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Assessment of HIF-1α Function and
Identification of a Novel Degradation Mechanism

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Stockholm 2009
To my family,

For all their support
ABSTRACT

HIF-1 is a heterodimeric complex of two bHLH/PAS transcription factors, HIF-1α and ARNT. In contrast to the constitutively expressed ARNT, HIF-1α protein levels are regulated by oxygen tension. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway. Upon exposure to hypoxia, HIF-1α is stabilized and translocated to the nucleus where it heterodimerizes with ARNT and binds hypoxia-responsive elements present in target genes. HIF-1 functions as a master regulator of adaptive responses to hypoxia by activating genes important for physiological processes. These processes range from oxygen supply, cellular metabolism, and cell growth and apoptosis.

The interaction between the von Hippel-Lindau tumor suppressor gene product (pVHL, part of an E3 ubiquitin-ligase) and HIF-1α has been shown to be regulated by three soluble dioxygenases. These dioxygenases are termed prolyl hydroxylase domain proteins (PHD). They hydroxylate two conserved proline residues (Pro\(^{402}\) and/or Pro\(^{563}\) within mouse HIF-1α), targeting HIF-1α for degradation. Initially, it was suggested that mutagenesis of these prolines to alanine residues that cannot be hydroxylated generates a constitutively stabilized form of HIF-1α, allowing the protein to be expressed independently of oxygen levels.

We have investigated the effects on neoangiogenesis by mHIF-1α(P402A/P563A), using adeno-associated virus (AAV) gene delivery to skeletal muscle. Additionally, we have compared these effects to those produced by previously known vascular growth factors, such as the VEGF. mHIF-1α(P402A/P563A) was shown to be capable of inducing formation of functional neovascularure without increased leakiness (a well-characterized side-effect of VEGF). Our data further suggest that the use of the mHIF-1α mutant in pro-angiogenic gene therapy may have the ability to circumvent most of the present problems in cardiovascular gene transfer studies. This is due to the fact that HIF-1α is capable of inducing endogenous angiogenic cascades that lead to the activation of multiple and necessary vasculo-generative growth factors.

We have also evaluated the expression of mHIF-1α and mHIF-1α(P402A/P563A) in cultured cells and observed normoxia-dependent degradation of these proteins. Our studies show that degradation of these proteins is mediated by the ubiquitin-proteasome system, through a pVHL-dependent mechanism. Furthermore, PHDs failed to induce normoxia-dependent degradation of the mHIF-1α(P402A/P563A) mutant. pVHL-mediated degradation of both mHIF-1α and the double proline mutant proved to be intrinsically independent of the hydroxylation status of the HIF-1α proteins. Taken together, these data suggest the existence of yet unraveled mechanisms of degradation of HIF-1α.

Subsequently, we evaluated the contribution of the small ubiquitin-like modifier (SUMO) and the SUMO-specific protease 1 (SENP1) to HIF-1α degradation. Our results show that RNAi directed to SENP1 impairs protein accumulation of both mHIF-1α and mHIF-1α(P402A/P563A) at hypoxia. At normoxia, silencing of the SUMO conjugating enzyme (Ubc9) resulted in stabilization of endogenous HIF-1α in cells where PHD2 was down regulated. Additionally, silencing of Ubc9 is sufficient to promote stability of the HIF-1α double proline mutant at normoxia.

In conclusion, our studies indicate that mHIF-1α(P402A/P563A) is a bona fide pro-angiogenic factor with potential therapeutic effects. Still, this protein is regulated in an oxygen-dependent manner by proteasome-mediated degradation. The degradation of mHIF-1α(P402A/P563A) proved to be pVHL-dependent yet PHD-independent. Further investigations indicated a role for SUMO modification in the HIF-1α degradation pathway. The presence of SUMO moieties on mHIF-1α(P402A/P563A) are sufficient for the recruitment of pVHL and thus promote degradation of the protein at normoxia, suggesting SUMOylation as the novel mechanism for degradation of HIF-1α.
LIST OF PUBLICATIONS


Stabilized HIF-1α Is Superior to VEGF for Angiogenesis in Skeletal Muscle via Adeno-Associated Virus Gene Transfer.

*These authors contributed equally for this publication


II. Helder André, and Teresa S. Pereira.

Identification of an Alternative Mechanism of Degradation of the Hypoxia-Inducible Factor-1α.

J. Biol. Chem. 2008 Oct 24; 283(43): 29375-84

III. Helder André, and Teresa S. Pereira.

Modification of HIF-1α by the Small Ubiquitin-like Modifier (SUMO) Targets the Protein for Degradation at Normoxia.

Manuscript

These papers are the base for this thesis, and will be referred to by their Roman numerals.
# LIST OF ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>C-TAD</td>
<td>C-terminal activation domain</td>
</tr>
<tr>
<td>C-TDB</td>
<td>C-terminal degradation box</td>
</tr>
<tr>
<td>CBP</td>
<td>Cyclic AMP response element binding protein (CREB)-binding protein</td>
</tr>
<tr>
<td>FIH-1</td>
<td>Factor inhibiting HIF-1</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>ID</td>
<td>Inhibitory domain</td>
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<tr>
<td>IPAS</td>
<td>Inhibitory PAS protein</td>
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<tr>
<td>N-TAD</td>
<td>N-terminal activation domain</td>
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<tr>
<td>N-TDB</td>
<td>N-terminal degradation box</td>
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<tr>
<td>ODD</td>
<td>Oxygen-dependent degradation domain</td>
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<tr>
<td>OS-9</td>
<td>Amplified in osteosarcomas gene 9 product</td>
</tr>
<tr>
<td>PAS</td>
<td>Per/ARNT/Sim</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain protein</td>
</tr>
<tr>
<td>pVHL</td>
<td>von Hippel-Lindau tumor suppressor gene product</td>
</tr>
<tr>
<td>SENP</td>
<td>Sentrin/SUMO-specific protease</td>
</tr>
<tr>
<td>Siah</td>
<td>Seven in absentia homolog</td>
</tr>
<tr>
<td>SIM</td>
<td>SUMO-interacting motif</td>
</tr>
<tr>
<td>SRC-1</td>
<td>Steroid receptor coactivator-1</td>
</tr>
<tr>
<td>STUbL</td>
<td>SUMO-targeted ubiquitin ligase</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>Ubc9</td>
<td>SUMO-conjugating enzyme</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VEC</td>
<td>pVHL/Elongins/Cullin2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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INTRODUCTION

The ability to adapt to surrounding environments has always been critical for the evolution of species. The capacity to maintain oxygen (O$_2$) homeostasis is essential for the survival of all organisms. Accordingly, organisms have developed a variety of mechanisms to regulate the oxygen levels of their tissues. These mechanisms range from simple diffusion in lower invertebrates where few cells are present to the development of complex physiological systems including the hematopoietic, cardiovascular, and respiratory systems in mammals (Covello and Simon, 2004).

The Hypoxia-Inducible Factors

The analysis of the molecular mechanisms by which erythropoietin (EPO) gene transcription is induced in response to hypoxia resulted in the initial discovery of hypoxia-inducible factor (HIF)-1 as a regulator of O$_2$ homeostasis. Subsequent research has revealed that a large number of hypoxia-inducible genes are regulated by HIF-1, suggesting that HIF-1 functions as a master transcriptional regulator of the adaptive response to hypoxia (Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998).

Biochemical purification and cloning of the HIF-1 protein has resulted in the identification of a heterodimeric complex formed by one α subunit and one β subunit (Wang and Semenza, 1995). The β subunit (HIF-1β) represents a previously identified heterodimerization partner of the aryl hydrocarbon receptor (AhR), named AhR nuclear translocator (ARNT) (Hoffman et al., 1991; Wang et al., 1995). The α subunit represents a novel protein (HIF-1α) and was further characterized as a labile nuclear factor induced in hypoxic cells, while ARNT was known to be a stable and ubiquitously expressed protein (Hirota and Semenza, 2005; Kallio et al., 1997; Salceda and Caro, 1997).

In mammals, six genes encode proteins of the HIF transcription factors – three genes encoding for HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) and another three encoding for HIF-β subunits (ARNT, ARNT2, and ARNT3). However, not all family members are involved in hypoxia-induced transcriptional activation. HIF-1α and HIF-2α are capable of activating the transcription of target genes, whereas HIF-3α appears to be only capable of weak transcriptional activation (Gu et al., 1998; Jelkmann, 2004; Jiang et al., 1996; Tian et al., 1998; Tian et al., 1997). Regarding the HIF-β subunits, only ARNT and ARNT2 can form functional HIF complexes, while ARNT3 cannot (Cowden and...
Additionally, dimerization between the different family members can be limited by expression patterns. The HIF-1α and ARNT proteins are ubiquitously expressed (the dimerization of which represents the canonical form of HIF). Their paralogs HIF-2α, HIF-3α, ARNT2, and ARNT3 have more restricted expression patterns (Covello and Simon, 2004; Kaelin, 2005; Weidemann and Johnson, 2008; Wenger, 2002).

More recently, a splice variant of the HIF-3α gene has been identified as a novel inhibitory PAS protein (IPAS) (Makino et al., 2001; Makino et al., 2002). IPAS lacks a transactivation domain and functions as a repressor of HIF signaling.

![Figure 1 – Schematic representation of HIF-1 and its subunits structural domains.](image)

At their N-termini, both HIF-1α and ARNT share basic helix-loop-helix (bHLH) and PAS domains that are involved in DNA binding and dimerization. The more variable C-termini are responsible for the transactivation functions, spanning a glutamine-rich (Q) transactivation domain in ARNT and two independent transactivation domains, named relatively to their position on the protein (NTAD & CTAD). HIF-1α has two additional characterized domains: the oxygen-dependent degradation domain (ODD) and the inhibitory domain (ID), responsible for oxygen-mediated degradation and inhibition of transcriptional activity, respectively. Note also the residues that undergo oxygen-dependent modifications within HIF-1α.

**HIF-1 protein domains**

The heterodimer HIF-1 is composed of two members of the family of transcription factors characterized by their bHLH/PAS domain (Figure 1). HIF-1α and ARNT share high homology within their N-termini, where both subunits contain a basic DNA-binding domain and a helix-loop-helix domain (bHLH), a dimerization interface, immediately followed by the PAS domain (an acronym for Per/ARNT/Sim, the first cloned members of the family). The last is formed by two contiguous repeats (PAS-A and PAS-B) and their functions have been target of investigation. Early reports suggested that they contribute to the dimerization specificity of the subunits in bHLH/PAS dimers formation (Lindebro et al., 1995; Pongratz et al., 1998). Subsequently, the solution structures of one of the PAS repeats, namely the PAS-B of both ARNT and HIF-2α, have been resolved, confirming their relevance for the
formation of the HIF heterodimer (Card et al., 2005; Erbel et al., 2003; Yang et al., 2005).

The C-termini of HIF-1α and ARNT have much higher variability and are involved in transcriptional regulation (Figure 1). ARNT has a potent glutamine-rich transactivation domain close to its carboxyl-end which is capable of driving transcription in the context of ARNT homodimers (Antonsson et al., 1995; Sogawa et al., 1995). Regarding HIF-1α, two independent transactivation domains can be identified in its C-terminal region. These two transactivation domains are commonly named after their relative position in the protein: N-terminal activation domain (N-TAD), and C-terminal activation domain (C-TAD) (Jiang et al., 1996; Jiang et al., 1997; Pugh et al., 1997; Wenger et al., 1997; Wenger et al., 1996). These domains have been shown to independently induce transactivation in a hypoxia-inducible manner, and recruit coactivators such as the p300/CREB-binding protein (CBP) and the steroid receptor coactivator-1 (SRC-1) (Carrero et al., 2000; Ema et al., 1999; Pereira et al., 2003; Ruas et al., 2002, 2005).

HIF-1α contains two additional domains within its C-terminus (Figure 1). The oxygen-dependent degradation domain (ODD), immediately preceded by the PAS domain and including the N-TAD, has been characterized to confer the oxygen-dependent degradation of this subunit (Huang et al., 1998). More C-terminally, the inhibitory domain (ID), located between the two transactivation domains, is the least characterized domain of HIF-1α (Jiang et al., 1997). Deletion analysis of the ID has demonstrated that gradual removal coincides with a progressive increase in transcriptional activity of HIF-1α transactivation domains (Jiang et al., 1997).

**Post-Translational Modifications and HIF-1 Regulation**

Much research in the field of hypoxia has focused on understanding the molecular mechanisms by which cells are capable of sensing O₂ levels and transducing this signal to HIF-1. ARNT protein levels and its activity as a transcription factor do not seem to be significantly influenced by O₂ levels. Instead ARNT is constitutively expressed and localized to the nucleus due to a constitutively active N-terminal nuclear localization signal (NLS) (Eguchi et al., 1997; Pollenz et al., 1994). On the other hand, HIF-1α stabilization and nuclear localization can only be achieved under hypoxic conditions (Kallio et al., 1998).
Taken together, the following observations characterize regulation of HIF-1α stability as an extremely dynamic process, capable of shifting from degradation to stabilization according to a multitude of parameters and based on the availability of several enzymes.

**Ubiquitylation**

Cellular proteins exist in a dynamic state. Under physiological conditions, protein degradation is approximately equal to protein synthesis. Modification of proteins by addition of ubiquitin moieties (ubiquitylation) plays a crucial role in the degradation of most cellular proteins. The best-studied modification involves proteolysis of the target substrate and occurs by covalent attachment of multiple ubiquitin molecules to the protein substrate. This modification acts as a signal for recognition of the protein by the 26S proteasome and subsequent degradation of the target molecule with recycling of free and reusable ubiquitin (Schwartz and Ciechanover, 2009).

The ubiquitin-proteasome system (UPS) appears to have a highly hierarchical structure. A single ubiquitin-activating enzyme (E1) provides the ubiquitin moieties required for all modifications, and can transfer ubiquitin to several ubiquitin-conjugating enzymes (E2). These latter enzymes act in concert with ubiquitin-protein ligases (E3). Each E2 enzyme can transfer ubiquitin to one or, more typically, to several E3 enzymes. Two major families of E3-ligases have been described: the HECT domain-containing and the RING-finger domain-containing E3s. In the latter, the RING-finger protein is a common component yet recognition is mediated by a different protein, such as an F-box protein in the SCF (Skp2, Cullin, F-box) ubiquitin ligase family. Following conjugation, the targeted protein is degraded, but free and reusable ubiquitin can be released via the activity of deubiquitylating enzymes (DUBs) (Schwartz and Ciechanover, 2009).

Until recently, ubiquitylation has been viewed as a complex molecular mechanism for proteolysis. However, it is now clear that ubiquitylation also serves as a signal for a variety of nonproteolytic functions (Mukhopadhyay and Riezman, 2007; Schwartz and Ciechanover, 2009).

Initial biochemical analyses of HIF-1α protein expression levels revealed a rapid turnover in cells under atmospheric oxygen conditions (21% O₂). However, under hypoxia (1% O₂) HIF-1α accumulates due to the inhibition of its degradation (Huang et al., 1998; Kallio et al., 1997). In addition, inhibition of the proteasomal degradation machinery has been shown to result in the accumulation of HIF-1α under non-hypoxic conditions, suggesting that HIF-1α is degraded by the UPS (Huang et al., 1998; Kallio et
al., 1999; Salceda and Caro, 1997). Subsequent studies have led to the identification of the ODD as being necessary for normoxia-dependent degradation of HIF-1α, since removal of this domain renders the protein stable under normal oxygen conditions (Huang et al., 1998; Sutter et al., 2000).

The observation that von Hippel-Lindau syndrome patients have a predisposition to develop highly vascularized tumors raised the question of HIF involvement in this disease. These tumors constitutively express vascular endothelial growth factor (VEGF) among other known hypoxia-inducible genes (Hirot and Semenza, 2005; Kaelin, 2005), confirming the initial hypothesis. The von Hippel-Lindau susceptibility gene, Vhl, encodes a product that acts as a tumor suppressor (pVHL) (Latif et al., 1993; Seizinger et al., 1988). Tumor cells that lack pVHL express high levels of HIF-1α and HIF-2α (Maxwell et al., 1999). Subsequently, it was shown that, via its β domain, pVHL binds directly to the ODD and polyubiquitylates HIF-1α, targeting it for degradation by the 26S proteasome (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Furthermore, pVHL has been characterized as the substrate-recognizing component of a complex with E3 ubiquitin-ligase activity. In order to form the VEC complex (HIF-α E3 ligase), pVHL interacts through its α domain with elongins B and C, cullin-2, and Rbx-1 (Figure 2). Structurally and functionally, the VEC is analogous to the SCF complex and is dependent on the recruitment of Ubch5a as its E2 ubiquitin-conjugating enzyme (Ohh, 2006).

Moreover, the pVHL-interacting deubiquitylation enzyme 2 (VDU2) has been shown to deubiquitylate HIF-1α, promoting protein stabilization (Li et al., 2005). In addition, both VDU2 and its homolog VDU1 are targeted to the UPS by pVHL (Li et al., 2002a; Li et al., 2002b), uncovering an intricately complex loop in HIF-1α protein regulation.

**Hydroxylation**

It has been demonstrated that HIF-1α must be hydroxylated on at least one of two conserved proline residues for pVHL recognition (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001a, b). Hydroxylation of human HIF-1α on Pro402 (N-terminal degradation box, N-TDB) and Pro564 (C-terminal degradation box, C-TDB) is intrinsically dependent on oxygen and is catalyzed by members of the prolyl hydroxylase domain (PHD) family of proteins (Bruick and McKnight, 2001; Epstein et al., 2001; Hirsila et al., 2003; Taylor, 2001) (Figure 2). Identification of these enzymes was greatly supported by C. elegans genetics, where the egl-9 gene was found to encode
an orphan prolyl hydroxylase. Consequently, *egl-9* mutant worms have been shown to constitutively express HIF-1α (Epstein et al., 2001). In silico analysis of the *egl-9* sequence led to the identification and cloning of three mammalian prolyl hydroxylase domain enzymes (PHD1-3) (Bruick and McKnight, 2001; Epstein et al., 2001), as regulators of the HIF-1α, -2α, and -3α subunits (Hirsila et al., 2003; Taylor, 2001). A protein named P4H-TM has been characterized as an additional member (PHD4) of these enzymes (Koivunen et al., 2007). Albeit anchored to the ER with its catalytic domain located in the lumen, PHD4 effectively hydroxylates and triggers HIF-1α degradation. Furthermore, the product of one of the genes amplified in osteosarcomas (OS-9) was identified as a novel HIF-1α interacting protein (Baek et al., 2005). OS-9 was demonstrated to bind both HIF-1α and the PHDs, namely PHD2 and PHD3. These data have suggested the formation of a multiprotein complex that is capable of stimulating HIF-1α hydroxylation and the subsequent recruitment of pVHL and proteasome-mediated degradation.

The oxygen-dependence of the PHDs is attributable to the fact that these are dioxygenases requiring molecular oxygen and 2-oxoglutarate as co-substrates. PHDs contain iron bound to their active centers and O₂ binding requires ascorbate-dependent maintenance of this iron molecule in its ferrous state. HIF-1α prolyl 4-hydroxylation occurs by transference of one oxygen atom to Pro⁴⁰² and/or Pro⁵⁶⁴, while the second oxygen atom reacts with 2-oxoglutarate. This yields succinate and carbon dioxide as reaction products, rendering this catalysis irreversible. The observation that HIF-α subunit hydroxylation is reduced under hypoxic conditions, similar to those known to stabilize HIF-1α, has confirmed that the PHDs are in fact functioning as oxygen sensors in the HIF signaling pathway (Epstein et al., 2001).

Moreover, the transactivation function of HIF-1α is negatively regulated by oxygen, independently of protein stability. Exposure of cells to hypoxia induces not only HIF-1α protein stability but also transactivation (Jiang et al., 1997; Pugh et al., 1997). These findings suggest the existence of a common molecular pathway between protein stabilization and transactivation.

Biochemical analysis of HIF-1α ID resulted in the identification of the factor inhibiting HIF-1 (FIH-1), a novel HIF-1α interacting protein that acts as a negative regulator of the HIF-1 transactivation function (Mahon et al., 2001). Subsequent studies identified a conserved asparagine residue (Asp⁸⁰³ of hHIF-1α) within the C-TAD that was found to be hydroxylated under normoxic conditions, inhibiting the binding of the coactivators CBP/p300 to HIF-1α (Lando et al., 2002b). Further analysis identified FIH-
1 as the HIF-1α asparaginyl hydroxylase (Hewitson et al., 2002; Lando et al., 2002a), belonging to the same dioxygenase family and sharing the same cosubstrates and mechanisms of action as the PHDs. Structural studies have shown that FIH-1 homodimers form in solution and are essential for catalysis of the β-carbon hydroxylation on Asp⁸⁰₃ (Dann et al., 2002). Interestingly, the $K_m$ of FIH-1—$O_2$ interaction is approximately 100 µM (Koivunen et al., 2004), compared to approximately 200 µM for the PHDs (Hirsila et al., 2003), suggesting the existence of a window where $O_2$ availability is insufficient to promote protein degradation but is still sufficient for inhibition of transcriptional activation (Figure 2).

Figure 2 – The hypoxia-inducible factor pathway.

HIF-1α is ubiquitously expressed. However, under normal oxygen conditions this protein is rapidly hydroxylated on specific proline residues by a family of prolyl hydroxylase domain proteins (PHD). Hydroxylation of the prolines is the signal for the binding of pVHL (VHL) and recruitment of elongins B and C (B & C), cullin-2 (Cul2), and Rbx1, to form an E3 ubiquitin-ligase complex. Consequently, HIF-1α is polyubiquitylated and degraded by the 26S proteasome. At reduced levels of oxygen PHD function is inhibited and regulation by degradation is compromised. An additional regulatory mechanism is still active at lower concentrations of $O_2$, this time through the inhibition of HIF-1α transcriptional activity. Due to a lower $K_m$ for $O_2$, the factor inhibiting HIF-1 (FIH-1) is capable of hydroxylating an asparagine residue within the C-TAD of HIF-1α and blocking its ability to recruit coactivators. Finally, under hypoxic conditions HIF-1α is stabilized, transcriptionally active, and free to translocate into the nucleus. In the nuclear compartment HIF-1α dimerizes with ARNT and forms the HIF-1 transcription factor complex. This complex is then capable of recognizing hypoxia response elements (HRE) and initiating the transcription of target genes through the recruitment of CBP/p300 and other coactivators.
Another level of complexity was illustrated by the fact that hypoxia leads to elevated levels of PHD2 and PHD3 mRNA expression in a HIF-1-dependent manner (Metzen et al., 2005; Pescador et al., 2005). These studies have underscored the importance of these enzymes during the reoxygenation processes (Berra et al., 2003; D'Angelo et al., 2003; Marxsen et al., 2004; Stiehl et al., 2006). However, PHD1 and PHD3 protein levels are sensitive to oxygen concentrations. These enzymes have been shown to interact with Siah (seven in absentia homolog) proteins, which contain a RING-domain and possess E3 ubiquitin-ligase activity (Nakayama et al., 2004). Siah2 is therefore capable of down-regulating PHD1 and -3 protein levels. In similarity to the PHDs, Siah1 has been reported to target FIH-1 for degradation by the UPS (Fukuba et al., 2008; Fukuba et al., 2007). These findings suggest that Siah ubiquitin-ligases might play a role as upstream regulators of both HIF-1α hydroxylases (PHDs and FIH-1) by targeting these dioxygenases for proteasome-mediated degradation under hypoxic conditions, thereby facilitating HIF-1α stabilization and transcriptional activity (Fukuba et al., 2007; Nakayama et al., 2009; Nakayama and Ronai, 2004).

SUMOylation

In addition to ubiquitin, a series of ubiquitin-like proteins have been identified in humans. This sub-family of proteins has been the subject of much interest, with members such as NEDD8, ISG15, and the small ubiquitin-like modifier (SUMO).

In vertebrates three paralogs of SUMO have been identified (SUMO-1, -2, and -3). The pathway of SUMOylation is reported to affect many biological processes and is required for cell viability from yeast to higher eukaryotes (Hay, 2005; Wilson and Heaton, 2008; Yeh et al., 2000). Curiously, SUMO-1 seems to be capable only of monoSUMOylation (single molecule conjugations), while the two highly homologous SUMO-2/-3 can form polySUMOylation chains (Tatham et al., 2001).

Similar to the ubiquitin pathway, SUMOylation is a dynamic process that is mediated by E1 activating, E2 conjugating, and E3 ligating enzymes. For the SUMO pathway, the E1 activating enzyme is a heterodimeric complex formed by the proteins SAE1 and SAE2 (SUMO-activating enzyme) (Desterro et al., 1999; Gong et al., 1999). In contrast to the more complex ubiquitin pathway, SUMOylation utilizes only one conjugating enzyme, Ubc9 (Gong et al., 1997), and a limited number of ligases (Johnson and Gupta, 2001; Kahyo et al., 2001; Pichler et al., 2002; Takahashi et al., 2001). One particularity of the SUMO pathway is the ability of Ubc9 to directly interact with substrate proteins.
through recognition of a specific SUMO-conjugating motif. This motif is characterized by a consensus sequence surrounding the acceptor lysine residue, $\psi$KxE (where $\psi$ is a large hydrophobic residue, K is the acceptor lysine, x is any amino acid, and E is glutamic acid). Although it is clear that modification of most substrates takes place within the SUMO modification consensus, many substrates are modified on non-consensus lysines (Hay, 2005; Wilson and Heaton, 2008). Furthermore, the presence of a SUMO modification consensus does not directly imply SUMOylation of a protein.

The SUMOylated status of a protein is not static. A dynamic equilibrium between SUMOylation and deSUMOylation is granted by the SUMO-specific proteases (SENP). SENPs are cysteine proteases and are involved in both the activation of SUMO precursors (endopeptidase cleavage) and deconjugation of the SUMO moieties from the targets (isopeptidase cleavage) (Drag and Salvesen, 2008; Yeh, 2009). Again when compared to the ubiquitin pathway, deSUMOylation shows a higher simplicity since only six SENPs have been identified in humans. These enzymes denote high divergence at their N-termini and a conserved catalytic domain C-terminally. Furthermore, they differ in their primary subcellular localization, as well as substrate specificity, and their activities range from endo- and isopeptidases of SUMO molecules to chain editing for SUMO-2/3.

Additionally, many non-SUMOylated proteins contain SUMO-interacting motifs (SIM) that promote association with SUMO molecules, and therefore SUMOylated proteins (Perry et al., 2008; Wilson and Heaton, 2008). Subsequently, proteomic analyses of these motifs led to the discovery of a novel family of E3 ubiquitin-ligases that are specifically targeted to SUMO-conjugated proteins (Prudden et al., 2007; Sun et al., 2007; Uzunova et al., 2007). These SUMO-targeted ubiquitin-ligases (STUbL) are RING-finger ubiquitin-ligases that contain SIMs and bind SUMO non-covalently, allowing degradation of SUMOylated substrates by the UPS (Perry et al., 2008). As such, STUbLs provide an unanticipated direct cross-talk between the ubiquitin and SUMO pathways, against the classical view that SUMO antagonizes ubiquitylation by competing for acceptor lysine residues on the target proteins.

SUMO modification of HIF-1$\alpha$ has been reported, albeit with contradictory results. SUMOylation on HIF-1$\alpha$ transcriptional activity has been suggested to mediate either repression (Berta et al., 2007; Cheng et al., 2007) or activation (Bae et al., 2004; Carbia-Nagashima et al., 2007). In a similar fashion, regulation of HIF-1$\alpha$ protein levels by SUMO is also a matter of controversy. Studies involving SENP1 indicate that this enzyme contributes to stabilization of HIF-1$\alpha$ at hypoxia (Cheng et al., 2007) and
therefore connects SUMO with degradation of the protein. Contrastingly, another study investigating the function of a protein that enhances SUMOylation (RSUME) suggested that SUMO modification mediates stability of HIF-1α (Carbia-Nagashima et al., 2007).

Although the results regarding the function of SUMOylation on HIF-1α are contradictory, it is clear that HIF-1α is modified by SUMO conjugation in at least two consensus-centered lysine residues (Lys$^{391}$ and Lys$^{477}$ of hHIF-1α) (Bae et al., 2004; Berta et al., 2007). Further research is undoubtedly needed to assess the functions of SUMO modification on HIF-1α regulation.

**Other modifications**

Besides to the previously described, additional HIF-1α modifications have been identified. A report suggested that acetylation on Lys$^{532}$ by arrest defective 1 (ARD1) promotes HIF-1α polyubiquitylation by enhancement of pVHL interaction (Jeong et al., 2002). However, the biological effects of ARD1 on HIF-1α have been challenged by other groups (Bilton et al., 2005; Fisher et al., 2005).

Further modifications have been characterized on either HIF-1α or HIF-2α, namely reduction of Cys$^{25}$ (Lando et al., 2000) and Cys$^{800}$ (Ema et al., 1999), as well as S-nitrosation of Cys$^{800}$ (Yasinska and Sumbayev, 2003). A phosphorylation modification has also been mapped to Thr$^{844}$ of HIF-2α (Gradin et al., 2002). These modifications have been associated with effects on DNA-binding and recruitment of CBP/p300 family of coactivators by the HIF-α proteins.

**Hypoxia and Physiology**

Under hypoxic conditions HIF-1α is stabilized against proteasome-mediated degradation, followed by nuclear translocation due to the presence of a C-terminal NLS (Kallio et al., 1998) (Figure 2). Once in the nucleus, HIF-1α heterodimerizes with ARNT, forming the transcriptionally active form of HIF-1. Formation of HIF heterodimers seems to be favored to other bHLH/PAS nuclear factors, due to a preferred affinity of ARNT to HIF-1α (Gradin et al., 1996). In this way, hypoxia may indirectly limit the availability of ARNT as a dimerization partner and have implications on their signaling pathways.
**HIF-induced transcription**

HIF binds DNA at hypoxia response elements (HRE), which are minimum cis-acting elements required for hypoxic induction of gene transcription (Semenza and Wang, 1992) which are present in enhancer regions of HIF-regulated genes (Figure 2). Analysis of HREs of known hypoxia-inducible genes led to the identification of a conserved core motif that can be characterized as 5’-RCGTG-3’ (where R is A or G) (Weidemann and Johnson, 2008; Wenger et al., 2005). Although microarray experiments have identified over 200 genes as targets for HIF regulation, subsequent experiments have so far mapped HREs to about 70 known HIF target genes. This may indicate HIF-dependent regulation of hypoxia-inducible genes through interactions with other transcription factors through yet unknown mechanisms.

Multiple studies have reported specific regulation of some HIF target genes by either HIF-1 or HIF-2 (Covello et al., 2006; Elvert et al., 2003; Holmquist-Mengelbier et al., 2006; Raval et al., 2005; Scortegagna et al., 2005; Warnecke et al., 2004). The mechanism by which HIF-1 and HIF-2 distinguish their respective target genes remains unclear. It does not seem, however, to be dependent on the core HRE sequence, but possibly on expression patterns of the α subunits or even on cooperation with other transcription factors (Ruas and Poellinger, 2005; Wenger et al., 2005).

The list of HIF target genes is constantly expanding (Wenger et al., 2005). Known HIF-regulated genes are commonly listed under categories such as: oxygen supply (Epo, VEGF and its receptors, inducible and endothelial nitric-oxide synthases, heme oxygenase 1, transferrin and its receptor, endothelin-1); cellular metabolism (aldolase A, phosphoglycerate kinase 1, lactate dehydrogenase A, glucokinase, glucose transporter 1, carbonic anhydrase IX); cell growth and apoptosis (tumor growth factor-β3, connective tissue growth factor, telomerase, myeloid cell factor-1, nucleophosmin); and others (Cited2, inhibitor of differentiation 2, furin, matrix metalloproteinase-1, PHD2, PHD3).

These lists continue to mark the relevance of HIF in physiological processes, including embryonic development, adaptation to high altitude, wound healing, and inflammation, as well as its contribution to the pathophysiology of cancer and ischemic diseases.
Hypoxia-mediated transcriptional repression

HIF-1 activity has also been shown to be subject to negative regulation at the DNA-binding level. The expression of IPAS in the corneal epithelium of the eye is important for the avascular phenotype of this tissue (Makino et al., 2001). Here, IPAS directly competes with ARNT for the HIF-α subunits forming an abortive complex, incapable of DNA-binding. IPAS is the product of a hypoxia-induced splice variant of HIF-3α (Makino et al., 2002), which reveals a direct hypoxia-inducible negative-feedback loop in hypoxic tissues where this inhibitory protein is expressed. Another negative-feedback mechanism has been suggested for HIF activation of transcription. Hypoxia-inducible expression of Cited2 leads to a competitive binding to CBP (Bhattacharya et al., 1999; Freedman et al., 2003), reducing the availability of this coactivator for the hypoxic response (Yin et al., 2002).

Three other naturally occurring potential HIF-1 antagonists have been reported: aHIF, an antisense RNA complementary to the 3’ untranslated region of HIF-1α (Thrash-Bingham and Tartof, 1999); HIF-1αZ, a zinc-inducible isoform lacking exon 12 (Chun et al., 2001); and a dominant-negative HIF-1α isoform lacking exons 11 and 12 (Chun et al., 2002). The physiological relevance of these antagonists remains unclear.

These findings highlight the need to tightly regulate the HIF signaling pathway, either by interfering with its capacity to fully induce transcription or by rapidly shutting-down this pathway during the reoxygenation of the tissues.

HIF in development

During embryogenesis, hypoxia plays an essential role for normal embryonic development. Initially, diffusion is sufficient to provide nutrients and O₂ needed for cellular metabolism. However, as cells continue to divide and embryonic growth reaches the gastrulation stage, O₂ and nutrients can no longer reach all cells by passive diffusion. Consequently, development of the vascular system begins.

Development of the vascular system is a complex process that involves multiple stages. Firstly vasculogenesis, a process involving de novo differentiation and formation of vascular structures from endothelial progenitor cells or stem cells, gives rise to vascular channels. This is followed by angiogenesis, where new blood vessels sprout from the previously formed ones. Finally, vessel maturation involves recruitment of supporting cells, such as pericytes and smooth muscle cells, in a process commonly
known as arteriogenesis. Many of the components involved in these processes have been identified and involve factors such as VEGF and its receptors (Flt-1 and Flk-1), angiopoietins (Ang) and their receptors (Tie-1 and Tie-2), and fibroblast growth factor (FGF) (Weidemann and Johnson, 2008; Yla-Herttuala and Alitalo, 2003). Interestingly, the expression of many of these growth factors and receptors is activated by HIF. The involvement of this transcription factor in such mechanisms is well-documented in loss-of-function studies (Doedens and Johnson, 2007), where both HIF subunits have proven essential for embryonic development.

Mice deficient in ARNT die within 10.5 days of embryonic development (E10.5) and are characterized by defective vascularization of the yolk sac, brachial arches, and placenta (Adelman et al., 2000; Kozak et al., 1997; Maltepe et al., 1997). Although initial development of vessels does not seem to be affected, vessel remodeling is impaired in the Arnt<sup>−/−</sup> embryos. In addition, these findings also revealed the relevance of HIF during placentation.

Nullizygous Hif-1α mutants also display embryonic lethality by E11, with vascular defects and malformations in cardiovascular morphogenesis (Iyer et al., 1998; Ryan et al., 1998). Vasculogenesis of Hif-1α<sup>−/−</sup> embryos occurs normally until E9.5, when a marked regression of the endothelium is noted. These embryos also exhibited defects in neural tube closure and extensive cell death, particularly in the cephalic and brachial regions.

The effects of HIF-2α inactivation are less clear. Three independent groups have reported strikingly different results including defects in catecholamine biosynthesis and perinatal death due to respiratory distress syndrome (Compernolle et al., 2002; Peng et al., 2000; Tian et al., 1998).

Additionally, knockout strategies have been employed to determine the role of many of the proteins involved in orchestrating the HIF pathway (Doedens and Johnson, 2007). pVHL, the recognition component of the VEC ubiquitin-ligase responsible for HIF-α subunits’ degradation is also essential for embryonic viability. Embryos with inactivation of Vhl died in utero at about E12 due to defects in placental development (Gnarra et al., 1997). Subsequent studies however, revealed a defect in fibronectin deposition (Ohh et al., 1998; Tang et al., 2006), suggesting that pVHL deletion might have both HIF-dependent and -independent roles.

The PHD enzymes play a key role in HIF regulation by hydroxylating and leading to pVHL recognition of the HIF-α subunits. Genetic ablation of PHD1, -2, and -3 has been carried out in mice (Takeda et al., 2006). Although Phd1<sup>−/−</sup> and Phd3<sup>−/−</sup> mice were viable
and apparently healthy, \textit{Phd2} was embryonic-lethal at E12.5 to E14 due to defects in heart and placenta development. While the placental defects were related to HIF-\(\alpha\) subunit levels, no increase of HIF-\(\alpha\) subunits was detected in the heart, suggesting roles for the PHDs other than HIF-\(\alpha\) regulation. Furthermore, both HIF-\(\alpha\) hydroxylases (PHDs and FIH-1) are negatively regulated by Siah ubiquitin ligases. Deletion of \textit{Siah1} and \textit{Siah2} genes, isolated or concomitantly, resulted in no observed phenotype (for \textit{Siah2}\(^{-/-}\)) to perinatal lethality (for \textit{Siah1a}\(^{-/-}\) and \textit{Siah1a}\(^{-/-}\)/\textit{Siah2}\(^{-/-}\)) (Frew et al., 2003). These phenotypes did not seem to be directly associated with the HIF pathway. However, subsequent studies with \textit{Siah2}\(^{-/-}\) mice showed that these animals have responses to chronic hypoxia comparable to those of \textit{Hif-1\(\alpha\)} heterozygous mice (Nakayama et al., 2004; Yu et al., 1999).

The observation of embryonic lethality of \textit{SENPI}\(^{-/-}\) mice due to placental abnormalities and severe fetal anemia arising from deficient EPO production, led to the discovery that \textit{SENPI} plays a critical role in regulating HIF-1\(\alpha\) stability during hypoxia (Cheng et al., 2007).

Taken together, these mouse models indicate that HIF plays an important role during postvasculogenesis stages of embryonic development and is required for the remodeling of the primary plexus into a vascular network.

**HIF and postnatal development**

HIF is also involved in physiological responses to \(O_2\) levels during postnatal life and is detectable in many adult tissues under normal oxygen conditions.

Analysis of adult \textit{Hif-1\(\alpha\)}\(^{+/+}\) mice demonstrated impaired responses to chronic hypoxia such as right ventricular hypertrophy, pulmonary artery hypertension, and pulmonary vascular remodeling (Yu et al., 1999). Cartilage is a naturally hypoxic tissue and conditional gene targeting of \textit{Hif-1\(\alpha\)} by floxed alleles in chondrocytes results in skeletal malformations and perinatal death (Schipani et al., 2001). Many other tissue-specific studies indicate functions of HIF-1\(\alpha\) in secretion and epithelial differentiation in the mammary gland (Seagroves et al., 2003), control of adipocyte differentiation (Yun et al., 2002), neuronal differentiation and survival (Jogi et al., 2002; Tomita et al., 2003), and control of inflammatory responses by the myeloid lineage (Cramer et al., 2003).

Generation of adult \textit{Hif-2\(\alpha\)} null mutant mice was achieved by out-breeding two heterozygous animals from different genetic backgrounds (Scortegagna et al., 2003a; Scortegagna et al., 2003b). Analysis of these mice revealed pancytopenia, multiorgan...
pathology, including cardiac hypertrophy and hepatomegaly, and other pathologies related to mitochondrial dysfunction. In another study, partial deficiency of \textit{Hif-2\(\alpha\)} reduced retinal vascularization, and protected the retina from hypertrophy of the vasculature in a model of hyperoxia-induced retinopathy (Morita et al., 2003).

Furthermore, tissue specific constitutive activation of HIF-\(\alpha\) has been achieved (Elson et al., 2001; Kim et al., 2006). In the skin, this resulted in a drastic increase of dermal capillary density and VEGF expression, without inflammation, edema, or vascular leakage. In the liver, these studies confirmed the phenotypes observed with tissue specific pVHL ablation (Haase et al., 2001), resulting in hepatocellular steanosis along with vascular tumors and local angiogenesis. Curiously, the liver studies showed that although HIF-1\(\alpha\) and HIF-2\(\alpha\) shared many target genes, the genes induced by either of HIF-\(\alpha\) subunits were not identical, indicating again the specificity of each subunit to different targets.

**HIF in pathology**

HIF is also implicated in pathological angiogenesis, such as neovascularization in the ischemic myocardium, hypoxia-induced pulmonary vascular remodeling, and tumor vascularization. The latter has been subject of intense research since HIF activation is a common feature of tumors. The classic example are the tumors associated with the von Hippel-Lindau syndrome (Maxwell et al., 1999), due to the loss of the well-characterized tumor suppressor pVHL function with subsequent activation of HIF-1\(\alpha\). Nevertheless, the mechanisms contributing to HIF activation in tumors are complex and difficult to dissect. They can range from the hypoxic tumor microenvironment itself to the activation of oncogenes and the inactivation of tumor suppressors (Weidemann and Johnson, 2008).

In genetic models, HIF-1\(\alpha\) has been identified as a positive factor for tumor growth (Ryan et al., 2000). Increased HIF-1\(\alpha\) activation was also correlated with the development of a more aggressive phenotype in carcinogenesis models (Elson et al., 2000; Mazzone et al., 2009). As such, HIF-1\(\alpha\) appears to be highly involved in the development of a characteristic tumor phenotype influencing growth rate, invasiveness, and metastasis. Although, it has to be taken into account that HIF-1\(\alpha\) does not only regulate downstream processes but is instead influenced by the tumor microenvironment in many different ways, allowing many possible scenarios.
Pharmacological manipulation of HIF

The central role of HIF in physiology, as well as in pathology, makes it an attractive target for pharmacological manipulation. Inhibitors of HIF could have potential as anticancer therapeutics, whereas activators of HIF might be useful for the treatment of ischemic disease (Weidemann and Johnson, 2008).

HIF and HIF-regulated genes are often detected in cancer, either by the classic example of von Hippel-Lindau syndrome or by the presence of hypoxic foci in the tumors. HIF inhibitors tend to target the HIF pathway by decreasing its mRNA or protein levels, and by inhibiting DNA binding or HIF-mediated transactivation (Fukuda et al., 2007; Weidemann and Johnson, 2008).

On the other hand, the physiological HIF response in ischemic, hypoxic and inflammatory conditions is well documented. Different chemical compounds have been utilized to activate HIF, particularly inhibitors of PHDs (Warnecke et al., 2003; Weidemann and Johnson, 2008). The PHDs are non-heme Fe(II)- and 2-oxoglutarate-dependent dioxygenases, and compounds competing with either of these components compromise their enzymatic activity, therefore stabilizing HIF at the protein level (Fong and Takeda, 2008). The best studied of these chemicals is dimethyloxalylglycine (DMOG), a 2-oxoglutarate antagonist. Other commonly used chemicals, particularly in in vitro studies, comprise iron-chelators and iron-oxidizing agents such as 2,2’-dipyridyl, deferoxamine, and divalent ions (cobalt and nickel salts).

Nonetheless, a general activation or inhibition of HIF will most likely produce pronounced side effects. Thus, it is mandatory a highly specific targeting of the organ or tissue of interest.

Gene Therapy and Vasculogenesis

Gene therapy is defined by the transfer of nucleic acids to somatic cells of an individual with resulting therapeutic effects. Among existing therapeutic approaches, gene therapy has advantages such as selective local treatment, the possibility of using endogenous proteins locally in cases where systemic delivery would lead to severe side effects, and the possibility of long-term effects after one single administration (Yla-Herttuala and Alitalo, 2003). Some of the few approved clinical trials for gene therapy involve vascular growth and pro-angiogenic therapy.
Gene transfer vectors

The administration of naked DNA represents the simplest form of gene transfer. Nonetheless, with the exception of skeletal muscle, only a small amount of the DNA is taken into a cell resulting in very low gene transfer efficiency (Laitinen et al., 1997). The use of liposomes or polymer complexes improves the delivery of plasmid DNA to the cytoplasm, although only a small fraction enters the nucleus, where the plasmids remain extra-chromosomal (Yla-Herttuala and Alitalo, 2003). Plasmids are expressed transiently in target cells, usually for 1-2 weeks.

Due to the low transfer efficiency of plasmid DNA, the use of modified viral vectors represents one option to increase efficiency. One of the most commonly used viral vectors is a virus that can transduce both dividing and non-dividing cells, the adenovirus. In target cells, adenoviruses escape endosomes, releasing the transgenes to the cytoplasm where they are transported into the nucleus. Once in the nuclear compartment, the transgenes remain extra-chromosomal and provide transient expression for 1-2 weeks (Yang et al., 1994).

Other vectors used in gene therapy involve the use of different viruses that integrate into the host genome and produce long-lasting transgene expression. For instance, retrovirus can only transduce dividing cells (Laitinen et al., 1997). For that reason, their utility has been limited and they have mainly been used in the ex vivo transduction of cells that are later returned to the host. Lentiviruses have been used for the central nervous system and liver due to their high transduction in these tissues (Trono, 2000). Adeno-associated viruses (AAV) show higher transduction in skeletal muscle, myocardium, and blood vessels (Monahan and Samulski, 2000) and are becoming the most used vector for gene delivery to the cardiovascular system. Although at a preliminary stage, nanoparticles have been used in gene therapy and proved efficient vectors in applications such as perivascular delivery (Kubik et al., 2005).

In spite of the promising results, the use of gene therapy is limited at this time due to possible adverse effects observed in vivo (Markkanen et al., 2005).

Gene delivery in vascular disease

The cardiovascular system presents specific physiological and anatomical characteristics that permit the improved delivery of genes. This includes the possibility to catheterize blood vessels (Laitinen et al., 1998) or make use of the perivascular
compartments for local delivery (Arras et al., 1998). In addition, intramuscular injection can lead to sustained transgene expression, providing a simple and efficient way to induce therapeutic vascular growth (Coura and Nardi, 2007; Daya and Berns, 2008; Yla-Herttuala and Alitalo, 2003). Notably, transient gene expression by extra-chromosomal transgenes should be sufficient for vascular gene therapy.

Factors used to stimulate therapeutic vascular growth include, among others, VEGFs (Rissanen et al., 2003), angiopoietins (Shyu et al., 1998), and FGFs (Giordano et al., 1996). Interestingly, the concomitant use of two or more of these factors has so far produced the best pre-clinical results (Arsic et al., 2003; Chae et al., 2000), revealing the possibility of a common upstream regulator of vascular growth. In fact, most of the factors used in therapeutic vascular growth are upregulated in response to hypoxia.

Transgenic mice expressing a skin-specific constitutive form of HIF-1α (K14-HIF-1αΔODD) exhibit increased dermal capillarization that is indistinguishable from normal capillaries, both in diameter and in morphology (Elson et al., 2001). In a subsequent study, treatment of animals with a specific inhibitor of the endogenous HIF-α degradation pathway improved angiogenesis in an ischemic limb model (Milkiæwicz et al., 2004). Observations like these led to the assumption that HIF-1α might be a good candidate for gene therapy, being capable of stimulating both angiogenesis and arteriogenesis, for an improvement in tissue perfusion. Moreover, similar results were obtained with skin conditional transgenic animals expressing a constitutive form of HIF-2α (HIF-2α(P405A/P531A)) (Kim et al., 2006), as well as animals with conditional knockout of pVHL in this tissue (Boutin et al., 2008).

Early studies with constitutively active forms of HIF-1α in gene therapy used naked DNA in a rabbit model of limb ischemia (Vincent et al., 2000). Criticism could arise from the fact that the transgene used for HIF-mediated transcription (HIF-1α(1-390)-VP16) was highly modified in comparison to wild-type HIF-1α. Nonetheless, the same transgene was used in an acute myocardial infarction model, through naked DNA injection in the rat, and yielded similar results (Shyu et al., 2002). Subsequent studies using adenovirus-mediated transduction of another constitutively active form of HIF-1α (HIF-1αΔ(392-520)(P567T/P658G)) proved successful in the induction of angiogenesis in non-ischemic tissues by regulation of multiple angiogenic growth factors in vivo (Kelly et al., 2003).

These findings underscore the utility of HIF-1α as a bona fide candidate for therapeutic vascular growth, capable of mimicking endogenous responses to ischemic stress and induce the expression of a natural cascade of downstream growth factors.
AIMS

The present investigation is focused on the evaluation of HIF-1α protein stability and its implications in physiological events. We aim to analyze the mechanisms of degradation of HIF-1α, as well as the role of stabilized forms of the protein in the formation of functional neovasculature. Specifically, our goals are:

• To evaluate the neoangiogenic potential of HIF-1α in comparison with known angiogenic factors, using AAV gene delivery to skeletal muscle. (Paper I)

• To investigate the existence of previously uncharacterized mechanisms for degradation of HIF-1α. (Papers II & III)
RESULTS

In this study we have examined the mechanisms of degradation of HIF-1α and their implications for generating stabilized mutant forms of HIF-1α. In an effort to evaluate the function of such stabilized forms in vivo, we have analyzed their expression in murine skeletal muscle by AAV-mediated transduction. In order to maintain a species-matched approach, all the studies were performed using the mouse orthologue of HIF-1α.

Induction of Neoangiogenesis by HIF-1α Proline Mutants (Paper I)

Cardiovascular diseases are one of the highest causes of morbidity and mortality in the Western world. These diseases are often characterized by an inadequate blood supply to organs or tissues (ischemia). Pro-angiogenic gene therapy may represent a viable therapeutic alternative to conventional clinical approaches to induce the formation of new vasculature and improve the blood supply to the affected areas. Notably, the introduction of angiogenic growth factors, such as VEGF (Ferrara et al., 2003), Ang-1 (Thurston et al., 2000), and platelet-derived growth factor-B (PDGF-B) (Gerhardt and Betsholtz, 2003), have been used in pre-clinical trials with promising success.

In paper I, we compared the in vivo neoangiogenic potential of mHIF-1α(P402A/P563A) against other known angiogenic growth factors in AAV-transduced mouse tibialis anterior (TA) muscles. The use of AAV as the gene transfer vehicle has been shown to be advantageous due to an optimal transduction of muscle tissue and lack of inflammatory responses (Buning et al., 2004).

For expression analysis, HeLa cells were in vitro transduced with the different recombinant AAV (rAAV) vectors. The expression of secreted angiogenic growth factors (VEGF_{120} or VEGF_{164}, Ang-1, and PDGF-B) was analyzed by co-immunoprecipitation with the respective receptor-Ig molecules (VEGFR-1, Tie-2, and PDGFR-β), while expression of mHIF-1α(P402A/P563A) was analyzed by immunohistochemistry. An additional stabilized form of mHIF-1α was used to generate rAAV – mHIF-1α(1-771)(P402A/P563A)-VP16 – where the C-TAD of mHIF-1α was replaced with the Herpes simplex protein-16 transactivation domain. This hybrid protein
is a potent inducer of HIF-mediated transactivation, both at normoxia and hypoxia (unpublished observations). However, when transduced in vivo by injection into mouse TA muscles, mHIF-1α(1-771)(P402A/P563A)-VP16 showed a decline in expression over time, rendering this mutant unsuitable for further studies. To avoid this, subsequent studies were performed using only the full-length double proline mutant of mHIF-1α. Although this mHIF-1α mutant undergoes proteasome-mediated degradation when overexpressed in cell cultures (Paper II), overexpression in mouse muscle resulted in functional activity. This effect may either be due to saturation of the degradation process and/or derepression of the transactivation potential. Noticeably, these issues remain to be investigated in the mouse muscle in vivo model.

Analysis of mouse TA muscles transduced with mHIF-1α(P402A/P563A), VEGF_{120}, or VEGF_{164} was performed using immunohistochemical methods. Staining for PECAM-1 (a cell surface marker on endothelial cells) and for phosphohistone H3 (pHH3) (a marker of proliferating cells) showed a massive proliferation of capillary endothelial cells following exposure to both VEGF isoforms. Nonetheless, VEGF-induced proliferation of the endothelium was not associated with the proper formation of new capillaries, as demonstrated by the low colocalization signals for the staining for proteoglycan NG2 (a cell surface marker for pericytes) with PECAM-1. In contrast to these results, TA muscles transduced with mHIF-1α(P402A/P563A) showed colocalized signals for PECAM-1 and NG2, accompanied with organization in sprouting capillary-like patterns. These results suggest that HIF-1α is a more potent inducer of neoangiogenesis than to VEGF.

Ang-1 has been associated with vascular maturation (Thurston et al., 2000), and PDGF-B has been shown to recruit pericytes and smooth muscle cells to newly formed capillaries (Gerhardt and Betsholtz, 2003). We next examined the effects of combined expression of HIF-1α with these arteriogenic growth factors. For these experiments, mouse TA muscles were cotransduced with rAAVs encoding mHIF-1α(P402A/P563A) together with rAAVs expressing either Ang-1 or PDGF-B. Both combinations showed increased endothelial sprouting and capillary formation, similar to that produced by mutant mHIF-1α alone. However, an increased recruitment of pericytes was observed following concomitant transduction of mHIF-1α(P402A/P563A) with PDGF-B, as shown by NG2 staining. Visualization of thick sections of these muscles showed numerous, intact, newly formed capillaries interconnecting the muscle fibers.

To analyze the physiological function of the newly formed capillaries, vascular permeability (Evans Blue dye extravasation) and perfusion (Doppler ultrasound
imaging) were assessed. As observed previously (Larcher et al., 1998), mouse TA muscles overexpressing either isoform of VEGF showed high levels of vascular leakage. Vascular permeability could be reduced by cotransduction of VEGF and Ang-1. In contrast, muscles transduced with mHIF-1α(P402A/P563A), alone or in combination with Ang-1 or PDGF-B, showed comparable leakiness to control transduced muscles (rAAV-human serum albumin) and native muscles (non-transduced). Furthermore, mHIF-1α(P402A/P563A)-transduced TA muscles showed enhanced perfusion in comparison to control transduced muscles. Cotransduced muscles overexpressing mHIF-1α mutant and Ang-1 displayed further improved perfusion, whereas PDGF-B and mHIF-1α(P402A/P563A) generated perfusion rates comparable with control transduced mouse TA muscles. Previous studies using skin-specific transgenic mice overexpressing a stabilized form of HIF-1α (hHIF-1αΔODD) also support these findings (Elson et al., 2001). Curiously, in the referred studies, the improvement observed in vascular leakiness could not be correlated to neither Ang-1 nor Ang-2 overexpression, suggesting that the improvement observed in mouse TA muscles cotransduced with mHIF-1α(P402A/P563A) and Ang-1 might be due to a cooperative action between these two factors.

It is noteworthy that HIF is responsible for the activation of endogenous angiogenic cascades that lead to vascular growth and remodeling, vascular tonus, and cellular proliferation. HIF-1α has been shown to directly and indirectly induce a multitude of growth factors, including VEGF, involved in many of these mechanisms (Wenger et al., 2005). Therefore, HIF-1α is a potent inducer of angiogenesis and arteriogenesis in the adult, and its expression in pro-angiogenic gene therapy experiments may initiate the necessary conditions for the formation of functional new blood vessels.

**Oxygen-dependent Degradation of HIF-1α Proline Mutants (Paper II)**

The regulation of protein stability plays an important role in the HIF signaling pathway. Under normal oxygen conditions, the ubiquitously expressed HIF-1α protein is rapidly degraded by the UPS (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Subsequent studies led to the identification of two independent degradation boxes (N- and C-TDB) centered around two conserved proline
residues (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001a, b). Further analysis of these degradation boxes demonstrated that hydroxylation of Pro\textsuperscript{402} and/or Pro\textsuperscript{563} in mHIF-1\(\alpha\) (Pro\textsuperscript{402} and Pro\textsuperscript{564} in hHIF-1\(\alpha\)) by the PHDs (Bruick and McKnight, 2001; Epstein et al., 2001) serve as the limiting step in the recruitment of the VEC ubiquitin-ligase complex. Therefore, mutation of these two proline residues could render HIF-1\(\alpha\) stable even under normoxic conditions.

In the present study, we have analyzed the expression pattern of such a mHIF-1\(\alpha\)(P402A/P563A) mutant in human embryonic kidney (HEK) 293A and in human hepatoma cells (HepG2). Surprisingly, transient expression of constructs containing mutations of the two previously identified degradation boxes showed oxygen-dependent degradation. Similar experiments have been performed in additional cell types (unpublished observations) rendering similar results, suggesting additional mechanisms of degradation of mHIF-1\(\alpha\)(P402A/P563A). Using an HRE-driven luciferase assay, the transactivation potential of the mHIF-1\(\alpha\) proline mutant was assessed. mHIF-1\(\alpha\)(P402A/P563A) exhibited a more potent transactivational activity when compared to the wild-type protein but, consistent with its hypoxia-dependent stabilization, it also showed hypoxia-dependent increase in transactivation. Taken together, these results show that mutation of the two previously identified degradation boxes, responsible for interaction with pVHL and the VEC complex, failed to produce a stable and constitutively active protein.

Furthermore, we have generated stable cell lines expressing either mHIF-1\(\alpha\) or mHIF-1\(\alpha\)(P402A/P563A) in HEK 293H cells. After monoclonal selection, clones were assayed by Western blot against CoCl\(_2\) treatment (a well established hypoxia-mimicking agent). As for overexpression, both wild-type and mutant mHIF-1\(\alpha\) showed stabilization to Co(II) treatment, suggesting normoxia-dependent degradation. Interestingly, when exposed to hypoxia, mHIF-1\(\alpha\)(P402A/P563A) showed only a transient stabilization, returning to normoxic levels after 2h of treatment.

In order to evaluate the ubiquitylation status of the HIF-1\(\alpha\) proteins, we coexpressed mHIF-1\(\alpha\)(P402A/P563A) with ubiquitin plasmids. Detectable levels of ubiquitylation could be identified in both mHIF-1\(\alpha\) and mHIF-1\(\alpha\)(P402A/P563A). Additionally, the ubiquitylation status of the proteins was increased by inhibition of the catalytic activity of the proteasome. Furthermore, we analyzed the levels of expression of both mHIF-1\(\alpha\) and mHIF-1\(\alpha\)(P402A/P563A) in HepG2 under similar conditions and observed comparable results to HEK 293A cells. These data suggest that degradation of the HIF-1\(\alpha\) proline mutant is UPS-mediated, and that the process is cell type-independent.
Both Pro$^{402}$ and Pro$^{563}$ have been shown to be hydroxylated by PHDs (Bruick and McKnight, 2001; Epstein et al., 2001). To assess the sensitivity of the double proline mutant of mHIF-1α to PHD-mediated degradation, we overexpressed mHIF-1α and mHIF-1α(P402A/P563A) to levels that lead to stabilization of the protein at normoxia, presumably due to saturation of the endogenous degradation machinery (Tanimoto et al., 2000). Expression of either of the PHDs (PHD1, -2 and -3) led to the degradation of wild-type mHIF-1α, while mHIF-1α(P402A/P563A) showed resistance to PHD-induced degradation. Curiously, PHD2-mediated degradation of mHIF-1α could be observed both at normoxia and under CoCl$_2$ treatment. This effect could be attributed to the overexpression conditions, where the activity of PHD2 is not completely abolished, even in the presence of Co(II). Additionally, we have exposed the stably transfected cell lines to known chemical inhibitors of PHDs action. In contrast to wild-type mHIF-1α, the protein levels of mHIF-1α(P402A/P563A) were not changed by any of the hydroxylase inhibitors. Taken together these results imply that mHIF-1α(P402A/P563A) degradation is not dependent on PHD-mediated hydroxylation. Importantly, the role of other putative PHDs (such as PHD4) in mHIF-1α(P402A/P563A) degradation seems unlikely due to the failure to achieve stabilization of this mutant using hydroxylase inhibitors. In addition, the presence of another yet unknown pathway inhibited by Co(II) appears to participate in this mechanism.

Since the only known HIF-1α ubiquitin-ligase is the VEC, we addressed the question of the involvement of pVHL in mHIF-1α(P402A/P563A) degradation. Our first observation was that in a renal cell carcinoma where pVHL is non-functional (SKRC-7), the levels of endogenous HIF-1α were not changed by CoCl$_2$ treatment. This observation indicates that functional pVHL is required for Co(II)-dependent regulation of HIF-1α. With that, we targeted endogenous pVHL using RNAi in stably transfected HEK 293H cells in order to assess the role of pVHL in the degradation and ubiquitylation of the mHIF-1α double proline mutant. Reduction of the Vhl transcript led to an increase of both mHIF-1α and mHIF-1α(P402A/P563A) protein levels. Furthermore, a reduction in ubiquitylation levels of these proteins was observed after transfection with pVHL siRNA in both wild-type- and mutant mHIF-1α-expressing cells, indicating that both proteins are ubiquitylated and degraded by a pVHL-dependent mechanism.

Subsequently, analysis of a mHIF-1α mutant lacking the ODD (mHIF-1αΔODD) showed positive binding to pVHL, ubiquitylation, and normoxia-induced regulation. Taken together, these findings show that additional pVHL-interacting interfaces,
independent of prolyl hydroxylation, are present on HIF-1α and are correlated to degradation of this protein under normal oxygen conditions.

In summary, paper II shows that mHIF-1α(P402A/P563A), previously described as a stable form of mHIF-1α, is degraded through the UPS, in a pVHL-dependent manner. The findings that PHDs failed to induce degradation of mHIF-1α(P402A/P563A) indicates that the mechanism through which pVHL acts on HIF-1α double proline mutant degradation is independent of hydroxylation. Additionally, our data suggests that pVHL is the major, if not the only, E3 ubiquitin-ligase for HIF-1α. Even if other mechanisms exist they do not overcome pVHL function.

**SUMO-mediated Degradation of HIF-1α (Paper III)**

We have shown previously that it is plausible that pVHL works as an HIF-1α E3 ubiquitin-ligase via proline hydroxylation-dependent and -independent mechanisms (Paper II). Furthermore, recent studies have shown that SENP1 contributes to HIF-1α stabilization at hypoxia (Cheng et al., 2007), and therefore SUMO is correlated to HIF degradation. In this study, we aimed to investigate the role of SUMO and SENP1 in HIF-1α degradation, particularly in a PHD-independent manner using HIF-1α double proline mutant.

To assess the contribution of SENP1 to the accumulation of mHIF-1α(P402A/P563A) at hypoxia or following CoCl₂ treatment, we targeted expression of SENP1 using siRNA in HEK 293H cells stably expressing wild-type or mHIF-1α double proline mutant. In either treatment (hypoxia or Co(II)), a decrease in protein levels of both mHIF-1α and mHIF-1α(P402A/P563A) was observed by knocking down SENP1 expression. This confirms that the SUMO protease is necessary for the stabilization of the proteins in response to these treatments. Next we examined if SUMOylation of HIF-1α was present at both normoxia and hypoxia. To this end, we transfected HEK 293A cells with plasmids encoding mHIF-1α and SUMO-1, and performed immunoprecipitation assays. We observed that mHIF-1α is efficiently modified by SUMO-1 at both normoxia and hypoxia, as long as the pool of free SUMO was sufficient to allow detection.

Following our observation that the SUMOylation machinery is functional on HIF-1α at normoxia, we addressed the question if this modification is able to mediate degradation of the protein at normoxia. As such, we performed RNAi targeting the E2 SUMO-conjugating enzyme Ubc9 and PHD2 in U2OS cells. Silencing of Ubc9 alone
was not sufficient to stabilize endogenous HIF-1α, while PHD2 knockdown alone reached low yet detectable levels of HIF-1α protein. However, when combined knockdown of both Ubc9 and PHD2 was performed, notably higher levels of endogenous HIF-1α were detected, suggesting a synergistic effect of these two enzymes on HIF-1α degradation pathway at normoxia.

Impairment of SUMO conjugation can be achieved by the silencing of Ubc9 or by the overexpression of SENPs. In this fashion, we transfected HEK 293A cells with mHIF-1α and mHIF-1α(P402A/P563A) with or without siRNA for Ubc9 and plasmids encoding SENP1. The protein levels of both wild-type and mutant mHIF-1α were increased in cells where Ubc9 expression was silenced, as well as in cells overexpressing SENP1. Moreover, we investigated the impact of knocking down Ubc9 in a cell line stably expressing mHIF-1α(P402A/P563A) and observed increased levels of expression of the mHIF-1α double proline mutant, particularly significant at normoxia. Taken together, these data point out a major role of SUMO in degradation of HIF-1α(P402A/P563A).

SUMO conjugation of proteins usually occurs at specific consensus sequences. On mHIF-1α three lysine residues (Lys^{391}, Lys^{476}, and Lys^{531}) are within mapped consensus motifs for SUMOylation. In an attempt to generate a SUMO-resistant mutant of mHIF-1α(P402A/P563A) all these residues were substituted to arginine, and their SUMOylation status was assessed in HEK 293A cells. SUMO-1 is conjugated to mHIF-1α(P402A/P563A), and mutation of the three mapped lysines did not inhibit SUMOylation of this mutant mHIF-1α(P402A/P563A). Furthermore, the triple lysine mutant mHIF-1α(P402A/P563A) is still degraded at normoxia and stabilized by Co(II). These results suggest the existence of additional lysines in mHIF-1α(P402A/P563A) that can be targeted by SUMO and mediate degradation of the protein.

We have previously shown that pVHL is able to bind to mHIF-1α(P402A/P563A), as well as to mHIF-1αΔODD (Paper II). In this context, we transfect HEK 293A cell with plasmids encoding different HIF-1α domains (bHLH/PAS, ODD(P402A/P563A), ID, and C-TAD) in order to investigate which domains are capable of pVHL recognition. All domains with the exception of the C-TAD interacted with pVHL, indicating the presence of several interfaces for pVHL on HIF-1α. Additionally, the same domains that bind pVHL were modified by SUMO, and subsequently showed upregulation of the protein levels either by the knocking down of Ubc9 or overexpressing SENP1. In contrast, the C-TAD that did not bind pVHL failed to be SUMOylated and remained almost unchanged upon the knocking down of Ubc9 or the overexpression of SENP1. Such observations show that HIF-1α bHLH/PAS, ODD(P402A/P563A), and ID domains can
interact with pVHL, and that several independent degradation domains of HIF-1α are able to mediate SUMO-dependent degradation of the protein.

We next generated a series of lysine-substitution mutants in each domain that is modified by SUMO. The choice of lysine residues was carried out according to the homology of the surrounding sequences to the SUMO consensus using the SUMOplot prediction software from Abgent (www.abgent.com/sumoplot). We observed that mutation of potential SUMOylation sites in mHIF-1α bHLH/PAS, ODD(P402A/P563A), and ID did not abrogate modification of these domains by SUMO. Accordingly, the corresponding lysine mutants were able to interact with pVHL as did their wild-type counterparts. These results suggest that, since HIF-1α can be SUMOylated in lysine residues that are not within predicted consensus motifs, the generation of a SUMO-resistant mutant can only be achieved by generating a lysine-deficient mutant.

Due to the fact that mutation of consensus lysine residues does not abrogate SUMO conjugation of HIF-1α, and therefore binding of pVHL to a SUMO-resistant mutant cannot be assessed, we decided to investigate if pVHL binding could be impaired upon SUMO deconjugation of HIF-1α by SENP1. For this purpose, we selected the ODD(P402A/P563A) domain because it was the most sensitive domain to SENP1 overexpression. Our data show that expression of SENP1 impaired pVHL binding to ODD(P402A/P563A), indicating that interaction of pVHL with this domain of HIF-1α is dependent on modification by SUMO.

Taken together, our results show that HIF-1α SUMOylation mediates the degradation of the protein. We present the first evidence that the SUMO conjugation contributes to HIF-1α destabilization at normoxia and is involved in the degradation mechanism targeting HIF-1α(P402A/P563A) (Paper II). Finally, we show that several HIF-1α domains that interact with pVHL can be SUMOylated, and that mutation of lysine residues in potential SUMO-acceptor sites does not prevent SUMO modification of these domains.
CONCLUSIONS

The present study has been focused on the evaluation of mechanisms underlying the protein degradation of HIF-1α. In addition, we aimed to investigate the functional potential of mutant forms of HIF-1α in physiological events, namely the induction of newly formed vasculature. To address these issues, we have used a series of biochemical approaches to further characterize the existence of additional degradation mechanisms, as well as cell biology methods and in vivo studies to assess the potential of HIF-1α as an inducer of neoangiogenesis. The results of this study can be summarized as outlined below:

• A HIF-1α mutant (mHIF-1α(P402A/P563A)) was shown to be a potent inducer of de novo angiogenesis and arteriogenesis by in vivo transduction of mouse tibialis anterior muscles with AAV. Moreover, this construct proved to be a good candidate for pro-angiogenic gene therapy since it was capable of circumventing most of the previously identified problems in cardiovascular gene transfer studies, by inducing endogenous angiogenic cascades responsible for the mechanisms. (Paper I)

• We have uncovered the existence of additional mechanisms of degradation of HIF-1α, involving proteasome-mediated degradation induced in a pVHL-dependent yet PHD-independent manner. Furthermore, our results indicate the presence of functional pVHL-interacting interfaces outside the ODD and the previously characterized degrons (both N-TDB and C-TDB). Collectively, these data present the existence of a novel mechanism of degradation of HIF-1α. (Paper II)

• We show that SUMOylation of HIF-1α contributes to the degradation of the protein at normoxia. Additionally, we show that several HIF-1α domains can be modified by SUMO and interact with pVHL, and that substitution of the potential SUMO-acceptor lysine residues does not prevent SUMO conjugation of these domains. Together, these data suggest that SUMOylation of HIF-1α occurs in a non-consensus restricted manner and that this modification is responsible for pVHL recognition of HIF-1α. (Paper III)
DISCUSSION

Gene transfer using rAAVs of species-matched HIF-1α carrying mutations of the two previously identified critical proline residues in the two degradation boxes, resulted in generation of functional neovasculature in adult mouse skeletal muscle (Paper I). The fact that HIF-1α itself is capable of inducing a multitude of endogenous growth factors required for the angiogenic response most likely explains the success obtained with this construct. However, our subsequent studies show that this mutant form of HIF-1α (mHIF-1α(P402A/P563A)) is not yet fully stabilized (Paper II). Thus, the stability of this mutant in gene therapy experiments needs to be more closely examined. The generation of a fully stabilized and competent HIF-1α mutant is needed to assess the complete array of action of HIF-1α in animal studies, and further implications in pro-angiogenic therapy, will therefore be very interesting.

The observation that the mutation of Pro^{402} and Pro^{564} in hHIF-1α generates a protein that can still be stabilized in response to blockage of the proteasome catalytic activity has been suggested (Kim et al., 2006). Though without further investigation of the mechanism of action, such an effect was attributed to a pVHL-independent mechanism. However, our data show that pVHL is capable of mediating ubiquitylation and subsequent proteasomal degradation of a HIF-1α double proline mutant, suggesting the existence of additional mechanisms of degradation of the HIF-1α protein (Paper II). Moreover, the failure to induce degradation of mHIF-1α(P402A/P563A) mutant by the PHDs indicates the possibility that pVHL is recognizing HIF-1α through proline hydroxylation-independent mechanisms.

In accordance with previous studies (Cheng et al., 2007), we have shown that modification of HIF-1α by SUMO can act as a signal for recruitment of pVHL, and we present first-hand evidence that SUMOylation of HIF-1α is relevant at normoxia, even in the absence of the proline-centered degrons (Paper III). These findings suggest that SUMOylation, and subsequent pVHL recognition represents a parallel mechanism to proline hydroxylation in the degradation of HIF-1α. Additionally, we have found several domains in HIF-1α that can be targeted by SUMO, bind pVHL, and are normoxia-dependently regulated at the protein level.

We also observed that mutation of putative SUMO-acceptor lysine residues (according to www.abgent.com/sumoplot) failed to prevent HIF-1α SUMOylation (Paper III). Recognition of SUMO consensus sequences by Ubc9 is mediated by direct
binding of the enzyme through a pocket near its active site (Bernier-Villamor et al., 2002). However, in proteins where SUMOylation is not restricted to consensus lysine residues but instead there is a promiscuous selection of acceptor lysines, a model involving SIMs has been proposed (Eladad et al., 2005; Lin et al., 2006). Our results indicate that there is a promiscuous selection of lysine residues mediating HIF-1α SUMOylation, therefore suggesting the possibility of an analogous mechanism regulating the SUMO modification of HIF-1α.

The recent discovery of STUbLs opened the possibility that SUMO-mediated degradation of proteins is a more general mechanism than previously assumed (Lallemand-Breitenbach et al., 2008; Perry et al., 2008). Regarding pVHL, it remains unclear how this E3 ubiquitin-ligase recognizes SUMOylated HIF-1α. The possibility of adaptor proteins, or a STUbL-like façade of pVHL, could be the putative mechanisms for recognition of SUMO modified HIF-1α. However, further research is necessary to investigate such assumptions.

Our data also indicate some noticeable differences between degradation of HIF-1α(P402A/P563A) and the wild-type protein. Although both proteins are stabilized upon CoCl₂ treatment, the HIF-1α mutant showed only a transient stabilization when exposed to hypoxia (Paper II). The mechanism of HIF-1α stabilization by Co(II) is not fully understood. Several mechanisms have been proposed that range from PHD inhibition to direct binding to HIF domains (Bruick and McKnight, 2001; Epstein et al., 2001; Yuan et al., 2003), ultimately inhibiting pVHL-mediated degradation. It is tempting to speculate that, in addition to PHD activity inhibition, CoCl₂ might act on another protein that requires divalent ions to be functional, and that such a protein might also be involved in the transient stability of HIF-1α(P402A/P563A) to hypoxia.

Collectively, our studies add to the knowledge that HIF-1α is a tightly regulated protein. The availability of multiple enzymes, post-translational modifications, and intricate pathways all combine to a complex and dynamic regulation of this protein. Such processes can easily be comprehended by the immediate necessity that arises in a cell when oxygen availability is limiting, as well as the necessity to rapidly terminate hypoxic signals upon reoxygenation. With this, it seems impossible to generate a fully stabilized form of HIF-1α and interference on HIF regulatory pathways is virtually unthinkable due to its vast complexity. Moreover, the generation of such a mutant might instigate adverse responses in tissues or organs at their normal oxygen tension, activating pathways that are otherwise physiologically silenced. Such studies are highly compelling and further investigation of HIF as a therapeutic tool is needed.
ACKNOWLEDGEMENTS

My supervisor, Teresa Pereira, a science mentor with ready ideas and a memory for raw data that is envious. Many thanks are in order, particularly in all the help to finish this thesis. Learning from you was an engaging and very fast-paced experience that I truly enjoyed.

Lorenz Poellinger, my co-supervisor I thank the opportunity for joining your group and presenting me to so many scientific events, allowing me to grow in this field.

To MOB past: Arunas, Petra, Ingmar, Ken, Bill, Jacky, Patrik, Sara. To MOB present: Katta, Xiaowei, Xiaofeng, Sakari, Michael, Markus F., Norio, Hideaki. In no particular order, thank you all for creating such a nice environment and for all the small talk that you so often need in science. Hannele, thank you for always having the time to keep all the bureaucracy out of my hands, you have no idea how grateful I am.

To all CMB personnel, thank you for making things work. Special thanks to: Ewa, for keeping all the thesis process running smoothly; Zdravko, for having everything working; Rosa-Amanda, gracias por todo.

Matti, thank you for making me believe there is always a way. And for the teaching, which I value greatly.

Getting to a new country empty handed is scary, but you made the transition the easiest thing ever. Jorge, you’re missed immensely! Always been great support and a good friend. Many stories, many memories, and more to come for sure. Thanks for all the company and discussions, and for somehow always finding a way to get me crying laughing!

And what a great way to start a day... Cecilia you’re a force of nature, a true standard. Super to have a friend close by when hard times come around, when you least expect it. And of course, Pablo, always keep cool. Love, thanks for making me realize that my Swedish is worst than yours. And a great future for baby Leo.

Inga, the joy of a country girl in the city. Don’t even know where to start and maybe it’s best if I don’t. There’s just so much... One I’ll have to say: thanks for “if you don’t have anything nice to say, just don’t.” Virgis and Alex, you’re incredibly lucky to have her, but I know you know it.

José, the friendliest critic. And I really mean this in the best way possible. Not only you’ve been an amazing friend, I could always rely on you for an opinion. And know that it would be truthful and to the point.

Thank you for the company and support, Markus. And for always understanding. And for finally, after all these years, showing me Sweden as it is.

Friendships really are unpredictable and at the turn of every corner. Cage, you truly are a ball of sunshine. Nick, for choosing to be my great friend. Mia, thank you for listening. Emma H., the polyglot, you never know which language to use. Malin, my inspiration for optimism. Carlos, for being fun. Emma A., Julianna, for staying close when it was so hard. Ana T., for always having a comforting word. Kyle, for all the good times and immense help.

And the CBMers. Ales, thanks for always find time for a break, buddy. Raju, Florian, Mario, Esther, Elisabet, thank you for keeping it dynamic. And the volleyball crew, of course.

A special thanks to Teresa, Lorenz, Cage, José for having the time to help me revising this thesis.

And a silly thanks to WOTC, for Magic, the Gathering. A mind-boggling hobby.
Vamos ver se eu ainda sei escrever português minimamente.

**João e Rosa André**, por muito que eu gostasse é impossível agradecer. Não há como começar sequer... Vocês são pura e simplesmente incríveis! O apoio incondicional e amor com que sempre me acompanharam não tem forma de agradecimento. Obrigado por sempre acreditar em mim, e tantas vezes por me fazerem acreditar em mim.

**Claudio** para além do facto de seres meu irmão (que não tens escolha) sempre foste um amigo e companheiro. Uma presença na minha vida que não tem igual. **Teresa**, por não só seres uma amiga mas por teres escolhido fazer parte da minha família. **Gabriel e Leonor**, um orgulho ter-vos como sobrinhos. Adoro-vos.

Muito poucas pessoas conheço que podem fazer isto: agradecer aos quatro avós. **João Martins, Ana Pires, Francisco André e Rosa Barata**, acompanharam-me a crescer e é mesmo com muito orgulho que vos agradeço por tudo o que sempre fizeram por mim.

**Tio Vicente** e tia Vina, sempre dispostos a uma boa aventura. Todos aqueles verões que passamos juntos serão para sempre inesquecíveis.

**Pauluxa**, uma mana. Obrigado por estares sempre lá. E por teres tido a paciência de me apresentar a noite quando todos os outros andavam no parque, incrível! **João Paulo**, genuinamente boa pessoa, alguém que consegue sorrir com o mundo que se nos apresenta e que me faz sempre sentir bem. **Andrézinho**, sabes que podes sempre contar comigo, independentemente de tudo. Se bem que ainda tenho de ensinar uns truques na x-box... Curte bem!

**Prima Lurdes**, sempre energética. Sabes bem como é a vida de emigrante, mesmo que outros tempos e padrões, sabes como é... E 'vó Teresa, a nossa matriarca, Maria da Conceição. Cá para as curvas!

Madrinha, **Fátima**, um marco de coragem. Uma voz amiga. Um abraço forte. E vez se eu não te agarrasse, Madrinha?


**Fátima C.**, do nada se criou uma amizade de que tanto me orgulho.

**Susa**, a um telefonema de distância, sempre. Passamos muito. E vamos passar. Mas estamos lá um para o outro.

**Ana**, incrível amiga. Companheira de tanto, é que não somos nós, são os outros... Sabes que nunca te conseguirei agradecer o facto de me fazeres sentir único.

**Lina e Darren** (sorry Darren, this one goes in portuguese). Vocês são uma fonte de inspiração. Um ombro amigo a qualquer instante. Sei que o **Hugo** não vos esta a dar muito trabalho. Vocês merecem!

**Susana**, a minha “afilhada”, ainda hoje. Sabes, acho que tenho mesmo de te agradecer o facto de seres alguém com quem se pode ter a mais indecente conversa e rir perdidamente da banalidade!

À malta, todos, sem ordem: **Pinto, Mafalda, Ricardo, Miau, Cláudia, Ricardo, Sofia** (a verdadeira afilhada), **Ritinha, Xana, Marta, Pedro** (Bro), e todos os que me passam agora... Obrigado por me fazerem sentir em casa por mais tempo que passe.
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