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Oxygen-dependent Regulation of HIF-1alpha Expression and Function

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To my family
Hypoxia is a state of reduced oxygen levels below normal values. It occurs in the pathophysiology of many diseases and plays important roles in mammalian physiology and development. The hypoxia-inducible factors (HIFs) are transcription factors that mediate adaptive cellular responses to hypoxia by activating the transcription of target genes. HIF is a heterodimer consisting of an oxygen-regulated α subunit and a constitutively expressed HIF-1β (ARNT) subunit. Both HIF subunits belong to the bHLH/PAS domain-containing protein family. The bHLH and PAS domains mediate DNA binding and dimerization. HIF-α contains a unique oxygen-dependent degradation (ODD) domain and two transactivation domains (TADs: N-TAD and C-TAD). The oxygen-dependent degradation of HIF-α is mediated by PHD-dependent hydroxylation, and subsequent pVHL-dependent ubiquitylation and proteasomal degradation. Under hypoxia, HIF-α is stabilized and accumulates in the nucleus where it dimerizes with ARNT and activates target gene expression by recruiting coactivators. FIH-1 inhibits the transcriptional activity of HIF-α by inhibiting the recruitments of coactivators such as CBP/p300 through the hydroxylation of Asparagine residue in C-TAD.

The aim of this study is to investigate in detail oxygen-dependent regulation of HIF-1α degradation and function. In paper I, we identified residues that are critical for the degradation and transactivation functions of HIF-1α N-TAD. In paper II, we demonstrated that the expression of pVHL-interacting peptides activates endogenous HIF-1α function, providing a potential pro-angiogenic stimulus. The degradation of HIF-1α is regulated by subcellular compartmentalization in a cell-type-dependent manner. The compartment in which degradation of HIF-1α occurs dictates where the pVHL-interacting peptide must be located in order to efficiently increase HIF-1α function at normoxia.

In paper III, we identified filamin A (FLNa) as a novel modulator of the HIF-1α pathway and presented a novel nuclear function of this actin-binding protein. Hypoxia enhances calpain-mediated cleavage of FLNa and the nuclear localization of the cleaved C-terminal fragment of FLNa. The C-terminal fragment of FLNa mediates the function of FLNa by facilitating the nuclear localization and enhancing the transcriptional activity of HIF-1α. FLNa promotes VEGF-A-mediated angiogenesis through activating HIF-1α-mediated hypoxic responses.

In paper IV, we demonstrated how hypoxia controls the differentiation status of a cell and demonstrates a crosstalk between the HIF-1α and Notch signaling pathways. Hypoxia blocks differentiation in myoblasts, satellite cells and primary neural stem cells in a Notch-dependent manner. Hypoxia stabilizes the Notch intracellular domain, activates Notch-responsive promoters and increases expression of Notch-regulated downstream genes. The modulation of the Notch pathway by hypoxia is mediated by HIF-1α. HIF-1α interacts with Notch ICD, binds to Notch-responsive promoter, and a transcriptionally active form of HIF-α increases Notch signaling.

The results from these studies contribute to a better understanding of the mechanism, which controls oxygen-dependent regulation and function of HIF-1α, and provide therapeutic implications by novel means to modulate the HIF-1α signaling pathway.
LIST OF PUBLICATIONS

This thesis is based on the following papers:

   Identification of residues critical for regulation of protein stability and the transactivation function of the hypoxia-inducible factor-1alpha by the von Hippel-Lindau tumor suppressor gene product.

II. Xiaowei Zheng, Jorge L. Ruas, Renhai Cao, Florian A. Salomons, Yihai Cao, Lorenz Poellinger, and Teresa Pereira.
    Cell-Type-Specific Regulation of Degradation of Hypoxia-Inducible Factor 1α: Role of Subcellular Compartmentalization.

III. Xiaowei Zheng, Xianghua Zhou, Meit Björndahl, Hidetaka uramoto, Lakshmanan Ganesh, Elizabeth G. Nabel, Yihai Cao, Jan Borén, Teresa Pereira, Lorenz Poellinger, and Levent M. Akyürek.
    Filamin-A promotes VEGF-A activity through the HIF-1α-mediated hypoxic response.
    *Manuscript.*

IV. Maria V. Gustafsson, Xiaowei Zheng, Teresa Pereira, Katarina Gradin, Shaobo Jin, Johan Lundkvist, Jorge L. Ruas, Lorenz Poellinger, Urban Lendahl and Maria Bondesson.
    Hypoxia Requires Notch Signaling to Maintain the Undifferentiated Cell State.
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LIST OF ABBREVIATIONS

ABD  actin-binding domain  
ActD  actinomycin D  
ADM  adrenomedullin  
AR   androgen receptor  
ASB2  ankyrin repeat and SOCS box-containing 2  
CA9  carbonic anhydrase-9  
PKA  cAMP-dependent protein kinase  
CBP  CREB-binding protein  
DMOG  dimethylxaloylglycine  
DUB  HIF-1α de-ubiquitylating enzyme  
ECSM2  Endothelial cell-specific molecule-2  
EGF  epidermal growth factor  
EPF  E2-endemic pemphigus foliaceus  
EPO  erythropoietin  
FD   familial dysautonomia  
FIH-1  factor-inhibiting HIF-1  
FILIP  FLNa-interacting protein  
FLN  filamin  
FMD  frontometaphyseal dysplasia  
GEF  guanine nucleotide-exchange factor  
GLUT-1  glucose transporter 1  
GSK3β  glycogen synthase kinase 3β  
HAF  hypoxia associated factor  
HD   heterodimerization domain  
HIFs  hypoxia-inducible factors  
HREs  hypoxia-responsive elements  
HSP90  90 kDa heat-shock protein  
IPAS  inhibitory domain PAS protein  
IRES  internal-ribosome-entry-site  
LDHA  lactate dehydrogenase-A  
LNR  cysteine-rich Lin12-Notch repeats  
MAPK  mitogen-activated protein kinase  
MFM  myofibrillar myopathies  
Mdm2  mouse double minute 2  
MNS  Melnik-Needles syndrome  
mTOR  mammalian target of rapamycin  
NEMO  NF-κB essential modulator  
NES  nuclear export signal  
NLS  nuclear localizing signal  
Notch ICD  Notch intracellular domain  
NPCs  nuclear pore complexes
ODD  O$_2$-dependent degradation domain
OPD1  otopalatodigital syndrome types 1
PAS  PER-ARNT-SIM
PEST  proline/glutamic acid/threonine-rich motifs
PGK1  phosphoglycerate kinase
PH  periventricular heterotopia
PHDs  prolyl hydroxylase domain proteins
PI3K-Akt  phosphatidylinositol 3-kinase-Akt
PTB  RNA-binding proteins polypyrimidine tract-binding protein
pVHL  von Hippel-Lindau tumor suppressor
RanGEF  Ran guanine exchange factor
RCC1  Chromosome condensation 1
RACK1  receptor of activated protein kinase C
ROCK  Rho kinase
RSK  ribosomal S6 kinase
RSUME  RWD-containing SUMOylation enhancer
SCT  spondylocarpotarsal synostosis syndrome
SphK1  sphingosine kinase 1
SSAT2  spermidine/spermine-N1-acetyltransferase 2
SUMO  small ubiquitin-like modifier
TAD  transactivation domain
TGF-$\alpha$  transforming growth factor-alpha
TIF-2  transcription intermediary factor 2
EPF-UCP  E2-endomic pemphigus foliaceus ubiquitin carrier protein
VDU2  pVHL-interacting de-ubiquitylating enzyme
VDUP1  Vitamin D3 upregulated protein 1
VEGF  vascular endothelial growth factor
XMVD  X-linked myxomatous valvular dystrophy
1. INTRODUCTION

1.1 HIF pathway

1.1.1 Physiology of oxygen and hypoxia

The importance of oxygen (O\textsubscript{2}) for the survival of aerobic organisms was first demonstrated in 1774 by Joseph Priestley who demonstrated that a mouse kept in a jar couldn’t survive in the absence of air renewal. Most organisms, including bacteria, yeasts, invertebrates and vertebrates, require O\textsubscript{2} for aerobic metabolism. O\textsubscript{2} serves as the primary electron acceptor in many intracellular biochemical reactions and is essential for the generation of ATP by mitochondria.

Normal O\textsubscript{2} levels inside the human body are variable depending on the organ and are substantially lower than the 21% O\textsubscript{2} in the air we breathe. It is 4-14% in the lung parenchyma and in circulation, as well as well-vascularized parenchyma organs such as liver, kidneys and heart. It varies from 0.5-7% in the brain, 1-5% in the eye (retina and corpus vitreous) and 0-4% in the bone marrow. Cornea and cartilage are almost anoxic due to their vascular feature (110).

Hypoxia is defined as a state of reduced O\textsubscript{2} levels below normal values. It can be caused by a reduction in oxygen supply, happening for example as consequence of high altitude, or because of localized ischemia caused by the disruption of blood flow to a given area such as during myocardial infarction and stroke. Many solid tumors contain hypoxic regions owing to the inability of the local vasculature to supply sufficient oxygen to the rapidly growing tumor and because of the severe structural abnormality of tumor micro vessels (204). Hypoxia also plays essential roles in the pathophysiology of anemia, pulmonary and hematological diseases, kidney diseases (acute kidney injury, chronic kidney disease and diabetic kidney), wound healing, inflammation and infection. However, hypoxia has also an important and beneficial role in mammalian physiology. Its presence is crucial for proper embryogenesis of developing embryos and is important for controlling stem cell differentiation and cell fate (BOX 1).

### BOX1: Hypoxia in Physiological and Pathophysiological Conditions

<table>
<thead>
<tr>
<th>Hypoxia in physiological conditions:</th>
</tr>
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<tbody>
<tr>
<td>increased altitude</td>
</tr>
<tr>
<td>exercising muscles, cornea, cartilage</td>
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<tr>
<td>developing embryos (normal fetal development and stem cell differentiation)</td>
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</tbody>
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<table>
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<tr>
<th>Hypoxia in pathophysiological conditions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ischemia (myocardial infarction and stroke)</td>
</tr>
<tr>
<td>solid cancer</td>
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<tr>
<td>pulmonary and hematological diseases</td>
</tr>
<tr>
<td>kidney diseases (acute kidney injury, chronic kidney disease and diabetic kidney)</td>
</tr>
<tr>
<td>anemia, wound healing, inflammation and infection</td>
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</tbody>
</table>
1.1.2 HIFs mediate adaptive responses to hypoxia

In order to cope with hypoxia, organisms undergo a variety of systemic and local changes to restore oxygen homeostasis and limit the effect of low O\textsubscript{2} levels. This is mediated by hypoxia-inducible factors (HIFs). As transcription factors, HIFs activate transcription of target genes in hypoxia. There are more than 70 HIF target genes identified so far (Fig. 1). HIF target genes mediate systemic and local adaptation to hypoxia including enhanced O\textsubscript{2} delivery by increasing erythropoietin, blood vessel dilation, and angiogenesis. The most noticeable response at the cellular level is the metabolic adaptation achieved by the reduction in oxidative phosphorylation and concomitantly increased anaerobic glycolysis to ensure production of ATP in an oxygen-independent manner. Other HIF target genes are involved in pH homeostasis, oxygen sensing, cell migration, cell survival and autophagy (227). HIF has been shown to play essential roles in promoting an aggressive cancer phenotype by increasing cell proliferation and generation of metastasis as well as contributing to the resistance to chemo- and radiotherapy (31, 208). Therefore, modulation of HIF pathway has become a promising therapeutic strategy for both ischemic disease and cancer.

Fig. 1 HIF mediates adaptive responses to hypoxia. HIF activates transcription of its target genes to keep oxygen homeostasis under hypoxic conditions. Examples of HIF target genes (blue) and corresponding cellular processes are shown. EPO: erythropoietin; SDF1: stromal-derived factor 1; VEGF: vascular endothelial growth factor; ANG: angiopoietin; MMP: matrix metalloproteinase; NOS: nitric oxide synthase; HO-1: heme oxygenase-1; ET-1: endothelin-1; ADM: adrenomedullin; GLUT: glucose transporter; LDH-A: lactate dehydrogenase A; PGK1: phosphoglycerate kinase-1; PDK1: pyruvate dehydrogenase kinase 1; GAPDH: glyceraldehyde-3-P dehydrogenase; CA9: carbonic anhydrase 9; NHE1: Na\textsuperscript{+}/K\textsuperscript{+} exchanger; MCT4: monocarboxylate transporter; AMF: autocrine motility factor; CTGF: connective tissue growth factor; CXCR4: chemokine receptor; LOX: lysyl oxidase; PAI-1: plasminogen activator inhibitor-1; SDF-1: stromal-derived factor-1; IGF: insulin-like growth factor; TGF-\alpha: transforming growth factor-\alpha.

1.1.3 The Hypoxia-inducible Factors (HIFs)

HIF is a heterodimer that consists of an oxygen-regulated \(\alpha\) subunit and a constitutively expressed HIF-\(\beta\) subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT) (Fig. 2). Both \(\alpha\) and \(\beta\) subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain-containing transcription factors (249). BHLH and PAS domains mediate DNA binding and dimerization. Other domains in the \(\alpha\) subunits include a unique O\textsubscript{2}-dependent
degradation domain (ODD) and transactivation domain (TAD). ARNT is the general binding partner for all bHLH/PAS family members. It lacks the ODD domain and is therefore constitutively expressed in all tissues under hypoxic conditions.

Figure 2. Schematic representation of human HIF proteins. HIFs are members of the basic helix-loop-helix (bHLH) / PER-ARNT-SIM (PAS) domain family of transcription factors. They bind to DNA as heterodimers composed of an oxygen-sensitive HIF-α subunit (HIF-1α, -2α, or -3α) and a constitutive HIF-1β (ARNT) subunit. The bHLH and PAS domains mediate DNA binding and dimerization, respectively. HIF-α subunits contain a unique oxygen-dependent degradation domain (ODD) that controls HIF-α stability in an oxygen-dependent manner. The transactivation domains (TADs) mediate transcriptional activation of HIF target genes. Percentage shows the percentage of homology between HIF-1α and HIF-2α for indicated domains. Two nuclear localization signals (NLS), one nuclear export signal (NES) and the location of amino acids essential for hydroxylation (Prolyl 402, 564 and Asparagine 803) in HIF-1α are shown.

To date, three HIF-α isoforms (HIF-1α, HIF-2α and HIF-3α) have been described among which HIF-1α and HIF-2α are the best characterized. HIF-1α is ubiquitously expressed, whereas HIF-2α displays tissue-specific expression in endothelial cells, glial cells, type II pneumocytes, cardiomyocytes, fibroblasts of the kidney, interstitial cells of the pancreas and duodenum, and hepatocytes (258). HIF-3α mRNA and protein are primarily detected in the thymus, kidney, cerebellar Purkinje cells and corneal epithelium of the eye (90). Similar to HIF-1α and HIF-2α, HIF-3α can also dimerize with ARNT and bind to hypoxia-responsive elements (HREs) in vitro (90). However, the role of HIF-3α in the hypoxic regulation of gene expression in vivo is not well understood. HIF-3α has several splice variants, of which the inhibitory domain PAS protein (IPAS) is the best characterized. IPAS is a truncated form of HIF-3α that lacks a transactivation domain and functions as a negative regulator by binding to HIF-1α and preventing the formation of HIF-1α/ARNT heterocomplexes (162, 163). Two ARNT homologues, ARNT2 and ARNT3 (also known as bMAL), have also been described; however, they mainly participate in O2-independent pathways such as development of the hypothalamus and regulation of circadian clocks, respectively. The HIF-1 heterodimer binds to a conserved HIF-binding sequence motif within HREs in the promoter or enhancer regions of target genes, thereby eliciting their transcriptional activation and the adaptive hypoxic response.
1.1.4 Regulation of HIF-α synthesis

To enable the rapid responses to changes in O₂ levels, cells have developed a highly sophisticated mechanism for both oxygen sensing and adaptation to hypoxia. HIF-α is continuously transcribed and translated in hypoxia despite an overall decrease in global protein translation. HIF-1α synthesis during hypoxia is largely regulated at the level of translation rather than transcription (74, 144). However, the underlying mechanism remains controversial and incompletely understood. It has been suggested that internal-ribosome-entry-site (IRES) elements are involved in selective HIF-α translation at hypoxia (142, 225, 280). However, this mechanism is challenged by other studies, in which cryptic promoter activity rather than IRES is suggested to mediate HIF-1α translation at hypoxia (24, 272). Other mechanisms involving the RNA-binding proteins polypyrimidine tract-binding protein (PTB) and HuR have been also reported (74). In addition, a variety of oncoproteins, growth factors and cytokines regulate HIF-1α translation under normoxic conditions, mainly through the activation of the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) mammalian target of rapamycin (mTOR) and the mitogen-activated protein kinase (MAPK) pathway (144, 279).

1.1.5 Regulation of HIF-α protein stability

In contrast to ARNT which is constitutively expressed, HIF-α subunits are highly regulated by oxygen at protein levels which have a half-life about 5 min under normoxic conditions. Hypoxia and other stimuli such as growth factors and cytokines inhibit the degradation of HIF-α, resulting in the activation of HIF-mediated transcription. The mechanism underlying the regulation of HIF-α protein stability is intensively investigated. The oxygen-dependent regulation of HIF-α expression is mainly mediated by prolyl-hydroxylation and pVHL-dependent ubiquitylation and proteasomal degradation. Other mechanisms such as RACK1 or GSK3β-mediated degradation are oxygen-independent and seem to be regulated by other stimuli.

1.1.5.1 Oxygen-dependent regulation of HIF-α stability

Under normoxic conditions, HIF-α is hydroxylated by specific prolyl hydroxylase domain proteins (PHD1, 2 and 3) at two conserved proline residues (Pro402 and
Figure 3. Oxygen-dependent regulation of HIF-1α stability

Under normoxic conditions, HIF-1α is hydroxylated by PHDs, a process that is facilitated by OS-9. This event leads to the binding of the pVHL E3 ubiquitin ligase complex to HIF-1α, a process facilitated by SSAT2 that binds to HIF-1α, pVHL and elongin C of the E3 ubiquitin ligase complex. The pVHL E3 ubiquitin ligase complex ubiquitylates HIF-1α, resulting in its degradation by proteasome. Ubiquitylated HIF-1α can be rescued from degradation by VDU2-mediated de-ubiquitylation. EPF UCP can specifically ubiquitylates pVHL, leading to its degradation and, resulting in stabilization of HIF-1α. In hypoxia, HIF-1α cannot be hydroxylated, but can be SUMOylated through interaction with SUMO1. SUMOylation mediates the recognition of HIF-1α by the pVHL E3 ubiquitin ligase complex by an unknown mechanism and leads to HIF-1α ubiquitylation and degradation. SENP1 deconjugates SUMOylated HIF-1α, enabling it to escape pVHL-mediated degradation. Hypoxia also induces RSUME expression that increases overall SUMO-1, -2, and -3 conjugation by interacting with the SUMO E2 enzyme Ubc9, and in an alternative proposed model for the role of SUMO in HIF-1α regulation increased HIF-1α SUMOylation results in an increase in HIF-1α protein stability. Thus, the role of SUMOylation in regulating HIF-1α stabilization is still unclear.

Abbreviations: PHDs: prolyl hydroxylases; OS9: osteosarcoma-9; SSAT2: spermidine/spermine-N1-acetyltransferase 2; VDU2: de-ubiquitylating enzyme; EPF UCP: E2-endemic pemphigus foliaceus ubiquitin carrier protein; SUMO1: the small ubiquitin-like modifier 1; SENP1: SUMO/sentrin specific peptidase 1; RSUME: RWD-containing SUMOylation enhancer.
Pro564) situated within the ODD domain (Fig. 3). This reaction requires oxygen, 2-oxoglutarate and ascorbate (62, 111). The prolyl hydroxylation mediates binding of the von Hippel-Lindau tumor suppressor (pVHL) to the HIF-α ODD (194). PVHL forms the substrate-recognition module of an E3 ubiquitin ligase complex comprising elongin C, elongin B, cullin-2 and ring-box 1, which mediates poly-ubiquitylation and proteasomal degradation of HIF-α. The central role of pVHL in regulation of HIF-α is manifested in von Hippel-Lindau (VHL) disease where the inactivation of the VHL gene results in the development of highly vascularized tumors of the kidney, retina and central nervous system (118).

PHDs are regarded as oxygen sensors that are essential to keep the proper level of HIF-α. While previous studies demonstrated that PHD2 is a primary hydroxylase for both HIF-1α and HIF-2α, genetic studies suggest that PHD3 is mainly responsible for HIF-2α hydroxylation (21, 27). Moreover, a recent study showed that all three PHDs have an additive effect on the stability of both HIF-1α and HIF-2α proteins (79). Thus, it seems that different PHD isoforms may be used to regulate HIF-α abundance depending on specific cell types, duration of hypoxic stress, and relative abundance of PHD proteins.

1.1.5.2 Modulation of pVHL-mediated ubiquitylation and proteasomal degradation

Modulation of PHD activities
The catalytic activity of PHDs requires iron and 2-oxoglutarate as cofactors, therefore compounds such as cobalt chloride, iron chelators such as dipyridyl and 2-oxoglutarate analogues such as dimethylloxalylglycine (DMOG) inhibit PHD activity and have been widely used to mimic hypoxia leading to HIF-α stabilization (Fig. 4). In addition to the enzymatic inhibition of the PHDs, hypoxia increases the levels of cytoplasmic reactive-oxygen species (ROS), which convert Fe$^{2+}$ to Fe$^{3+}$. This alteration inhibits PHD activity and promotes HIF-1α stabilization. Thus, the disruption of mitochondrial function using either pharmacological, genetic inhibition or knockout of the mitochondrial electron-transport chain prevents HIF-1α stabilization during hypoxia (94, 233). In addition, the PHDs are subject to regulation by other factors including intracellular calcium concentrations (20), nitric oxide (170) and Siah2 (seven in absentia homologs 2) E3 ubiquitin ligase. Siah2 targets PHD3 and to a less degree PHD1 for degradation via the ubiquitin-proteasome pathway, leading to HIF-1α stabilization (184). Recently, a new group of PHD inhibitors was discovered, and these substances bind to the active site of the PHDs, thereby inducing angiogenesis and exerting tissue protection against ischemia (185). Interestingly, expression of PHDs is reciprocally up-regulated by HIF-
α proteins under hypoxia, acting as a negative feedback loop of regulation to fine-tune HIF activities (4, 21).

**Figure 4. Modulation of PHD activity.** The catalytic activity of PHDs requires O\textsubscript{2}, Fe\textsuperscript{2+} and 2-oxoglutarate (2-OG) as cofactors. Therefore hypoxia, iron chelators and reactive-oxygen species (ROS), as well as the 2-oxoglutarate analogue dimethyloxalylglycine (DMOG) inhibit PHDs activity by restricting the availability of the cofactors. In addition, calcium chelator, nitric oxide, Siah2 and other PHD inhibitors can also inhibit PHDs activity resulting in HIF-α protein stabilization. Stabilized HIF-α increases PHD expression, resulting in negative feedback regulation.

**OS-9 facilitates HIF-α prolyl hydroxylation**

The osteosarcoma-9 (OS-9) protein has been suggested to form a ternary complex with HIF-1α and PHD2 or PHD3, thereby promoting HIF-1α proline hydroxylation and directing pVHL binding and subsequent proteasomal degradation (12) (Fig. 3). Furthermore it has been shown that overexpression of OS-9 decreases HIF-1α levels, whereas knockdown of OS-9 by siRNA increases HIF-1α protein levels and expression of its target genes under both normoxia and hypoxia (12).

**Modulation of pVHL activity**

Prolyl-hydroxylation of HIF-α by PHDs is a prerequisite to pVHL-mediated ubiquitylation of HIF-α. Hypoxia inhibits HIF-α prolyl-hydroxylation resulting in the inhibition of HIF-α ubiquitylation, resulting in HIF-α stabilization. In addition, the activity of pVHL is also regulated by its subcellular compartmentalization. Acidosis (low pH) resulting from hypoxia/ischemia or an extracellular milieu of cancer sequesters pVHL in nucleoli, preventing it from degrading HIF-α (168). pVHL is a predominantly cytoplasmic protein and is shuttling between the nucleus and cytoplasm.
Instead of a classical nuclear export signal that can be inhibited by leptomycin B, it has been proposed that pVHL contains a transcription-dependent nuclear export motif (TD-NEM) since its nuclear export can be abrogated by a RNA polymerase II inhibitor actinomycin D (ActD). Treatment with ActD or cancer-causing mutations in TD-NEM inhibits the nuclear export of pVHL, resulting in the stabilization of HIF-1α (124). However, it has also been shown that both nuclear and cytoplasmic forms of pVHL are capable of ubiquitylating and degrading HIF-1α under normal cell growth conditions (147), and HIF-1α can be degraded in either the nucleus or cytoplasm (22). Therefore, the exact mechanism underlying how ActD and mutations in TD-NEM regulate HIF-α stabilization remains to be further elucidated.

PVHL is also regulated by the ubiquitylation and proteasomal degradation pathway, and this is mediated by the E2-endemic pemphigus foliaceus (EPF) ubiquitin carrier protein (UCP). EPF-UCP is an E2 ubiquitin-conjugating enzyme. Although a cognate E3 ligase for UCP has not been identified, UCP can specifically ubiquitylate pVHL leading to its degradation (117) (Fig. 3). UCP overexpression causes the proteasomal-dependent degradation of pVHL, resulting in the accumulation of HIF-1α in normoxia and in increased tumor growth and metastasis, whereas UCP knockdown increases pVHL levels, thereby decreasing HIF-1α levels and inhibiting tumor growth. UCP expression correlates with decreased pVHL and increased HIF-1α expression in a panel of cell lines and primary and metastatic tumors (117). This may provide an explanation for the elevated levels of HIF-1α that are observed in non-hypoxic regions of such tumors.

**SSAT2 promotes pVHL-mediated ubiquitylation of HIF-1α**

The role of the spermidine/spermine-N1-acetyltransferase 2 (SSAT2) on HIF-1α degradation has been investigated (10). This study shows that SSAT2 binds simultaneously to pVHL and elongin C, thereby stabilizing their interaction and promoting HIF-1α ubiquitylation (Fig. 3). Accordingly, SSAT2 overexpression decreases HIF-1α levels, whereas SSAT2 knockdown increases HIF-1α levels under both normoxia and hypoxia. SSAT2 acetylates a thialysine, a naturally occurring modified amino acid, and mutant SSAT2 defective in thialysine acetyltransferase activity, although still able to interact with HIF-1α, is substantially less effective at reducing HIF-1α protein levels compared with wild-type SSAT2. Hence, the inhibitory activity of SSAT2 on HIF-1α is believed to result from the combined effect of its acetyltransferase activity and its physical interactions with HIF-1α, pVHL and elongin C.
De-ubiquitylation of HIF-α

The pVHL-interacting de-ubiquitylating enzyme (VDU2; also called USP20) has been identified as a HIF-1α de-ubiquitylating enzyme (DUB) (150) (Fig. 3). VDU2 binds and de-ubiquitylates HIF-1α in a pVHL-dependent manner, hence salvaging it from proteasomal degradation. The region of pVHL, which is commonly altered in VHL disease, harbors the binding sites for both VDU2 and HIF-1α (149). VDU2 itself can be ubiquitylated and degraded by the pVHL E3 ligase complex (151). Because pVHL can ubiquitylate both HIF-1α and VDU2, cellular HIF-1α levels might be determined by the balance between pVHL-mediated ubiquitylation and VDU2-mediated de-ubiquitylation.

SUMOylation of HIF-1α

Several studies indicate that hypoxia induces the small ubiquitin-like modifier (SUMO)-1 expression (228) and increases HIF-1α SUMOylation, a process that has been suggested to lead to either stabilization or degradation of HIF-1α according to a number of contradictory reports (9, 37, 44). Contribution of SUMO to stabilization of HIF-1α is supported by a recent study where the RWD-containing SUMOylation enhancer (RSUME), which increases overall SUMO-1, -2, and -3 conjugation by interacting with the SUMO E2 enzyme Ubc9, interacts with and increases HIF-1α SUMOylation resulting in increased HIF-1α protein levels and transactivation (37). RSUME is induced by hypoxia in tumors and is expressed in the necrotic zone of gliomas, underscoring its importance during hypoxia and in tumor maintenance and growth. By contrast, other studies indicate that HIF-1α SUMOylation leads to HIF-1α degradation at hypoxia. It has been shown that hypoxia-induced HIF-1α SUMOylation can promote hydroxyproline-independent HIF-1α-pVHL E3 ligase complex binding, thus leading to HIF-1α ubiquitylation and proteasomal degradation (Fig. 3) (44). SENP1 (SUMO1/sentrin specific peptidase 1), a nuclear SUMO protease that deconjugates SUMOylated HIF-1α, enables it to escape pVHL-mediated degradation during hypoxia. Accordingly, SENP1 knockdown decreases HIF-1α expression in a pVHL-dependent manner. Furthermore, SENP1-/- embryos exhibit severe fetal anemia owing to deficient erythropoietin production, thus providing strong evidence for a physiological role of SENP1 in regulating HIF-1α (44). Since the present results are controversial, further investigations are necessary to elucidate the role of SUMOylation in the regulation of HIF-1α stability.

1.1.5.3 Oxygen-independent regulation of HIF-α stability

Increasing evidence indicates that mechanisms other than pVHL-dependent HIF-1α degradation have an important role in controlling HIF-1α levels. These pathways seem
to be less dependent on oxygen availability and more on specific cellular conditions such as calcium or the presence of growth factors and appear to be particularly important in the context of cancer.

**Figure 5. Oxygen-independent regulation of HIF-1α stability**

RACK1 binds to HIF-1α as a dimer and recruits components of the E3 ligase complex through a process facilitated by SSAT1, leading to the ubiquitylation and subsequent proteasomal degradation of HIF-1α. Hsp90 inhibits RACK1-mediated ubiquitylation of HIF-1α by competing with RACK1 for the binding to HIF-1α. Calcineurin A inhibits RACK1-dependent degradation of HIF-1α in a calcium-dependent manner by inhibiting RACK1 dimerization. GSK3β phosphorylates HIF-1α and leads to its ubiquitylation. FOXO4 also induces pVHL-independent HIF-1α ubiquitylation. Both GSK3β and FOXO4 are inactivated by the PI-3K pathway, which is itself activated by growth factors and cytokines. P53 promotes Mdm2-mediated ubiquitylation and degradation of HIF-1α, but the results are still controversial. Abbreviations: Ub, ubiquitin; RACK1: receptor of activated protein kinase C; SSAT1: spermidine/spermine-N1-acetyltransferase 1; GSK3β: the glycogen synthase kinase 3β; FOXO4: Forkhead box O4; PI-3K: Phosphatidylinositol 3-kinase-Akt; Mdm2: mouse double minute 2.
**RACK1-mediated ubiquitylation and proteasomal degradation**

RACK1 (receptor of activated protein kinase C) has been proposed to homodimerize and to recruit the E3 ligase complex to HIF-1α, leading to HIF-1α ubiquitylation and proteasomal degradation in an oxygen- and pVHL-independent manner (Fig. 5) (152). RACK1-HIF-1α binding is dependent upon the presence of SSAT1, which stabilizes the RACK1–HIF-1α interaction, and its acetyltransferase activity is required in this process (11). The molecular chaperone 90 kDa heat-shock protein (HSP90) competes with RACK1 for the binding to HIF-1α, and inhibition of HSP90 can cause oxygen- and pVHL-independent degradation of HIF-1α (152). The RACK1 pathway is also regulated by calcium through the activity of calcineurin, a calcium- and calmodulin-dependent and serine/threonine-specific protein phosphatase. Calcineurin A dephosphorylates RACK1 in a calcium-dependent manner, thus blocking RACK1 dimerization and inhibiting RACK1-mediated HIF-1α degradation (153).

**GSK3 or FOXO4-dependent ubiquitylation and degradation**

The glycogen synthase kinase 3β (GSK3β) mediates the phosphorylation of HIF-1α, resulting in a prolyl-hydroxylation and pVHL-independent HIF-1α ubiquitylation and proteasomal degradation (Fig. 5) (68). Similarly, overexpression of forkhead box (FOX)O4 down-regulates HIF-1α by inducing pVHL-independent HIF-1α ubiquitylation and degradation (239). Both GSK3 and FOXO4 are negatively regulated by the phosphatidylinositol 3-kinase–Akt (PtdIns3K–Akt) signaling pathway. Hence, it is possible that prolonged hypoxia might inhibit the PtdIns3K pathway, resulting in increased GSK3β and FOXO4 activity, which then leads to decreased HIF-1α levels and activity (173).

**Mdm2-mediated proteasomal degradation**

The tumor suppressor p53 has been suggested to promote mouse double minute 2 (Mdm2, known as Hdm2 for human homolog)-mediated ubiquitylation and proteasomal degradation of HIF-1α (210) (Fig. 5). Loss of p53 in tumor cells enhances HIF-1α levels and the transcriptional activation of the VEGF gene in response to hypoxia (210). This is supported by another study showing that the orphan nuclear receptor Nur77 enhances the stabilization of HIF-1α, and is correlated with the down-regulated expression of MDM2 by Nur77 in hypoxia (270). However, this mechanism is challenged by the results from other studies showing that Mdm2 does not promote HIF-1α protein degradation, but increases HIF-1α protein in either normoxic (15) or hypoxic tumor cells (188). In normoxic pVHL-deficient renal carcinoma cells, down-regulation of Hdm2 by siRNA leads to decreased levels of both HIF-1α and HIF-2α protein levels (40). The apparent discrepancies in these results may be due to different
experimental settings. Thus, the role of Mdm2 in regulation of HIF-α protein stability is still controversial and needs to be further elucidated.

Isoform-specific regulation of HIF-α degradation

Although HIF-1α and HIF-2α display 48% amino acid identity and have similar protein structure and mechanism of regulation, it has been proposed that they regulate both common and unique target genes (101, 103), and are differentially regulated depending on the duration and severity of hypoxia exposure (100). Recently, isoform-specific and pVHL-independent pathways of regulation of HIF-α ubiquitylation and proteasomal degradation have been identified. The hypoxia-associated factor (HAF), also known as SART1800 (squamous cell carcinoma antigen recognized by T-cells), is an E3 ligase specific for HIF-1α, and mediates the ubiquitylation and proteasomal degradation of HIF-1α (132). While the murine mammary tumor integration site 6 (Int6), also known as EIF3E, interacts specifically with HIF-2α and mediates its proteasomal degradation (41). These isoform-specific mediators of HIF-α degradation may provide a mechanism for the selective activation of HIF-α in order to fulfill precise cellular requirements.

1.1.6 Regulation of HIF-α subcellular localization

1.1.6.1 Nucleocytoplasmic transport of proteins

Transport of macromolecules into and out of the nucleus occurs through the nuclear pore complexes (NPCs) (Fig. 6). Nuclear pore complexes allow passive diffusion of ions and small proteins but restrict passage of larger molecules (>40-60 kDa) to those containing an appropriate targeting signal (200). The active transport of macromolecular cargo through NPCs is facilitated by specific soluble carrier proteins, collectively referred to as karyopherins (206). Karyopherins involved in nuclear import and nuclear export are termed importins (82) and exportins (235), respectively.

Nuclear import and nuclear import signals

Classical nuclear import begins in the cytoplasm, with importin α-mediated recognition of a nuclear localization signal (NLS) present in the cargo. Importin α links the cargo to importin β (Fig. 6). Importin β then mediates interaction of the trimeric complex with the NPC as it translocates into the nucleus. Once the import complex reaches the nucleus, RanGTP binds to importin β resulting in the release of cargo. Importin α and β are recycled to the cytoplasm through separate RanGTP-dependent events. Classical NLSs have been identified in many nuclear proteins. They are either a single stretch (monopartite) of basic amino acids like the one from SV40 large T antigen (PKKKRRK; critical residues underlined) (58, 120), or bipartite (two short sequences
with spacer) basic amino acid stretches like the one from nucleoplasmin (KRPAATKKAGQAKKKLDK) (99).

**Figure 6: Nucleoplasmic transport of HIF-1α.** Nucleoplasmic transport of HIF-1α occurs through the nuclear pore complexes (NPCs). During nuclear import, the NLS motif of HIF-1α is recognized by importin α (Impα). Impα links HIF-1α to importin β (Impβ). Impβ mediates the HIF-1α/Impα/Impβ complex to transport to the nucleus via NPC, where the HIF-1α/Impα/Impβ complex is dissociated upon the formation of the Impβ/RanGTP complex. During nuclear export, the NES of HIF-1α binds to exportin CRM1 in the presence of RanGTP. Upon binding, HIF-1α/CRM1/RanGTP complex translocates through NPC to the cytoplasm where RanGTP is hydrolyzed to RanGDP and the whole complex dissociates. CRM1-mediated nuclear export can be inhibited by leptomycin B, but facilitated by VDUP1. Additionally, MAPK phosphorylates HIF-1α and inhibits CRM1-mediated nuclear export, resulting in the nuclear accumulation of HIF-1α. In the drosophila HIF-α Sima an oxygen-dependent NES has been identified. Prolyl hydroxylation and pVHL-binding are required for the interaction between NES and CRM1, therefore hypoxia inhibits the nuclear export of Sima. Nuclear HIF-α dimerizes with ARNT on hypoxia responsive element (HRE) and recruits coactivators such as CBP/p300 leading to the transcription of target genes. Abbreviations: VDUP1: Vitamin D3 up-regulated protein 1; MAPK: mitogen-activated protein kinase.
Nuclear export and nuclear export signals

In classical nuclear export, the cargo protein binds to exportin in the presence of RanGTP (Fig. 6). Upon binding, the cargo/exportin/RanGTP complex translocates through NPC to the cytoplasm where RanGTP is hydrolyzed by RanGAP (GTPase-activating protein) to RanGDP. RanGDP no longer binds to the exportin, and the whole complex dissociates. RanGDP is imported to the nucleus by NTF2. Chromosome condensation 1 (RCC1), a chromatin-bound Ran guanine exchange factor (RanGEF) in the nucleus, converts RanGDP to RanGTP. The exact localization of RanGAP in the cytoplasm and of RanGEF in the nucleus results in the localization of RanGTP in the nucleus and RanGDP in the cytoplasm. As a consequence, a RanGTP gradient appears which ensures the direction of the transport.

One of the most studied exportins is CRM1 (also known as Xpo1 in yeast or exportin-1). CRM1 cargos generally contain a leucine-rich nuclear export signal (NES) (67, 255). CRM1-dependent export is sensitive to leptomycin B, a small molecule that disrupts the interaction between CRM1 and the NES and has therefore been used as a tool to identify CRM1-dependent export targets (137, 260).

1.1.6.2 Regulation of HIF-α subcellular localization

As a transcription factor, HIF localizes to the nucleus to exert its function. The nuclear accumulation of HIF-1α was reported to be induced by hypoxia (121). However, the mechanism by which HIF subcellular localization is regulated is not fully understood. ARNT has been shown to be a constitutively nuclear protein and an NLS in the bHLH domain mediates its nuclear import, but the dimerization of HIF-1α and ARNT is not a prerequisite for HIF-1α nuclear translocation (45). Recent data suggest that HIF-α is continuously imported to the nucleus and re-exported to the cytoplasm, and the subcellular localization of HIF-α emerges from a dynamic equilibrium between nuclear import and nuclear export (107). The oxygen level and other stimuli can promote the nuclear accumulation of HIF-α by increasing the nuclear import or preventing the nuclear export.

Nuclear import of HIF-α

Nuclear translocation of HIF-α has been shown to be mediated by the classical nuclear import pathway (Fig. 6). Several importins α have been demonstrated to be able to bind to HIF subunits (-1α, -2α and ARNT) with similar binding affinity (56). In addition, HIF-2α can bind directly to importin β in vitro with higher affinity than to importin α (56). The direct interaction of HIF-1α and HIF-2α to importin α is dependent on a functional NLS within the C-terminal region of the proteins (56, 121, 159) (Fig. 2). Additionally, a bipartite NLS is predicted for the N-terminus of HIF-α, however it might be masked by PAS-B and is therefore not effective (Fig. 2) (121, 269).
Nuclear export of HIF-α

Studies in mammalian cell lines have recently demonstrated that the nuclear export of HIF-α is CRM1-dependent, and therefore leptomycin B treatment results in the nuclear accumulation of HIF-α (176, 177, 232) (Fig. 6). A nuclear export signal locates in a MAPK target domain (aa 616-658) (Fig. 2) and interacts with CRM1 in a phosphorylation-sensitive manner (176). Phosphorylation of HIF-1α Ser 641/643 by MAPK blocks CRM1-dependent nuclear export resulting in the nuclear accumulation and increased transcriptional activity of HIF-1α (177). These data suggest that control of HIF-1α nuclear export represents an important MAPK-dependent regulatory mechanism. In addition, it was also shown that Vitamin D3 up-regulated protein 1 (VDUP1), a novel tumor and metastasis suppressor, enhances CRM1-mediated nuclear export of the pVHL/HIF-1α complex and promotes HIF-1α degradation (232).

An oxygen-dependent nuclear export signal located in ODD domain has been identified in the *drosophila* HIF-α homologue, Sima (107). Prolyl hydroxylation and pVHL binding are required for the efficient interaction between NES and CRM1 during nuclear export (Fig. 6). Thus, degradation and nuclear export of Sima appear to take place simultaneously in an oxygen-dependent manner, thereby synergizing in the reduction of Sima nuclear concentration and preventing hypoxia-inducible transcription. Hypoxia inhibits Sima degradation and blocks CRM1-dependent nuclear export, resulting in the nuclear accumulation of Sima and increased transcriptional activity (107). In addition, nuclear export of Sima depends on two other functional NESs localized in the bHLH domain that are critically required for Sima steady-state subcellular localization and for nuclear export upon reoxygenation. These functional NESs are conserved in the bHLH domain of mammalian HIF-α subunits, as well as in several other bHLH-PAS proteins (215). The fact that Sima shuttles continuously between the nucleus and the cytoplasm raises the possibility that oxygen-dependent nuclear export is a physiological mechanism that contributes to the regulation of Sima transcriptional activity. It remains to be investigated whether the nuclear export of mammalian HIF-α subunits are regulated by oxygen through a similar mechanism.

1.1.7 Dimerization and DNA binding

Once in the nucleus, HIF-α and ARNT dimerize and bind to specific DNA sequences. Dimerization is an absolute prerequisite for DNA binding and is mediated by bHLH and PAS domains of each subunit, whereas DNA binding occurs through the bHLH domains. The specific DNA sequences that are targeted by HIF, known as hypoxia-response elements (HREs), are composed of the 5′-(A/G)CGTG-3′ consensus core motif and are found in the promoter, intron and/or enhancer regions of target genes.
IPAS, an alternative splicing variant of HIF-3α with no transactivation domains, is hypoxia-inducible and inhibits HIF-1 heterodimer formation and DNA-binding by competing with ARNT for the alpha subunit, thus acting as a physiological dominant negative regulator of HIF-1 (Fig. 7). This mechanism has been proposed to contribute to the avascular phenotype of cornea with low levels of VEGF (162, 163).

**1.1.8 Regulation of HIF Transcriptional activity**

**1.1.8.1 HIF transactivation domains**

Once HIF is assembled on the HRE, it must recruit transcriptional coactivators to form an intact initiation complex, a process mediated by transactivation domains (TADs). Although ARNT has its own well-defined TAD, it appears to be dispensable for transcription (148). HIF-1α and -2α contain two TADs, an N-terminal TAD (N-TAD, aa 531-575 of hHIF-1α) within the ODD domain and a C-terminal TAD (C-TAD, aa 786-826) at the end of C-terminus. N-TAD and C-TAD are highly conserved between species, with more than 90% and almost 100% homology, respectively, between mice and humans. However, N-TAD and C-TAD share no more than 20% sequence similarity, implying divergent yet highly important roles for each transactivation domain (32).

Both N-TAD and C-TAD employ recruitment of coactivators such as p300 and its parologue CREB-binding protein (CBP), SRC-1, and the transcription intermediary factor 2 (TIF-2) (6, 60, 38, 217, 218). CBP/p300 are essential for linking transcription factors with coactivator complexes and the basal transcriptional machinery, and are thus indispensable for robust transcriptional activation. Additionally, CBP/p300 proteins have histone acetyltransferase activity that is necessary for chromatin modification prior to transcription. HIF transcription activity can also be enhanced by the interaction with other transcription factors such as Smad3 and STAT3 due to stabilization of HIF-CBP/P300 complex (Fig. 7) (86, 219).

**1.1.8.2 FIH-1-mediated hydroxylation represses HIF activity**

Transcriptional activity of HIF-α is regulated by hydroxylation of asparagine residues 803 at the end of the C-terminus of HIF-1α (N847 in HIF-2α) (141). The hydroxylation reaction is carried out by an asparaginyl hydroxylase termed factor-inhibiting HIF-1 (FIH-1), and this modification prevents interaction of the HIF-α C-terminal TAD with coactivators such as CBP/p300, resulting in the repression of HIF activity (Fig. 7) (98, 140)

**1.1.8.3 Differential HIF-α target gene expression regulated by FIH-1**

Under hypoxic conditions, PHDs and FIH-1 are inactive due to the lack of oxygen. Both of these enzymes use oxygen and 2-OG as substrates in the hydroxylation
reaction. However, measurements of the $K_m$ of FIH-1 for O2 revealed that it is almost three times less than that of the PHDs (133). Thus, there would be a hypoxic window where HIF-1$\alpha$ is stable due to the inhibition of prolyl hydroxylation but is transcriptionally less active due to the hydroxylation of N803 by FIH-1. Maximal activation of the HIF pathway requires a further decrease in the oxygen concentration that inactivates FIH-1 and fully activates HIF transcriptional activity (Fig. 8). The activity of the C-TAD can be inhibited by FIH hydroxylation, but the activity of the N-TAD is independent of FIH-1. Thus, HIF-\(\alpha\) has bifunctional transcriptional activity, which, depending on the activity of FIH, allows for differential gene activation that is either N- or C-TAD controlled. Indeed, Dayan et al. has shown a differential regulation of gene expression by either N-TAD or C-TAD in response to the hypoxic gradient.

In this study it was proposed that transcription of some genes such as PGK1 and BNIP3 is solely activated by the N-TAD and not regulated by FIH-1, while transcription of other genes such as CA9, GLUT1, VEGF, PHD3, CITED2 and p21 is regulated by FIH-1 (Fig. 8) (54).

Fig. 7. Regulation of HIF transcriptional activity. Nuclear HIF-\(\alpha\) dimerizes with ARNT on hypoxia responsive element (HRE) and recruits coactivators such as CBP/p300 to activate the transcription of target genes. HIF activity can be enhanced by interaction with Smad3 and STAT3 due to stabilization of the HIF-CBP/p300 complex. The inhibitory domain PAS protein (IPAS), which is a hypoxia-induced splicing variant of HIF-3$\alpha$, inhibits HIF dimerization by competing with ARNT for binding to HIF-\(\alpha\) subunit. FIH-1 inhibits HIF-\(\alpha\) transcriptional activity through hydroxylation of Asp803 that in turn inhibits the recruitments of the coactivators CBP/p300. Activation of p53 and CITED2 sequesters CBP/p300 from HIF resulting in the inhibition of HIF activity. Forkhead transcription factor 3a (FOXO3a) inhibits HIF activity both through p300-dependent mechanism and through increasing the transcription of CITED2 in response to hypoxia. Interestingly, both FOXO3a and CITED2 are induced by HIF, acting as negative feedback regulators.
1.1.8.4 Target gene specificity of HIF-α subunits

Expression profiling and functional studies have revealed that HIF-1α and HIF-2α regulate both shared and unique target genes. For instance, HIF-1α uniquely stimulates the expression of glycolytic enzymes, such as phosphoglycerate kinase (PGK1) and lactate dehydrogenase-A (LDHA), carbonic anhydrase-9 (CA9), and the pro-apoptotic gene BNIP-3 (85, 103, 209, 251). In contrast, the embryonic transcription factor Oct-4 (Pou5f1, Oct-3/4), Cyclin D1, TWIST1, transforming growth factor-alpha (TGF-α), erythropoietin (EPO), CITED2, and PAI-1 are up-regulated under hypoxia in a HIF-2α-dependent manner (8, 50, 84, 89, 92, 102, 209, 251, 254). A third group of genes, including vascular endothelial growth factor (VEGF), adrenomedullin (ADM), and glucose transporter 1 (GLUT-1) are regulated by both alpha subunits (103, 259).

Both HIF-α subunits can bind to the endogenous HREs of hypoxia-responsive genes; however, binding is not sufficient for target gene activation (102, 143). In contrast, experiments using deletion and domain swap mutants reveal that N-TAD contributes to HIF-1α and HIF-2α target gene specificity (102). Replacement of the HIF-2α N-TAD with the analogous region of HIF-1α switches the specificity of these proteins (102). However, some genes such as prolyl hydroxylase-3 (PHD3) require additional regions of the HIF-2alpha protein to be induced (143).

![Fig. 8. Model of differential HIF target gene expression regulated by FIH-1](Image).

Fig. 8. Model of differential HIF target gene expression regulated by FIH-1. Km of FIH-1 for O2 is almost three times less than that of PHDs. Thus, as the O2 concentration drops, the PHDs lose catalytic activity first, increasing HIF-α stability, but C-TAD activity is still inhibited due to the hydroxylation of Asp803 by FIH-1. At this point the transcription of N-TAD-dependent genes such as PGK1 and BNIP3 is activated. At lower O2 concentrations, catalytic activity of FIH-1 is reduced leading to the full activation of HIF. The transcription of genes such as CA9, GLUT1, VEGF, PHD3, CITED2 and p21 will be activated.
Cooperation with other transcription factors may also contribute to selective activation of target genes by HIF-α subunits. For example, HIF-2α but not HIF-1α binds the NF-κB essential modulator (NEMO) resulting in increased recruitment of CBP/p300 and enhanced transactivation activity of HIF-2α at normoxia (30). The ETS family members such as EST-1, EST-2, GABP and ELK may confer target gene selection to HIF-2α (5, 59, 102). Interestingly, HIF-1α and HIF-2α appear to play strikingly distinct roles in modulating the c-Myc pathway. HIF-1α regulates cell cycle and DNA repair genes by counteracting c-Myc activities through c-Myc displacement. In contrast, HIF-2α enhances c-Myc activities by stabilizing c-Myc-Max complexes, increasing expression of c-Myc targets involved in cell cycle progression (104, (205).

1.1.8.5 Competition-mediated regulation of HIF activity

HIF-α CAD and CBP/p300 interaction is also regulated by competition. Coactivators CBP/p300 interact with a large number of proteins, leading to limiting availability of these coactivators in the cells, e.g. CITED2 binds p300/CBP CH1 with high affinity and competitively inhibits other p300/CBP CH1-dependent transcription factors, including HIF (25). FOXO3a inhibits HIF activity by increasing transcription of CITED2 in response to hypoxia (14). FOXO3a also interferes with p300-dependent HIF-1 transcriptional activity by directly binding to the promoter of HIF target genes (61). Interestingly, both FOXO3a and CITED2 have been shown to be induced by HIF, indicating another mechanism of negative feedback regulation. Activated p53 also inhibits HIF function by sequestering p300/CBP from HIF (28). In addition, FIH-1 has been shown to be able to modify a number of other targets, all of which contain ankyrin repeats, such as IκB (46) and Notch intracellular domain (Notch ICD) (47, 278). Although the effect of FIH-1 on the NFκB pathway is not so clear, FIH-1 negatively regulates Notch ICD activity and accelerates myogenic differentiation. By binding to FIH-1 with higher affinity, Notch ICD can sequester FIH-1 away from HIF-1α and thus derepresses HIF-1α function (278).

1.2 FILAMINS

Filamins (FLNs) are large actin-binding proteins with a relative molecular mass of 280 kDa. The name filamin refers to its filamentous colocalization with actin stress fibers (250). FLNs are widely expressed and cross-link with F-actin to form orthogonal networks that are responsible for cellular integrity and mechanics. FLNa anchors actin network to membrane receptors including adhesion molecules and ion channels. Filamins also act as scaffolds for a growing list of proteins with diverse functions. Therefore they function as integrators of cell mechanics and signaling (236).
Filamin comprises an actin-binding domain (ABD) and a rod domain composed of up to 24 immunoglobulin (Ig)-like repeats of ~96 amino acids each (Fig. 9). ABD is composed of two calponin homology domains, CH1 and CH2. The amino acid sequence of filamin ABD is representative of ABDs of the α-actinin or spectrin superfamily, with the exception that the filamin ABD has a unique calmodulin-binding site positioned in the CH1, and therefore calcium-activated calmodulin competes with F-actin for binding to CH1 (180). The Ig-like repeats fold into antiparallel β-pleated sheet domains that overlap so as to generate a rod. The rod domain is interrupted with two flexible loops between repeats 15 and 16 (hinge 1 domain, H1) and between repeats 23 and 24 (hinge 2 domain, H2). Each hinge domain contains around 30 amino acids. Rod domain 1 consists of Ig-like repeats 1-15, and rod domain 2 is composed of Ig-like repeats 16-24. Filamins form Y-shaped dimers via Ig repeat 24 and bind to F-actin through both actin-binding domain and Ig-like repeats 9-15 (182). The dimerization accounts for rigid high-angle F-actin branching and high avidity to F-actin binding and cross-linking. In contrast to the extended flexible structure of rod domain 1, rod domain 2 has a more compact configuration with three globular units. Rod domain 2 does not contribute to F-actin binding or branching. It is thus potentially free to interact with other partner proteins enabling filamin to act as scaffolds for a growing list of transmembrane receptors, signaling and adapter proteins.

Fig. 9 Schematic representation of the domain organizations of filamins.
1.2.1 The Filamin Protein Family

Mammals have three highly homologous filamins: filamin A, B and C (FLNa, FLNb and FLNc). The three filamin proteins show strong homology over their entire sequence (60-80% homology), with the exception of the two hinge domains that show 45% homology. FLNc contains an 81 aa insertion in repeat 20 that is not present in FLNa and FLNb. Orthologues of human filamins have been identified in other species, such as mouse, chicken, rat, drosophila, Xenopus, and C. Elegans. (245). While FLNa and FLNb are the most ubiquitously expressed filamin isoforms, expression of FLNc is largely restricted to skeletal and cardiac muscle. The diversity of the filamin family is increased by alternative splicing of filamin mRNA. The hinge 2 domain between repeats 23 and 24 is present in all human filamin isoforms, while hinge 1 between repeats 15 and 16 is absent in chicken filamin (16) and in some splice-variants of human FLNb (ΔH1) and FLNc (ΔH1) (266, 267).

1.2.2 Filamins, human genetic diseases, and genetic models

Filamins are essential for human development, and mutations of filamins have been associated with human genetic diseases with abnormal development of brain, bone, cardiovascular system and many other organs. FLNa is encoded on the X chromosome. The loss-of-function mutations of FLNa result in abnormal mRNA splicing or early truncation of the FLNa protein leading to an X-chromosome linked congenital disease called periventricular heterotopia (PH) (66, 71, 172, 230). X-linked PH is mainly confined to females, indicating a predominant prenatal lethality for males who are hemizygous for FLNa mutations. A typical PH brain has neuronal cells along the lateral ventricles as a result of failed neuronal migration to the cortex during development. The major clinical syndrome of PH is late-onset epilepsy that often starts in the second decade of life. The patients have an unusually high incidence of vascular complications including ductus arteriosus, aortic aneurysm and premature strokes. Males with FLNa mutations are embryonic lethal and rare live born males have severe cardiovascular defects and intractable hemorrhages. Not only loss-of-function mutations of FLNa cause PH, but also improper FLNa localization, expression, phosphorylation and function, induced by the mutation or dysfunction of FLNa regulators such as BIG2, FILIP and MEKK4, contribute to the pathogenesis of PH (221).

Clustered missense point mutations in FLNa have been identified in a diverse spectrum of other X-linked human disorders generally characterized by skeletal dysplasias, including otopalatodigital syndrome types 1 (OPD1) and 2 (OPD2), frontometaphyseal dysplasia (FMD) and Melnik-Needles syndrome (MNS) (214). OPD1 mutations and most of OPD2 mutations occur in the CH2 domain. OPD2 mutations also occur in repeats 3, 14 and 15. All known FMD and MNS mutations
affect repeat 10 or 14. In contrast to loss-of-function mutations of FLNa that cause periventricular heterotopia, these mutations are predicted to increase actin binding of filamin and either disorganize actin or create toxic products that function in a dominant-negative fashion (66). In addition, mutations in FLNa repeats 1 to 7 are linked to the X-linked myxomatous valvular dystrophy (XMVD) (138). The diversity in phenotypes associated with different filamin mutations reveals that filamins perform a variety of essential functions and specific phenotype will result from disruption of specific interactions between different domains of filamin with different partners.

FLNa-deficient human melanoma cell line (M2) due to markedly reduced FLNa mRNA levels were identified by Cunningham (51). M2 cells do not undergo locomotion in response to factors that induce migration of FLNa-expressing melanoma cells. M2 cells have an unstable surface and display extensive continuous blebbing of the plasma cell membrane and poor pseudopod protrusion.

A mutation of FLNa in mice induced by a chemical N-ethyl-N-nitrosourea led to the absence of the FLNa protein (95). This loss-of-function mutation causes mild skeletal abnormalities in female carrier mice. In males high lethality occurs due to incomplete septation of the heart during gestation and is accompanied by other cardiac, skeletal and palate defects. In a mouse model of complete FLNa deficiency, embryonic lethality has been observed due to severe cardiac structural defects involving ventricles, atria, and outflow tracts, as well as widespread aberrant vascular patterning and integrity (65). The abnormalities in cardiac vasculature are the most common phenotypes exhibited in both models of FLNa deficiency, indicating that FLNa plays crucial role in cardiac morphogenesis. None of the FLNa-deficient mice exhibit impaired neuronal migration and motility defects in other cell types harvested from these mice were not observed. Whether FLNb, which is also expressed in mice (231), can compensate for the loss of FLNa function in these mice is unknown.

The gene encoding FLNb is located on chromosome 3. Mutations in the FLNb gene are found in five human skeletal disorders: autosomal recessive spondylocarpotarsal synostosis syndrome (SCT), atelosteogenesis I, atelosteogenesis III, autosomal dominant Larsen syndrome (135), and Boomerang dysplasia (26). Severe skeletal malformations have been documented in FLNb-deficient mice (64, 158, 277, 282), indicating a critical role of FLNb during skeletogenesis. The phenotypes of these FLNb-mutation-caused disorders have some overlap with the OPD spectrum diseases caused by FLNa mutations.

FLNc gene is located on chromosome 7, and the expression of FLNc is largely restricted to skeletal and cardiac muscle. A heterozygous nonsense mutation in repeat 24 of FLNc protein is known to cause myofibrillar myopathies (MFM), a autosomal dominant disease manifesting clinically as skeletal and cardiac myopathy (129, 247). MFM is characterized by focal myofibrillar destruction and pathological cytoplasmic protein aggregations. Massive FLNc immunopositive aggregates were found in
decaying myofibrils. Functional studies showed that the mutant FLNc is incapable of forming dimers with wild-type FLNc (156). A in-frame four-residue deletion located within repeat 7 of FLNc is also found to cause MFM (229). A mouse model carrying a deletion of repeats 20-24 has less muscle mass and decreased number of primary fibers, indicating that FLNc is critical for normal myogenesis as well as maintaining the structural integrity of the muscle fibers (52).

1.2.3 FLNa functions and interacting partners

Up to date, more than 70 filamin-binding partners have been reported. By interacting with these proteins, FLNa plays essential roles in for example cytoskeleton remodeling, cell migration, signaling transduction, lymphocyte function, proteolysis and cell membrane excitation (281). The accumulation of filamin ligands at the C terminus supports the concept that filamin serves as a scaffold allowing crosstalk between different signaling pathways.

**FLNa regulates cell migration**

Cell migration is a fundamental process that is essential for both development and homeostasis. It is also involved in many pathological processes, such as angiogenesis, inflammation and tumor metastasis. Cell migration involves several highly orchestrated processes, including polarization and protrusion in the direction of movement, formation of adhesion to stabilize the protrusion, and retraction and release of the attachments at the rear. The turnover and reorganization of the actin cytoskeleton are the driving forces of cell migration. Filamins exist at both the leading and the rear of the migrating cells and are essential for cell migration by regulating actin cytoskeletal remodeling and interacting with a number of partners. Cells lacking FLNa are incapable of locomotion and can only migrate after they are transfected with FLNa cDNA (51). Loss-of-function mutations of FLNa in human results in failed neuronal migration during development causing the X-linked disease PH.

FLNa regulates cell polarization by coordinating the small GTPase family as well as its upstream and downstream pathways. During cell polarization, inducers of protrusion activate the Rho small GTPase family members Rac and Cdc42 resulting in actin polymerization through Pak1 and other pathways. Simultaneously, activation of the GTPase RhoA promotes retraction through its effector Rho kinase (ROCK) (195). Rac inhibits RhoA through its activation of p190RhoGAP. RhoA activation of ROCK inhibits Rac and restricts spontaneous lamellae formation through FilGAP phosphorylation. Both protrusion and retraction involve actin remodeling. FLNa has a central role in coordinating these reactions. It crosslinks actin filaments and anchors the small RhoGTPases, such as RhoA, Rac, Cdc42 and RalA in proximity to the cell membrane where they are activated. It associates with both Rac GEF Trio (18) and Rho
GEF Lbc (202) to activate Rac and Rho. It binds to and activates Pak1, which is necessary for Pak1-mediated membrane ruffling. It forms complex with ROCK and co-localizes at protrusive cell membranes (243). Furthermore, the interaction with FLNa is necessary for inactivation of Rac by FilGAP (181), and the suppression of Rho activity by P190RhoGAP is modulated by the cleavage of FLNa (164). It was recently demonstrated that a tri-directional communication between Pak1, FLNa, and sphingosine kinase 1 (SphK1) in a feed-forward mechanism is critical for the regulation of the dynamics of the actin cytoskeleton at the leading edge of the cell (160).

Functional connections between transmembrane adhesion receptors and the intracellular cytoskeleton permit transmission of biochemical signals and mechanical force across the plasma membrane, and are essential for regulation of cell shape, motility, and signaling. FLNa crosslinks F-actin to three-dimensional orthogonal networks and anchors it to cell-extracellular matrix adhesion sites by binding to β-integrins (36). FLNa binds to integrins and competes with talin for the binding site, resulting in the inhibition of talin-dependent integrin activation (125). Migfilin localizes at the cell-matrix adhesion sites and competes with integrin for binding to FLNa. The competition between the different filamin-binding partners may provide a mechanism to regulate filamin interactions and downstream signaling (139, 242).

In addition, FLNa mediates the regulation of cell adhesion and locomotion by interacting with a number of other proteins. The association of FLNa and Ror2 is required for Wnt5a-induced JNK activation and polarized cell migration (189, 190). IKAP co-localizes with FLNa to membrane ruffles and regulates actin cytoskeleton organization and cell migration, which underlies the mechanism for the pathogenesis of familial dysautonomia (FD) caused by the loss-of-function mutations of IKAP (116). In vasculature, activation of FLNa is a prerequisite for the G protein-coupled P2Y2 nucleotide receptor-mediated spreading and migration of aortic smooth muscle cells (274). Endothelial cell-specific molecule-2 (ECSM2), an endothelial-specific membrane protein that regulates endothelial chemotaxis and tube formation, was also found to interact with FLNa (7). These studies implicate a role for FLNa in angiogenesis. Caveolin-1 interacts with FLNa and promotes both transcription and phosphorylation of FLNa in human breast cancer cells, mediating the effects of caveolin-1 on IGF-1-induced cancer cell migration (211). CEACAM1-L binding to FLNa reduces cell migration by competing with the small GTPase RalA for the binding sites in FLNa (128). FLNa-interacting protein (FILIP) interacts with FLNa and induces its degradation. FILIP determines the migration of cortical neurons during development by controlling the amount of FLNa at the ventricular zone (178, 223). Furthermore, elevated expression levels of FLNa resulting from resistance of phosphorylated FLNa to calpain-mediated degradation underlie the mechanism for the impaired neuronal migration in MEKK4/- mice (220). These two studies provide alternative mechanisms
for the pathogenesis of periventricular heterotopia besides the loss-of-function mutations of FLNa.

**FLNa regulates the localization and function of transcription factors**

There is growing evidence implicating the role of FLNa in the regulation of nuclear functions including transcription, DNA repair and nuclear import. FLNa was originally identified as a cytoplasmic cytoskeletal protein. However, several recent studies have demonstrated nuclear localization of both full-length and the C-terminal cleaved fragment of FLNa (57, 157, 199, 222, 275). FLNa increases the DNA damage response by interacting with nuclear protein BRCA2 (275). FLNa facilitates the nuclear localization of several nuclear proteins and regulates their nuclear function. The C-terminal fragment of FLNa interacts with the androgen receptor (AR), facilitates its nuclear translocation, and represses its transactivation activity by disrupting AR interdomain interactions and the recruitment of the coactivator TIF2 (157, 199). FLNa interacts with the transcription factor FOXC1 and partitions FOXC1 to a heterochromatin-rich region of the nucleus, resulting in an inhibition of FOXC1 transcriptional activation. In addition, FLNa is required for the efficient nuclear localization of PBX1 and the formation of a transcriptionally inactive FOXC1-PBX1 complex (23). FLNa activates the transforming growth factor-β signaling pathway by facilitating the nuclear localization and receptor-induced serine phosphorylation of Smads (222). On the other hand, FLNa retains PEBP2β in the cytoplasm and prevents it from functioning as a partner of the Runx1 transcription factor (271). Similarly, FLNa is also shown to sequester p73a in the cytoplasm (127).

Nuclear actin plays an important role in diverse nuclear processes such as chromatin remodeling, transcriptional regulation, RNA processing, and nuclear export. It has been shown recently that a number of actin-binding proteins such as spectrin, filamin, and actinin also exist in the nucleus (57). However, how they get into the nucleus and their precise functions in the nucleus remain to be elucidated. There is a putative nuclear localizing signal (NLS) present in FLNa repeat 20 (residues 2146-2149), although it is not yet elucidated whether FLNa translocates into the nucleus utilizing its own NLS or NLS motifs of interacting partners (or both).

**1.2.4 Regulation of FLNa function**

**1.2.4.1 Proteolysis**

*Calpain-mediated cleavage of FLNa*

Filamins are highly susceptible to proteolysis. FLNa can be cleaved by calpain at residues 1761–1762 in the H1 domain into 190 and 100 kDa fragments. The C-terminal 100 kDa fragment can be further cleaved less efficiently by calpain in the H2 domain.
into 90 and 10 kDa fragments (83). However, only a proportion of full-length FLNa is expected to be processed by proteolysis. After cleavage, FLNa would allow actin cytoskeleton reorganization. In vitro cleavage of FLNa by calpain causes irreversible loss in its ability to cross-link actin filaments into networks although the 190 kDa N-terminal fragment retains the capability to interact with F-actin. Cleavage of FLNa could allow for the disassembly of actin filaments and permit the subsequent reorganization of these filaments into bundles. Among the over 70 FLNa-interacting partners, a large number of them binds to the C-terminus of FLNa (Ig-like repeats 16-24) which can be cleaved by calpain. Moreover, the 100 kDa cleaved fragment has a compact structure distinct from the N-terminal fragment, implying that calpain-dependent cleavage of FLNa may play an essential role in mediating the functions of the C-terminal interacting partners. Indeed, it has shown that calpain activity and calpain-mediated cleavage of FLNa are important for cell migration (164, 165, 192, 265). In addition, the C-terminal fragment of FLNa has been demonstrated to co-localize with androgen receptor in the nucleus and plays important roles in regulation its function (157, 199). Moreover, the cleavage and subcellular localization of FLNa are important for androgen-dependency and metastasis of prostate cancer (17, 252). Taken together, these data implicate an essential role of calpain-mediated cleavage of FLNa in regulating its diverse functions.

Calpains are nonlysosomal calcium-dependent cysteine-proteases. The calpains constitute a family of at least 14 members of which µ-calpain (calpain 1) and m-calpain (calpain 2) are ubiquitous expressed. Calpain activity is crucial for a diverse spectrum of cellular responses, including cell adhesion and motility, apoptosis, proliferation and protein turnover (80). Calcium is the most important activator of calpains, and calpain activity can be influenced by phosphorylation. An additional important regulator of calpain activity is the endogenous inhibitor calpastatin (81). Calpain has also been shown to be activated by hypoxia (3, 105, 276). µ-Calpain is responsible for the cleavage of FLNa. Calpain-mediated cleavage of FLNa is regulated by intracellular calcium levels. Wnt5A increases FLNa cleavage by a mechanism mediated by the release of calcium from intracellular stores (192). Phosphorylation of FLNa at Ser 2152 makes it resistant to cleavage by calpain (43, 75). Reduced FLNa cleavage resulting from increased Ser2152 phosphorylation leads to defective neuronal migration that is responsible for periventricular heterotopia in MEKK-/− mice (220). FLNa-interacting protein (FILIP) inhibits neuronal cell migration out of the ventricular zone during development by enhancing calpain-dependent cleavage of FLNa (178, 223).
Cleavage by caspase-3 and granzyme B

It was also reported that FLNa could be cleaved by caspase-3 and granzyme B during the process of apoptosis, generating 170, 150 and 120 kDa N-terminal fragments and 135, 120, and 110 kDa C-terminal fragments (35, 69, 119).

Ubiquitylation and proteasome-mediated degradation of FLNa

It has recently been identified that the ASB2 (ankyrin repeat and SOCS box-containing 2) is a subunit of a multimeric E3 ubiquitin ligase of the cullin-RING ligase family, and targets FLNa and/or FLNb for ubiquitylation and proteasome-mediated degradation during retinoic acid-induced differentiation of myeloid leukemia cells (97) or during muscle differentiation (19).

1.2.4.2 Phosphorylation of FLNa

FLNa is a phosphoprotein that can be phosphorylated by PKA (cAMP-dependent protein kinase) (39, 42, 53, 112, 113, 248), Pak1 (244), ribosomal S6 kinase (RSK) (196, 262), and PKC (protein kinase C) (123, 240). Phosphorylation is important for regulating FLNa functions such as its binding capacity for several proteins and actin-crosslinking activity. Interestingly, the phosphorylation of FLNa at Ser 2152 mediated by PKA, Pak1, and RSK protects FLNa from calpain-dependent cleavage (75, 220).

1.3 Notch signaling pathway

The Notch gene was discovered in 1919 and was named for the phenotype of a mutant Drosophila with an indentation in the wings (171). The Notch signaling pathway is highly conserved during evolution and plays a fundamental role in embryonic development and adulthood.

1.3.1 Components of Notch pathway and domains organization

In mammals, four Notch receptors (Notch 1-4) and five structurally similar Notch ligands (Delta-like 1, 3, 4, and Jagged 1, 2) have been identified. The domain organization of Notch is schematically represented in figure 10. The extracellular domain of Notch proteins contains 29-36 tandem epidermal growth factor (EGF)-like repeats. Productive interactions with ligands presented by neighboring cells via trans interaction are mediated by repeats 11-12. The EGF repeats are followed by a unique negative regulatory region (NRR), which is composed of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD). The NRR plays an essential role in preventing receptor activation in the absence of ligands. Notch is synthesized as a large entire molecule that is subsequently cleaved by a furin-like
convertase at site 1 (S1 cleavage) during their transport to the plasma membrane (154) (29). This cleavage converts Notch into a heterodimer held together through HD. Notch contains a single transmembrane domain. The Notch intracellular domain (NICD) contains a RAM (RBPjk associate molecule) domain followed by a number of motifs named Ankyrin repeats, which mediate the interaction with the CSL (also known as RBPjk, CBF1, Su(H) and Lag-1) transcription factor. Additionally, there is a TAD, two NLS and a proline/glutamic acid/threonine-rich motifs (PEST). The PEST domain mediates Sel-10-dependent ubiquitylation and degradation of NICD, thus negatively regulates NICD protein stability (93, 193, 263).

Fig. 10 Notch domain organization. A, before S1 cleavage; B, after S1 cleavage.

Fig. 11 Notch signaling pathway. (1) Signal initiated by the interaction between the Notch receptor and ligand. (2) Two successive proteolytic cleavages release NICD. (3) NICD translocates into the nucleus. (4) NICD binds to CSL, displaces corepressors from CSL and recruits a coactivator complex to activate Notch target genes.
1.3.2 The Notch signaling cascade

Notch signaling is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Fig. 11). This is mediated by two successive proteolytic cleavages. The first cleavage occurs extracellularly at site 2 (S2 cleavage) and is mediated by a metalloprotease of the ADAM family (34, 174). As a result of this processing, the remaining membrane-tethered Notch fragment called Notch extracellular truncation (Notch ΔE) undergoes γ-secretase-mediated cleavage within the transmembrane domain at site 3 and site 4 (S3/S4 cleavage). The γ-secretase-mediated cleavage releases NICD, which then translocates to the nucleus (134, 226, 237). NICD cannot bind directly to DNA but heterodimerizes with the DNA binding protein CSL and activates the transcription of genes containing CSL-binding sites (5’-CGTGGGAA-3’) (238). In the absence of ligands and Notch signaling, CSL represses Notch target genes through the recruitment of corepressor complexes (238). NICD binding displaces corepressors from CSL and subsequently recruits a coactivator complex including Mastermind-like proteins (MAML) and CBP/p300 to activate transcription of Notch target genes (73, 114, 264).

1.3.3 Notch target genes

The best-characterized Notch target genes is the family of transcription factors known as Hairy enhancer of split (Hes) (13, 115) and the closely related Hey family (Hey1, Hey2 and HeyL) (161). Hes and Hey proteins are helix-loop-helix transcription factors that negatively regulate transcription of genes that are involved in neuronal differentiation (108). Thus, through regulating the transcription of Hes and Hey genes, the Notch signaling pathway functions as a gatekeeper of differentiation. Additionally, Notch signaling has been found to play important roles in promoting the identity of various types of glial cells by activating GFAP gene expression (77). CD25 and GATA3 that are involved in T-cell development (1, 55, 63, 212) were also shown to be Notch target genes. NRARP and Deltex-1, two potent negative regulators of Notch signaling are also Notch target genes, demonstrating a mechanism of negative feedback regulation (136, 203, 268). Furthermore, CSL-binding sites have been identified in many other genes that are accepted as Notch target genes including NF-kB2 (198), c-Myc (201, 224, 257), GATA2 (213), p21 (207), and cyclin D1 (216), indicating versatile functions of Notch signaling. However, it should be noted that regulation of these genes by Notch activation is tissue-specific and highly dependent on the context.
1.3.4 Notch function

Notch signaling is activated upon cell-to-cell contacts as a result of interactions between Notch receptors and their ligands. The signals that are transduced by the Notch pathway have a central role in cell-fate decisions both during embryonic development and in adult tissue homeostasis. In a context-dependent manner, Notch signals can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs (70).

1.3.4.1 Notch regulates binary cell fate decisions

Notch is mainly involved in a binary cell fate decision that will ultimately lead to pattern formation in the organism. Lateral inhibition is one process of binary cell fate choice. It occurs where a group of equipotent cells expressing equal amounts of Notch receptor and ligand begin to express either Notch receptor or Notch ligand induced by a combination of intrinsic and extrinsic signals. Consequently, Notch signaling is activated in neighboring cells and differentiation is inhibited. For example, the pattern of bristles of Drosophila is established by lateral inhibition (78). In the mammalian system, lateral inhibition plays also an important role in hair cell development in the inner ear (126).

Another way of binary cell fate decision is associated with asymmetric cell division and relies on cell polarization. In this case, the direction of Notch signaling is determined by the asymmetrical distribution during cell division of a number of factors that influence Notch activity in daughter cells. For example, one of the factors capable of modifying the Notch signal is Numb, a protein that binds Notch directly to avoid Notch activation. Therefore, although these two cells are competent for Notch signaling since both express ligands and receptors, only the cell without Numb protein expression will be able to receive the signal via Notch, while the other cell with Numb will send the signal (130).

1.3.4.2 Notch regulates stem cell maintenance

An example of Notch signaling in vertebrates involved in stem cell maintenance is the intestine where Notch prevents the crypt progenitor transient amplifying (TA) cells from differentiating (246). Post-natal gut specific inactivation of CSL results in the complete loss of proliferating TA cells (246). On the other hand, expression of activated NICD in the gut inhibits differentiation of crypt progenitors (72). Upon chronic treatment with a γ-secretase inhibitor that inhibits cleavage of Notch leads to the loss of TA cells (261).
1.3.4.3 Notch determines boundary formation
Inductive Notch signaling typically occurs between non-equipotent cell populations. This mechanism is involved in the boundary establishment during development. In this case, one group of cells signals to a distinct adjacent group of cells to create a new cell fate along the interface between these two distinct cell populations. Well-understood examples include Drosophila wing development and vertebrate somitogenesis (48, 49).

1.3.4.4 Notch induces terminal differentiation
Another role of Notch is to promote the development of a given cell type or body region. For example, in mouse keratinocytes, Notch signaling stimulates expression of early differentiation markers and enhances the expression of the cell cycle regulator p21/Waf1, causing cell-cycle arrest of basal cells and thus allowing onset of differentiation (207).

1.3.4.5 Crosstalk with other signaling pathways
Activation of Notch has been demonstrated to crosstalk with multiple other pathways, such as NF-kB, mTOR, Wnt, PI3K-AKT, and VEGF signaling pathways to exert a broader range of functions (175, 179, 183, 187, 197, 253).

1.3.5 Notch and disease
Gain or loss of Notch signaling components has been directly linked to multiple human disorders characterized by abnormal cell or tissue differentiation. These disorders include developmental diseases (such as Alagille syndrome, spondylocostal dysostosis, and familial aortic valve disease) (76, 87) and adult-onset diseases (such as CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (155). Notch signaling is also involved in pathogenesis of cancer (131), and has been suggested to be a therapeutic target for such as T cell acute lymphoblast leukemia (256) and colon cancer (246).

1.3.6 Regulation of Notch signaling pathway
Regulation of endocytic trafficking and post-translational modifications has emerged as an important mechanism that controls ligand and/or receptor availability and productive ligand-receptor interactions. Ubiquitylation and endocytic trafficking of Notch ligands plays a critical role in enhancing Notch signaling (145, 186). The activity of Notch receptor is regulated through post-transcriptional mechanisms. The important role of proteolytic cleavage in Notch activation, Sel-10 mediated ubiquitylation and degradation, and the regulations of transcriptional activity by recruitment of
coactivators have been mentioned above. Additionally, glycosylation and receptor trafficking also play important role in regulating Notch activation (33).
2. AIMS OF THE PRESENT INVESTIGATION

The overall focus of the present study is to investigate the oxygen-dependent regulation and function of HIF-1α. We aimed to analyze the structure/function relationship of HIF-1α N-TAD and to characterize a pVHL-interacting peptide for a potential use as a tool to activate HIF-dependent signaling, possibly resulting in pro-angiogenesis. We also aimed to identify novel modulators of HIF-1α and novel pathways that crosstalk with HIF-1α and to provide novel therapeutic avenues to modulate the HIF-1α signaling pathway.

**Specific aims:**

- To identify amino acid residues defining the degradation and transactivation functions of the HIF-1α N-TAD.

- To investigate the possibility of increasing HIF-1α activity at normoxia using pVHL-interacting peptides thus providing a potential pro-angiogenic stimulus, and to investigate the impact of subcellular compartmentalization on the regulation of HIF-1α function.

- To characterize Filamin A as a novel modulator of HIF-1α signaling pathway and to investigate the mechanism of the modulation and the physiological relevance of this mechanism.

- To elucidate the mechanism by which hypoxia maintains the undifferentiated cell fate and to investigate the crosstalk between Notch and HIF-1α signaling pathways in this process.
3. RESULTS AND DISCUSSION

3.1 Analysis of the bifunctional N-TAD that mediates both transactivation and protein degradation (Paper I).

HIF-1α has two transactivation domains, N-TAD and C-TAD. In contrast with the C-TAD protein, which is constitutively stable, the protein stability of N-TAD is strictly regulated by oxygen (60, 234). The N-TAD (residues 531-584 and 532-585 in mouse and human HIF-1α, respectively) is located in the ODD domain of HIF-1α, and harbors one of the two proline residues (Pro563 in mouse HIF-1α) that can be hydroxylated by PHDs and mediate pVHL-dependent degradation of HIF-1α (109, 111, 166, 273). In this paper, we have characterized by mutation analysis residues within the N-TAD that are important in mediating transactivation and degradation functions.

We have found that residues Tyr564, Ile565, Asp568-Asp569-Asp570, Phe571 and Leu573 are important for both protein degradation and transcriptional activity of N-TAD, since mutations of these residues interfere with the binding of pVHL and resulted in protein stabilization but reduced transactivation. One exception to this was mutation of Pro563 to Ala (P563A) that abolished pVHL binding and stabilized the protein, but resulted in a dramatic increase in transactivation activity, both at normoxia and hypoxia. CBP/p300 enhanced the activity of both wild-type N-TAD and the P563A mutant. It remains to be investigated if the increased activity of P563A mutant is exclusively due to protein stabilization or if hydroxylation of Pro563 per se, affects the transcriptional activity of N-TAD.

In this study, we also identified residues Leu556-Leu558 and Met560-Leu561 that were essential for transactivation, but dispensable for protein degradation of NTAD, since mutations of these residues had no effect on pVHL binding but significantly reduced N-TAD-mediated transactivation. A smaller region of the N-TAD containing mHIF-1α (546-574) binds efficiently to pVHL, but shows a significantly weaker transactivation activity. This indicates that residues that compose the minimal degradation box can be limited to a central N-TAD region, whereas full transactivation activity is mediated by a longer peptide.

The importance of these residues on protein stabilization and transcriptional activity were also investigated in the context of full-length HIF-1α. In agreement with what was observed with N-TAD, mutations of Leu556-Leu558 did not affect HIF-1α protein stability but resulted in decreased HIF-1α activity shown by decreased expression of an HRE-driven luciferase reporter gene. Mutations of residues Tyr564, Ile565, and Phe571-Leu573 that are important for both protein degradation and transcriptional activity of N-TAD resulted in reduced transactivation activity of the full-
length HIF-1α, but did not affect HIF-1α protein stability. The inability of N-TAD mutants to affect protein degradation in the context of the full length HIF-1α can be explained by the presence of another independent degradation box centered on Pro402 (166) or the existence of additional degradation mechanisms. The same mechanisms may underlie the phenomenon that mutation of Pro563 in the context of full-length HIF-1α did not result in a fully stabilized mutant protein (166). Furthermore, in contrast to the constitutively active character of the P563A mutant in the context of the isolated N-TAD, the same mutation did not affect the transactivation of the full-length protein. Interestingly, a HIF-1α mutant containing both mutated Pro402 and Pro563 (HIF-1α (P402A/P563A)) showed increased protein levels at normoxia but is still hypoxia-responsive, implying that an alternative degradation mechanism that is independent of prolyl hydroxylation of Pro402 or Pro563 targets this mutant. This is further verified by a recent report demonstrating that HIF-1α (P402A/P563A) can be degraded by a pVHL-dependent and PHDs-independent mechanism (2). However, the detailed degradation mechanism of this mutant needs to be further elucidated.

3.2 Cell type-specific compartmentalization of HIF-1α degradation: implications for therapeutic strategy targeting HIF-1α (Paper II)

3.2.1 A pVHL-interacting peptide induces angiogenesis in vivo

In the previous study, we have shown that a region of HIF-1α N-TAD (amino acids 546-574) interacts with pVHL, and we have identified the residues in this region that are essential for pVHL-binding and pVHL-dependent protein degradation. In this study, we have demonstrated that expression of pVHL-interacting fusion proteins following transient transfection activates endogenous HIF-1α activity at normoxia. HIF-1α-derived pVHL-interacting peptides increased expression of an HRE-driven luciferase reporter gene, while mutants that were unable to bind pVHL had no effect. Among a series of deletion mutants of this region, mHIF-1α (559-573) is the most effective one inducing HIF-1α activity. In vitro experiments demonstrated that mHIF-1α (559-573) sequesters pVHL from endogenous HIF-1α resulting in increased HIF-1α activity. Prolyl-hydroxylated mHIF-1α (559-573) is 10 times more efficient than an unhydroxylated peptide in competing with HIF-1α for binding pVHL. When fused to human HIV-1 TAT protein transduction domain and implanted into mouse cornea, the hydroxylated mHIF-1α (559-573) peptide significantly induced angiogenesis. These results provide evidence for the possibility of using pVHL-interacting subfragments of HIF-1α in therapeutic angiogenesis to activate endogenous HIF-1α activity at normoxia.
3.2.2 Cell type-specific regulation of subcellular compartmentalization and degradation of HIF-1α

We next restricted the expression of mHIF-1α (559-573) peptide into the nucleus or cytoplasm by fusion with GST-GFP-NLS or GST-GFP-NES, respectively. The results from HRE-driven luciferase reporter expression analysis demonstrated that the induction of HIF-α activity by the mHIF-1α (559-573) peptide is dependent on its subcellular localization in a cell type-specific manner. In hepatoma HepG2 cells, the peptide only induced HIF-1α activity when it is located in the cytoplasm; while in mouse brain endothelial cells (MBEC), both nuclear and cytoplasmic located peptides induced HIF-α activity, however they are less efficient than the peptide with homogeneous distribution in the two compartments. Since the peptides induced HIF-α activity by competing with pVHL-mediated degradation of HIF-1α, these results indicate that HIF-1α maybe degraded in different compartments of different cell types at normoxia. Utilizing a fluorescent proteasome sensor protein that is rapidly degraded by the proteasome without requiring prior ubiquitylation and is stabilized and detected at the time and place where HIF-1α is degraded, we showed that the degradation of HIF-1α occurs predominantly in the cytoplasm of HepG2 cells, while in MBEC cells it happens equally in the nuclear and cytosolic compartments. Consistent with this finding, western blot analysis showed that the half-life of cytoplasmic HIF-1α is much shorter than nuclear HIF-1α in HepG2 cells during reoxygenation; whereas in MBECs, the degradation rate of HIF-1α was almost the same in both compartments. In addition, the proteasome was more active in the cytoplasm of HepG2 cells, but slightly more active in the nucleus in MBECs. Taken together, our results present the first evidence that degradation of HIF-1α can preferably occur in different cellular compartments in a cell type-specific manner.

Degradation of HIF-α by the proteasome depends on prolyl-hydroxylation by PHDs and subsequent pVHL-mediated ubiquitylation. It has been shown that PHD1 appears exclusively located in the nucleus, PHD2 mainly in the cytoplasm and PHD3 in both compartments. The subcellular localization of PHDs is not affected by hypoxia (169). There are two main forms of pVHL displaying distinct subcellular localization, pVHL30 located mainly in the cytoplasm and pVHL19 present in the nucleus (96). Both are biologically active products of the VHL gene (106). Other studies show that pVHL is predominantly cytoplasmic but shuttles between the nucleus and cytoplasm (88) (146). Moreover, restricted localization of expressed pVHL in either the nucleus or cytoplasm does not inhibit HIF-1α degradation at normoxia (147), and HIF-1α can be degraded in either the nucleus or cytoplasm (22). Thus, it appears that HIF-1α is readily hydroxylated and ubiquitylated in both the nucleus and cytoplasm. This is supported by our results that a pVHL-binding peptide distributed throughout the cell was the most efficient one in rescuing HIF-1α from degradation. However, our data
suggested that compartmentalization of HIF-1α degradation is cell type-specific, and proteasomal activity levels in different subcellular compartments are relevant to determine where HIF-1α is efficiently degraded at normoxia or during reoxygenation.

In addition, we also observed a cell type-specific differential subcellular distribution of GFP-tagged HIF-1α. In hypoxic HepG2 cells HIF-1α showed an exclusively nuclear localization and a redistribution of HIF-1α into the cytoplasm during reoxygenation. In contrast, HIF-1α was distributed in both the nucleus and cytoplasm in hypoxic MBECs, and there was no obvious redistribution during reoxygenation. Since the subcellular localization of HIF-1α was shown to be influenced by the MAPK pathway, the differential subcellular localization of HIF-1α observed in HepG2 and MBEC cells may result from differential status of MAPK activity or other unknown mechanisms that need to be further investigated.

Taken together, the results from this paper indicate new levels of complexity in the regulation of HIF-1α protein levels by cell-type-specific parameters such as subcellular distribution of the proteins and compartmentalized degradation of HIF-1α (Fig. 12). It may be important to consider this in efforts to enhance HIF-1α activity (e.g., in pro-angiogenic therapy) or inhibit HIF-1α function (e.g. in tumor therapy) in a tissue-specific manner.

**Fig. 12** Cell-type-specific regulation of subcellular compartmentalization and degradation of HIF-1α. In primary endothelial cells (MBECs) (A and B), HIF-1α is present in both nuclear and cytoplasmic compartments under conditions of normoxia, hypoxia, and reoxygenation, and proteasome-dependent degradation occurs at a similar rate in both compartments. In this context pVHL-interacting peptides are able to inhibit pVHL-mediated degradation of HIF-1α in both compartments. In contrast, in highly proliferating or transformed cell types (e.g., HepG2 cells) (C and D), proteasome activity dominates in the cytoplasmic compartment. HIF-1α translocates from the cytoplasm to the nucleus in hypoxia. Upon reoxygenation, the protein needs to be transported back to the cytoplasm to be efficiently degraded by the proteasome. In this type of cells, pVHL-interacting peptides can only inhibit pVHL-mediated degradation of HIF-1α when targeted to the cytoplasmic compartment.
3.3 Filamin A enhances HIF-1α-mediated hypoxic response: a new role of a cytoskeleton protein (Paper III)

3.3.1 Filamin A is a novel HIF-1α-interacting protein

In this study, we identified filamin A (FLNa) as a novel modulator of the HIF-1α-mediated hypoxic response. We first identified FLNa as a HIF-1α-interacting protein using yeast two-hybrid system and the interaction was confirmed using immunoprecipitation assays. The interaction domains were Ig repeats 20-24 in the C-terminus of FLNa and residues 1-390 containing bHLH and PAS domains of HIF-1α. There is a very weak interaction interface corresponding to residues 390-531 of HIF-1α as well.

3.3.2 Hypoxia enhanced calpain-mediated cleavage of FLNa and the nuclear localization of C-terminal fragment of FLNa

The modulation of FLNa by hypoxia and the role of FLNa in HIF-1α signaling pathway was investigated using FLNa-deficient M2 and FLNa-supplemented A7 human melanoma cell lines (51) as well as COS 1 cells. In whole cell extracts of A7 and COS1 cells, we detected two major FLNa subfragments in addition to full-length FLNa that are 190 kDa and 100 kDa, respectively. A calpain inhibitor calpeptin rather than a caspase-3/7 inhibitor Ac-DEVD-CHO inhibited the generation of the subfragments, indicating these subfragments were generated by calpain-mediated cleavage. The 190 kDa and 100 kDa fragments were recognized by antibodies against the N-terminus, and the C-terminus of FLNa, respectively. Expression of an FLNa construct corresponding to Ig repeats 16-24 presented the same size as the 100-kDa fragment, indicating that the 100-kDa fragment corresponds to this region. This is in agreement with a previous findings that calpain cleaves FLNa at the H1 domain generating a 100 kDa C-terminal fragment (FLNa\textsuperscript{CT}) containing Ig repeats 16-24 (83). Interestingly, we found that the generation of FLNa\textsuperscript{CT} is significantly enhanced by hypoxia in both A7 and COS1 cells. Moreover, in contrast to full-length FLNa that is predominantly cytoplasmic, FLNa\textsuperscript{CT} presented a much stronger expression in the nucleus, which was further increased by hypoxia. Calpain was shown before to be activated by hypoxia (3, 105, 276) presumably as a consequence of increased intracellular calcium levels (241). In our study we showed for the first time that hypoxia enhances calpain-mediated cleavage of FLNa generating FLNa\textsuperscript{CT} containing Ig repeats 16-24, and increases nuclear localization of FLNa\textsuperscript{CT}. Since calpain-mediated cleavage of FLNa is important for cell migration (164, 165, 192, 265), our data imply that the hypoxia-mediated enhancement of FLNa cleavage may contribute to cell migration and cancer metastasis. Moreover, a majority of FLNa-interacting partners
such as androgen receptor interact with FLNa\textsuperscript{CT}. Therefore hypoxia may play important roles in modulating the functions of FLNa\textsuperscript{CT}-interacting proteins by promoting the generation and nuclear localization of FLNa\textsuperscript{CT}.

### 3.3.3 C-terminal fragment of FLNa facilitates the nuclear accumulation of HIF-1\(\alpha\)

We next investigated the role of FLNa in HIF-1\(\alpha\) signaling. The expression of FLNa had no obvious effect on HIF-1\(\alpha\) protein stability shown by a similar degradation rate of endogenous HIF-1\(\alpha\) in M2 and A7 cells during reoxygenation. However, FLNa facilitated the nuclear accumulation of HIF-1\(\alpha\), as demonstrated by increased nuclear localization of both GFP-tagged HIF-1\(\alpha\) and endogenous HIF-1\(\alpha\) in hypoxic A7 cells than M2 cells. Inhibition of FLNa cleavage and FLNa\textsuperscript{CT} generation by calpain inhibitor calpeptin diminished the increased nuclear accumulation of HIF-1\(\alpha\) caused by hypoxia in both A7 and COS1 cells, while they produced no effect in M2 cells. In addition, GFP-tagged HIF-1\(\alpha\) presented a significantly enhanced nuclear localization in cells stably expressing full-length FLNa and FLNa\textsuperscript{CT} when compared with cells expressing an FLNa mutant (\(\Delta\text{H1}\)) that is resistant to calpain-mediated cleavage due to the deletion of H1 domain. These results indicate that the hypoxia-inducible, calpain-mediated cleavage of FLNa and the generation of FLNa\textsuperscript{CT} play essential roles in the nuclear accumulation of HIF-1\(\alpha\) at hypoxia. Since the subcellular localization of HIF-1\(\alpha\) was shown to be influenced by the MAPK pathway, we evaluated the subcellular distribution of GFP-tagged HIF-1\(\alpha\) after the treatment with the MAPK inhibitor PD98059. As expected the MAPK inhibitor decreased the nuclear localization of GFP-tagged HIF-1\(\alpha\), however, the reduction was not significantly different between M2 and A7 cells. Thus, our results confirm that MAPK inhibits nuclear export of HIF-1\(\alpha\) but it seems that FLNa does not participate in this process. The exact mechanism by which FLNa promotes the nuclear accumulation of HIF-1\(\alpha\) requires further elucidation.

### 3.3.4 FLNa enhances HIF-1\(\alpha\) transcriptional activity

We next investigated the impact of FLNa on HIF-1\(\alpha\) transactivation activity. We found that HIF-1\(\alpha\) target genes such as VEGF-A, GLUT1, GLUT3, PGK1 and BNIP3 showed higher levels of mRNA expression at hypoxia in A7 cells when compared with M2 cells. Although to a lesser extent, FLNa also enhanced mRNA expression of some of HIF-1\(\alpha\) target genes at normoxia. Down-regulation of FLNa expression by siRNA significantly reduced the expression of HIF-1\(\alpha\) target genes at hypoxia. Similar results were observed in human embryonic kidney 293T cells and human osteosarcoma U2OS cells. Interestingly, inhibition of calpain-mediated cleavage of FLNa by calpeptin significantly reduced HIF-1\(\alpha\) target gene GLUT1 mRNA expression in A7 cells, but
had no effect in M2 cells. Taken together, these results indicate that FLNa enhances the transactivation activity of HIF-1α, and the calpain-mediated cleavage of FLNa is playing an important role in this process. This is further supported by the findings that the expression of an HRE-driven luciferase gene and endogenous GLUT1 gene were significantly higher in cells stably expressing full-length FLNa and FLNa CT than in cells expressing ΔH1 FLNa mutant or control cells. Interestingly, our more recent results (not included in the manuscript) showed that FLNa is recruited to the HIF-1α transcriptional complex on HRE-containing promoters. Chromatin immunoprecipitation (ChIP) experiments using M2, A7 and COS-1 cells cultured under normoxic and hypoxic conditions demonstrated that both HIF-1α and FLNa were detected within the same HRE-containing region of the VEGF-A promoter at hypoxia in A7 and COS1 cells. These results correlate with the increase in HIF-1α-dependent transcriptional activity observed in A7 versus M2 cells. There is growing evidence implicating the role of FLNa in the regulation of nuclear functions such as transcription, DNA repair and nuclear transport. However, how FLNa translocates into the nucleus and the exact mechanisms of FLNa regulating HIF-1α transcriptional activity is still not fully understood.

3.3.5 FLNa promotes VEGF-A-mediated angiogenesis

HIF has been suggested to be the master switch of angiogenesis by activating the transcription of VEGF-A. We therefore investigated the role of FLNA in VEGF-A-mediated endothelial activation, an initial step of angiogenesis. We found that the secretion of VEGF-A protein was significantly higher in A7 cells than in M2 cells both at normoxia and hypoxia, which is significantly reduced after down-regulation of FLNa by siRNA. We then demonstrated that media from both normoxic and hypoxic A7 cells increased the morphological changes, migration and proliferation of porcine aortic endothelial cells expressing VEGF receptor 2. Taken together, these results indicate an important role of FLNa in VEGF-A-mediated angiogenesis. This is in agreement with previous reports that FLNa is essential for proper cardiovascular development, and cardiovascular defects have been associated with the loss of FLNa in both humans and mouse genetic models (refs). A recent report shows that overexpression of FLNa in peripheral cholangiocarcinomas is correlated with increased VEGF-A expression and increased vascular density (91).

In summary, our unexpected discovery of FLNa as a regulator of HIF-1α function supports the new concept that the actin-crosslinking protein FLNa may also exist as a nuclear protein (57). Based on our observations, we propose a model in which FLNa mediates a previously unrecognized mechanism of regulation of HIF-1α function and thus control of VEGF-A activity. Under hypoxic conditions, calpain activity is
increased and higher levels of FLNa<sup>CT</sup> are present in the nucleus, facilitating the nuclear localization of HIF-1α. The functional consequence of this interaction is an enhanced transactivation function of HIF-1α, resulting in increased levels of expression of HIF-1α target genes including VEGF-A. Thus, this new interaction of HIF-1α with FLNa represents another potential mechanism of regulating angiogenic responses in various diseases, notably those involving cardiovascular and tumor disease.

3.4 Crosstalk between Notch and HIF pathways (Paper IV)

In this study, we identified a crosstalk between Notch and HIF pathways, a mechanism that mediated the maintenance of the undifferentiated cell fate by hypoxia.

3.4.1 Hypoxia inhibits cell differentiation

In this study, we demonstrated that hypoxia inhibits cell differentiation of myogenic C2C12, satellite and primary neural stem cells. The inhibition depends on the Notch signaling pathway since a γ-secretase inhibitor which blocks the Notch pathway by preventing the generation of the Notch intracellular domain (ICD), substantially abrogated hypoxia-induced inhibition of differentiation. These results implicate Notch signaling in mediating the effect of hypoxia on cell differentiation.

3.4.2 Hypoxia enhances Notch signaling

We next showed that the expression of Notch immediate downstream genes such as Hes-1 and Hey-2 were significantly increased by hypoxia in C2C12 cells and neural stem cells. To further investigate the relationship between hypoxia and Notch signaling, we employed the embryonic carcinoma cell line P19 (167), which can undergo neuronal differentiation controlled by Notch signaling (191). In P19 cells, hypoxia resulted in elevated expression of Hes-1 mRNA, Hes-1 promoter-driven luciferase reporter gene (Hes-1-luc) as well as a highly Notch-specific promoter-driven luciferase reporter, gene 12XCSL-luc. The promoter in the 12XCSL-luc construct is composed of six multimerized dimeric CSL binding sites and thus responds only to Notch ICD-CSL-mediated activation (122). The increased reporter gene expression by hypoxia was abolished by a γ-secretase inhibitor, suggesting that hypoxia enhances endogenous Notch signaling through the activation of Notch ICD. This is confirmed by the finding in transient transfection experiments that Notch 1 ICD upregulated the expression of both Hes-1-luc and 12XCSL-luc reporters at hypoxia. Moreover, hypoxia increased the half-life of Notch 1 ICD generated from Notch 1 ΔE, a membrane-tethered form of Notch. Notch 1 ΔE mimics an S2-cleaved receptor and rapidly undergoes S3 processing in the cells, thus generating Notch 1 ICD. Taken together, these results
suggest that hypoxia enhances Notch signaling through increasing the stability and activity of Notch 1 ICD.

3.4.3 HIF-1α interaction with Notch 1 ICD regulates Notch signaling at hypoxia

Since hypoxia responses are mainly mediated by HIF, we investigated the potential role of HIF-1α in modulating Notch pathway by hypoxia. Indeed, while hypoxia substantially enhanced Hey-2 gene expression in wild-type mouse embryonic fibroblast cells (MEFs), it did not affect Hey-2 gene expression in HIF-1α null MEFs. These results implicate that HIF-1α is essential for the increased Notch activity during hypoxia. We next found that HIF-1α physically interacts with Notch 1 ICD using in vitro translated proteins, and two interaction domains were identified in HIF-1α, which are residues 1-390 and 390-531. Interestingly, although both HIF-1α and HIF-1α (1-390) increased Notch 1 ICD expression at both normoxia and hypoxia, only full-length HIF-1α enhanced Notch 1 ICD-induced expression of 12XCSL-luc reporters either at normoxia or hypoxia. Neither HIF-1α (1-390) nor HIF-1α (390-531) had similar effects. These results indicate that although the HIF-1α interaction interface with Notch 1 ICD is located in the N-terminal region of HIF-1α, hypoxia-dependent enhancement of Notch signaling requires the C-terminal transactivation domain of HIF-1α. We were thus encouraged to perform Notch 1 ICD- or HIF-1α-specific chromatin immunoprecipitation (ChIP) experiments under various combinations of hypoxia and Notch activation. Notch activation was achieved in C2C12 cells after coculture with 293T cells stably expressing the Jagged 1 ligand. Interestingly, under conditions of both hypoxia and ligand-activation of Notch, binding of HIF-1α to the Hey-2 promoter was observed. HIF-1α was not detected at the Hey-2 promoter when Notch signaling was not activated. In contrast, we did not detect any interaction between Notch 1 ICD on the HIF-1α target gene PGK1 promoter. Taken together, these data suggest that HIF-1α in response to hypoxia and Notch signaling is recruited to Notch-responsive promoter and enhances the transcription of Notch target genes.

3.4.4 FIH-1 reduces Notch 1 ICD activity

It has been shown that FIH-1 inhibits the transcriptional activity of HIF-1α through the hydroxylation of Asp803 in the C-TAD. We found in this study that FIH-1 interacts with Notch 1 ICD and reduced Notch 1 ICD-induced activation of 12X CSL-luc expression under either normoxia or hypoxia conditions. This suggests that FIH-1 either affects endogenous HIF-1α activity or that FIH-1 plays a direct role in the regulation of Notch signaling. Recent studies confirmed that FIH-1 not only regulates HIF-1α activity, but also hydroxylates Notch 1 ICD and negatively regulates Notch
activity leading to the acceleration of myogenic and neural differentiation (47, 278). In addition, our results show that although Notch 1 ICD was not detected on the PGK1 promoter, Notch activation enhanced binding of HIF-1α on the PGK1 promoter. Moreover, inhibition of Notch activity by a γ-secretase inhibitor reduced PGK1 mRNA expression levels. These results imply that Notch activation enhanced HIF-1α activity by an unknown mechanism. Interestingly, a recent study has provided evidence that Notch ICD enhances recruitment of HIF-1α to its target promoters and derepresses HIF-1α function by sequestering FIH-1 away from HIF-1α (278).

In conclusion, the data from this paper demonstrate a link between hypoxia and Notch signaling and provide insights into how hypoxia maintains the undifferentiated cell state, by a functional interplay with the Notch signaling pathway. HIF-1α plays an important role in this process by interacting with the Notch intracellular domain to link hypoxic information to a Notch response (Fig. 13).

**Fig. 13** Schematic Depiction of Crosstalk between Notch and Hypoxia. In this model, two modes of the hypoxic response are shown: (1) the canonical hypoxic signaling leading to activation of downstream genes such as VEGF, PGK1, and Epo through HIF1α/HIF-1β binding to HRE elements in the corresponding promoters; and (2) maintenance of the stem cell state through interaction with the Notch signaling pathway. In the latter case, HIF-1α and Notch ICD form a point of convergence between the two signaling mechanisms, leading to stabilization of Notch ICD, recruitment of HIF-1α to Notch-responsive promoters, and activation of Notch downstream genes (Hey and Hes).
4. CONCLUSIONS

Paper I and II
- Identification of N-TAD residues critical for regulation of the degradation and transactivation function of HIF-1α by pVHL.
- Expression of pVHL-interacting peptides activates endogenous HIF-1α function, providing a potential pro-angiogenic stimulus.
- Degradation of HIF-1α is regulated by subcellular compartmentalization in a cell type-specific manner.
- The compartment in which degradation of HIF-1α occurs dictates where the pVHL-interacting peptide must be located in order to efficiently increase HIF-1α function at normoxia.

Paper III
- FLNa is identified as a novel HIF-1α-interacting protein.
- Hypoxia enhances calpain-mediated cleavage of FLNa and the nuclear localization of C-terminal fragment of FLNa.
- FLNa facilitates the nuclear localization of HIF-1α, binds to HRE-containing promoters and enhances the transcriptional activity of HIF-1α. These functions are mediated by C-terminal fragment of FLNa.
- FLNa promotes VEGF-A-mediated angiogenesis.

Paper IV
- Hypoxia blocks differentiation in myoblasts, satellite cells and primary neural stem cells in a Notch-dependent manner.
- Hypoxia stabilizes Notch intracellular domain, activates Notch-responsive promoters and increases expression of Notch-regulated downstream genes.
- HIF-1α interacts with Notch ICD, binds to Notch-responsive promoter, and a transcriptionally active form of HIF-α increases Notch signaling.
- Taken together, these data provide a model for how hypoxia controls the differentiation status of a cell and demonstrates a crosstalk between the HIF-1α and Notch signaling pathways.
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