Research Project

Bachelor Biomedische wetenschappen, Universiteit Utrecht
E. M. V. Gradenwitz, Std. Nr. 3407985
Utrecht, july 22th 2011
Supervisor: A.S.A. van Brussel

Literature part:

The differential regulation of the transcription factors HIF1α and HIF2α by posttranslational modifications

Practical part:

Isolation of Glut1 from erythrocyte ghosts

ABSTRACT

The hypoxia regulated transcription factors HIF-1α and HIF-2α have different targets and are differentially regulated. Literature describes a number of mechanisms that regulate each of the two paralogues in a different way. The aim of this thesis is to provide an overview of how cells distinguish between HIF-1α and 2α regarding regulation of protein activity by posttranslational modifications and interactions with other proteins. We describe five mechanisms, that may serve as a target for genotype based prognosis and therapy.
Glut1 which is a downstream target of HIF-α, is used as a candidate epitope to find variable domains of heavy chain-only lama antibodies (VHH)s that bind specifically to cancer cells. In the context of the practical part of this thesis, Glut1 has been isolated of from purified erythrocyte ghosts.

The differential regulation of the transcription factors HIF1α and HIF2α by posttranslational modifications

Introduction

Lack of Oxygen on cellular level leads to activation of transcription factors of the Hypoxia induced factor (HIF) family, which orchestrate the cellular adaptation to hypoxia. Many of these adaptations are linked to cancer development and progression, leading to a profound therapeutic interest in HIFs.

The HIF family of transcription factors includes α-subunits, among which HIF-1α, and HIF-2α and β-subunits such as HIF-1β. HIF-1β is also called ARNT (Aryl hydrocarbon receptor nuclear translocator) for its function in Aryl hydrocarbon receptor signalling.

The common structure of al members includes basic Helix-Loop-Helix (bHLH) and Per-Arnt-Sim (PAS) motives, last of which are named after the drosophila proteins period and single minded and the HIF-β-subunit ARNT (Pillai *et al.*, 2011). The PAS motives enable dimerization of α- and β-subunits and the bHLH motives form the DNA binding domain that binds to hypoxia-responsive element (HRE) which is a local regulatory sequence in the promoter of hypoxia induced genes (Fig. 1).

HIF-α contains two domains that activate transcription, an N-terminal transactivation domain (NTAD), which is C-terminal to the bHLH-PAS domain and a C-terminal transactivation domain (CTAD). These domains interact in trans with the primary transcription machinery at the core promoter and enable the activation of transcription of genes close to the HIF binding site (Yan *et al.*, 2007). The CTAD for instance, interacts with p300, a general co-activator of transcription, to drive expression of HIF responsive genes (Sang *et al.*, 2002; Yan *et al.*, 2007).

The regulation of HIF activity takes place largely through direction of the α-subunit protein levels in the cell. HIF-α gets readily hydroxylated during normoxia by prolyl hydroxylases domain (PHD) family members which are both oxygen and iron (Fe(II)) dependant (Kaelin and Ratcliffe, 2008). Hydroxylation of proline residues creates a binding site for the substrate binding subunit of an E3-ligase complex called von Hippel Lindau protein (pVHL). Specific binding of pVHL to hydroxylated HIF-α forms a bridge between HIF-α and the less specific components of the ubiquitination machinery, leading to polyubiquitination and proteasomal degradation of HIF-α (Fig. 2 left panel) (Yan *et al.*, 2007). Hydroxylation and following pVHL induced ubiquitination is not thought to incorporate feedback. The proline hydroxylation persists and is not reversed under hypoxia. Stabilization is accomplished through *de novo* synthesis of HIF-α, which cannot be hydroxylated by PDH under hypoxic conditions. Subsequently the unhydroxylated HIF-α is not prone to pVHL and proteasomal degradation (Chan *et al.*, 2002). Upon stabilization of HIF-α, heterodimerization with the constitutionally stable β-subunit occurs. Hetrodimerization of HIF proteins leads to a functional transcription factor. In the nucleus the transcription factor binds to HREs of hypoxia induced genes (Fig. 2 right panel) (Kaelin and Ratcliffe, 2008; Yan *et al.*, 2007).

In addition to the oxygen and PHD dependant hydroxylation that is directed against proline residues of HIF-α and results in its degradation, HIF-α is also hydroxylated on asparagine residues by the oxygen dependant Factor inhibiting HIF 1 (FIH1). Asparagine hydroxylation of HIF-α inhibits the interaction of its CTAD with p300. (Fig. 2) (Sang *et al.*, 2002; Yan *et al.*, 2007).

Mutation of *VHL* is common in several human cancers and is the cause of van Hippel Lindau disease, an autosomal dominant disorder bearing increased risk for neoplasms of various kinds. Dysfunctional pVHL leads to uncontrolled levels of HIF-α proteins and enhanced expression of HIF responsive genes (Barontini and Dahia, 2010). Although pVHL may have different tumor suppressing capabilities, its role in HIF-α subunit degradation has been shown to be a critical component in kidney cancer. The most prominent kind of neoplasms that arise frequently in company of defective HIF-α signalling are clear cell renal cell carcinoma (CCRCC) (Li *et al.*, 2007; Kondo *et al.*, 2003).

Although HIF-1α and -2α have been shown to not only share structural similarities but also share a large set of target genes, this seams to not exclusively be the case. The targets of both subunits do not entirely overlap,

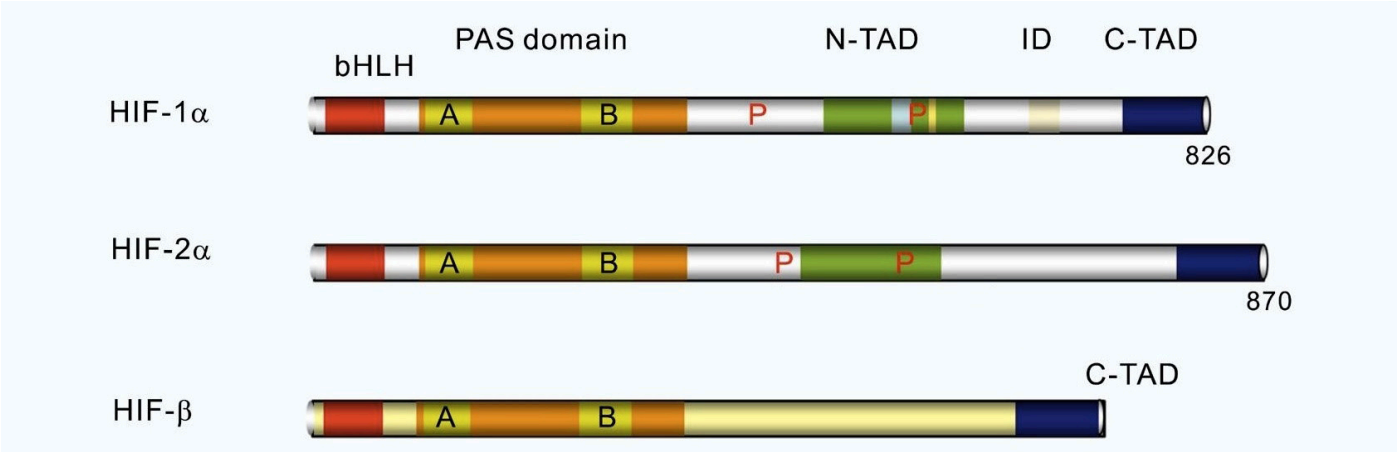

Fig. 1 (adapted from Chen ***et al.***, 2009) Schematic representation of Human HIF-1 α, HIF-2α and HIF-α. All three HIF members contain a N-terminal Helix-Loop-Helix (bHLH) domainto interact with DNA, two Per-Arnt-Sim (PAS) domains (A and B) for dimerization, and a C-terminal transactivation domain (CTAD) to co-activate transcription. HIF-1α and HIF-2α include an N-terminal transactivation domain (NTAD) for additional transcriptory interaction. Before and within the NTAD, both α-subunits contain two proline residues (P), which can be hydroxylated. HIF-1α contains an additional hydroxylation site consisting of an asparagine within the inhibitory domain (ID) in-between the NTAD and the CTAD.

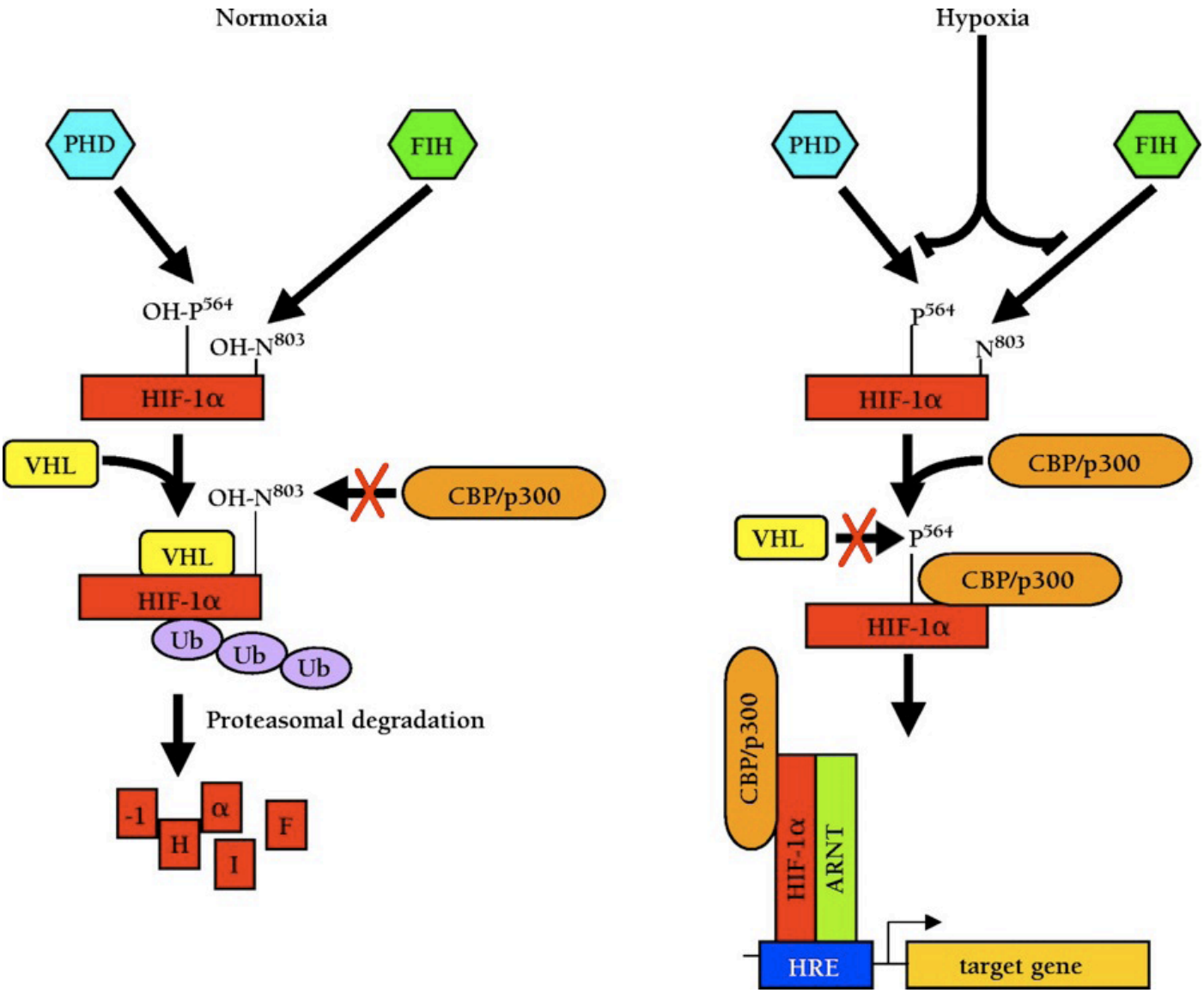

Fig. 2 (adapted from Fedele *et al.*, 2002) Simplified mechanism of how prolyl hydroxylase domain (PHD) enzymes and factor inhibiting Hif (FIH) both inhibit HIF-1α through hydroxylation. The inhibitory effect under normoxia is however accomplished by two completely different mechanisms. Left panel: Under normoxia Asparagine hydroxylation of the C-terminal transactivation domain (CTAD) by FIH blocks the binding site of CBP/p300, a general co-activator of transcription that is necessary to drive expression of HIF responsive genes. Hydroxylation on proline residues by prolyl hydroxylase domain (PHD) enzymes creates a binding site for von Hippel Lindau protein (pVHL, VHL in the figure). pVHL is an E3 ligase, which causes specific ubiquitination of HIF-1α targeting it for proteasomal degradation. Right panel: Under hypoxia the action of both PHD and FIH is hindered because they both need oxygen for their enzymatic activity. VHL cannot bind to the unhydroxylated proline residues leading to stabilization of HIF-1α protein. Stabelized HIF-1α forms heterodimers with Aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to local regulatory sequences of Hif-responsive genes called Hif responsive elements (HRE). CBP/p300 can bind to the unhydroxylated CTAD enabling HIF-1α to drive expression of Hif-responsive genes.

bearing subsets of genes that are mainly regulated by one of the two. Glycolytic proteins have been shown to be exclusively induced by HIF-1α. (Hu *et al.*, 2003; Nagao and Oka, 2011). The same applies to transforming growth factor alpha and pro-apoptotic factors, such as *BNIP3* and *BNIP3L*, the last of which are even inhibited by HIF-2α in *VHL* defective CCRCC cells (Raval *et al.*, 2005). On the other hand, genes responsible for iron absorption in murine enterocytes seam to be exclusively induced by HIF-2α (Mastrogiannaki *et al.*, 2009). Also *OCT-4* a transcription factor, which is well-known for keeping stem cells in a non-differentiating, self-renewing state is expressed in a HIF-2α but not -1α dependent way. Replacing *HIF-1α* for *HIF-2α* markedly increased the growth and lessened the differentiation of terratomas grown from murine embryonic stem cells in an *OCT-4* dependant way (Covello *et al.*, 2006; 2005). While Vascular endothelial growth factor (*VEGF*) is induced by both paralogues (Hu *et al.*, 2003; Chen *et al.*, 2007), *angiopoietin* and *bFGF*, two angiogenetic factors also necessary for correct vascularization are exclusively induced by HIF-2α (Chen *et al.*, 2007). Finally HIF-2α activity has been shown to interfere with p53. The tumor suppressor protein p53 induces cell cycle arrest and eventually apoptosis in response to stress and DNA damage (Bertout *et al.*, 2009).

Apparently both HIF-α paralogues have distinct targets in addition to the shared target genes. These differences potentially have great implications for therapies and diagnosis, because the effect of medical inhibition of one or the other subunit has different consequences. Also the diagnostic conclusions drawn from the mutation status of one or the other may lead to different prognoses (Turner *et al.*, 2002).

Since the recognition of the importance of HIF-α's, much

Regulator name in search term	Search term: HIF AND Cancer AND Regulator	Search term: HIF AND Regulator	Differeces in posttranslational 1α/2α regulation	Publications with new findings
none used	309		16	11
PHD	49		5	3
FIH	32		2	2
HAF	3	3	3	3
INT6 / eif3e	6	6	5	2
YY1 / cf1 / nf-e1	1	1	1	1

Table 1, Representation of a methodical search in PubMed for posttranslational regulators that differentiate between HIF-1α and -2α. A pubmed search was performed with the search term "HIF" and " Cancer". The 309 search results were analysed by title and abstract if they included investigation of differences in posttranslational HIF-1α and -2α regulation. In total five regulators were found that matched these criteria. These 5 Regulators were then again included in the search query to find missed publications. After the second round 11 publications were found investigating 5 different HIFα paralogue specific regulators. HIF =((hif-2alpha[Title/Abstract] OR hif 2 alpha[Title/Abstract]) OR hif2alpha[Title/Abstract]); Cancer=("neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields]); Regulator= name(s) of Regulator as given in first column [Title/Abstract].

research has been done on the regulation of the two and also on the differences between the two. The first level of distinct regulation is the difference in HIF-α protein expression on transcription- and translation levels. After protein synthesis however, the activity of both HIF-1α and -2α is determined by repression, inactivation and targeting for breakdown through covalent modification. Especially the posttranslational regulation has been subject to extensive investigation, leading to an array of proposed mechanisms how the two prologues HIF-1α and HIF-2α are differentially regulated.

The aim of this thesis is to provide an overview of how cells distinguish between HIF-1α and HIF-2α regarding regulation of protein activity by posttranslational modifications and interactions with other proteins. To reach a representative reflection of the literature, a methodical search was performed on PubMed t May 10th 2011 for posttranslational regulators that differentiate between HIF-1α and -2α (Table 1).

PHDs have different preferences for HIF-α isoforms

The different PHDs do not share the same hydroxylation activity for each HIF-α subunit. Up until now four Prolyl 4-Hydroxylases, mostly referred to as prolyl hydroxylase domain (PHD), have been described to hydroxylate HIF-α subunits (Berra *et al.*, 2003; Appelhoff *et al.*, 2004; Koivunen *et al.*, 2007). One of these, a recently found PHD is part of an endoplasmic reticulum transmembrane protein and specificity for an HIF-α isoforms has not been characterized (Koivunen *et al.*, 2007). The other three have been more extensively examined. *PHD1*, *PHD2* and *PHD3* probably arose from one common gene that yet fulfils overall function in *Drosophila melanogaster* and *Caenorhabditis elegans*. Duplication events, leading to three different genes throughout all mammals, point to separate roles for each *PHD* paralogue. Of these PHD3 has been demonstrated to only interact with HIF-2α (Appelhoff *et al.*, 2004; Bishop *et al.*, 2008) as genetic study in mural neurons indicate. Mice with homozygous deleted *PHD3* were intercrossed with mice with heterozygous deletions for either *HIF-1α* or *HIF-2α*. Only the heterozygous los of *HIF-2α* and not of *HIF-1α* decreased the survival of *PHD3* deficient neurons. (Bishop *et al.*, 2008). PHD2 on the other hand has been proposed as main hydroxylase of HIF-1α, after being sufficient and necessary to drive HIF-1α breakdown in a variety of human cell lines (Appelhoff *et al.*, 2004; Berra *et al.*, 2003).

However, to soundly establish the distinctive role of

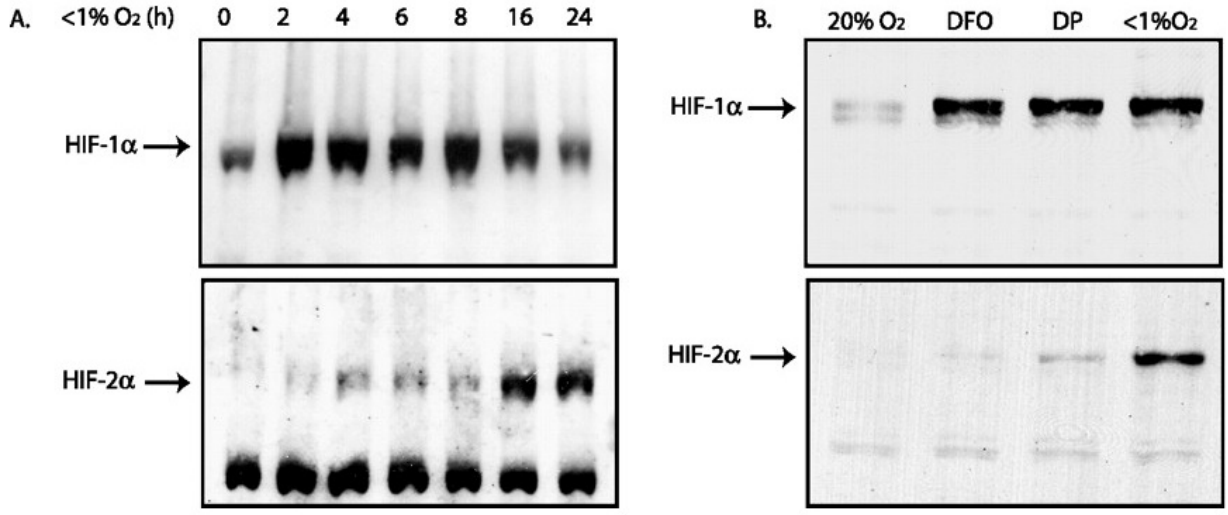

Fig. 3 (from Bracken ***et al.***, 2006) Panel A: Western blot of PC12 Cells which were exposed to severe hypoxia during 0-24h. While HIF-1α was stabilized almost instantaneously HIF-2α was only stabilized after 8 to 16h of oxygen starvation. Panel B: Western blot of PC12 Cells which were exposed to hypoxia or iron-chelating chemicals desferroxamine (DFO) and dipyridyl (DP) respectively. Unlike HIF-1α, HIF-2α stabilization was not achieved by iron chelating chemicals.

each PHD in HIF-1α and HIF-2α hydroxylation, more substantiated and broader investigation is needed.

Differences in HIF-1α and HIF-2α activation, stabilization and transactivation by FIH1

Although all three inhibitors PHD, pVHL and FIH1 prevent the oncogenic function of HIF, not all of them are exclusively tumorsuppressors. FIH specifically inhibits HIF-1α but plays thereby an oncogenic role in the context of deregulated HIF-1α stabilization. In clear cell renal cell carcinoma (CCRCC) loss of *VHL* is an often-recurring major step in tumor development. In these tumors HIF activity is much higher than in healthy renal tissue, but the function of HIF is still attenuated by the oxygen dependent hydroxylase FIH1. Some CCRCC lines exclusively express *HIF-2α*. Although in these cells FIH1 mRNA and protein are present in similar amounts as in healthy renal cells, knockdown of *FIH1* did not change expression of HIF responsive genes. Introduction of *HIF-1α* into these cell lines rendered them again susceptible to *FIH-1* knockdown, leading to increased HIF activity including expression of the proapoptotic gene *BNip3*. Hence knockdown of *FIH1* in CCRCC further unleashes HIF activity, specifically HIF-1α, but ultimately leads to apoptosis revealing the oncogenic function of FIH1. FIH1 seams to enable an oncogenic balance between fully regulated and completely unregulated HIF-1α (Khan *et al.*, 2011).

A mechanism supported by evidence has been unravelled for how FIH distinguishes between HIF-1α and HIF-2α. While both paralogues bear an asparagine residue at which they can be hydroxylated by FIH, HIF-2α seams to be less prone for FIH activity (Khan *et al.*, 2011; Yan *et al.*, 2007; Park *et al.*, 2003). Neither deletion of the CTAD nor substitution of the asparagine with an alanine in *VHL*$^{+/+}$ U2OS osteosarcoma cells did substantially influence transcriptional activity of HIF-2α in a luciferase assay. In contrast, the corresponding mutations in *HIF-1α* resulted in substantially enhanced HIF-1α dependant transcriptional activity (Yan *et al.*, 2007). Also Overexpression of *FIH1* severely impaired normal HIF-1α mediated transcription in MEFs and human embryonic kidney 293 cells, while HIF-2α mediated transcription was reduced by a small extend (Bracken, 2006).

In a comparative study regarding differences in HIF-1α and HIF-2α activation, stabilization and CTAD transactivation thresholds, 6 cell lines were compared under variable oxygenation, leading to profound evidence for differential regulation of HIF-α paralogues by FIH1.

Generally the observed hypoxia depended stabilization of HIF-1α and HIF-2α were comparable but in the PC12 cell line, which is a noteworthy candidate to have the ability to distinguish between HIF-1α and HIF-2α mediated responses. PC12 cells are commonly used to study oxygen sensing because of their similarities to cells of the carotid body, the peripheral oxygen sensing chemoreceptors. While 2h of severe hypoxia (<1% O_2) was sufficient to maximally stabilize HIF-1α protein in PC12 cells, HIF-2α was only stabilized after 8h to 16h. The same applied to treatment of PC12 cells with DP and desferroxamine, another hypoxia imitative. Both chemicals stabilized HIF-1α but not HIF-2α (Fig. 3). These results indicate the presence of a separate oxygen dependent stabilization mechanism for each HIF-α isoform in PC12 cells (Bracken, 2006). A more recently proposed model which is discussed in the next section may be accountable for the observed temporal differences between HIF-α paralogues (Koh *et al.*, 2011).

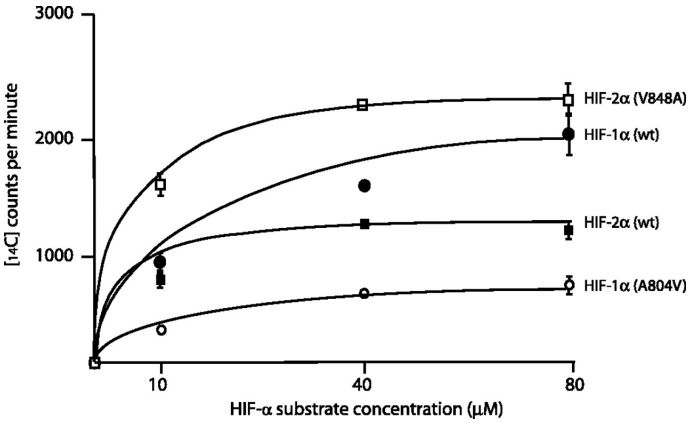

Fig. 4 (from Bracken *et al.*, 2006) *In vitro* aspariginyl hydroxylation essay of both wilt type (WT) HIF-α and their mutants (A804V and V848A) differing in one amino acid substitution corresponding to the paralogue. Enzyme activity was measured by [14C] counts per minute of the radioactively labelled 2-oxogluterate, which is a cofactor of the hydroxylation enzyme. The single amino acid substitution just C-terminal to the hydroxylation site is sufficient to swap the Vmax between the paralogues while the Km stays the same.

However the absolute CTAD activation level of HIF-α isoforms at normoxia was higher for HIF-2α in most cells, varying from 2 to 10 fold increase, independently from CTAD expression and turnover determined by western blot and translation block. So the different functions observed of both HIF-α paralogues may be partially caused by differences in normoxic CTAD activation.

To unravel the mechanism behind this specific CTAD activation, both HIF-α proteins were structurally analysed for differences of functional importance. A single amino acid substitution, conserved cross species, may account for this difference in activation. The asparagine residue at position 803 in HIF-1α that is hydroxylated by FIH-1 is preceded by a Valine that interacts through a hydrogen bond with the Alanine C-terminal of the asparagine (Val^{802} Asp^{803} Ala^{804}). At the corresponding position in the paralogue HIF-2α the alanine is exchanged for a Valine, which is not able to interact in the same way with the N-terminal Valine (Val^{846} Asp^{847} Val^{848}). Both Residues are specific for the respective 1α and the 2α paralogues and they are conserved throughout all known orthologues. Experiments measuring FIH1 dependent asparagine hydroxylation with wild type and mutant CTADs both *in vitro* and *in vivo* showed, that a single amino acid substitution was sufficient to change the Vmax but not the Km for FIH1 hydroxylation of HIF-1α to close to HIF-2α levels and vice versa (Fig. 4), indicating that FIH1 binding is unchanged but Catalysis of the hydroxylation is impaired.

The cellular results were however not entirely interchangeable, indicating that FIH1 hydroxylation does not alone account for the differenced in activation after stabilization. Hence for those cells where protein stability is greatly increased by moderate hypoxia, and CTAD activation is only achieved after severe hypoxia, a model for PHD inactivation followed by FIH-1 inactivation,

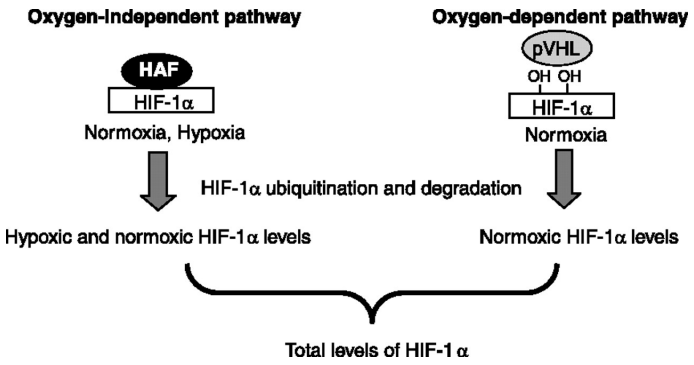

Fig. 5 (from Koh *et al.*, 2008) Schematic comparison of HIF-1α degradation by Hypoxia associated factor (HAF) and von Hippel Lindau protein (pVHL) Both HAF and pVHL cause HIF-1α ubiquitination and subsequent proteasomal degradation. While the action of pVHL depends on oxygen and only plays a role during normoxia, the action of HAF is oxygen independent and is the major cause of HIF-1α degradation under hypoxia.

leading to a stepwise activation of HIF-1α, is supported by experiments (Bracken, 2006).

The Hypoxia associated factor (HAF) is an E3 ligase of HIF-1α and a necessary coactivator for HIF-2α

In addition to the ubiquitination orchestrated by pVHL, which is dependant on oxygen and hydroxylation by PDH, a second ubiquitination pathway for HIF-α has been identified which is completely oxygen independent. The Hypoxia associated factor (HAF) has originally been characterized as part of the splicing machinery, were it contributes to the correct formation of splicosomes. HAF seems to have opposite effects on each HIF-α paralogue. It causes breakdown of HIF-1α and is necessary for HIF-2α function.

Overexpression and knockdown of *HAF* in a variety of cell lines, including renal and colon cancer, caused respective lower and higher levels of HIF-1α protein, completely independent of pVHL or oxygen. SiRNA mediated knockdown of HAF yielded a similar increase in HIF-1α levels as the proteasomal inhibitor MG132. Decreased HIF-1α protein stability in cells, which had been stably transfected with *HAF* could be reversed by MG132 and was therefore proteasome dependant. HAF has subsequently been identified to be an E3 ligase, which interacts with other parts of the ubiquitination machinery to drive ubiquitination and subsequent breakdown of HIF-1α. During normoxia, HAF and pVHL contributed comparably to the breakdown of HIF-1α in PANC-1 and PC-3 cells. During hypoxia, absence of PHD mediated hydroxylation increased the relative role of HAF to many times the effect of pVHL (Fig. 5) (Koh and Powis, 2009; Brusselmans *et al.*, 2001; Koh *et al.*, 2008; 2008). The regulative role of HAF thus arises especially under hypoxic conditions. Opposed to HIF-1α, HIF-2α is completely unsusceptible to inhibition by HAF and stayed active despite HAF mediated degradation of HIF-1α in al described cellular settings (Brusselmans *et al.*, 2001; Koh *et al.*, 2008; Koh and Powis, 2009).

HAF has been shown to not only promote breakdown of HIF-1α but also promote stability and transcriptional activity of HIF-2α by a mechanism independent of the E3 ligase domain and HIF-1α. Since knockdown of HAF had the same consequences for HRE activity as knockdown of HIF-2α, HAF may even be required for HIF-2α activity, at least in the used 786-0 cells. *In vitro* immune precipitation (IP) of flagged, complete and partial HIF-1α, HIF-2α and HAF revealed a distinct binding location for each of the two paralogues. HAF binds with its C-terminus E3 domain to HIF-1α residues just C-terminal of the N-terminal DNA binding domain. In the contrary HAF binds with more central residues distinct from its E3 domain to HIF-2α at residues just N-terminal to the CTAD. Overexpression of the minimal fragment of *HIF-2α* in 786-0 cells competitively inhibited HIF-2α, supporting the necessity of HAF binding for HIF-2α function. These data indicate that HAF binds with two distinct domains, at two different regions on HIF-1α and HIF-2α respectively, whereby it causes breakdown

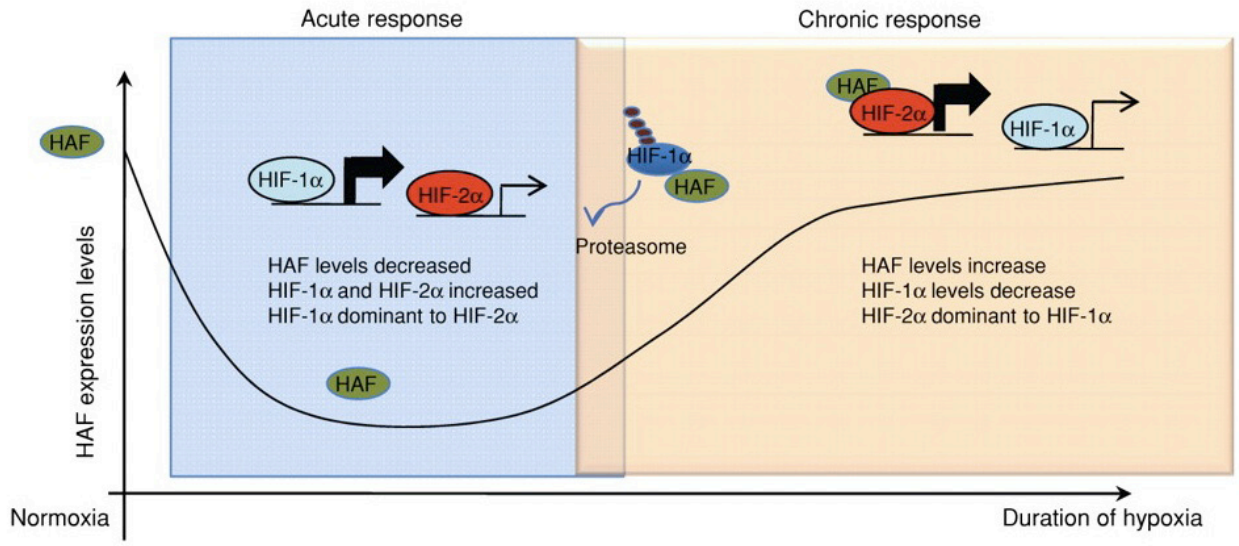

Fig. 6 (from Koh *et al.*, 2011) Shortly after onset of Hypoxia HAF protein levels decline leaving HIF-α activity unrestrained with dominating HIF-1α activity. Recovery of HAF protein, observed after 8h of hypoxia, may explain the temporal differences between HIF-1α and 2α. Increased levels of HAF restrain HIF-1α and co-activate HIF-2α.

of HIF-1α and enables HIF-2α transactivation (Koh *et al.*, 2011).
HAF may represent a molecular switch that regulates the temporal differences between HIF-2α and HIF-2α regulation. Repeatedly HIF-1α has been reported to be early activated by moderate hypoxia, while HIF-2α is most active after prolonged and severe hypoxia (Bracken, 2006; Uchida *et al.*, 2004; Holmquist-Mengelbier *et al.*, 2006; Löfstedt *et al.*, 2007). Recently HAF protein levels have been shown to decline shortly after the onset of severe hypoxia (>1% O_2) and independent of HAF mRNA. After 8h of severe hypoxia however, HAF protein levels recover. Confirmation of these results and unravelling of the mechanism would offer an explanation of the temporal discrepancy of the two HIF-αs. Shortly after onset of hypoxia, HIF-1α mediated expression dominates while it is not repressed by HAF and HIF-2α mediated expression is low because the activating cofactor HAF is not present. Recovery of HAF during hypoxia over time results in HAF mediated HIF-1α breakdown and co-activation of HIF-2α (Fig. 6) (Koh *et al.*, 2011). HAF thus unveils a switching mechanism how cells can specifically regulate which of the two HIF-α subunits stays active.
HAF may drive tumor progression by switching the main activity from one paralogue to the other. The switch from HIF-1α to -2α is repeatedly observed in CCRCC where this event often marks a transition to a more aggressive and undifferentiated phenotype. Players for this phenotype include HIF-2α responsive proto-oncogenes *OCT-4*, and c-*Myc* and also HIF-1α dependant pro-apoptotic factors such as *BNIP3* and *BNIP3L*. BNIP3 and BNIP3L might drive apoptosis in late tumor cells that do not switch to HIF-2α under some conditions. Only tumor cells switching to HIF-2α might overcome the apoptotic barrier in addition to the advantage of expressed *OCT-4*, and *c-Myc*. (Raval *et al.*, 2005; Brusselmans *et al.*, 2001; Koh *et al.*, 2008; Koh and Powis, 2009). Conclusively HAF activity may represent the mechanism of how CCRCC undergo the switch form HIF-1α to -2α leading to more dangerous carcinoma.

INT6 selectively causes breakdown of HIF-2α

Unlike HIF-1α, HIF-2α is also targeted for proteasomal breakdown by a mechanism independent of *VHL* and hydroxylation. A new role in HIF-α paralogue regulation has been identified for integrator subunit 6 (INT6), which has been previously categorized as the e subunit of the eukaryotic initiation factor 3 (eIF3e). A yeast two-hybrid screening system unveiled the N-terminal INT6 domain of 124 amino acids (372 bp) binding to HIF-2α in-between its NTAD and its CTAD with high affinity but not to HIF-1α. Binding of functional int6 resulted in destabilization of HIF-2α protein levels even in 886-O cells that lack pVHL. This destabilization was shown not to be dependent on pVHL mediated breakdown, but on the INT6 C-terminal Proteasome/Int6/Nip-1/TRIP-15 (PINT) domain, that enables INT6 to interact with the E3 ligase Ret and with the proteasome.
Under normoxia the HIF-2α protein stability and likewise expression of HIF responsive angiogenic factors was increased by both inhibition of *INT6* expression with siRNA and also by competitive inhibition with deficient INT6 lacking the PINT domain. The same applied to the use of proteasome inhibitors.
The INT6 binding site was distinct to the pVHL binding site and yeast two-hybrid analysis revealed no competition between pVHL and int6. The pVHL pathway is oxygen dependent wile INT6 was working likewise under hypoxia and normoxia. Although HIF-2α protein is present in various cells under normoxia, protein levels are still higher under hypoxia, indicating that both proteasomal pathways can regulate HIF-2α protein levels independently (Chen *et al.*, 2007). *In vivo*, siRNA against INT6 induced the formation of new blood vessels in tissue surrounding a wound and in subcutaneous tissue in mice (Chen *et al.*, 2010b). Taken together this data supports a model where HIF-2α is selectively prone to proteasomal breakdown by interaction with INT6, introducing another mechanism for specific regulation of HIF-α subunits.

In ES cells HIF-2α is repressed by the transcriptional repressing cofactor YY1

Another mechanism to selectively regulate each HIF-α isoform has been shown in ES cells. ES cells express a number of genes that can be induced by either of the two paralogues in normal cells. Knockout of *HIF-1α* but not of *HIF-2α* abolished the expression of these genes and additional HRE reporters completely. Since the expression and even binding of HIF-2α to the HRE of target genes was the same or even higher in ES cells than in 786-O control cells lacking pVHL, the existence of a transcriptional repressing cofactor was postulated. Overexpression of *HIF-2α* and HIF-2α transcription defective mutant in *HIF-1α* knockouts partially rescued HIF-2α function and transcription of common HIF-α genes, indicating a repressor that can be titrated out. *HIF-1α* knockouts were also treated with HIF-1α transcription defective mutants, which could not out titrate the postulated repressor, as hypoxic transcription remained silent. The repressor binding to the transcription defective mutant together with activating cofactors, which are then simultaneously out titrated, may be an explanation for the rescue by titration being only partially. Taken together this data strongly supports the existence of a transcriptional repressor selectively disabling transactivation of HIF-2α already bound to HREs in ES cells (Hu *et al.*, 2006).
More recently, Yin Yang 1 (YY1) a transcriptional cofactor has been proposed to repress activity of HIF-2α and its downstream targets. While PHDs and FIH are regulated by oxygen and Fe(II), upstream regulators of HAF and INT6 and their role in tumorgenesis has not yet been clarified. YY1 on the other hand is regulated by the RAS AKT/PI3 kinase pathway, which is frequently deregulated in many kinds of neoplasms. YY1 is a constitutively expressed protein bearing different functions. Depending on the cell type and promoter context it can act as a co-repressor or as a co-activator of transcription. In the context of HREs and HIF-2α YY1 has been shown to act as a co-repressor, which may be responsible for the observations in ES cells described above. YY1 is in turn repressed by Phosphatase and tensin homologue (PTEN). PTEN has been described to function as a tumor suppressor by antagonizing the AKT/PI3 kinase pathway (Petrella and Brinckerhoff, 2009). In the context of deregulated HIF-2α the tumor-suppressing

role of PTEN is not clear since repression of YY1 relieves the restriction on HIF-2α, driving the expression of genes promoting tumor progression.

Conclusion and perspectives

Summarizing, five mechanisms of posttranslational regulation have been described which differentiate between HIF-1α and -2α. Of the PHDs, at least one has higher specificity for one paralogue. PHD3 mainly acts on HIF-2α. Secondly, FIH1 inhibits transactivation by asp hydroxylation, which is only efficient in the case of HIF-1α and not of HIF-2α. Then, HAF is a necessary cofactor for HIF-2α and at the same time E3 ligase for HIF-1α. YY1 functions as a transcription repressing cofactor of HIF-2α. At last INT6 specifically targets HIF-2α for proteasomal breakdown.

Each of these mechanisms needs more profound investigation to determine to which tumors and healthy tissues they apply. The relative role of each of the regulators should be investigated in a combined study that takes each of these five mechanisms into account. A more accurate model would allow predicting the progression of tumors more precisely based on the mutation status of each of these regulators. A combined study could also help to clarify which of them should be targeted for therapy.

The differentiated regulation of HIF-1α and -2α allows tumors to switch from a HIF-1α dominated response to a HIF-2α dominated response. A phenotype in which HIF-2α is more active than HIF-1α has been shown to be more progressed in CCRCCs, colorectal cancer and neuroblastomas (Koh and Powis, 2009). On the other hand, inhibition of *HIF-2α* expression enhanced p53 function, increasing the susceptibility to radiation (Bertout *et al.*, 2009), cell death induced by death receptors and chemotherapy (Roberts *et al.*, 2009). Analyzing the mutation status of all the HIF-α regulators in patients may provide more insight in the course of cancer progression. This would enable more precise prognosis and more accurate choice, which methods of treatment are to prefer. Potentially this may prevent unnecessary aggressive treatment and on the other hand prevent underestimation of the risk, during more moderate treatment. In addition the activity of the HIF-αs and their regulating proteins in response to therapy could provide validation for the applied therapy.

The therapeutic aim should be to keep HIF-2α at normal levels, because total knockdown of HIF-2α also disables its tumor suppressing functions. For instance one of the many HIF-2α-responsive genes Scgb3a1 inhibits Akt/BPK signalling which is essential in Kras tumors (Mazumdar *et al.*, 2010). To keep HIF-2α activity in check without totally shutting it down, the regulating proteins described in this paper may be targeted for therapy.

Unspecific interference of the HIF pathway, like inhibitors of the HIF-α/p300 complex, (Fath *et al.*, 2006) would while lowering the angiogenic effect of HIF-2α, also suppress the apoptotic effect of HIF-1α. Inhibition of the HIF-1α specific inhibitor FIH1 alongside unspecific HIF repression, may favour the HIF-1α response. HAF may potentially even be more promising because HAF has opposite effects on each HIF-α paralogue. Drugs targeting HAF may prevent the transition from HIF-1α to -2α retaining a less aggressive phenotype.

While the down regulation of HIF, mainly HIF-2α, is desirable in cancer therapy, patients suffering from impaired angiogenic functions may benefit from HIF up regulation. Interference of the HIF-2α inhibitor INT6 with siRNA has been proposed (Chen *et al.*, 2007), while PHD inhibitors are already tested in human clinical trials against a variety of disorders including infarctions, anaemia, whole body hypoxia and ischemia (Kaelin and Ratcliffe, 2008).

The HIF-α transcription factors are each differentially regulated by a number of interactions allowing for specific and gradual regulation, which is essential for healthy cellular function. Deregulation may be treated by inhibition of specific combinations of the described regulators, allowing for correct restoration of HIF-α activity in a gradual and paralogue specific manner.

Isolation of Glut1 from erythrocyte ghosts

Introduction

One of the HIF responsive genes, Glut1 is up regulated in some tumors including breast cancer (Chen *et al.*, 2010a). Glut1 is thought to be a 12-transmembrane protein expressed at he plasma membrane and may sever as a surface tumor marker (Boulter and Wang, 2001). Antibodies against Glut1 may serve as an instrument to label tumors with higher specificity than conventional scans.

Our approach involves the development of a single domain antibody against Glut1. This single domain consists of a variable heavy chain domain of a heavy chain-only lama antibody (VHH) and is frequently called nanobody. As part of the approach to find VHHs that bind specifically to Glut1, isolation of Glut1 was performed, which could serve as an epitope to screen for binding VHHs *in vitro*. For the Glut1 purification, a protocol was used from a previously described isolation of Glut1 (Boulter and Wang, 2001). After anion exchange chromatography of purified erythrocyte ghosts, the highly diluted flow through showed substantial staining with antibodies against Glut1. Review of the literature questioned the applicability of the used protocol because the theoretical Pi of Glut1 did not point to the use of an anion exchange column in at the pH described in the protocol. To assess this, cation exchange chromatography was performed instead. Although a band correlate with the elution peak, the results are not very clear due to unspecificity of the labelling antibody, contamination with grass and probably breakdown of the protein of interest.

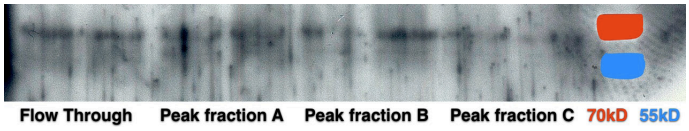

Figure 2. Western blot of anion exchange chromatography fractions and flow through of solubilized human ghosts. The band at 65 kD with a smear down to 55 kD is visible both in the fractions and in the diluted flow trough.

Methods

Preperation and solubilisation of erythrocyte membranes (Boulter and Wang, 2001). Erythrocytes were isolated from full blood by centrifugation and lysed in hypotonic buffer. Ghost membranes were separated from hemoglobin by repeated centrifugation at >10000g in lysis buffer. The Ghost membranes were stripped of cytoskeleton by incubation at 37°C and pH 7,5 and washed with 1mM NaOH of pH11 to remove extrinsic membrane proteins. The pellet was then solubilized in 2% decylmaltoside.

Isolation of Glut1 from ghost membranes by ion-exchange chromatography (Boulter and Wang, 2001). After spinning down of the large aggregates and unsolubilized materials the supernatant of solubilized membranes was loaded onto a ion exchange column and washed with 15ml loading buffer. UV absorption was measured during elution at 0.2ml/min with a 0-500mM NaCl-gradient. The fractions around occurring peaks were loaded on SDS-page gel and labelled with a polyclonal antibody against Glut1 on western blot.

Calcualtion of theoretical Pi. The theoretical Pi of Glut1 was determined by inserting its amino acid sequence, derived

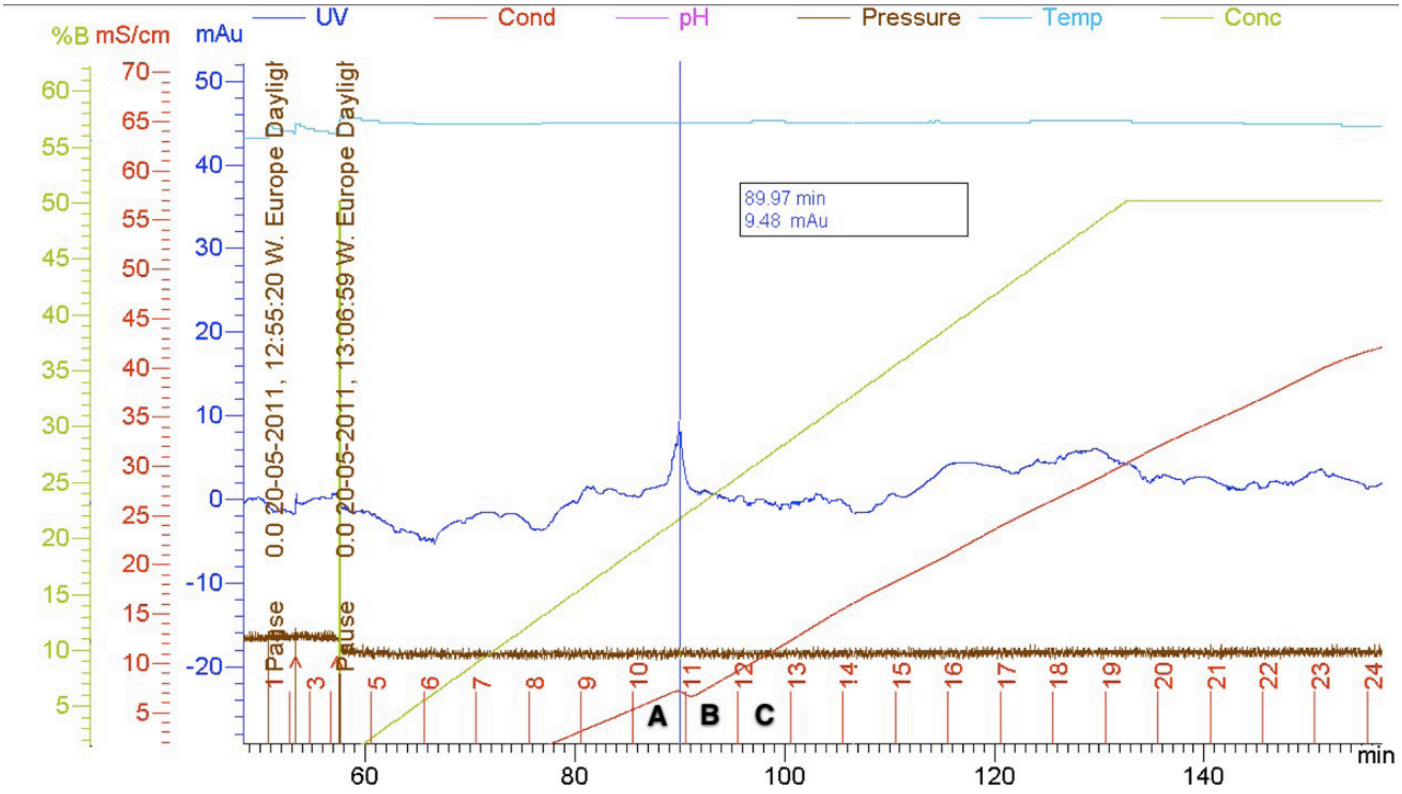

Figure 1. Elution of anion-exchange chromatography of human ghosts solubilized in decylmaltoside. During the elution one peak occurred of 7mAu around 7.04 mS/cm. UV; ultra violet absorption scaled in mAu, Cond; conduction scaled in mili Siemens (mS)/cm, Conc; concentration scaled in %B, B; elution buffer with 1M NaCl, pH; pH 6.0 scale not shown, Pressure; around 0.4mP scale not shown, Temp; temperature 21°C scale not shown.

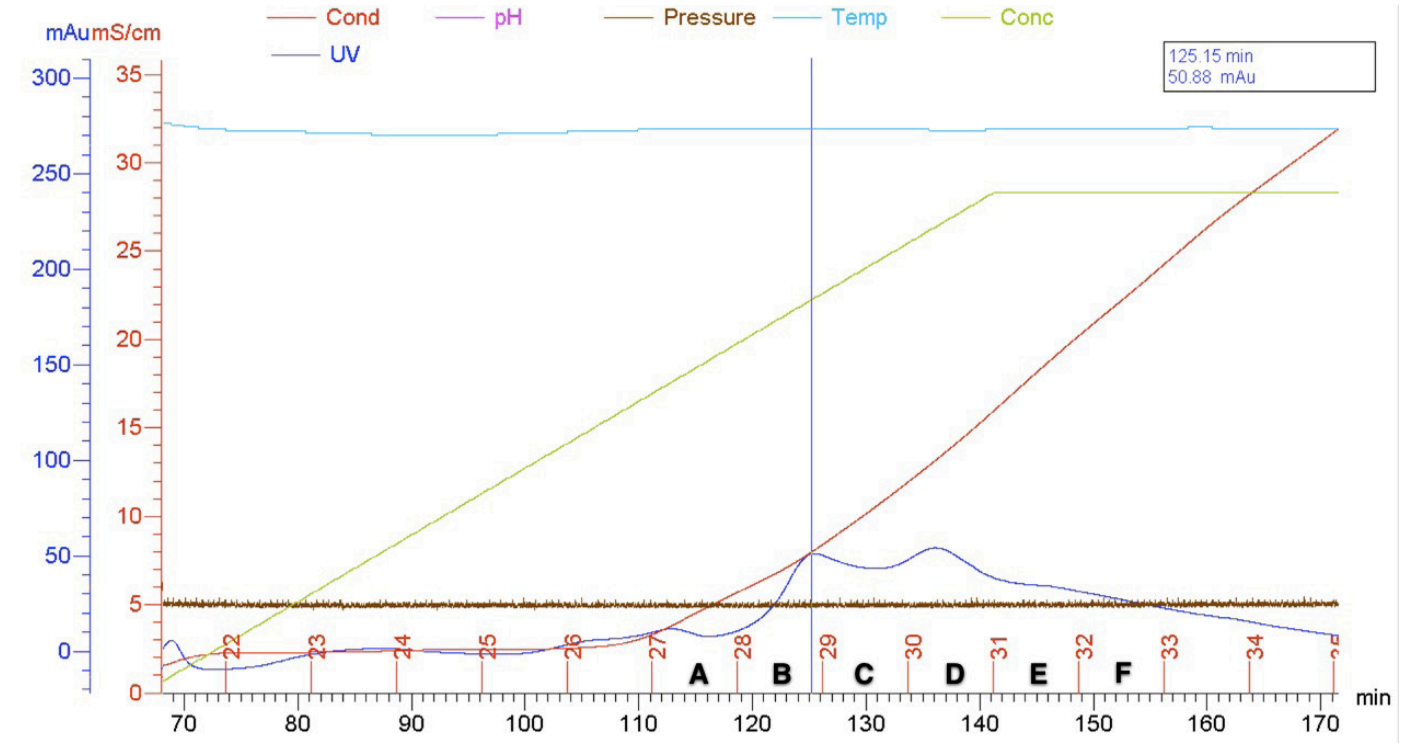

Figure 3. Elution of cation exchange chromatography of human ghosts solubilized in decylmaltoside. During the elution two UV peaks of 30 mAu appeared around 8 mS/cm and 12,5 mS/cm. UV; ultra violet absorption scaled in mAu, Cond; conduction scaled in mili Siemens (mS)/cm, Conc; concentration of elution buffer with 1M NaCl scale not shown, maximal concentration of B is 50%, pH; pH 6.0 scale not shown, Pressure; around 0.4mP scale not shown, Temp; temperature 21°C scale not shown.

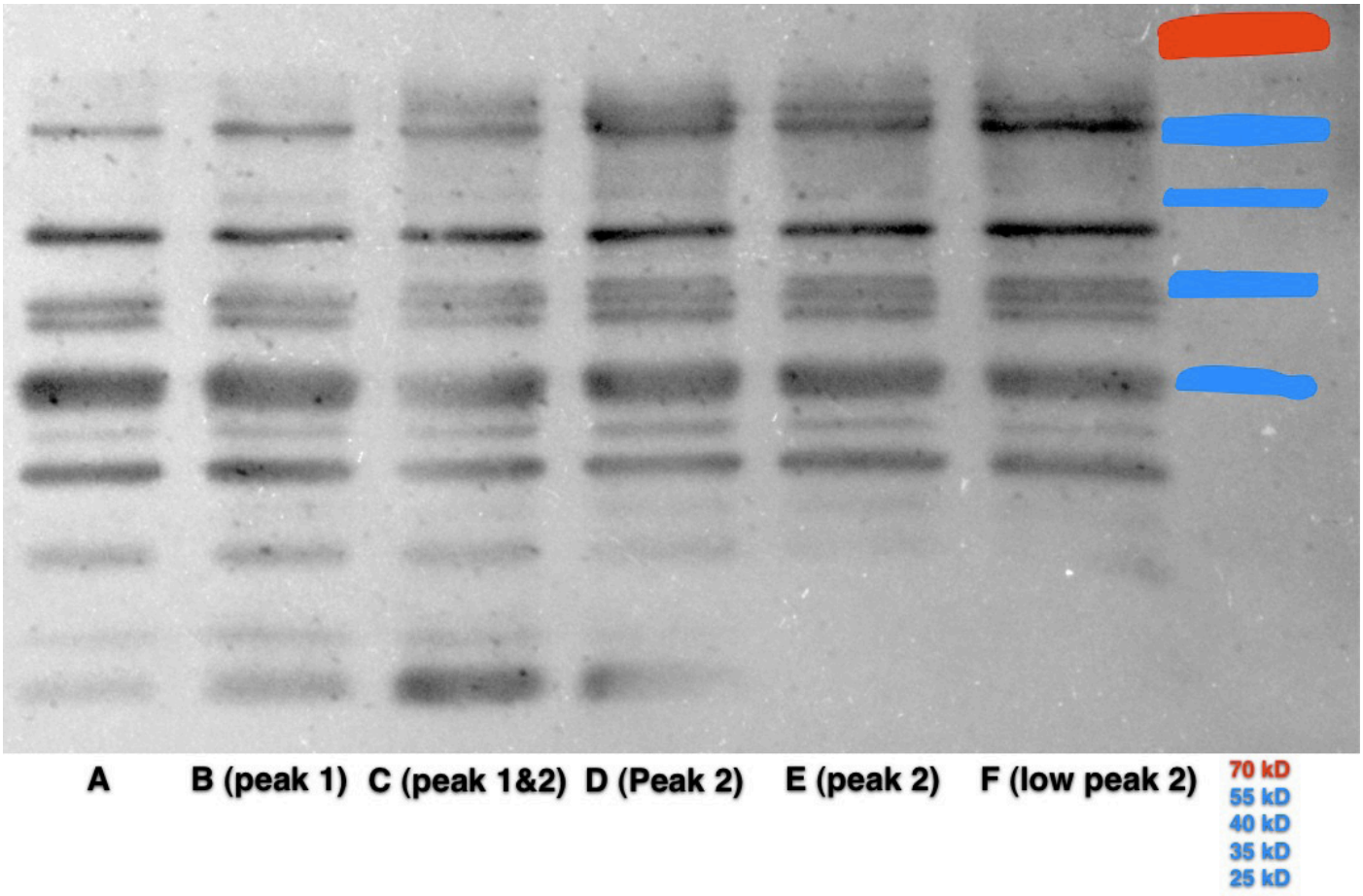

Figure 4. Western blot of cation exchange chromatography fractions of flow through from anion exchange chromatography of solubilized human ghosts.Various very clear bands of different sizes between 45 kD and 7 kD are stained by anti Glut1.

from ensemble.org (Embl EBI, 2011) into the Compute pI/Mw tool on expasy.org (Swiss Institute of Bioinformatics, 2011). Pi, 8.93 / Mw 54083.78

Results

Anion-exchange column of solubelized human ghosts. During the elution, one UV peak of 7 mAu appeared (Fig. 1). There was no band on the western blot that correlated

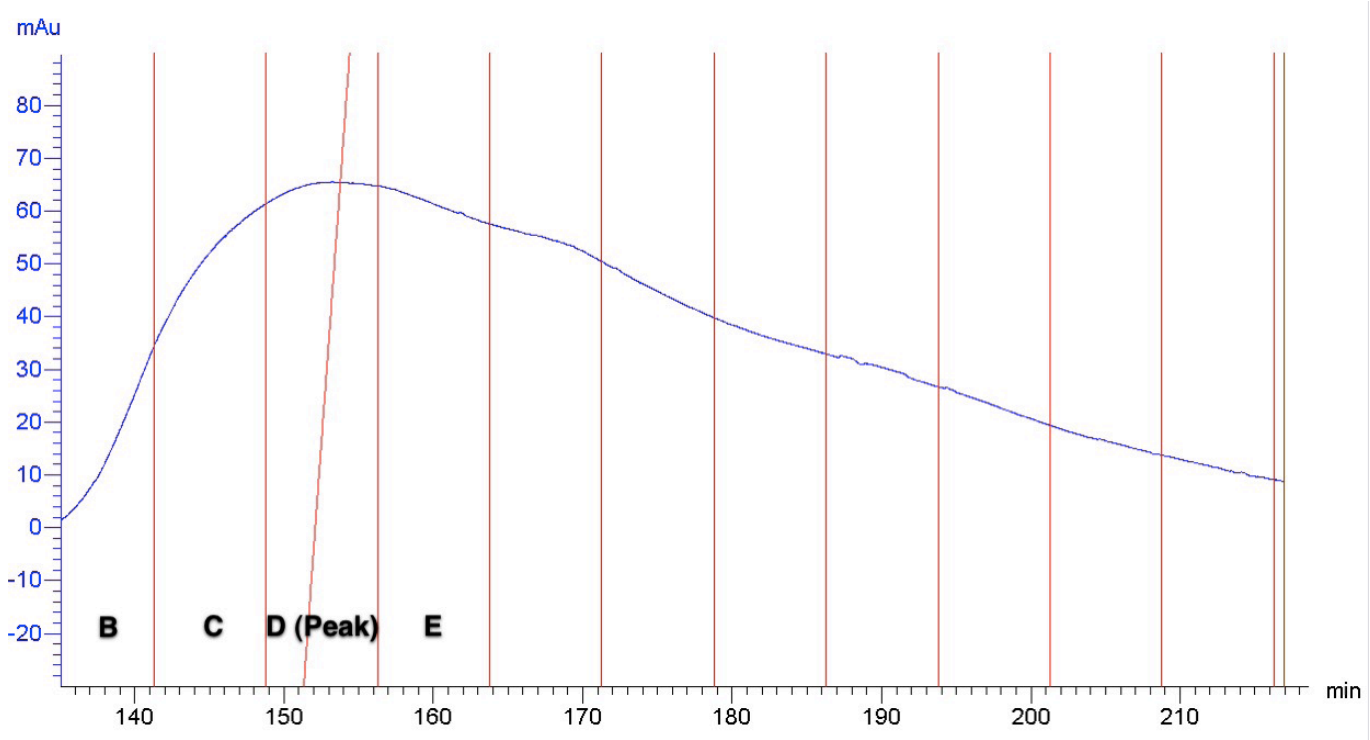

Figure 5. Elution of cation-exchange chromatography of bovine ghosts solubilized in decylmaltoside. During the elution one extended peak of 60 mAu around 15 mS/cm. UV; ultra violet absorption scaled in mAu, mS; millisiemens.

with the peak. Instead there was a band at 65kD with a smear down to 50kD, which was visible in all labelled fractions. Additionally this band was clearly visible in the flow through which had been diluted many times. While there were other bands in the fractions, the only band in the flow trough was the band at 65kD with a smear down to 50kD (Fig. 2).

Cation-exchange column of anion-exchange flow through of solubelized human ghost membranes. During the elution, two UV peaks of 30 mAu occurred(Fig. 3). The highest bands visible in the western blot of the corresponding fractions started at 50 kD which was correlating with the second peak of the elution. A lot of other bands with sizes between 45 kD and 7 kD where visible in all fractions (Fig. 4). These bands had not been visible in the diluted flow through of the anion exchange column before the cation exchange column.

Cation-exchange column of solubelized bovine ghost membranes. During the elution, one UV peak of 60 mAu with en extended curve appeared (Fig. 5). On western blot a band between 65 kD and 60 kD correlated with the peak (Fig. 6). The mass of this band corresponded to the mass of recombinant Glut1 produced by insects (not shown).

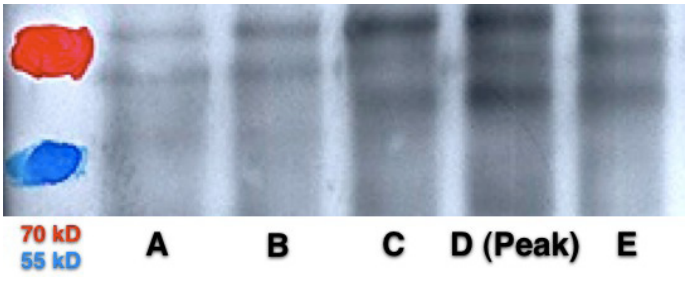

Figure 6. Western blot of cation exchange chromatography fractions of solubilized bovine ghosts. A band between 65 kD and 60 kD correlates with the peak of the curve at D.

Conclusions and Discussion

The results from the anion exchange column showed only one band around 60 kD, which was clearly visible in the diluted flow trough. Since the theoretical Pi of Glut1 calculated from its amino acid sequence is around 8.93 (Embl EBI, 2011; Swiss Institute of Bioinformatics, 2011) and the protocol used a loading buffer at pH 6, the approach from the original protocol was questioned. To assess this, the flow through of the first column was loaded onto a cation exchange column to bind Glut1, which would be positively charged according to its theoretical Pi. The resulting numerous intense labelled bands could be due to breakdown of the protein during several steps at which de solution was neither at <4°C nor mixed with protease inhibitors. Some of these steps were several hours long during the chromatography, which was passed twice by this sample.

To get clearer results, an increased amount of solubilized bovine ghosts was directly loaded onto a cation exchange column. The bands that correlated with the peak were at the same height as the recombinant protein derived from insects.

Assuming that all the intense bands after two columns originated either from human Glut1 or one of its breakdown products, the combined use of both the anion exchange and the cation exchange column could be an efficient method to isolate Glut1. The anion exchange flow through would be loaded on the cation exchange column to get purified Glut1 in the fractions. To minimize breakdown, chromatography should be performed at 4°C.

Limitations

No quantitative measurements have been performed, and therefore there is no indication of protein purity inside the fractions. Furthermore, the antibody used against Glut1 has a low specificity and may have cross-reacted with other

proteins. Finally, the bovine ghosts were contaminated with a substantial amount of grass, washed from the pharynx of the animals, which might have caused interference.

Acknowledgments

Much thanks goes to Aram who literally gave blood sweat and tears for my project. I enjoyed working with you! Also I would like to thank Martha, Andrea, Rachid, Eric, and all other lab members that helped me when I had questions, and Melisse also for her inspiration. The slaughterhouse van Kooten in Montford is very much appreciated for letting me take blood from cattle. Also thanks to everyone else who made my thesis possible. Since this is the last part of my bachelor, I would like to thank all the people that motivated me during the last 3 years. Thanks to Lisanne for everything. Thanks to al the fascinating and motivating teachers of my bachelor.

References

Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem *279*, 38458–38465.

Barontini, M., and Dahia, P. L. M. (2010). VHL disease. Best Pract Res Clin Endocrinol Metab *24*, 401–413.

Berra, E., Benizri, E., Ginouvès, A., Volmat, V., Roux, D., and Pouysségur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J. *22*, 4082–4090.

Bertout, J. A., Majmundar, A. J., Gordan, J. D., Lam, J. C., Ditsworth, D., Keith, B., Brown, E. J., Nathanson, K. L., and Simon, M. C. (2009). HIF2alpha inhibition promotes p53 pathway activity, tumor cell death, and radiation responses. Proceedings of the National Academy of Sciences *106*, 14391–14396.

Bishop, T., Gallagher, D., Pascual, A., Lygate, C. A., de Bono, J. P., Nicholls, L. G., Ortega-Saenz, P., Oster, H., Wijeyekoon, B., Sutherland, A. I., *et al.* (2008). Abnormal sympathoadrenal development and systemic hypotension in PHD3-/- mice. Mol Cell Biol *28*, 3386–3400.

Boulter, J. M., and Wang, D. N. (2001). Purification and characterization of human erythrocyte glucose transporter in decylmaltoside detergent solution. Protein Expression and Purification *22*, 337–348.

Bracken, C. P. (2006). Cell-specific Regulation of Hypoxia-inducible Factor (HIF)-1 and HIF-2 Stabilization and Transactivation in a Graded Oxygen Environment. Journal of Biological Chemistry *281*, 22575–22585.

Brusselmans, K., Bono, F., Maxwell, P., Dor, Y., Dewerchin, M., Collen, D., Herbert, J. M., and Carmeliet, P. (2001). Hypoxia-inducible factor-2alpha (HIF-2alpha) is involved in the apoptotic response to hypoglycemia but not to hypoxia. J Biol Chem *276*, 39192–39196.

Chan, D. A., Sutphin, P. D., Denko, N. C., and Giaccia, A. J. (2002). Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1alpha. J Biol Chem *277*, 40112–40117.

Chen, C.-L., Chu, J.-S., Su, W.-C., Huang, S.-C., and Lee, W.-Y. (2010a). Hypoxia and metabolic phenotypes during breast carcinogenesis: expression of HIF-1alpha, GLUT1, and CAIX. Virchows Arch. *457*, 53–61.

Chen, L., Endler, A., Uchida, K., Horiguchi, S.-I., Morizane, Y., Iijima, O., Toi, M., and Shibasaki, F. (2010b). Int6/eIF3e silencing promotes functional blood vessel outgrowth and enhances wound healing by upregulating hypoxia-induced factor 2alpha expression. Circulation *122*, 910–919.

Chen, L., Uchida, K., Endler, A., and Shibasaki, F. (2007). Mammalian tumor suppressor Int6 specifically targets hypoxia inducible factor 2 alpha for degradation by hypoxia- and pVHL-independent regulation. J Biol Chem *282*, 12707–12716.

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

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

Embl EBI, W. (2011). Ensembl Genome Browser. ensembl.org. Available at: http://www.ensembl.org/index.html [Accessed June 7, 2011].

Fath, D. M., Kong, X., Liang, D., Lin, Z., Chou, A., Jiang, Y., Fang, J., Caro, J., and Sang, N. (2006). Histone deacetylase inhibitors repress the transactivation potential of hypoxia-inducible factors independently of direct acetylation of HIF-alpha. J Biol Chem *281*, 13612–13619.

Holmquist-Mengelbier, L., Fredlund, E., Löfstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K., *et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell *10*, 413–423.

Hu, C.-J., Iyer, S., Sataur, A., Covello, K. L., Chodosh, L. A., and Simon, M. C. (2006). Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 alpha (HIF-1alpha) and HIF-2alpha in stem cells. Mol Cell Biol *26*, 3514–3526.

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

Kaelin, W. G., and Ratcliffe, P. J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Molecular Cell *30*, 393–402.

Khan, M. N., Bhattacharyya, T., Andrikopoulos, P., Esteban, M. A., Barod, R., Connor, T., Ashcroft, M., Maxwell, P. H., and Kiriakidis, S. (2011). Factor inhibiting HIF (FIH-1) promotes renal cancer cell survival by protecting cells from HIF-1α-mediated apoptosis. British Journal of Cancer *104*, 1151–1159.

Koh, M. Y., Darnay, B. G., and Powis, G. (2008). Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1alpha, leading to its oxygen-independent degradation. Mol Cell Biol *28*, 7081–7095.

Koh, M. Y., Lemos, R., Liu, X., and Powis, G. (2011). The Hypoxia-Associated Factor Switches Cells from HIF-1{alpha}- to HIF-2{alpha}-Dependent Signaling Promoting Stem Cell Characteristics, Aggressive Tumor Growth and Invasion. Cancer Research *71*, 4015–4027.

Koh, M. Y., and Powis, G. (2009). HAF : the new player in oxygen-independent HIF-1alpha degradation. Cell Cycle *8*, 1359–1366.

Koivunen, P., Tiainen, P., Hyvärinen, J., Williams, K. E., Sormunen, R., Klaus, S. J., Kivirikko, K. I., and Myllyharju, J. (2007). An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. J Biol Chem *282*, 30544–30552.

Kondo, K., Kim, W. Y., Lechpammer, M., and Kaelin, W. G. (2003). Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol. *1*, E83.

Li, L., Zhang, L., Zhang, X., Yan, Q., Minamishima, Y. A., Olumi, A. F., Mao, M., Bartz, S., and Kaelin, W. G. (2007). Hypoxia-inducible factor linked to differential kidney cancer risk seen with type 2A and type 2B VHL mutations. Mol Cell Biol *27*, 5381–5392.

Löfstedt, T., Fredlund, E., Holmquist-Mengelbier, L., Pietras, A., Ovenberger, M., Poellinger, L., and Påhlman, S. (2007). Hypoxia inducible factor-2alpha in cancer. Cell Cycle *6*, 919–926.

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

Mazumdar, J., Hickey, M. M., Pant, D. K., Durham, A. C., Sweet-Cordero, A., Vachani, A., Jacks, T., Chodosh, L. A., Kissil, J. L., Simon, M. C., *et al.* (2010). HIF-2alpha deletion promotes Kras-driven lung tumor development. Proceedings of the National Academy of Sciences *107*, 14182–14187.

Nagao, K., and Oka, K. (2011). HIF-2 directly activates CD82 gene expression in endothelial cells. Biochem. Biophys. Res. Commun. *407*, 260–265.

Park, S.-K., Dadak, A. M., Haase, V. H., Fontana, L., Giaccia, A. J., and Johnson, R. S. (2003). Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha (HIF-1alpha): role of cytoplasmic trapping of HIF-2alpha. Mol Cell Biol *23*, 4959–4971.

Petrella, B. L., and Brinckerhoff, C. E. (2009). PTEN suppression of YY1 induces HIF-2 activity in von-Hippel-Lindau-null renal-cell carcinoma. Cancer Biol Ther *8*, 1389–1401.

Pillai, R., Huypens, P., Huang, M., Schaefer, S., Sheinin, T., Wettig, S. D., and Joseph, J. W. (2011). Aryl hydrocarbon receptor nuclear

translocator/hypoxia-inducible factor-1{beta} plays a critical role in maintaining glucose-stimulated anaplerosis and insulin release from pancreatic {beta}-cells. J Biol Chem *286*, 1014–1024.

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

Roberts, A. M., Watson, I. R., Evans, A. J., Foster, D. A., Irwin, M. S., and Ohh, M. (2009). Suppression of Hypoxia-Inducible Factor 2 Restores p53 Activity via Hdm2 and Reverses Chemoresistance of Renal Carcinoma Cells. Cancer Research *69*, 9056–9064.

Sang, N., Fang, J., Srinivas, V., Leshchinsky, I., and Caro, J. (2002). Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent, hydroxylation-regulated association with p300/CBP. Mol Cell Biol *22*, 2984–2992.

Swiss Institute of Bioinformatics ed. (2011). ExPASy - Compute pI/Mw tool Swiss Institute of Bioinformatics, ed. Available at: http://proxy.library.uu.nl/login?url=http://expasy.org/tools/pi_tool.html [Accessed June 6, 2011].

Turner, K. J., Moore, J. W., Jones, A., Taylor, C. F., Cuthbert-Heavens, D., Han, C., Leek, R. D., Gatter, K. C., Maxwell, P. H., Ratcliffe, P. J., *et al.* (2002). Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel-Lindau gene mutation. Cancer Research *62*, 2957–2961.

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

Yan, Q., Bartz, S., Mao, M., Li, L., and Kaelin, W. G. (2007). The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. Mol Cell Biol *27*, 2092–2102.