in human cells can activate transcription of target genes (Dioum et al., 2009), so acetylation modifications provide a possible mechanism for the differing functions of HIF-1 and HIF-2."

Page 15: In contrast to the first paragraph, HIF-2 has also recently been implicated in regulation of *Glut-1* in colorectal carcinoma cells (Li et al., 2011). The second sentence of the second paragraph should read:

"The *Epo* gene (which encodes Erythropoietin) is currently considered to be the best characterised *in vivo* HIF-2 specific target gene, as its expression is disrupted in HIF-2α knockout kidneys (Scortegagna et al., 2005)."

Page 19: The following sentence and reference should be added to the end of the first paragraph:

"Stabilisation of the HIF-α subunits by hypoxia can also be caused by signalling by reactive oxygen species produced by the functioning mitochondria, although the effect of hydroxylase inhibition is more pronounced at severe hypoxic or anoxic conditions (Schroedl et al., 2002)."

Page 25: On the seventh line, the word "heterozygous" should be replaced with "homozygous".

Page 26: The following references should be added to the twelfth line: (Gruber et al., 2007; Scortegagna et al., 2005; Scortegagna et al., 2003).

Page 28: On the first line of the second paragraph, the word "oncogenic" should be replaced with "tumour supportive".

Page 73: The following sentences should be inserted at the end of the first paragraph:

"After transfection, tagged control siRNA was observed to be colocalised with cells, although some fluorescence was detected on unpopulated regions of the growing surface. Cellular fluorescence was concentrated in speckles which appeared in all cells observed, so optimisation was undertaken to further decrease levels of fluorescence detected on unpopulated growth surfaces, in order to increase cell transfection specificity."

Page 76: The following sentences should be inserted after the second sentence of the second paragraph:

"Incomplete knockdown of HIF-α protein was observed by western blot, while visual detection of fluorescently-labelled control siRNA localised to all cells. As these siRNA sequences have been used to silence HIF-α messages in other cell types previously (Sowter et al., 2003), high knockdown efficiency should be possible if transfection efficiency is high. Therefore, despite colocalisation of siRNA complexes to cells, it seems likely that some siRNA complexes bind to but do not enter SK-N-BE(2)C cells effectively, or are being sequestered within cells."

Page 80: The following paragraph should be inserted after the third sentence of the second paragraph:

"It has been noted previously that between in moderate hypoxia the HIF-α subunits can be differently stabilised and activated (Bracken et al., 2006), by not only the PHD and FIH hydroxylase enzymes but also mitochondrial activity (Schroedl et al., 2002). To ensure complete activation of both HIF-1 and HIF-2, severe hypoxia of less than 1% environmental oxygen was used in treatment."

Page 80: The first paragraph should be replaced by the following paragraph:

"Rgs4 is known to be expressed in the cardiovascular system (*Chapter 1.3.6*), and both RGS4 and RGS5 protein are detectable in Human Umbilical Vein Endothelial Cells (HUVECs). Jin et al. (2009) describe experiments showing hypoxic induction of RGS5, but not RGS4 protein in HUVECs, so I decided to test the hypoxic response of the *Rgs4* and *Rgs5* genes in the same cell type by qRT-PCR (*Figure 3.4*). Both 3 and 16 hour hypoxic treatments were tested to allow direct comparison to the previously published data (Jin et al., 2009), and also to the other experiments described earlier in this thesis. My results disagree with the data of Jin et al. (2009) for *Rgs5*, as *Figure 3.4* shows no positive change in either *Rgs4* or *Rgs5* mRNA, and actually a modest but statistically significant decrease in levels of both after 16 hours of treatment relative to *Pol2a*, in contrast to positive control *Vegf*. However, the lack of hypoxic induction of RGS4 described previously by Jin et al. (2009) is supported by my experiments on *Rgs4* message levels in HUVECs. This shows that the response of *Rgs4* to hypoxia is not a general property of RGS encoding genes, and also that *Rgs4* responds to hypoxia only in a subset of those cell types which express it at a basal level."

Addendum References

Bilton, R., Mazure, N., Trottier, E., Hattab, M., Dery, M.A., Richard, D.E., Pouyssegur, J., and Brahimi-Horn, M.C. (2005). Arrest-defective-1 protein, an acetyltransferase, does not alter stability of hypoxia-inducible factor (HIF)-1alpha and is not induced by hypoxia or HIF. J Biol Chem *280*, 31132-31140.

Bracken, C.P., Fedele, A.O., Linke, S., Balrak, W., Lisy, K., Whitelaw, M.L., and Peet, D.J. (2006). Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. J Biol Chem *281*, 22575-22585.

Dioum, E.M., Chen, R., Alexander, M.S., Zhang, Q., Hogg, R.T., Gerard, R.D., and Garcia, J.A. (2009). Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. Science *324*, 1289-1293.

Fisher, T.S., Etages, S.D., Hayes, L., Crimin, K., and Li, B. (2005). Analysis of ARD1 function in hypoxia response using retroviral RNA interference. J Biol Chem *280*, 17749-17757.

Geng, H., Harvey, C.T., Pittsenbarger, J., Liu, Q., Beer, T.M., Xue, C., and Qian, D.Z. (2011). HDAC4 Protein Regulates HIF1alpha Protein Lysine Acetylation and Cancer Cell Response to Hypoxia. J Biol Chem *286*, 38095-38102.

Gruber, M., Hu, C.J., Johnson, R.S., Brown, E.J., Keith, B., and Simon, M.C. (2007). Acute postnatal ablation of Hif-2alpha results in anemia. Proc Natl Acad Sci U S A *104*, 2301-2306.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., and Kim, K.W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell *111*, 709-720.

Jin, Y., An, X., Ye, Z., Cully, B., Wu, J., and Li, J. (2009). RGS5, a hypoxia-inducible apoptotic stimulator in endothelial cells. J Biol Chem *284*, 23436-23443.

Kim, S.H., Park, J.A., Kim, J.H., Lee, J.W., Seo, J.H., Jung, B.K., Chun, K.H., Jeong, J.W., Bae, M.K., and Kim, K.W. (2006). Characterization of ARD1 variants in mammalian cells. Biochem Biophys Res Commun *340*, 422-427.

Li, Q.Q., Sun, Y.P., Ruan, C.P., Xu, X.Y., Ge, J.H., He, J., Xu, Z.D., Wang, Q., and Gao, W.C. (2011). Cellular prion protein promotes glucose uptake through the Fyn-HIF-2alpha-Glut1 pathway to support colorectal cancer cell survival. Cancer Sci *102*, 400-406.

Lim, J.H., Lee, Y.M., Chun, Y.S., Chen, J., Kim, J.E., and Park, J.W. (2010). Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. Mol Cell *38*, 864-878.

Schroedl, C., McClintock, D.S., Budinger, G.R., and Chandel, N.S. (2002). Hypoxic but not anoxic stabilization of HIF-1alpha requires mitochondrial reactive oxygen species. Am J Physiol Lung Cell Mol Physiol *283*, L922-931.

Scortegagna, M., Ding, K., Zhang, Q., Oktay, Y., Bennett, M.J., Bennett, M., Shelton, J.M., Richardson, J.A., Moe, O., and Garcia, J.A. (2005). HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood *105*, 3133-3140.

Scortegagna, M., Morris, M.A., Oktay, Y., Bennett, M., and Garcia, J.A. (2003). The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood *102*, 1634-1640.

Sowter, H.M., Raval, R.R., Moore, J.W., Ratcliffe, P.J., and Harris, A.L. (2003). Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res *63*, 6130-6134.

Xenaki, G., Ontikatze, T., Rajendran, R., Stratford, I.J., Dive, C., Krstic-Demonacos, M., and Demonacos, C. (2008). PCAF is an HIF-1alpha cofactor that regulates p53 transcriptional activity in hypoxia. Oncogene *27*, 5785-5796.