Jaana Hyvärinen

ENZYMES INVOLVED IN HYPOXIA RESPONSE

CHARACTERIZATION OF THE IN VIVO ROLE OF HIF-P4H-2 IN MOUSE HEART, OF A NOVEL P4H IN HUMAN AND ZEBRAFISH AND OF THE CATALYTIC PROPERTIES OF FIH

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ENZYMES INVOLVED IN HYPOXIA RESPONSE
Characterization of the \textit{in vivo} role of HIF-P4H-2 in mouse heart, of a novel P4H in human and zebrafish and of the catalytic properties of FIH

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Abstract
Oxygen homeostasis is critical to all animals, as both excess (hyperoxia) and reduced (hypoxia) levels of oxygen can result in pathological changes and ultimately in the loss of cellular and organismal viability. Complex systems have evolved to sense and adapt to changes in cellular oxygen availability, and the hypoxia-inducible factor HIF plays a pivotal role in this elaborate molecular network. In normoxic conditions the α-subunit of HIF becomes hydroxylated by HIF prolyl 4-hydroxylases (HIF-P4Hs 1-3), earmarking HIF-α for proteasomal degradation. Additionally, in the presence of oxygen the hydroxylation of an asparagine residue by the HIF asparaginyl hydroxylase FIH inhibits the transactivation of HIF-target genes by blocking the interaction of HIF-α with a transcriptional coactivator. In addition to being a feature of an organism’s normal life, hypoxia is also characteristic of many common diseases such as severe anemia and myocardial infarction, and it notably decreases these hydroxylation reactions, as HIF-P4Hs and FIH have an absolute requirement for oxygen as a cosubstrate. HIF-α thus escapes degradation and translocates into the nucleus, where it dimerizes with HIF-β and recruits transcriptional coactivators to the hypoxia-response elements of target genes, inducing their transcription and triggering the hypoxia response aimed at restoring cellular oxygen homeostasis.

In this study we generated a genetically modified HIF-P4H-2 hypomorphic mouse line that expresses only 8% of the wild-type HIF-P4H-2 mRNA in the heart. We showed that chronic cardiac HIF-P4H-2 deficiency leads to stabilization of HIF-1α and HIF-2α and protects the heart against acute ischemia-reperfusion injury without causing any adverse effects.

Furthermore, we identified and cloned a novel human transmembrane prolyl 4-hydroxylase P4H-TM and showed that it regulates HIF-1α protein levels in cellulo and hydroxylates HIF-1α in vitro similarly to the HIF-P4Hs, but may also have other physiological substrates. Using forward genetic tools we showed that lack of P4H-TM during development leads to basement membrane defects and compromised kidney function in zebrafish embryos.

Finally, we demonstrated that FIH displays substrate selectivity in terms of hydroxylation and binding of HIF-1α and novel substrates Notch1-3. We showed that FIH has higher affinity for oxygen with Notch1 than with HIF-1α as a substrate, implying that FIH-mediated hydroxylation of Notch can continue in oxygen concentrations where HIF-1α hydroxylation would be markedly reduced.

Keywords: HIF asparaginyl hydroxylase, hypoxia-inducible factor, oxygen homeostasis, prolyl 4-hydroxylase
Hyvärinen, Jaana, Entsyymit elimistön happitasapainon säätelyssä
Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja molekyylilibiologia, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Biocenter Oulu, Center for Cell-Matrix Research, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto


Oulu

Tiivistelmä
Happitasapainon ylläpito on edellytys elimistön normaalille toiminnalle, koska sekä liian korkea (hyperoksia) että liian matala (hypoksia) happipitoisuus ovat elimistölle stressitiloja ja johtavat pitkittyessään haitallisii seurauksiin. Happipitoisuuden muutosten havaitseksi ja niihin reagoimiseksi on elimistössä kehitetty monimutkainen säätelyjärjestelmä, jossa avainmassa on hypoksia-indusoituva tekijä HIF. Solun happipitoisuuden ollessa normaali yksi kolmesta HIF prolyylti 4-hydroksylaasi-isoentsyyminä (HIF-P4Ht 1-3) katalysoi kahden proliinitähteen hydroksylaation HIF-α-alayksikössä. 4-hydroksiproliini toimii signaalin HIF-α:n nopealle proteasomaalaiselle hajotukselle. Lisäksi HIF asparaginyyli hydroksylaasi FIH:n katalysoi HIF-α:n asparaginyltähteen hydroksylaation estää transkriptiovaikutuksesta. Koska HIF-P4Ht ja FIH tarvitsevat kouluvaativaan happea, nämä hydroksylaatioreaktiot vähenevät happipitoisuuden laskiessa, jolloin HIF-α stabiloituu ja siirtyy solun tumaan, jossa se muodostaa kompleksin HIF-β-alayksikön kanssa ja houkuttelee paikalle tarvittavat kofaktorit. HIF-kompleksi tehostaa hypoksiavasteessa tarvittavien geenien lukemaa sitoutumalla niiden promootoreihin ja pyrkii näin palauttamaan solun happipitoisuuden normaalksi.


FIH:n katalysoinnan hydroksylaatioreaktion kineettisiä ominaisuuksia verrattiin tässä tutkimuksessa ensimmäistä kertaa aiemmin tunnetun HIF-α substraatin ja uusien Notch substraattien kesken. Tulokset osoittivat, että substraatin sitomisessa ja hydroksylaatiotissa on merkittäviä eroja eri substraattien välillä.

Asiatanat: asparaginyyli hydroksylaasi, happitasapainon säätely, hypoksiaindusoituva tekijä, prolyylti-4-hydroksylaasi
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Tampere, April 2010
Jaana Hyvärinen
<table>
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<tbody>
<tr>
<td>ANK/ARD</td>
<td>ankyrin repeat domain</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon nuclear translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CKO</td>
<td>conditional knockout</td>
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<td>collagen prolyl 4-hydroxylase</td>
</tr>
<tr>
<td>CTAD</td>
<td>C-terminal transactivation domain</td>
</tr>
<tr>
<td>dpf</td>
<td>day(s) post-fertilization</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FIH</td>
<td>factor inhibiting HIF</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HIF-P4H</td>
<td>HIF prolyl 4-hydroxylase</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia response element</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>IP</td>
<td>ischemic preconditioning</td>
</tr>
<tr>
<td>K_i</td>
<td>inhibitory constant</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis-Menten constant</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>MO</td>
<td>morpholino antisense oligomer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NTAD</td>
<td>N-terminal transactivation domain</td>
</tr>
<tr>
<td>ODDD</td>
<td>oxygen-dependent degradation domain</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>real time quantitative PCR analysis</td>
</tr>
<tr>
<td>P4H</td>
<td>prolyl 4-hydroxylase</td>
</tr>
<tr>
<td>P4H-TM</td>
<td>transmembrane prolyl 4-hydroxylase</td>
</tr>
<tr>
<td>p53</td>
<td>tumor suppressor protein p53</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulphide isomerase</td>
</tr>
<tr>
<td>pVHL</td>
<td>von Hippel-Lindau tumor-suppressor protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximal initial velocity</td>
</tr>
<tr>
<td>X in -Leu-X-X-Leu-Ala-Pro-</td>
<td>any amino acid</td>
</tr>
<tr>
<td>X and Y in –Gly-X-Y-</td>
<td>any amino acid</td>
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List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


*Equal contribution
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1 Introduction

The maintenance of oxygen homeostasis is critical to all aerobic organisms, and some oxygen-sensing systems are found even in prokaryotes. In animals, complex systems have evolved to sense and adapt to changes in cellular oxygen availability. During the last two decades the hypoxia-inducible transcription factor HIF has been identified as the master regulator triggering the intricate response to lowered oxygen concentration, i.e. hypoxia. The stability and transcriptional activity of HIF are stringently controlled by a family of HIF prolyl 4-hydroxylases (HIF-P4Hs 1-3) and HIF asparaginyl hydroxylase (FIH). In normoxic conditions the 4-hydroxylation of two conserved proline residues in the α-subunit of HIF by HIF-P4Hs earmarks HIF-α for proteasomal degradation, while hydroxylation of an asparagine by FIH prevents the interaction of HIF-α with a transcriptional coactivator, thereby inhibiting the transactivation of target genes. When cellular oxygen concentration becomes limiting due to physiological or pathological conditions such as high altitude, anemia or myocardial infarction, these hydroxylation reactions diminish considerably as HIF-P4Hs and FIH belong to the family of 2-oxoglutarate and Fe(II)-dependent dioxygenases and thus have an absolute requirement for molecular oxygen as a cosubstrate. Hence, HIF-α escapes degradation, translocates into the nucleus, dimerizes with the HIF-β subunit, recruits transcriptional coactivators and binds the hypoxia response elements (HRE) of more than a hundred target genes involved in the hypoxia response. Another family of prolyl 4-hydroxylases, collagen prolyl 4-hydroxylases (C-P4Hs I-III), has long been known to catalyze the formation of 4-hydroxyproline (4-Hyp) in newly synthesized or growing collagen polypeptide chains. 4-Hyp is essential to the thermal stability of collagens at body temperature.

We studied the role of the most important HIF-α stability regulating HIF-P4H isoenzyme, HIF-P4H-2, in cardioprotection against ischemia-reperfusion (I/R) injury using a genetically modified hypomorphic mouse line expressing only 8% of the wild-type HIF-P4H-2 mRNA in the heart. Our results suggest that the chronic cardiac deficiency of HIF-P4H-2 and the subsequent stabilization of HIF-1α and HIF-2α provide the heart with protection against acute I/R injury without causing any observable adverse effects.

We identified and characterized a novel transmembrane prolyl 4-hydroxylase (P4H-TM), which more closely resembles the C-P4Hs in terms of sequence identity and subcellular localization, but similarly to the HIF-P4Hs regulates HIF-1α protein levels in cellulo and hydroxylates HIF-1α in vitro. To broaden our
understanding of the role of the novel P4H-TM, we exploited the antisense morpholino oligomer technique to generate P4H-TM knockdown in zebrafish embryos. Analysis of the embryos revealed that deficiency of P4H-TM during zebrafish development leads to dysfunction of the pronephric kidney. Moreover, basement membrane defects were observed in the kidney and eye.

FIH has recently been shown to hydroxylate asparagines in many proteins containing ankyrin repeats, including Notch1-3. We studied the kinetic properties of FIH using HIF-1α and Notch1-4 as substrates, and observed that hydroxylation of Notch1-3 by FIH differs considerably in terms of affinity and dependence on oxygen from that of HIF-1α. We also observed that the K_m of FIH for oxygen is markedly lower with Notch1-3 than with HIF-1α as a substrate, implying that FIH-mediated hydroxylation of Notch can continue in cells in oxygen concentrations where HIF-1α hydroxylation would be notably reduced.
2 Review of the literature

2.1 Oxygen sensing in animals

The maintenance of cellular oxygen homeostasis is critical to all aerobic organisms, as both excess and reduced levels of oxygen (hyperoxia and hypoxia, respectively) pose threats to the organism. Hyperoxia may result in excessive oxidative damage of biomolecules, while prolonged hypoxia eventually leads to cessation of adenosine triphosphate (ATP) production through oxidative phosphorylation. Cellular hypoxia can arise under a variety of circumstances, including physiological (e.g. embryogenesis, high altitude) as well as pathophysiological (e.g. ischemic diseases, anemia, solid tumors) conditions, and the degree of hypoxia tolerated by different cells varies considerably. Elaborate molecular mechanisms have evolved in higher organisms to sense and adapt to changes in oxygen levels. The master regulator of the cellular hypoxia response pathway is a heterodimeric transcription factor, hypoxia-inducible factor (HIF), which consists of a constitutively expressed $\beta$ subunit and an oxygen-sensitive $\alpha$ subunit. HIF is stabilized in cells in hypoxic conditions, leading to subsequent transcriptional upregulation of a number of genes promoting cellular adaptation to hypoxic stress. More than a hundred HIF target genes have been characterized to date, and the total number of HIF-regulated genes has been proposed to exceed 5% of all human genes in endothelial cells (Manalo et al. 2005). Classical HIF target genes encode proteins that act to restore oxygen homeostasis either by enhancing oxygen delivery to tissues or by decreasing oxygen consumption. (For reviews, see Kaelin & Ratcliffe 2008, Lendahl et al. 2009, Semenza 2009.)

For a long time, the mechanism by which changes in cellular oxygen availability are translated into regulation of both stability and transcriptional activity of HIF remained unknown. Through analysis of oxygen-dependent posttranslational modifications in HIF-$\alpha$, it became evident that selective hydroxylation of two proline residues within HIF-$\alpha$ earmarks the $\alpha$ subunit for rapid proteosomal degradation under normoxic conditions. A novel family of prolyl 4-hydroxylases, the HIF prolyl 4-hydroxylases (HIF-P4Hs 1-3), containing three cytoplasmic and nuclear isoenzymes, was identified as being responsible for this modification. Shortly after, hydroxylation of HIF-$\alpha$ was shown to extend to the transactivation domain located in the C-terminus of HIF-$\alpha$, where hydroxylation of an asparagine residue by a novel HIF asparaginyl hydroxylase,
factor inhibiting HIF (FIH), inhibits the interaction of HIF-α with the coactivator p300, thereby repressing the transactivation function of HIF-α. HIF-P4Hs and FIH belong to the group of 2-oxoglutarate and Fe(II)-dependent dioxygenases, which require molecular oxygen as a cosubstrate. These hydroxylation reactions thus diminish considerably in hypoxia, allowing for stabilization of HIF-α and the recruitment of coactivators, and providing a link between cellular oxygen concentration and HIF regulation. Since even the changes occurring within the physiological range of oxygen concentrations are effectively reflected in the catalytic activity of these enzymes, they can be considered as bona fide oxygen sensors, distinguishing them from other members of the same group of enzymes, e.g. the collagen prolyl 4-hydroxylases. (See Fig. 1; for reviews, see Fraisl et al. 2009, Kaelin & Ratcliffe 2008, Myllyharju 2008, Schofield & Ratcliffe 2005, Semenza 2009.)

Fig. 1. Oxygen-dependent regulation of HIF. In normoxic conditions a family of three cytoplasmic and nuclear HIF-P4Hs catalyzes the 4-hydroxylation of two conserved proline (Pro) residues in HIF-α. The 4-hydroxyproline (4-Hyp) allows the recognition of HIF-α by pVHL, leading to polyubiquitination and proteasomal degradation of HIF-α. In addition, a conserved asparagine residue (Asn) becomes hydroxylated by FIH (Asn-OH), blocking the interaction of HIF-α with the transcriptional coactivator CBP/p300 and thereby inhibiting the transactivation of HIF target genes. In hypoxia, HIF-P4Hs and FIH are inhibited by the lack of O₂. HIF-α escapes degradation, translocates into the nucleus, dimerizes with HIF-β and binds CBP/p300. The complex then binds to the hypoxia-response elements (HRE) of a vast number of target genes and induces their expression.
2.2 Hypoxia-inducible factor

2.2.1 Structure of HIF

HIFs are heterodimeric transcription factors composed of a constitutively expressed β subunit and an oxygen-sensitive α subunit, both of which belong to the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) protein superfamily (Wang et al. 1995). HIF-α was identified less than two decades ago (Wang et al. 1995), while the HIF-β subunit is identical to the previously characterized mammalian aryl hydrocarbon receptor nuclear translocator (ARNT) (Reyes et al. 1992). The bHLH and PAS domains (see Fig. 2, for a review, see Kewley et al. 2004) comprising the amino-terminal half of HIF-1α and ARNT are required for heterodimerization and DNA binding, while the carboxyl-terminal half of HIF-1α is required for the transactivation function (Jiang et al. 1996). The two transactivation domains, the N-terminal and C-terminal transactivation domain (NTAD and CTAD, respectively) span residues 534-575 and 786-826 in HIF-1α, respectively (see Fig. 2, Jiang et al. 1997). The region mediating the oxygen-dependent regulation of HIF-1α stability, the oxygen-dependent degradation domain (ODDD), spans amino acids 401-603 in the central region of HIF-1α (see Fig. 2, Huang et al. 1998).

![Fig. 2. The domain structure of human HIF-1α. HIF-1α contains an oxygen-dependent degradation domain (ODDD) and two transactivation domains (N-terminal and C-terminal transactivation domains, NTAD and CTAD). The two conserved proline (P) residues hydroxylated by HIF-P4Hs are located in the ODDD, while the conserved asparagine (N) residue hydroxylated by FIH resides in the CTAD. The bHLH and PAS domains are required for dimerization and DNA binding.](image)

In mammals, the HIF-α subunits are encoded by three distinct genes, giving rise to HIF-1α, HIF-2α (also termed the endothelial PAS domain protein EPAS1, HIF-like factor (HLF), HIF-related factor (HRL) and member of PAS superfamily 2 (MOP2)) and HIF-3α (Ema et al. 1997, Flamme et al. 1997, Gu et al. 1998, Hara et al. 2001, Hogenesch et al. 1997, Tian et al. 1997). Both HIF-2α and HIF-3α display close sequence similarity to HIF-1α in the N-terminal part of the
polypeptide containing the bHLH and PAS domains, but HIF-3α lacks the CTAD (Ema et al. 1997, Flamme et al. 1997, Gu et al. 1998, Hogenesch et al. 1997, Tian et al. 1997). HIF-2α functions in a fashion similar to HIF-1α, and after dimerization with ARNT it binds to the HREs of target genes and induces their transcription (Ema et al. 1997, Flamme et al. 1997, Hogenesch et al. 1997, Tian et al. 1997). The human and mouse HIF-3α loci are subject to alternative splicing and some of the variants have been shown to act as weak transcription factors, while some act as dominant-negative regulators of HIF-1, the latter group including a splice variant HIF-3α4 in humans and Inhibitory PAS domain protein (IPAS) in mouse (Hara et al. 2001, Jang et al. 2005, Makino et al. 2001, Makino et al. 2002, Makino et al. 2007, Maynard et al. 2003, Maynard et al. 2005, Maynard et al. 2007). For simplicity, later on in this review of literature, HIF-α refers to HIF-1α unless otherwise specified.

2.2.2 Functions of HIF

HIFs induce the transcription of more than a hundred human genes involved in cell autonomous, tissue-restricted and systemic homeostatic responses to hypoxia (see Fig. 3; for reviews, see Chowdhury et al. 2008, Gordan & Simon 2007, and Semenza 2007a) by dimerizing with ARNT and subsequently binding to the hypoxia-response elements (HRE) in the promoters of target genes containing the recognition sequence 5’-(A/G)CGTG-3’ (Semenza et al. 1996). Of interest, a novel HRE differing from the canonical HRE sequence at one nucleotide position (5’-GATTGTG-3’), was recently characterized in the lactate dehydrogenase-B gene of the killifish Fundulus heteroclitus. Since the canonical HRE sequence also found in the LDH-B gene failed to confer hypoxia sensitivity in reporter assays, this finding suggests that the mere presence of the consensus HIF-binding sequence is a poor predictor of actual HIF-binding and hypoxic induction of gene expression. (Rees et al. 2009.) Interestingly, in addition to inducing the transcription of target genes, HIF-mediated gene repression has also been reported (Hammer et al. 2007, Jeong et al. 2007). HIF-1α and HIF-2α regulate both shared and unique target genes, and HIF-1α appears to be the major HIF-α isoform (for reviews, see Gordan & Simon 2007, Lendahl et al. 2009, Semenza 2009). Furthermore, HIF-1α is the key mediator of the hypoxia response in the acute phase, while HIF-2α predominantly regulates the long-term, chronic responses to hypoxia (Holmiquist-Mengelbier et al. 2006). The importance of HIF-1α in the response to physiological hypoxia is highlighted by the fact that
homozygous inactivation of the HIF-1α gene leads to embryonic lethality, which is preceded by severely impaired cardiovascular development in mouse (Iyer et al. 1998, Kotch et al. 1999, Ryan et al. 1998). Deficiency of HIF-2α in mouse leads to a group of mouse strain-specific phenotypes, including embryonic lethality due to bradycardia and vascular defects, perinatal lethality resulting from impaired lung maturation, and embryonic and postnatal lethality caused by multiorgan failure and mitochondrial dysfunction (Compernolle et al. 2002, Scortegagna et al. 2003, Tian et al. 1998).

While oxygen-dependent gene expression is important in the normal development and function of animals, HIF-mediated transactivation of genes also contributes to pathologic as well as protective responses in several major diseases. Stabilization of HIF-1 has been shown to be beneficial in anemia, myocardial and cerebral infarction and pulmonary hypertension (for a review, see Semenza 2000). Conversely, HIFs have been reported to promote oncogenesis and cancer progression, but while compelling evidence indicates that HIF-2α drives the development of pVHL-deficient (VHL−/−) renal carcinomas (RCC) and possibly promotes generation of nonrenal tumors as well (for a review, see Qing & Simon 2009), the role of HIF-1α in tumor progression remains somewhat ambiguous. HIF-1α has been shown to antagonize tumor progression in the context of VHL−/− clear cell RCC (Gordan et al. 2008), yet high expression of both HIF-1α and HIF-2α proteins has been associated with poor prognosis in several primary and metastatic human cancers. (For reviews, see Brahimi-Horn et al. 2007, Gordan & Simon 2007, Qing & Simon 2009, Semenza 2010.) Furthermore, recent data indicate that HIF-1α promotes malignant progression via inhibiting DNA repair genes by displacing c-Myc from the promoters of its target genes (also referred to as activation of the HIF-1α-c-Myc axis) independently of its DNA-binding and transactivation domains (Yoo et al. 2009). HIF-induced transcription of select genes has been proposed to contribute to the pathogenesis of atherosclerosis (for a review, see Sluimer & Daemen 2009). HIF has also been implicated in the development of sepsis in an experimental mouse model (for a review, see Gale & Maxwell 2010). Epithelial HIF-1 has been shown to promote renal fibrosis in an experimental model of chronic renal injury (for a review, see Haase 2009). Furthermore, HIF-1 takes part in the pathogenesis of age-related macular degeneration (AMD), which is a multifactorial retinal disease causing irreversible blindness in the elderly (for a review, see Arjamaa et al. 2009). Defective downregulation of HIF-1 may additionally play a role in the pathogenesis of pre-
eclampsia, a potentially lethal disorder affecting 5% of all pregnancies (for a review, see Semenza 2000).

HIFs have recently been implicated in the regulation of microRNAs (miRNAs), 21-23-nucleotide non-protein-coding RNA molecules, which act as negative regulators of gene expression by modulating the stability and translational efficiency of target mRNAs. miRNAs are involved in the regulation of at least one third of all translated genes and thus participate in controlling a wide range of biological processes and functions. (For reviews, see Bartel 2004, Fasanaro et al. 2010, Ivan et al. 2008.) Interestingly, a variety of hypoxia-induced miRNAs have been identified, and the claimed master miRNA of the hypoxic response, miR-210, has been shown to be induced by HIF-α in hypoxia (Crosby et al. 2009, Kulshreshtha et al. 2007). The hypoxia-inducible miRNAs thus represent a novel branch in the HIF-pathway of the hypoxia response, as hypoxia-induced HIF-stabilization triggers the expression of select miRNAs, which in turn contribute to the hypoxia response by downregulating specific target genes. (For reviews, see Fasanaro et al. 2010, Ivan et al. 2008.)

Recent reports suggest that HIFs also participate in epigenetic regulation by indirectly influencing gene expression at the level of histone methylation under hypoxia, as several Jumonji domain-containing histone demethylases (JHDMs) have been shown to be direct targets of HIF-α (Beyer et al. 2008, Krieg et al. 2010). HIF-mediated upregulation of JHDM genes such as JMJD1A reduces methylation of specific hypoxia-responsive promoters, leading to increased gene expression (Krieg et al. 2010). Since Jumonji-domain histone demethylases are 2-oxoglutarate-dependent dioxygenases (Loenarz & Schofield 2008) that are likely to become less active under hypoxia, hypoxic induction by HIF-α may serve to maintain sufficient demethylase activity in low oxygen concentrations. Considering that recent studies have demonstrated that histone demethylation plays an important role in modulating growth within the tumor microenvironment (Krieg et al. 2010), yet another link is provided between HIFs and malignant progression.
Fig. 3. HIF target genes. Genes transcriptionally activated by HIF-1 and HIF-2 can be classified into categories according to their functions. One or more genes are given as examples from each category. In addition to the classical target genes, HIFs regulate the expression of several miRNAs and function as epigenetic regulators by promoting histone demethylation. VEGF, vascular endothelial growth factor; PAI1, plasminogen-activator inhibitor-1; GLUT1, glucose transporter 1; PFKL, 6-Phosphofructo-1-kinase L; PGK1, Phosphoglycerate kinase-1; IGFBP1, Insulin-like growth factor binding protein-1; BNIP3, BCL2/adenovirus E1B protein-interacting protein 3; MMP2, matrix metalloproteinase 2; C-P4H α(I), collagen prolyl 4-hydroxylase α subunit I; IGF-2, Insulin-like growth factor 2; CITED2, CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain 2.

2.2.3 Expression and regulation of HIF

HIF-1α and HIF-β mRNAs are ubiquitously and constitutively expressed in all human, rat and mouse organs studied (Kallio et al. 1997, Wenger et al. 1996, Wiener et al. 1996). Two alternative promoters have been identified in the mouse HIF-1α gene, giving rise to two different mRNA isoforms, one of which is a ubiquitously expressed housekeeping-type major mRNA isoform corresponding to the one known in humans, while the other is differentially expressed in different mouse tissues (Wenger et al. 1998). HIF-2α expression was first reported to be limited to endothelial cells (Tian et al. 1997), but subsequent analysis showed expression in the parenchyma and interstitial cells of multiple tissues, suggesting more widespread expression (Tian et al. 1998, Wiesener et al. 2003). HIF-3α expression has been observed in the thymus, lung, brain, heart and
kidney of adult mice (Gu et al. 1998). Expression of the HIF-3α splice variant IPAS has been reported in the Purkinje cells of cerebellum and in corneal epithelium of the eye in mouse (Makino et al. 2001). Under hypoxic conditions IPAS accumulates in the mouse heart and lung (Makino et al. 2002).

Hypoxic treatment has been shown to increase HIF-1α mRNA levels in pulmonary artery smooth muscle cells (Belaiba et al. 2007), and brain, kidney and lung of mice or rats exposed to systemic hypoxia (Wiener et al. 1996), although contrary reports claim that HIF-1α mRNA levels are unaffected by hypoxia in vitro (Kallio et al. 1997, Wenger et al. 1996). HIF-2α mRNA appears not to be affected by hypoxic stimuli (Wiesener et al. 1998, Wiesener et al. 2003). HIF-3α mRNA is hypoxia-inducible, and the induction in HIF-3α transcription occurs rapidly (Heidbreder et al. 2003, Tanaka et al. 2009). Furthermore, HIF-3α is a HIF-1α target gene, but is unaffected by silencing of HIF-2α (Tanaka et al. 2009). IPAS is directly upregulated by HIF-1α through a mechanism distinct from RNA splicing (Makino et al. 2007). mRNA levels of HIF-1α and HIF-2α have recently been shown to be induced by certain cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (INF)-γ (Ortis et al. 2010) and oncogenes (a mutated Raf kinase BRAF in colon cancer cells) (Kikuchi et al. 2009). Oncogenes can also enhance hypoxic translation of HIF-1α and HIF-2α (Kikuchi et al. 2009).

The stability and transcriptional activity of HIF-1α and HIF-2α are tightly regulated by two separate posttranslational oxygen-dependent modifications (see Fig. 1). Hydroxylation of two specific proline residues (Pro-402 and Pro-564 in human HIF-1α) in the ODDDs of these HIF-αs mediates interaction with the protein von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex, which targets them to the ubiquitin-proteasome pathway for degradation in normoxia (Ivan et al. 2001, Jaakkola et al. 2001, Yu et al. 2001, Masson et al. 2001). Conversely, asparaginyl hydroxylation (Asn-803 in human HIF-1α) by factor inhibiting HIF (FIH) inactivates the C-terminal transactivation domain of HIF-1α and HIF-2α under normoxic conditions by blocking their interaction with a transcriptional coactivator p300/CBP (Hewitson et al. 2002, Lando et al. 2002a, Lando et al. 2002b). Since HIF-3α protein levels are detectable already in normoxia, it appears not to be regulated oxygen-dependently at the level of protein stability, thus differing from HIF-1α and HIF-2α (Tanaka et al. 2009).

A recent report demonstrated that the ODDD is not the only region in HIF-α contributing to the oxygen-dependent degradation of the protein, as a double mutant of HIF-α insensitive to the HIF-P4Hs was shown to be degraded in
normoxia in a pVHL-dependent manner (André & Pereira 2008). Additionally, degradation of HIF-α protein can be mediated by hypoxia-induced SUMOylation (small ubiquitin-like modifier) of HIF-α, which allows the binding of the pVHL complex to HIF-α regardless of the hydroxylation status of HIF-α (Cheng et al. 2007). Conversely, a hypoxia-induced SUMO/sentrin-specific protease (SENP) can prevent the pVHL-mediated degradation by deconjugating SUMOylated HIF-α (Cheng et al. 2007). Furthermore, HIF-α protein stability is enhanced by nitric oxide-mediated S-nitrosylation (Li et al. 2007) and interaction with heat shock protein 90 (HSP90) (Gradin et al. 1996, Kallio et al. 1997), while the receptor of activated protein kinase C 1 (RACK1) competes with HSP90 for binding to HIF-α and thereby promotes degradation of HIF-α (Liu & Semenza 2007, Liu et al. 2007). Spermidine/spermine-N-acetyltransferase-1 (SSAT1) also promotes ubiquitination and subsequent degradation of HIF-α by binding to it (Baek et al. 2007).

2.2.4 Role of HIF in myocardial ischemia-reperfusion injury

Myocardial infarction resulting from a restriction in the myocardial blood supply, i.e. ischemia, is a common cause of death in Western countries. In the past decades, it has become evident that the myocardial response to ischemia-reperfusion (I/R) can be manipulated to delay the injury (for a review, see Murphy & Steenbergen 2008). Ischemic preconditioning (IP) is a phenomenon in which short sub-lethal cycles of I/R trigger an adaptive physiological response that protects the myocardium from subsequent prolonged I/R injury (Murry et al. 1986). The protection associated with IP has an immediate component occurring within minutes or hours following IP and a delayed component observed several hours after the preconditioning trigger (for a review, see Yellon & Baxter 1995), involving de novo synthesis of stress-related proteins (Das et al. 1994). Many different signal transduction pathways are likely to interact or act in parallel to induce IP (for a review, see Halestrap et al. 2007). HIF-1 has recently emerged as an essential mediator of cardioprotection induced by IP (Cai et al. 2008, Eckle et al. 2008). HIF-1α appears to be required for both the immediate and delayed phases of IP, as acute cardioprotection induced by IP is completely lost in HIF-1α−/− mice and in mice treated with siRNA targeting HIF-1α (Cai et al. 2008, Eckle et al. 2008), while exposure of mice to intermittent systemic hypoxia leads to late-phase cardioprotection in wild-type but not HIF-1α−/− mice (Cai et al. 2003). Moreover, several studies have shown that high-level expression of HIF-
1α in the heart achieved by different means reduces infarct size and increases neoangiogenesis in peri-infarct and infarct regions in murine models of myocardial infarction (Eckle et al. 2008, Kido et al. 2005, Shyu et al. 2002). Furthermore, transfection of mice with HIF-1α or a HIF-target gene heme oxygenase prior to ischemic insult reduced infarct size and improved post-ischemic function (Czibik et al. 2009). In support of the proposed role of HIF-1α in cardioprotection, an increase in HIF-1α mRNA expression has been demonstrated in the infarcted myocardium (Lee et al. 2000), and a marked and persistent increase has been observed in HIF-1 protein levels following coronary artery ligation in the rat heart (Willam et al. 2006). HIF-1α protein is also detected in the peri-infarct and infarct regions in myocardium of patients with acute myocardial infarction even late after the acute event (Blanco Pampin et al. 2006, Parisi et al. 2005).

Cardioprotection promoted by HIF-1 is likely to be mediated through multiple mechanisms. Adenosine is the key regulator of the early phase of myocardial IP (for a review, see Cohen & Downey 2008), and the genes encoding CD73, the ecto-5′-nuclease generating adenosine, and the adenosine receptor A2B have been shown to be HIF-targets that are upregulated when HIF-P4H-2 is silenced by left ventricle siRNA (Eckle et al. 2008). Adenosine signalling has been proposed to activate the prosurvival kinases such as serine/threonine kinase AKT and Erk1/2, which may in turn prevent the opening of the mitochondrial permeability transition pore (MPTP). MPTP opening has been suggested to be responsible for the I/R injury by causing mitochondrial swelling, eventually leading to cell death. (For reviews, see Halestrap & Pasdois 2009, Halestrap 2009, Haunlooy et al. 2009.) Reactive oxygen species (ROS) have also been implicated in triggering both the acute and delayed phases of IP-induced cardioprotection (Halestrap et al. 2007), and IP-induced ROS generation in cardiac mitochondria requires HIF-1α (Cai et al. 2008). HIF-1α contributes to the generation of ROS by inducing the expression of inducible nitric oxide synthase, which in turn increases nitric oxide (NO)-mediated inhibition of the mitochondrial electron transport chain and thus promotes production of ROS (Palacios-Callender et al. 2004, Quintero et al. 2006). ROS then targets protein kinase C (PKC), which induces the opening of mitochondrial K_ATP channels, causing depolarization of the inner mitochondrial membrane and preventing opening of the MPTP (Murata et al. 2001, Murphy & Steenbergen 2008), while another ROS target, transcription factor NRF2, initiates an antioxidant gene expression program (Leonard et al. 2006). Furthermore, ROS can inactivate the main negative regulator of the AKT
pathway, the phosphatase and tensin homolog PTEN, resulting in cardioprotection (Cai & Semenza 2005; for a review, see Mocanu & Yellon 2007). Very recently, the hypoxia-inducible microRNA miR-210 was associated with IP in a stem cell model. miR-210 was shown to act downstream of HIF and to be induced by IP, and via suppression of Caspase-8-associated Protein 2 to markedly improve the survival of bone marrow-derived stem cells following their transplantation into infarcted rat heart (Kim et al. 2009).

On the other hand, the immediate phase of preconditioning involves a shift from aerobic to glycolytic metabolism, and HIF-1α has been shown to mediate the metabolic switch from oxidative phosphorylation to anaerobic glycolysis (i.e. the Pasteur effect) in mammalian cells (Seagroves et al. 2001). By inducing pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al. 2006, Papandreou et al. 2006), HIF-1 shunts pyruvate from the mitochondria to cytosolic glycolysis, resulting in enhanced glycolysis and decreased mitochondrial oxygen consumption and a consequent relative increase in intracellular oxygen tension (Kim et al. 2006, Kim et al. 2007, Papandreou et al. 2006). In addition, the majority of glycolytic enzymes are encoded by HIF-1 target genes (for reviews, see Semenza 2007a, 2009).

2.3 HIF hydroxylases

2.3.1 HIF prolyl 4-hydroxylases

Prolyl 4-hydroxylation in the ODDD of HIF-α was shown to mediate the interaction between the human pVHL and the HIF-α subunit in 2001 (Ivan et al. 2001, Jaakkola et al. 2001, Yu et al. 2001). Up until that time, 4-hydroxyproline had only been found in collagens or proteins containing collagen-like motifs, and collagen prolyl 4-hydroxylases (C-P4Hs) were the only identified enzymes catalyzing the formation of 4-hydroxyproline (for a review, see Myllyharju & Kivirikko 2004). However, recombinant human C-P4H did not hydroxylate a peptide representing the HIF-α hydroxylation site (Jaakkola et al. 2001) indicating that a separate class of P4Hs must exist, and a family of HIF prolyl 4-hydroxylases (HIF-P4H 1, 2 and 3, also known as prolyl hydroxylase domain (PHD)-containing proteins 1, 2 and 3, Egl-nine 2, 1 and 3, and HIF prolyl hydroxylases (PHFs) 3, 2 and 1, respectively) was rapidly identified and characterized (Bruick & McKnight 2001, Epstein et al. 2001, Ivan et al. 2002).
Three HIF-P4H isoenzymes exist in mammals (in human, rat and mouse) and in zebrafish, while only a single HIF-P4H is found in *C. elegans* and *D. melanogaster* (Bruick & McKnight 2001, Centanin *et al.* 2005, Epstein *et al.* 2001, Ivan *et al.* 2002, van Rooijen *et al.* 2009). HIF-P4H-2 is clearly the most abundant of the three mammalian isoenzymes in normoxic conditions, and it is also responsible for setting the normoxic steady-state levels of HIF-α (Appelhoff *et al.* 2004, Berra *et al.* 2003).

### 2.3.2 HIF asparaginyl hydroxylase FIH

Factor inhibiting HIF (FIH or FIH-1) was first identified in 2001 as a negative regulator of HIF-α transactivation domain function (Mahon *et al.* 2001). By hydroxylating a conserved asparagine residue in the CTAD of HIF-1α and HIF-2α, FIH prevents their association with a transcriptional coactivator p300, and thereby represses the transactivation function of HIF-1α and HIF-2α in normoxic conditions (Hewitson *et al.* 2002, Lando *et al.* 2002a, Lando *et al.* 2002b). Mouse and zebrafish homologs of FIH have been identified, and related protein sequences have also been found in rat, *C. briggsae* and *D. melanogaster*, defining a family of related proteins that has been conserved through invertebrate and vertebrate evolution (Mahon *et al.* 2001, van Rooijen *et al.* 2009). Since the identification of FIH as an asparaginyl hydroxylase regulating the HIF pathway, more than 20 additional proteins have been shown to interact with FIH, and 11 novel hydroxylation substrates have been characterized among these proteins, indicating that FIH-dependent asparaginyl hydroxylation may be a prevalent posttranslational modification of the mammalian proteome (for a review, see Cockman *et al.* 2009b).

### 2.3.3 Molecular properties of HIF hydroxylases

#### Structure of HIF-P4Hs

The polypeptides of the human HIF-P4H isoenzymes 1, 2 and 3 consist of 407, 426 and 239 residues, respectively, and display a 42–59% sequence identity to each other but no significant sequence similarity to the α subunits of the C-P4Hs (see Fig. 4, Bruick & McKnight 2001, Epstein *et al.* 2001, Ivan *et al.* 2002). The crystal structure has been described for HIF-P4H-2 (Chowdhury *et al.* 2009,
McDonough et al. (2006), and sequence comparisons and modelling studies suggest that the active site is highly conserved among the three human HIF-P4H isoenzymes (McDonough et al. 2006). Like other Fe(II)- and 2-oxoglutarate-dependent dioxygenases, HIF-P4Hs possess a core of eight β-strands folded into a “jelly-roll” motif (double-stranded β-helix) (for a review, see Schofield & Ratcliffe 2004). Iron is coordinated by a two-histidine, one-carboxylate motif (His-313, Asp-315 and His-374 in HIF-P4H-2), while the C-5 moiety of 2-oxoglutarate is bound by an arginine (Arg-383 in HIF-P4H-2) (Bruick & McKnight 2001, Epstein et al. 2001, Ivan et al. 2002, McDonough et al. 2006), differing from the C-P4Hs, where the 2-oxoglutarate binding residue is a lysine (Myllyharju & Kivirikko 1997). HIF-P4Hs have a sterically narrow active site (McDonough et al. 2006), restricting access of compounds and possibly explaining the tight binding constants for Fe(II) and 2-oxoglutarate (see section 2.3.5) (Hirsilä et al. 2005, Koivunen et al. 2004, 2007). HIF-P4H-2 crystallizes as a homotrimer, but is likely to exist predominantly as a monomer in solution (McDonough et al. 2006).

HIF-P4H-1 exists as two distinct isoforms resulting from alternative initiation of translation, and while the two isoforms display similar biological activity, the shorter form has a substantially reduced half-life (Tian et al. 2006). HIF-P4Hs 2 and 3 are subject to alternative splicing. At least two inactive forms of HIF-P4H-2 are generated by alternative splicing, and the mRNA expression levels of these variants are considerably lower than that of the full-length mRNA in all tissues studied (Hirsilä et al. 2003). In addition to the full-length mRNA, two alternatively spliced mRNA isoforms are generated from the HIF-P4H-3 gene. The shorter, approximately 500-bp, HIF-P4H-3 mRNA isoform is translated into a catalytically inactive protein, while the protein encoded by the longer mRNA splice variant retains its catalytic activity. (Cervera et al. 2006, Hirsilä et al. 2003.) The shorter splice variant is expressed in approximately equal amounts to the full-length mRNA in all adult and fetal tissues studied (Cervera et al. 2006, Hirsilä et al. 2003), while expression of the longer HIF-P4H-3 splice variant has only been detected in primary cancer tissues (Cervera et al. 2006).
Fig. 4. Schematic representation of the human HIF-P4Hs 1-3 and FIH. The two-histidine, one-carboxylate motif used for coordinating Fe$^{3+}$ is conserved in all HIF hydroxylases, while the C-5 moiety of 2-oxoglutarate is bound by an arginine (R) in the HIF-P4Hs 1-3 and by a lysine (K) in FIH.

Structure of FIH

The human FIH gene encodes a 349-amino acid polypeptide (See Fig. 4, Mahon et al. 2001). Similarly to the HIF-P4Hs, the structural core of FIH consists of a jellyroll-like β-barrel formed from eight β-strands, and the iron-binding site of FIH contains the conserved two-histidine, one-carboxylate triad (His-199, Asp-201 and His-279) (Dann et al. 2002, Elkins et al. 2003, Lee et al. 2003). However, the two histidine ligands were recently reported to be sufficient for iron binding and catalysis by FIH, indicating flexibility in the iron coordinating chemistry of 2-oxoglutarate-dependent dioxygenases (Hewitson et al. 2008). The residue interacting with 2-oxoglutarate is a lysine (Lys-214) in FIH (Dann et al. 2002, Elkins et al. 2003, Lee et al. 2003). FIH forms a homodimer in solution and the dimerization domain is found in its C-terminus (Dann et al. 2002, Lee et al. 2003). The dimerization has been shown to be crucial for the substrate recognition and activity of FIH, as a single point mutation leading to a predominantly monomeric state in solution abolishes the catalytic activity of FIH (Dann et al. 2002, Lancaster et al. 2004, Lee et al. 2003). Furthermore, FIH contains a long and wide groove at the center of the molecule ranging from the active site of the enzyme towards the dimerization domain, possibly serving as a HIF-binding site (Lee et al. 2003). FIH also contains a putative pVHL-binding site distinct from the HIF-binding site, supporting the proposed formation of a ternary complex by FIH, HIF and pVHL (Lee et al. 2003).
2.3.4 Reaction mechanism of HIF hydroxylases

HIF-P4Hs and FIH belong to the group of 2-oxoglutarate and Fe(II)-dependent dioxygenases and require Fe(2+), 2-oxoglutarate, O₂ and ascorbate (Bruick & McKnight 2001, Epstein et al. 2001, Hewitson et al. 2002, Ivan et al. 2002, Lando et al. 2002a, for a review, see Myllyharju 2008). During catalysis, the splitting of molecular oxygen is coupled to the hydroxylation of a proline residue or an asparagine residue in HIF-α and to the oxidative decarboxylation of 2-oxoglutarate to succinate and CO₂ (see Fig. 5). The reaction proceeds via the formation of a highly reactive ferryl intermediate (Fe⁴⁺ = O) that oxidizes the target amino acid residue. The binding of cosubstrates occurs in an ordered manner, so that Fe(2+) becomes enzyme-bound first, followed by subsequent binding of 2-oxoglutarate, peptide substrate and O₂. (For reviews, see Kaelin & Ratcliffe 2008, Schofield & Ratcliffe 2004, Schofield & Ratcliffe 2005.)

Fig. 5. Schematic representation of the reaction catalyzed by prolyl 4-hydroxylases. 2-oxoglutarate is stoichiometrically decarboxylated during prolyl hydroxylation (A). In an uncoupled reaction, ascorbate is stoichiometrically consumed either in the presence (B) or absence (C) of substrate.
2.3.5 Cosubstrates and inhibitors of HIF hydroxylases

Table 1. Km and Ki values of HIF-P4H-1, 2 and 3 and FIH for reaction cosubstrates, substrates and certain inhibitors.

<table>
<thead>
<tr>
<th>Cosubstrate, substrate or inhibitor</th>
<th>Constant</th>
<th>HIF-P4H-1</th>
<th>HIF-P4H-2</th>
<th>HIF-P4H-3</th>
<th>FIH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe^{2+}</td>
<td>Km</td>
<td>0.03(a)</td>
<td>0.03(a)</td>
<td>0.1(a)</td>
<td>0.5(a)</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>Km</td>
<td>2(b)</td>
<td>1(b)</td>
<td>12(b)</td>
<td>25(b)</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>Km</td>
<td>170(c)</td>
<td>180(c)</td>
<td>140(c)</td>
<td>260(c)</td>
</tr>
<tr>
<td>O_{2}</td>
<td>Km</td>
<td>230(c)</td>
<td>100(c)--250(c)</td>
<td>230(c)</td>
<td>90(c)--237(c)</td>
</tr>
<tr>
<td>HIF-1αODDD</td>
<td>Km</td>
<td>0.01--0.02(d)</td>
<td>0.14(d)</td>
<td>0.07(d)</td>
<td>No</td>
</tr>
<tr>
<td>HIF-2αODDD</td>
<td>Km</td>
<td>0.01--0.02(d)</td>
<td>0.06(d)</td>
<td>0.1(d)</td>
<td>No</td>
</tr>
<tr>
<td>HIF-1α^{788-822} CTAD</td>
<td>Km</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>100(d)--222(d)</td>
</tr>
<tr>
<td>HIF-2α^{832-866} CTAD</td>
<td>Km</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>160(d)--229(d)</td>
</tr>
<tr>
<td>Pyridine-2,4-dicarboxylate</td>
<td>K_{i}</td>
<td>40(f)</td>
<td>7(f)</td>
<td>8(f)</td>
<td>30(f)</td>
</tr>
<tr>
<td>Pyridine-2,5-dicarboxylate</td>
<td>K_{i}</td>
<td>&gt;300(f)</td>
<td>&gt;300(f)</td>
<td>&gt;300(f)</td>
<td>50(f)</td>
</tr>
<tr>
<td>3-Hydroxypridine-2-carbonyl-glycine</td>
<td>K_{i}</td>
<td>15(f)</td>
<td>2(f)</td>
<td>1(f)</td>
<td>&gt;300(f)</td>
</tr>
<tr>
<td>Oxalylglycine</td>
<td>K_{i}</td>
<td>50(f)</td>
<td>8(f)</td>
<td>10(f)</td>
<td>2(f)</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate</td>
<td>K_{i}</td>
<td>&gt;300(f)</td>
<td>&gt;300(f)</td>
<td>300(f)</td>
<td>10(f)</td>
</tr>
<tr>
<td>N-((3-Hydroxy-6-chloroquinolin-2-</td>
<td>K_{i}</td>
<td>0.8(f)</td>
<td>0.2(f)</td>
<td>0.2(f)</td>
<td>&gt;300(f)</td>
</tr>
</tbody>
</table>

No, does not act as a substrate

\(a\)Hirsilä et al. 2005, \(b\)Koivunen et al. 2007, \(c\)Hirsilä et al. 2003, \(d\)Koivunen et al. 2006, \(e\)Koivunen et al. 2004, \(f\)Ehrisman et al. 2007

Iron and iron chelators, metal ions

HIF-P4Hs have very low Km values for Fe^{2+}, suggesting unusually tight binding of iron at the active site of the enzyme (see Table 1, Hirsilä et al. 2005). It is well established that divalent metals such as cobalt and nickel can mimic hypoxia and stimulate the expression of hypoxia-inducible genes, such as erythropoietin (EPO) (Goldberg et al. 1988), and that recombinant HIF-P4Hs are sensitive to iron chelators in vitro (Epstein et al. 2001), but the mechanism of action has remained unclear. Cobalt has been reported to inhibit the activities of HIF-P4Hs and FIH, possibly by occupying their Fe^{2+} binding site (Epstein et al. 2001, Huang et al. 2002). It has also been suggested that cobalt stabilizes HIF-α by occupying the VHL-binding domain of HIF-α, thereby preventing its degradation (Yuan et al. 2003). Yet, the inhibitory action of cobalt and nickel has been proposed to be mediated through depletion of intracellular ascorbate levels, leading to
inactivation of the HIF-P4Hs (Salnikow et al. 2004). A study using purified recombinant HIF-P4Hs proved cobalt and nickel to be very ineffective inhibitors of the HIF-P4Hs in vitro, demonstrating that the stabilization of HIF-α by cobalt and nickel is mediated by mechanisms other than simple competitive inhibition of the HIF-P4Hs with respect to iron (Hirsilä et al. 2005). Among the other divalent metals studied, zinc was found to efficiently inhibit HIF-P4H-3, while being a poor inhibitor of HIF-P4Hs 1 and 2, and cadmium was found to inhibit all three HIF-P4Hs. Magnesium and manganese were the least potent inhibitors of all three HIF-P4Hs. (Hirsilä et al. 2005.) The iron chelator desferrioxamine (DFO) was also shown to be a poor inhibitor of HIF-P4Hs, while effectively inhibiting FIH and C-P4Hs (Hirsilä et al. 2005). The $K_m$ of FIH for Fe$^{2+}$ is approximately 5- to 15-fold higher than those of the HIF-P4Hs (see Table 1) (Hirsilä et al. 2005, Koivunen et al. 2004), likely providing an explanation for the differences observed in the inhibition of FIH and HIF-P4Hs by various metals. Namely, zinc was found to be the most potent inhibitor of FIH, and cobalt, nickel, cadmium and manganese all proved effective inhibitors of FIH, magnesium being the only poor inhibitor among the metals studied (Hirsilä et al. 2005). The inhibition of FIH by metals was found to be competitive with respect to iron (Hirsilä et al. 2005). Iron supplementation has been shown to be sufficient to reduce steady-state HIF-1α levels in normoxic cells, most likely by enhancing HIF-P4H activity (Knowles et al. 2003). Furthermore, a recent clinical trial demonstrated that iron supplementation can attenuate hypoxic pulmonary hypertension (HPH), while iron depletion exacerbates HPH (Smith et al. 2009). Taken together, these findings indicate that cellular iron availability does play a physiological role in the regulation of HIF hydroxylases.

2-oxoglutarate

2-oxoglutarate, a cosubstrate of the HIF hydroxylases, is mainly generated in the matrix of mitochondria as an intermediate of the citric acid cycle (also known as the tricarboxylic acid (TCA) cycle and Krebs cycle), which is the convergence point of the metabolic pathways involved in the chemical conversion of carbohydrates, fats and proteins into carbon dioxide and water to produce ATP in all eukaryotes. The HIF-P4H isoenzymes 1 and 2 have very similar $K_m$ values for 2-oxoglutarate, while the $K_m$ value of HIF-P4H-3 is about 10-fold higher (see Table 1) (Koivunen et al. 2007). $K_m$ values of FIH (see Table 1) and the C-P4Hs (see Table 2) for 2-oxoglutarate are distinctly higher than those of the HIF-P4Hs.
(Koivunen et al. 2004). Since the basic residue binding the carboxyl group of 2-oxoglutarate is a lysine in FIH (Dann et al. 2002, Elkins et al. 2003, Lee et al. 2003) and C-P4Hs (Myllyharju & Kivirikko 1997) while being an arginine in the HIF-P4Hs (Bruick & McKnight 2001, Epstein et al. 2001), it has been speculated that the differences in the $K_m$ values may be due to the differences in the 2-oxoglutarate binding residue, although other properties of the catalytic sites may also have an effect (Koivunen et al. 2004).

Other TCA cycle intermediates

Given that both the cosubstrate 2-oxoglutarate and the product succinate of the hydroxylation reaction catalyzed by the HIF hydroxylases are TCA cycle intermediates, a cross-talk between energy metabolism and hypoxic transcriptional responses has been proposed. Indeed, three TCA intermediates, succinate, fumarate and oxaloacetate, have been shown to function as *in vitro* inhibitors of all three HIF-P4Hs, acting in a competitive fashion that can be reversed by elevating the intracellular 2-oxoglutarate concentration (Hewitson et al. 2007, Isaacs et al. 2005, Koivunen et al. 2007, Lee et al. 2005, MacKenzie et al. 2007, Selak et al. 2005). Fumarate appears to be by far the most effective inhibitor of the HIF-P4Hs, while inhibition by succinate is intermediate and that by oxaloacetate markedly less efficient (Hewitson et al. 2007, Koivunen et al. 2007). Citrate inhibits HIF-P4H-3, but has no effect on the other two isoenzymes (Koivunen et al. 2007). In sharp contrast to the HIF-P4Hs, FIH is effectively inhibited by citrate and oxaloacetate, whereas succinate and fumarate have a much less pronounced effect on FIH activity (Hewitson et al. 2007, Koivunen et al. 2007). Pyruvate has also been shown to induce the HIF system in normoxia by presumably inhibiting the HIF-P4Hs (Dalgard et al. 2004, Lu et al. 2005). In a more recent study, however, pyruvate failed to inhibit any of the HIF-P4Hs (Koivunen et al. 2007), the discrepancy between the studies possibly reflecting different experimental setups.

Oxygen

for O₂ (see Table 1) (Ehrismann et al. 2007, Hirsilä et al. 2003, Koivunen et al. 2004, 2006). Using short peptides as substrates, the $K_m$ values of HIF-P4Hs for O₂ were initially reported to be in the range of 230–250 μM (Hirsilä et al. 2003), thus being slightly above the ambient oxygen concentration at sea level, but as longer recombinant polypeptides were used as substrates, the $K_m$ value of HIF-P4H-2 for oxygen was observed to vary between 76–100 μM, depending on the assay used (Ehrismann et al. 2007, Koivunen et al. 2006). The $K_m$ value of FIH for O₂ is in the range of 90–240 μM (Ehrismann et al. 2007, Koivunen et al. 2004, 2006). Despite the high $K_m$ values for O₂, HIF-P4Hs appear to target HIF-α for degradation even under severely hypoxic conditions (0.2% O₂). However, the hydroxylase activity of HIF-P4Hs under these conditions is significantly reduced, and the decreased O₂ availability is partially compensated for by HIF-mediated increases in the amounts of HIF-P4Hs 2 and 3. (Stiehl et al. 2006.) Also, FIH remains enzymatically active even under severely hypoxic conditions, but not in anoxic cells (Stolze et al. 2004). Taken together, these data indicate that although HIF-P4Hs and FIH remain functional even under severely hypoxic conditions, the changes in cellular oxygen availability are effectively reflected in the hydroxylase activity of HIF hydroxylases, fitting well with their proposed role as cellular oxygen sensors (Ehrismann et al. 2007, Hirsilä et al. 2003, Koivunen et al. 2006).

**Ascorbate**

Ascorbate is not essential to the reaction catalyzed by HIF-P4Hs and FIH. Lack of ascorbate leads, however, to a decrease in the reaction rate. During catalytic cycles in which decarboxylation of 2-oxoglutarate is not coupled to hydroxylation of the substrate (i.e. uncoupled decarboxylation, see Fig. 5B and C) iron becomes oxidized to the Fe(III) form, leading to subsequent inactivation of the enzyme. The rate of uncoupled decarboxylation presents approximately 1–4% of the rate of the complete reaction occurring in the presence of saturating substrate concentration. (For reviews, see Kivirikko & Pihlajaniemi 1998 and Myllyharju 2005.) Ascorbate is needed to reduce the enzyme-bound Fe(III) back to the active Fe(II) form within the active site of the enzyme (Myllylä et al. 1984, Schofield & Ratcliffe 2004). The importance of ascorbate in regulating HIF hydroxylase activity has been highlighted in recent years. Addition of ascorbate at a physiological concentration (25 μM) strikingly suppressed HIF-α protein levels and HIF transcriptional activity in normoxic tissue culture conditions, suggesting that without ascorbate supplementation HIF hydroxylase activity may be
submaximal and rate-limiting for degradation of HIF-α (Knowles et al. 2003). Moreover, HIF-α is upregulated in ascorbate-deficient neutrophils (Vissers & Wilkie 2007). However, as decreased intracellular ascorbate levels have been associated with increased reactive oxygen species (ROS) production, the subsequent reduction in HIF-P4H activity may at least in part be due to the concomitant increase in ROS (Gerald et al. 2004, Pagé et al. 2008).

**Reactive oxygen species**

The role of ROS in the hypoxic response remains somewhat controversial. Intracellular ROS have been reported to increase upon exposure to hypoxia (Brunelle et al. 2005, Guzy et al. 2005, Mansfield et al. 2005), but the direction and degree of the hypoxia-induced changes in ROS production may depend on the cell type, as a decrease in ROS production in response to hypoxic exposure has also been reported (for a review, see Cash et al. 2007). Furthermore, data from several groups suggest that mitochondrial ROS regulate HIF-α stabilization (for a review, see Cash et al. 2007). Several reports have demonstrated compromised hypoxic HIF-α stabilization in cells lacking functional mitochondria and consequently displaying reduced ROS generation, suggesting retained hypoxic HIF hydroxylase activity. (For a review, see Cash et al. 2007; Brunelle et al. 2005, Chandel et al. 1998, Chandel et al. 2000, Guzy et al. 2005, Mansfield et al. 2005). In accordance with the alleged role of ROS in HIF-α stabilization, JunD-deficient mouse embryonic fibroblasts exhibit normoxic HIF-α protein stabilization due to lower levels of ROS scavenging enzymes and subsequent higher amount of free ROS (Gerald et al. 2004). Furthermore, a very recent report showed that deficiency of fumarate hydratase (FH) leads to ROS-dependent HIF-α stabilization (Sudarshan et al. 2009). In contrast, it has also been shown that cells lacking functional mitochondria and subsequently producing markedly reduced amounts of ROS still stabilize HIF-α under hypoxia (Doege et al. 2005, Srinivas et al. 2001). Despite the obvious controversy, several mechanisms have been proposed for the action of ROS in HIF-α stabilization. It has been suggested that ROS may trigger a signal transduction cascade, which results in inhibitory posttranslational modifications of HIF-P4Hs, oxidize the enzyme-bound iron or alter HIF-P4H disulfide bond structure to regulate HIF-P4H activity. (For a review, see Cash et al. 2007.)
Nitric oxide (NO), a key mediator of the inflammatory response, also modulates the HIF pathway by regulating the activity of HIF-P4Hs in a complex, bi-phasic manner. Acute exposure of cells to high concentrations of NO inhibits HIF-P4H activity irrespective of the prevailing oxygen concentration, leading to stabilization of HIF-α, while prolonged exposure or low concentrations of NO reduce accumulation of HIF-α even under hypoxic conditions, most likely via induction of HIF-P4H activity. (For a review, see Berchner-Pfannschmidt et al. 2009.) Inhibition of FIH by NO has also been demonstrated, albeit only when iron and ascorbate are limiting (Park et al. 2008). The exact molecular mechanism by which NO inhibits HIF-P4Hs remains unclear. As studies on other non-heme Fe(II) containing enzymes have shown that NO can directly bind to the ferrous iron (Arciero et al. 1985, Hegg & Que 1997, Roach et al. 1995), competitive inhibition with respect to molecular oxygen has been suggested as a mechanism (Metzen et al. 2003b). Conversely, the NO-mediated induction of HIF-P4H activity in hypoxia has been suggested to result from redistribution of O₂ towards HIF-P4Hs following inhibition of mitochondrial respiration, or from increases in intracellular iron and 2-oxoglutarate. The involvement of ROS in this phenomenon has not been excluded either. (For a review, see Berchner-Pfannschmidt et al. 2009.)

2.3.6 HIF-P4H substrates and other interacting proteins

HIF-P4H substrates

HIF-P4Hs hydroxylate two prolines in HIF-1α and HIF-2α (Pro-402 and Pro-564 in human HIF-1α) in conserved –Leu-X-X-Leu-Ala-Pro- sequences (Bruick & McKnight 2001, Ivan et al. 2001, Jaakkola et al. 2001, Masson et al. 2001, Yu et al. 2001). All three HIF-P4Hs also hydroxylate a peptide corresponding to the one potential hydroxylation site in HIF-3α (Hirsilä et al. 2003). Substantial variation in the consensus sequence can, however, be tolerated in vitro, as alanine seems to be the only relatively, but still not absolutely, strict requirement in addition to the proline itself (Huang et al. 2002, Li et al. 2004). An increase in peptide substrate length results in a marked decrease in the apparent Kₘ values of HIF-P4Hs for the substrate (Ehrismann et al. 2007, Koivunen et al. 2006), and the shortest HIF-α-like peptide hydroxylated by all three recombinant human HIF-P4H isoenzymes
is 11 residues in length (Hirsilä et al. 2003). Determination of the crystal structure of the catalytic domain of HIF-P4H-2 in complex with the C-terminal part of the HIF-1α ODDD has shown that the proline residue is bound in a specific conformation and that a significant structural change involving a mobile loop likely occurs concomitant with HIF-1α binding (Chowdhury et al. 2009). It must therefore be born in mind that the in vitro $K_m$ values do not necessarily reflect the in vivo correlation of substrate length and binding affinity, since in vivo, the optimal length of the substrate is likely to depend on its three-dimensional structure.

The C-terminal prolines in the HIF-1α and HIF-2α ODDDs are much more efficiently hydroxylated than the N-terminal ones, demonstrating selectivity of the HIF-P4Hs within the two hydroxylation sites in the HIF-α polypeptides. HIF-P4H-3 is essentially inactive in suppressing N-terminal ODDD-bearing proteins from either HIF-1α or HIF-2α. (Appelhoff et al. 2004, Chan et al. 2005, Ehrismann et al. 2007, Epstein et al. 2001, Hirsilä et al. 2003, Koivunen et al. 2006, Villar et al. 2007.) Furthermore, hydroxylation at the C-terminal site seems to promote hydroxylation of the N-terminal proline, and in vivo Pro-564 hydroxylation precedes Pro-402 hydroxylation, the latter being attenuated in the absence of Pro-564 (Chan et al. 2005). HIF-P4H-2 has relatively more influence on HIF-1α than HIF-2α, the opposite being the true for HIF-P4H-3 (Appelhoff et al. 2004), but since the $K_m$ of HIF-P4H-2 for HIF-1α ODDD is slightly higher than that for HIF-2α ODDD (see Table 1), this appears not to be due to differences in binding affinity (Koivunen et al. 2006). A novel substrate was very recently identified for HIF-P4H-3. Hydroxylation of two distinct prolines in the type 2 β-adrenergic receptor β2AR by HIF-P4H-3 targets β2AR to proteasomal degradation via recognition by the pVHL-E3 ligase complex (Xie et al. 2009).

**Potential novel HIF-P4H substrates**

HIF-P4H-1 interacts with the IκB kinase-β (Iκkβ), which contains a conserved prolyl hydroxylation consensus sequence homologous to the conserved sites in HIF-αs, but hydroxylation of Iκkβ has not been demonstrated to date (Cummins et al. 2006). HIF-P4H-3 has been shown to interact with and regulate the protein levels of the activating transcription factor-4 (ATF-4) in an oxygen-dependent fashion (Köditz et al. 2007), proposing ATF-4 as a potential novel HIF-P4H-3 substrate. HIF-P4H-3 has also been shown to influence the kinesin KIF1Bβ in a manner dependent on its catalytic activity, leading to induction of neuronal
apoptosis during development (Schlisio et al. 2008). The large subunit of RNA polymerase II (Kuznetsova et al. 2003) and the iron regulatory protein IRP-2 (Hanson et al. 2003, Wang et al. 2004) have been shown to be regulated in a manner requiring prolyl hydroxylation, but direct hydroxylation by the HIF-P4Hs remains to be established.

**Non-substrate proteins interacting with the HIF-P4Hs**

Interactions of the HIF-P4Hs with several recently identified non-substrate molecules may regulate their amount or activity, thereby potentially providing an interface between oxygen sensing and other signalling pathways. Biochemical analysis indicated that HIF-P4H-2 might exist as a part of a larger heteromeric complex (Ivan et al. 2002). Indeed, several HIF-P4H-2 interacting proteins have been identified in recent years. The peptidyl prolyl cis/trans isomerase FK506-binding protein 38 (FKBP38) interacts with HIF-P4H-2 and regulates HIF-P4H-2 protein stability (Barth et al. 2007). It has been proposed that membrane-associated FKBP38 binds a fraction of the intracellular HIF-P4H-2 and mediates its ubiquitin-independent proteasomal degradation, while cytosolic HIF-P4H-2 is stable and able to function as an active HIF hydroxylase (Barth et al. 2009). The candidate tumor suppressor protein (ING4) directly interacts with HIF-P4H-2 and represses activation of HIF-1, but is not a HIF-P4H-2 substrate (Ozer et al. 2005). Furthermore, HIF-P4H-2 has been shown to interact with the melanoma-antigens MAGE-11 and MAGE-9 both in normoxic and hypoxic conditions, resulting in inhibition of HIF-P4H-2 activity accompanied by accumulation of HIF-α protein (Aprilekova et al. 2009). OS-9, the protein product of a widely expressed gene with no assigned function, interacts with HIF-P4H-2 and -3 through the formation of a ternary complex of OS-9, HIF-P4H-2/3 and HIF-α, promoting hydroxylation and subsequent degradation of HIF-α (Baek et al. 2005).

HIF-P4H-3 is a potential substrate of the cytosolic TRiC chaperonin (TCP-1 ring complex), which may regulate its activity (Masson et al. 2004). The ring finger E3 ligase Siah2 interacts with all three HIF-P4Hs, but primarily affects the stability of HIF-P4H-3 by targeting it to proteasome-dependent degradation under hypoxic conditions (Nakayama et al. 2004, 2007). HIF-P4H-3 can assemble into higher order complexes whose composition is controlled by the ambient oxygen tension, these high-molecular mass complexes being conceivably more stable but less active, while the low-molecular mass complexes retain more activity but are more susceptible to degradation by Siah2 (Nakayama et al. 2007). The mitogen-
activated protein kinase organizer Mrg1 interacts with HIF-P4H-3 and possibly functions as a molecular scaffold for HIF-P4H-3 (Hopfer et al. 2006). Furthermore, HIF-P4H-3 inhibits IκkB/NFκB signaling by binding to IκkB and thereby blocking the interaction of IκkB with Hsp90 in human colorectal cancer cells independently of its hydroxylase activity (Xue et al. 2010). HIF-P4H-3 also binds to and subsequently protects myogenin from degradation, thereby regulating skeletal muscle differentiation (Fu et al. 2007), but whether this regulatory action is hydroxylase-dependent or independent remains to be defined.

2.3.7 FIH substrates and other interacting proteins

**HIF**

FIH hydroxylates a conserved asparagine residue in HIF-1α and HIF-2α (Asn-803 HIF-1α) (Hewitson et al. 2002, Lando et al. 2002a, Lando et al. 2002b). The crystal structure of FIH in complex with the CTAD of HIF-1α shows that the FIH-CTAD interaction occurs via an induced fit process at two distinct sites, one involving the hydroxylation site itself (CTAD_{795-806}, site 1) and the other lying to the C-terminal side of this site (CTAD_{813-822}, site 2), both being of high importance to the interaction between FIH and HIF-1α (Elkins et al. 2003, Koivunen et al. 2004). The hydroxylation of Asn-803 by FIH can be prevented by phosphorylation of Thr-796, which probably leads to the disruption of the binding interaction between FIH and HIF-1α (Lancaster et al. 2004). With the exception of Val-802, the other conserved residues surrounding Asn-803 are not essential for efficient hydroxylation of HIF-1α in vitro or in cell-based assays (Linke et al. 2004). FIH appears to require particularly long peptide substrates, since the relative activities obtained with 13- and 30-mer HIF-1α CTAD substrate peptides were only about 5 and 30% of the activity obtained with a 52-mer HIF-1α CTAD peptide (Elkins et al. 2003). Furthermore, the K_m value of FIH for HIF-1α decreased from 395 μM to 222 μM and further to 154 μM with an increase in the peptide substrate length from 19 to 35 and to 173 residues (Ehrismann et al. 2007). Additionally, the V_max with a 19-residue HIF-1α CTAD fragment was approximately 10% of that with the 35-residue fragment (Koivunen et al. 2004). The K_m values of FIH for the 35-residue HIF-1α and HIF-2α polypeptides are very similar, if not identical (see Table 1) (Ehrismann et al. 2007, Koivunen et al. 2004). However, the activity obtained with the HIF-2α peptide substrate was less
than 25% of that obtained with HIF-1α peptide (Koivunen et al. 2004), and the $V_{\text{max}}$ values of FIH were more than 8-fold for HIF-1α compared to HIF-2α (Ehrismann et al. 2007), suggesting that FIH may act less effectively on HIF-2α than HIF-1α.

**ARD-containing proteins**

An alternative class of FIH substrates bearing a ubiquitous ankyrin repeat domain (ARD) has emerged in recent years (for a review, see Cockman et al. 2009b). IκB proteins p105 and IκBα were the first ARD-containing proteins to be identified as FIH substrates, and eight additional peptides derived from different ARD-containing proteins were simultaneously shown to be efficiently hydroxylated by FIH in vitro (Cockman et al. 2006). Since 2006, more than 20 ARD-containing proteins have been shown to interact with FIH, 11 of which have been confirmed as bona fide FIH substrates to date (for a review, see Cockman et al. 2009b). The ARD is one of the most common amino acid motifs in nature, being present in over 300 proteins encoded by the human genome and conserved in all kingdoms of life (for a review, see Li et al. 2006). Interestingly, some ARD-containing proteins have been shown to be hypoxia-sensitive: the muscle ankyrin repeat proteins (MARPs) that are known to be induced under stress conditions, display significant increases in their mRNA levels in response to hypoxia (Band et al. 2009). Bioinformatics alignment of ARD proteins with known FIH substrates indicates that most ARD proteins contain at least one candidate FIH hydroxylation site, suggesting that the hydroxylation of ARDs by FIH is a very common posttranslational modification (Webb et al. 2009).

ARDs are composed of a variable number of 33-residue repeats that individually fold into paired antiparallel $\alpha$-helices linked by a $\beta$-hairpin-type turn, and the hydroxylated asparagine residue is located within the hairpin loop that links individual repeats (for a review, see Li et al. 2006). The FIH consensus motif within the hydroxylated ARDs appears to be largely degenerate, with only the target asparagine showing absolute conservation, which is consistent with the ability of FIH to accommodate different ankyrin repeat (AR) sequences in its active site (Cockman et al. 2009a). ARDs frequently contain multiple hydroxylation sites, and the extent of hydroxylation between different sites varies (Cockman et al. 2006, Cockman et al. 2009a, Coleman et al. 2007). FIH binds ARDs in an elongated conformation which requires transient unfolding on the AR fold (Hardy et al. 2009), and it appears that high stability of the AR fold renders
proteins significantly less susceptible to FIH-dependent hydroxylation (Hardy et al. 2009, Kelly et al. 2009). Conversely, hydroxylation of ARD proteins by FIH stabilizes the ARD fold in solution (Kelly et al. 2009), decreasing the extent of ARD binding to FIH (Coleman et al. 2007).

The functional role of FIH-dependent hydroxylation of ARD-containing proteins remains unclear. FIH substrates include proteins involved in well-characterized signaling pathways such as NFκB (IkBα and p105) or Notch signaling (Notch1), and various other processes including actin-myosin contractility (MYPT1), ubiquitin-mediated proteolysis (ASB4), endocytosis (Rabankyrin) and telomere regulation/vesicle trafficking (Tankyrase-2) (Cockman et al. 2006, Cockman et al. 2009a, Coleman et al. 2007, Ferguson et al. 2007, Webb et al. 2009, Zheng et al. 2008). The diversity of the numerous pathways in which the recently identified FIH-substrates are involved suggests that a generic signalling role for ARD hydroxylation is unlikely (Cockman et al. 2009a).

Cross-competition between HIF-α and the ARDs for FIH-mediated asparagine hydroxylation has been suggested, and several lines of evidence support the hypothesis of ARDs functioning as a “sink” for FIH (Coleman et al. 2007, Shin et al. 2009, Webb et al. 2009, Zheng et al. 2008). First, it has been observed that overexpression of FIH is able to further suppress HIF transactivation function even in well oxygenated cells, where endogenous FIH activity should be sufficient to maximally repress HIF-α activity (Stolze et al. 2004). Second, binding studies indicate a more than 20-fold higher affinity of FIH for the unhydroxylated Notch ARD than for the unhydroxylated HIF-α CTAD, while competition assays show a striking preference of FIH for ARD hydroxylation over HIF-α CTAD hydroxylation (Coleman et al. 2007). Third, ARD-containing proteins are ubiquitous in nature and ARD hydroxylation appears to be widespread (Cockman et al. 2009a). Furthermore, Notch1-mediated derepression of HIF-α can be abrogated by increasing the level of FIH (Zheng et al. 2008). The extent to which each ARD substrate physically sequesters FIH away from HIF CTAD appears to be variable, possibly reflecting different affinities of FIH for each substrate (Shin et al. 2009, Webb et al. 2009). In conclusion, given the large number of ARD-containing candidate FIH substrates within the cell and the fact that FIH-dependent hydroxylation of ARDs decreases the extent of ARD binding to FIH, it is likely to be the hydroxylation status of the ARD pool within the cell rather than any one individual ARD protein that provides the effective competition and thereby protects HIF-α from inactivation (for a review, see Cockman et al. 2009b).
Other proteins interacting with FIH

FIH interacts with natural ankyrin repeats in cells also without hydroxylating them (Hardy et al. 2009). Siah-1 was recently shown to interact with and ubiquitinate FIH, promoting degradation of FIH under normoxic conditions (Fukuba et al. 2008). Mint3, a member of the X11 family of proteins that bind Alzheimer β-amyloid precursor protein, binds to FIH in an identical domain with HIF-α, and thereby inhibits the asparagine hydroxylation of HIF-α in a dose-dependent manner (Sakamoto & Seiki 2009).

2.3.8 HIF-P4Hs in cells and tissues

Tissue expression of HIF-P4Hs

The three HIF-P4H isoenzymes are widely expressed in various vertebrate tissues and cell lines, exhibiting unique and overlapping expression patterns. HIF-P4H-1 is most abundantly expressed in the testis and placenta, with relatively low levels of expression in other tissues studied, while HIF-P4H-3 has its strongest expression in the heart. The expression levels of HIF-P4H-2 are relatively stable in all tissues studied. (Appelhoff et al. 2004, Hirsilä et al. 2003, Lieb et al. 2002, Oehme et al. 2002, Soilleux et al. 2005, Willam et al. 2006.) Normoxic HIF-α levels are mainly regulated by the most abundant isoenzyme HIF-P4H-2, given that siRNA-mediated suppression of HIF-P4H-2 alone is sufficient to stabilize HIF-α (Appelhoff et al. 2004, Berra et al. 2003). Under moderately hypoxic conditions, however, HIF-P4H-3 primarily regulates HIF-α availability (Nakayama et al. 2004). Moreover, it seems that all three HIF-P4Hs contribute to the regulation of HIF-α depending to a substantial extent on their relative abundance under the conditions of analysis (Appelhoff et al. 2004).

Subcellular localization of HIF-P4Hs

At the cellular level, HIF-P4H-1 is exclusively nuclear and HIF-P4H-3 is homogeneously distributed in the cytoplasm and nucleus under both normoxic and hypoxic conditions, while HIF-P4H-2 appears to be able to shuttle between the cytoplasm and the nucleus (Metzen et al. 2003a, Steinhoff et al. 2009, Yasumoto et al. 2009). HIF-P4H-2 is mainly cytoplasmic in normal tissues (Metzen et al. 2003a, Soilleux et al. 2005), whereas increased nuclear expression
has been observed in cancer cells, with a strong correlation with tumor aggressiveness and poor differentiation (Jokilehto et al. 2006, Luukkaa et al. 2009). Similarly, increased nuclear HIF-P4H-2 levels have been observed under exposure to hypoxia and NO, the cytoplasmic-to-nuclear ratio of HIF-P4H-2 showing variation in different cell lines (Berchner-Pfannschmidt et al. 2008). Regulation of the subcellular distribution of HIF-P4H-2 seems to be an effective pathway for the control of the hypoxic response, as cytoplasmic HIF-P4H-2 has higher prolyl 4-hydroxylase activity than nuclear HIF-P4H-2 (Yasumoto et al. 2009). HIF-P4H-2 contains no typical nuclear export signal, but nuclear export appears to require the 100 N-terminal residues of HIF-P4H-2 and to be mediated by the main export receptor CRM1 (Steinhoff et al. 2009, Yasumoto et al. 2009). HIF-P4H-1 possesses a nuclear localization signal (NLS) (Steinhoff et al. 2009, Yasumoto et al. 2009), and the nuclear import occurs importin α/β-dependently relying on the NLS (Steinhoff et al. 2009).

**Inducibility of HIF-P4Hs by hypoxia and other stimuli**

HIF-P4Hs 2 and 3 have been shown to be induced by hypoxia at mRNA and protein levels both in vitro and in vivo, the hypoxia-inducibility being particularly striking with HIF-P4H-3, whereas HIF-P4H-1 mRNA and protein levels are not affected by hypoxia or hypoxic mimics, suggesting constitutive expression (Appelhoff et al. 2004, Berra et al. 2003, Cioffi et al. 2003, D'Angelo et al. 2003, del Peso et al. 2003, Epstein et al. 2001, Lieb et al. 2002, Marxsen et al. 2004, Metzen et al. 2003a, Stiehl et al. 2006). Tissue-specific differences in the hypoxia-inducibility may exist, however, as HIF-P4H-2 mRNA expression was not increased in response to hypoxia in cells of cardiovascular origin (Cioffi et al. 2003). HIF-P4Hs 2 and 3 both contain functional HREs and are direct HIF target genes (Metzen et al. 2005, Pescador et al. 2005). However, the induction of HIF-P4H-2 by hypoxia appears to have also HIF-independent components (Aprelikova et al. 2004). It has been concluded that induction of HIF-P4Hs 2 and 3 by hypoxia forms a functionally important negative feedback loop leading to rapid proteasomal degradation of HIF-α upon reoxygenation (D'Angelo et al. 2003, del Peso et al. 2003, Marxsen et al. 2004) and allowing the HIF-P4Hs to remain operative under a wide range of lowered oxygen concentrations, creating a flexible oxygen threshold for the induction of the HIF system (Stiehl et al. 2006). Conversely, HIF-P4H expression is downregulated at higher O2 concentrations, revealing dual regulation of HIF-P4Hs by ambient pO2 (Khanna et al. 2006).
addition to mRNA levels and protein abundance, HIF hydroxylase activity is also subject to regulation. Chronic hypoxia leads to “overactivation” of the HIF-P4Hs despite the reduced overall O₂ concentration, the probable explanation being that as mitochondrial respiration is inhibited by hypoxia, more of the intracellular oxygen is made available to the HIF-P4Hs (Ginouves et al. 2008).

Variable inducibility of the HIF-P4H genes by stimuli other than hypoxia has been reported as well. Estrogen stimulation induces HIF-P4H-1 but not HIF-P4H-2 or -3 in breast carcinoma cells (Seth et al. 2002, Zhang et al. 2009), whereas HIF-P4H-3 has been identified as a gene induced by p53 (Madden et al. 1996), by stimuli inducing smooth muscle differentiation (Plisov et al. 2000, Wax et al. 1994) and by nerve growth factor removal (Lipscomb et al. 2001).

2.3.9 FIH in cells and tissues

The data available on the in vivo role of FIH is limited. Based on EST data, FIH appears to be ubiquitously expressed (Mahon et al. 2001). More recently FIH protein has been shown to be widely distributed in the central nervous system in rat (Fukuba et al. 2008). Within the cell, FIH is mainly cytoplasmic with little if any staining visible in the nucleus (Fukuba et al. 2008, Linke et al. 2004, Metzen et al. 2003a). Treatment with a hypoxia mimic did not alter the subcellular localization of either endogenous or overexpressed FIH, suggesting that FIH does not translocate to the nucleus under hypoxic conditions (Linke et al. 2004). More recently, however, FIH has been reported to localize to the nucleus (Tan et al. 2007) and in the perinuclear region (Sakamoto & Seiki 2009), and cytoplasmic localization of FIH has been associated with enhanced hypoxic response and tumor aggressiveness (Tan et al. 2007). The perinuclear localization of FIH is dependent on the presence of the newly identified FIH interacting protein Mint3 (Sakamoto & Seiki 2009). In a rat hypoxia-induced pulmonary hypertension model, a decline was observed in FIH protein in lung tissues from seven days of hypoxia onwards, suggesting downregulation of FIH in response to chronic hypoxia (Fu et al. 2008).
2.3.10 HIF-P4Hs in disease and knockout mouse models

HIF-P4H-1

Loss of HIF-P4H-1 activity in mice leads to lowered oxygen consumption in skeletal muscle and protects myofibers against ischemic injury by preventing excess oxidative damage. This is achieved by reprogramming glucose metabolism from oxidative to more anaerobic ATP production through activation of a Ppara pathway leading to upregulation of Pdk4, which restricts the entry of glycolytic intermediates into the TCA cycle. (Aragones et al. 2008.) In a similar fashion, deficiency of HIF-P4H-1 in mice provides hepatocytes with tolerance to acute hypoxia and protects them against I/R injury by decreasing the production of oxidative stress through reduced oxygen consumption resulting from reprogrammed hepatocellular metabolism (Schneider et al. 2010). RNA interference-mediated knockdown of HIF-P4H-1 has been shown to prevent also normoxic oxidative death in neuronal cells independently of HIF-1, HIF-2 or CREB in vitro (Siddiq et al. 2009).

It has been reported very recently that loss of HIF-P4H-1 in mice decreases cyclin D1 mRNA and protein levels, causing impaired cell proliferation and decreased breast tumorigenesis (Zhang et al. 2009). The regulation of cyclin D1 and cell proliferation by HIF-P4H-1 was shown to be dependent on HIF-P4H-1 catalytic activity, yet independent of HIF (Zhang et al. 2009). In Drosophila, deficiency of the single HIF-P4H has been shown to suppress the growth but not the proliferative function of CycD/Cdk4, suggesting a role for HIF-P4H as a regulator of cellular growth and a key mediator for CycD/Cdk4 (Frei & Edgar 2004). Together these studies propose an additional role for HIF-P4Hs in controlling cell proliferation in a HIF-independent fashion.

HIF-P4H-2

Germ-line inactivation of HIF-P4H-2 in mouse is embryonally lethal and leads to development of severe placental and cardiac defects and embryonic death between E12.5 and E14.5 (Takeda et al. 2006), indicating an absolute requirement for HIF-P4H-2 in normal embryonic development. Very recently, this phenotype was reproduced by knockdown of HIF-P4H-2 through in vivo injection of one-cell murine zygotes with lentivirus-containing RNAi, and the observed defects
were suggested to be associated with an induction in insulin-like growth factor binding protein 1 (Igfbp1) in the placenta (Ozolins et al. 2009).

Conditional loss-of-function (conditional knockout, CKO) studies have defined an essential role for HIF-P4H-2 in the regulation of oxygen homeostasis of the adult vascular system (Minamishima et al. 2008, Takeda et al. 2007, 2008), as conditional broad-spectrum inactivation of HIF-P4H-2 leads to hyperactive angiogenesis and angiectasia in multiple organs in adult mice, whereas mice deficient in either HIF-P4H-1 or HIF-P4H-3 show no obvious angiogenic defects (Takeda et al. 2007). Additionally, HIF-P4H-2 inhibition by siRNA has been shown to effectively induce neoangiogenesis through the coordinate regulation of expression of multiple angiogenic growth factors in a HIF-dependent fashion (Wu et al. 2008). Furthermore, somatic inactivation of HIF-P4H-2 results in severe erythrocytosis associated with significant increases in hematologic parameters, a drastic increase in serum EPO levels and increased extramedullar hematopoietic activity (Minamishima et al. 2008, Takeda et al. 2008). The HIF-P4H-1/3 double-knockout mice exhibit a moderate increase in hematocrit values, but even the combined inactivation of these isoenzymes is not sufficient to cause erythrocytosis comparable to that following the loss of HIF-P4H-2 (Takeda et al. 2008). HIF-P4H-2 CKO mice die prematurely 70–90 days after the tamoxifen-induced gene knockout of congestive heart failure most likely developed on the basis of severe polycythemia and hyperviscosity of the blood (Minamishima et al. 2008, Takeda et al. 2008). However, the possibility of the heart failure being a direct consequence of the cardiac lack of HIF-P4H-2 cannot be ruled out and necessitates further studies. Interestingly, germ-line mutations leading to a marked decrease in HIF-P4H-2 enzyme activity have been described in the human HIF-P4H-2 gene in patients presenting familial erythrocytosis, further highlighting the role of HIF-P4H-2 as the major regulator of erythropoiesis (Percy et al. 2006, 2007). In contrast, HIF-P4H-2 has also been reported to reduce proliferative responses of human vascular smooth muscle cells (VSMC) to growth factors including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF-2) in a HIF-dependent manner (Schultz et al. 2009), and to suppress hypoxia-induced endothelial cell (EC) proliferation through a mechanism independent of the hydroxylase function (Takeda & Fong 2007).

The role of HIF-P4H-2 in malignant progression remains controversial. HIF-P4H-2 has been proposed to function as a tumor suppressor, but contrasting reports exist as well. In a screen for genetic mutations in endometrial cancers and endometrial cancer cell lines, 60% of the samples studied displayed structural
alterations (missense mutations or large deletions) in the HIF-P4H-2 gene that were not found in their normal counterparts. Furthermore, expression of the wild-type HIF-P4H-2 in mutated cancer cells induced senescence by negatively regulating HIF-1 expression, supporting the tumor-suppressor function of HIF-P4H-2. (Kato et al. 2006.) In another study HIF-P4H-2 was shown to negatively regulate tumor angiogenesis by limiting the NFκB-dependent secretion of two angiogenic factors, interleukin-8 (IL-8) and angiogenin (ANG), in a HIF-independent fashion. In line with this data, analysis of various human cancer cell lines showed decreased levels of HIF-P4H-2 correlating with an increase in mature blood vessels. In addition, immunohistochemical analysis of breast tumor samples revealed lower levels of HIF-P4H-2 in the tumor tissue compared to the matched normal tissue in 6/11 samples, implicating the clinical relevance of the loss of HIF-P4H-2. (Chan et al. 2009.) Moreover, a novel mutation leading to a reduction in enzyme activity was recently identified in the human HIF-P4H-2 gene in a patient with erythrocytosis and recurrent paraganglioma, a highly vascularized tumor, which displayed loss of heterozygocity of the wild-type HIF-P4H-2 (Ladroue et al. 2008). A biphasic role in modulating tumor-forming potential was suggested for HIF-P4H-2 by another report, as a modest decrease in HIF-P4H-2 was shown to lead to malignant transformation, while severe loss of HIF-P4H-2 function did not increase tumor-forming potential. In addition, overexpression of HIF-P4H-2 in malignant fibroblasts rescued the tumorigenic phenotype. (Lee et al. 2008.) On the contrary, haplo deficiency of HIF-P4H-2 was recently shown to normalize vessel maturation in tumor vessels, leading to improved tumor perfusion and oxygenation, and subsequent inhibition of invasion and metastasis in a HIF-1-dependent manner, suggesting that HIF-P4H-2 acts to create a dysfunctional tumor vasculature and to accelerate metastatic progression (Mazzone et al. 2009).

**HIF-P4H-3**

HIF-P4H-3 activity is required for the development of a normal sympathoadrenal system in mice, and the loss of HIF-P4H-3 results in an adrenergic phenotype characterized by alterations in blood pressure and cardiac contractility (Bishop et al. 2008). Although the mice lacking HIF-P4H-3 display no polycythemia (Takeda et al. 2008), the concurrent loss of HIF-P4H-3 in cells and tissues lacking HIF-P4H-2 enhances HIF-α accumulation and increases activation of HIF target genes, and exacerbates the phenotype observed in HIF-P4H-2 null mice, leading
to severe hepatic steatosis and worsening of the dilated cardiomyopathy and premature mortality (Minamishima et al. 2009). Interestingly, not all aspects of the phenotype observed in HIF-P4H-2 null mice are exacerbated by the loss of HIF-P4H-3, reflecting possible differences in the degree of compensation by HIF-P4H-3 for different HIF targets in different tissues (Minamishima et al. 2009). Given that the expression of HIF-P4H-3 was shown to be decreased in human colorectal cancer and that decreased HIF-P4H-3 expression was associated with higher tumor grade, also HIF-P4H-3 has recently been suggested to function as a tumor suppressor, however independently of its catalytic activity (Xue et al. 2010).

2.3.11 Therapeutic possibilities associated with inhibition of the HIF pathway

From the rapidly increasing number of reports on the pathophysiological role of HIF it has become clear that the HIF hydroxylases are an attractive therapeutic target. Since pharmacological inhibition of HIF-P4Hs has been shown to result in subsequent increases in HIF-target genes (Ivan et al. 2002), inhibition of the HIF-P4Hs is regarded as a promising way of promoting beneficial processes in diseases such as anemia, ischemic heart disease and stroke, while the controlled activation of these enzymes may restrain HIF-dependent responses in tumorigenesis. (For reviews, see Fraisl et al. 2009, Myllyharju 2008, Semenza 2010.) However, as already discussed above, some data indicate that stabilization of HIF-1α via inhibition of the HIF-P4Hs may also confer beneficial effects in some cancer types (Gordan et al. 2008, Mazzone et al. 2009). More in-depth studies are thus needed to clarify the effect of HIF-P4H inhibition/activation in cancers. The most effective inhibitors identified to date for the HIF-P4Hs are 2-oxoglutarate analogs (Epstein et al. 2001, Hewitson & Schofield 2004, Hirsilä et al. 2003), which are also very potent inhibitors of the C-P4Hs (for a review, see Myllyharju 2005). Development of novel inhibitors targeting the HIF hydroxylases and specific isoenzymes among the HIF-P4Hs has thus gained considerable interest in recent years. (For reviews, see Fraisl et al. 2009, Hewitson & Schofield 2004, Myllyharju 2008, Ratcliffe 2006, Schofield & Ratcliffe 2004.)

Recombinant human EPO is currently used for treating anemias associated primarily with renal disease and cancer (for a review, see Bunn 2008). Several lead compounds developed by the biotechnology-based drug discovery company
Fibrogen Inc. have been shown to inhibit HIF-P4Hs and effectively correct anemia and improve iron utilization in rodent models of anemia without promoting tumor progression. Furthermore, induction of erythropoiesis and increased hemoglobin concentrations have been achieved in anemic human patients suffering from chronic kidney disease in two phase 2 studies. Clinical trials are currently under way to assess the safety and efficacy of these compounds in different types of anemias. (For reviews, see Bunn 2008, Myllyharju 2008.) It is of interest that treatment of healthy mice with the same HIF-P4H inhibitor that was previously shown to augment EPO plasma levels in normal mice in vivo (Hsieh et al. 2007), was recently shown to lead to lasting improvement of hippocampal memory (Adamcio et al. 2010), suggesting that HIF stabilization may also be exploited to improve cognition.

Ischemic diseases such as myocardial infarction and stroke are among the most common causes of death in Western countries. Genetic or pharmacological inhibition of HIF-P4Hs has been shown to protect several organs against ischemia-reperfusion (I/R) injury (Aragones et al. 2008, Bernhardt et al. 2006, Hill et al. 2008, Schneider et al. 2010). Genetic deficiency of HIF-P4H-1 reduces the extent of oxidative damage during I/R in skeletal muscle and liver (Aragones et al. 2008, Schneider et al. 2010) and siRNA-mediated silencing of HIF-P4H-2 improves myocardial ischemic preconditioning (IP) (Eckle et al. 2008) in mouse models. Furthermore, pretreatment of tissues with HIF-P4H inhibitors reduces ischemia-induced injury in the brain (Baranova et al. 2007, Bergeron et al. 2000, Sarco et al. 2000, Siddiq et al. 2005) and kidney (Bernhardt et al. 2006, Rosenberger et al. 2008). In addition to alleviating the irreversible I/R injury, pharmacological inhibition of HIF-P4Hs enhances angiogenesis and therapeutic revascularization in vivo (Knowles et al. 2004, Loinard et al. 2009, Milkiewicz et al. 2004). Furthermore, HIF-1α gene transfer has been shown to improve functional recovery of ischemic tissues (Kido et al. 2005, Shyu et al. 2002, Vincent et al. 2000).

HIF-1 plays an important role also in the inflammatory response, and manipulation of the HIF pathway thus represents an option for the treatment of inflammatory diseases as well (for a review, see Zinkernagel et al. 2007). Inhibition of the HIF-P4Hs could prove therapeutic in inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease, as stabilization of HIF-α has been shown to be protective against mucosal inflammation, while a reduction in HIF-1 activity exacerbated the symptoms in a murine model of colitis (Karhausen et al. 2004). Moreover, pharmacological inhibition of the HIF-P4Hs
had a profoundly beneficial influence on clinical symptoms in two mouse models of colitis via reducing the levels of critical pro-inflammatory cytokines (Cummins et al. 2008, Robinson et al. 2008).

2.4 Collagen prolyl 4-hydroxylases

Collagens are the most abundant proteins in mammals, making up the majority of the extracellular matrix. Collagens act to maintain the integrity and architecture of tissues and provide them with mechanical strength. They are also involved in regulation of cell signalling, proliferation, migration and apoptosis (for reviews, see Gelse et al. 2003, Myllyharju & Kivirikko 2004, and Ortega & Werb 2002). Collagens were the first proteins in which 4-hydroxyproline was identified, and the 4-hydroxylation of the proline residues in the –X-Pro-Gly- repeats is required for the stabilization of the collagen triple helix at normal body temperature. Collagen prolyl 4-hydroxylases (C-P4Hs) are 2-oxoglutarate and Fe(II)-dependent dioxygenases located within the lumen of the endoplasmic reticulum (ER), that catalyze the formation of 4-hydroxyproline residues in –X-Pro-Gly-triplets in more than 40 different polypeptide chains in the 29 distinct collagen types and proteins containing collagen-like domains. (For reviews, see Myllyharju 2003, Myllyharju & Kivirikko 2004, and Myllyharju 2008.)

2.4.1 Molecular and catalytic properties of C-P4Hs

C-P4Hs have been characterized both in vertebrates and invertebrates. To date, three vertebrate C-P4H isoenzymes have been cloned from human, mouse, rat and chicken (Annunen et al. 1997, Bassuk et al. 1989, Helaakoski et al. 1989, Helaakoski et al. 1995, Hopkinson et al. 1994, Kukkola et al. 2003, Van Den Diepstraten et al. 2003). Mammalian C-P4Hs are α2β2 tetramers, the α subunit being the catalytically active subunit and the β subunit being identical to the protein disulfide isomerase (PDI) (Koivu et al. 1987, Pihlajaniemi et al. 1987). All three α subunit isoforms dimerize with the same β subunit, forming [α(I)]2β2, [α(II)]2β2 and [α(III)]2β2 tetramers named C-P4Hs I, II and III, respectively. Mixed tetramers with different types of human α subunits are not formed in vitro (Annunen et al. 1997). (For reviews, see Myllyharju 2003, Myllyharju & Kivirikko 2004, Myllyharju 2008.) The functional role of PDI as the β subunit of the C-P4H tetramers is to keep the highly insoluble α subunit in a catalytically active, non-aggregated conformation, and to mediate the retention of the enzyme
tetramer within the lumen of the ER via its C-terminal retention signal (Vuori et al. 1992a, Vuori et al. 1992b).

The human α(I)-(III) subunits consist of 517, 514 and 525 residues, respectively, all three of them comprising a signal peptide of 17–21 additional residues (Annunen et al. 1997, Helaakoski et al. 1989, Kukkola et al. 2003, Van Den Diepstraten et al. 2003). The overall amino acid sequence similarity between the human α(I) and α(II) subunits is 65%, while the sequence identities between the α(I) and α(III) and between α(II) and α(III) are 35–37%, the highest degree of identity being found in the C-terminal regions containing the catalytically important residues (Annunen et al. 1997, Kukkola et al. 2003). The Fe$^{2+}$ is bound by three residues in a conserved –His-X-Asp–…-His-motif, while the C5 carboxyl group of 2-oxoglutarate is bound by a lysine (Myllyharju & Kivirikko 1997). A peptide-substrate–binding domain consisting of residues Phe-144-Ser-244 in α(I) is found in the N-terminal region of the human α(I)-(III) subunits (Myllyharju & Kivirikko 1999). Alternative splicing generates two widely expressed forms of human α(I) and α(II) subunits, while no alternative splicing of the α(III) transcript has been reported (Kukkola et al. 2003, Nokelainen et al. 2001).

The catalytic properties of the three vertebrate C-P4Hs are very similar (see Table 2), but the existence of some distinct differences in their $K_m$ and $K_i$ values suggests variability in the structure of the peptide-substrate-binding domain between the isoenzymes (Annunen et al. 1997, Helaakoski et al. 1995, Kukkola et al. 2003). The minimum sequence requirement for vertebrate C-P4Hs is fulfilled by a –X-Pro-Gly– triplet, although the $K_m$ values for short peptides containing a single hydroxylatable unit are very high and decrease markedly with an increase in the number of repeated –X-Pro-Gly– triplets (for reviews, see Kivirikko et al. 1992 and Kivirikko & Pihlajaniemi 1998). Although the HIF-P4Hs and C-P4Hs require the same cosubstrates and are likely to have identical reaction mechanisms, differences in the $K_m$ and $K_i$ values for the cosubstrates and inhibitors (compare Tables 1 and 2) indicate distinct differences in the structures involved in the binding of cosubstrates. The most striking difference is seen in the $K_m$ values of HIF-P4Hs and C-P4Hs for oxygen: in line with their proposed role as effective oxygen sensors, HIF-P4Hs have a much higher $K_m$ value for $O_2$ compared to the C-P4Hs, which in turn have to retain activity even at poorly oxygenated sites during processes such as wound healing, and consequently have a higher affinity for $O_2$. 

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Table 2. *K*~m~, *K*~i~, and IC~50~ values of C-P4Hs I, II and III for reaction cosubstrates, a peptide substrate and certain competitive inhibitors.

<table>
<thead>
<tr>
<th>Cosubstrate, substrate or inhibitor</th>
<th>Constant</th>
<th>C-P4H-I μM</th>
<th>C-P4H-II μM</th>
<th>C-P4H-III μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe^2+</td>
<td><em>K</em><del>m</del></td>
<td>2^a</td>
<td>2^a</td>
<td>0.5^a</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td><em>K</em><del>m</del></td>
<td>20^a</td>
<td>22^a</td>
<td>20^a</td>
</tr>
<tr>
<td>Ascorbate</td>
<td><em>K</em><del>m</del></td>
<td>300^a</td>
<td>340^a</td>
<td>370^a</td>
</tr>
<tr>
<td>O₂</td>
<td><em>K</em><del>m</del></td>
<td>40^a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(Pro-Pro-Gly)₁₀</td>
<td><em>K</em><del>m</del></td>
<td>18^a</td>
<td>95^a</td>
<td>20^a</td>
</tr>
<tr>
<td>Pyridine 2,4-dicarboxylate</td>
<td><em>K</em><del>i</del></td>
<td>2^b</td>
<td>1^b</td>
<td>ND</td>
</tr>
<tr>
<td>Pyridine 2,5-dicarboxylate</td>
<td><em>K</em><del>i</del></td>
<td>0.8^a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3-Hydroxypridine-2-carbonyl-glycine</td>
<td>IC<del>50</del></td>
<td>0.4^b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td><em>K</em><del>i</del></td>
<td>5^b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Poly(L-proline), Mr 5000–7000</td>
<td>IC<del>50</del></td>
<td>6^c</td>
<td>300^d</td>
<td>30^d</td>
</tr>
</tbody>
</table>

ND, not determined


2.4.2 In vivo properties of C-P4Hs

While C-P4H-I is the main form in most cell types and tissues, C-P4H-II accounts for 70–80% of the total C-P4H activity in cultured mouse chondrocytes and cartilage, respectively, and represents the major C-P4H isoform in chondrocytes, osteoblasts and endothelial cells (Annunen et al. 1998, Nissi et al. 2001). α(III) mRNA is expressed in many adult and fetal human tissues, but at much lower levels than the α(I) and α(II) subunit mRNAs (Kukkola et al. 2003). Knockout mouse studies have demonstrated an absolute requirement for C-P4H activity in normal embryonic development, as the C-P4H-I null mice die in utero at E10.5 and display an overall developmental delay and rupture of the basement membranes due to a lack of collagen IV (Holster et al. 2007). In accordance with C-P4H-I being the main C-P4H isoform in majority of tissues, C-P4H-II null mice are viable and fertile with no obvious phenotypic abnormalities (for reviews, see M yllyharju & Kivirikko 2004, M yllyharju 2008).
3 Outlines of the present study

As several in vitro studies had indicated HIF-P4H-2 as the most important HIF-P4H isoform in regulating the normoxic HIF-α stabilization and as it was already known from a previous report that complete lack of HIF-P4H-2 in mouse leads to embryonic lethality, we set out to generate a mouse line in which the HIF-P4H-2 gene is disrupted by a Genetrap insertion cassette containing the β-galactosidase reporter, aiming at characterizing the in vivo expression pattern of HIF-P4H-2 by X-gal staining of tissues of heterozygous mice. However, very early on in our study we observed that heterozygous matings of these mice produced a small percentage of viable pups that had two mutated alleles. We were able to show that instead of being complete HIF-P4H-2 knockouts, these mice were hypomorphs producing varying amounts of wild-type HIF-P4H-2 mRNA in different tissues, the lowest levels, only 8%, being found in the heart. We therefore refocused our study to i) characterize the role of HIF-P4H-2 in the heart and to explore its role in the preconditioning phenomenon in the heart.

Until the identification and subsequent characterization of the family of HIF-P4Hs in 2001, 4-hydroxyproline had only been found in collagens and collagen-like proteins, and the family of C-P4Hs had been extensively studied. We identified a novel human C-P4H-like polypeptide in a database search. The novel P4H did not, however, show any homology to the distinct peptide substrate binding domain present in the identified C-P4Hs and contained a predicted transmembrane domain. We set out ii) to clone and characterize the novel P4H, which we named P4H-TM. Interestingly, based on database searches, a P4H-TM homolog was found in zebrafish, Danio rerio, but not in invertebrates C. elegans or D. melanogaster. Given the many advantages of using zebrafish as a model organism, including the feasibility of gene knockdown in zebrafish embryos, we decided to broaden our study to iii) characterize the in vivo role of P4H-TM in zebrafish embryos.

Studies on the catalytic properties of HIF-P4Hs and FIH have demonstrated distinct differences in their reaction kinetics. Moreover, the $K_m$ values of HIF-P4Hs for the HIF-substrate had been reported to markedly decrease with the use of longer recombinant polypeptide substrates instead of synthetic peptides. At the same time, a novel expanding group of substrates had been identified for FIH. Notch1-3 were among the first ARD-containing proteins shown to be efficiently hydroxylated by FIH. We set out iv) to study the effects of substrate sequence
composition and length and the concentration of oxygen on the binding and hydroxylation of Notch1-4 and HIF-1α by FIH.
4 Materials and methods

The materials and methods used in this thesis are summarized in the table below. Detailed descriptions with references can be found in the original articles I-IV.

Table 3. Methods.

<table>
<thead>
<tr>
<th>Level</th>
<th>Method</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Cloning techniques</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>Southern Blot analysis</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Rapid amplification of cDNA ends (RACE)</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Site-directed mutagenesis</td>
<td>IV</td>
</tr>
<tr>
<td>RNA</td>
<td><em>In situ</em> hybridization</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Northern Blot analysis</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>RNA isolation, RT-PCR and Quantitative real-time PCR</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td>Microarray analysis</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>siRNA</td>
<td>II</td>
</tr>
<tr>
<td>Protein</td>
<td>Expression and analysis of recombinant proteins in mammalian, insect and bacterial cells</td>
<td>II, IV</td>
</tr>
<tr>
<td></td>
<td>SDS-PAGE and Western blotting</td>
<td>I, II, IV</td>
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<tr>
<td></td>
<td>Enzyme activity assays</td>
<td>II, IV</td>
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<tr>
<td></td>
<td>Metal chelate affinity chromatography</td>
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5 Results

5.1 Hearts of HIF-P4H-2 hypomorphic mice show protection against acute ischemia-reperfusion injury (I)

5.1.1 HIF-P4H-2 hypomorphic mice are viable and express varying levels of wild-type HIF-P4H-2 mRNA in different tissues

To study the in vivo expression and role of HIF-P4H-2, we generated a mouse line having their HIF-P4H-2 gene disrupted in intron 1 by a GeneTrap insertion cassette containing a β-galactosidase reporter (Figure 1A in I). Surprisingly, genotyping of the pups from heterozygous matings (n = 470) indicated that 11.7% of the offspring completely lacked the wild-type HIF-P4H-2 allele and were thus named HIF-P4H-2\(2^{gt/gt}\) (gt stands for gene-trapped allele), while 28.9% were HIF-P4H-2\(2^{+/+}\) and 59.4% HIF-P4H-2\(2^{gt/+}\). The percentage of HIF-P4H-2\(2^{gt/gt}\) mice was lower than expected for normal Mendelian inheritance, but otherwise the HIF-P4H-2\(2^{gt/gt}\) mice appeared to be healthy and fertile and to have a normal lifespan. RT-PCR of their heart RNA showed that a small amount of wild-type HIF-P4H-2 mRNA was generated from the gene-trapped alleles (Figure 1C in I), indicating that recognition of the splicing acceptor sequence in the insertion cassette was not absolute. The lowest relative amounts of wild-type HIF-P4H-2 mRNA in HIF-P4H-2\(2^{gt/gt}\) tissues were seen in the heart and skeletal muscle, 8% and 15%, respectively, the values being 34–47% in the kidney, spleen, lung and bladder, 60% in the brain and 85% in the liver (Figure 3A in I). Western blot analysis showed corresponding decreases in the amounts of HIF-P4H-2 protein accompanied by a concordant stabilization of HIF-1\(\alpha\) and HIF-2\(\alpha\) in the heart, skeletal muscle and kidney (Figure 3B-C in I).

5.1.2 HIF-P4H-2\(2^{gt/gt}\) mice display no histologic or hematologic abnormalities

X-Gal staining, performed to study the HIF-P4H-2 promoter-driven β-galactosidase expression, showed that HIF-P4H-2 is ubiquitous in embryos at E12.5 and widely expressed in various adult tissues, with particularly intense staining in the heart (Figure 2 in I). Analysis of blood samples from the HIF-P4H-2\(2^{gt/gt}\) mice revealed no increase in the hematocrit, red blood cell or hemoglobin
values (Figure 4 in I). The mean serum EPO level was increased about 2-fold and there was a trend for an increase in the kidney EPO mRNA level in the HIF-P4H-2<sup>gt/gt</sup> mice, but these changes were not statistically significant nor sufficient to lead to increased erythropoiesis (Figure 4 in I). The HIF-P4H-2<sup>gt/gt</sup> hearts were similar in weight to the wild-type hearts, and histological analysis of H&E-stained sections showed no detectable alterations relative to the controls (Figure 5A in I). Likewise, no differences were observed in the number or size of blood vessels in anti-Pecam-1-stained sections of HIF-P4H-2<sup>gt/gt</sup> hearts relative to the wild-type (Figure 5B in I), despite the reported hyperactive angiogenesis in the heart and other organs of 6-week-old mice with conditional broad-spectrum HIF-P4H-2 inactivation (Takeda et al. 2007). In line with this, statistically significant differences were not found in the serum VEGF-A concentration, either. Transmission electron microscopy analysis revealed no differences in the ultrastructure of the capillaries, myofilaments or mitochondria in the HIF-P4H-2<sup>gt/gt</sup> hearts relative to the wild-type (Figure 5C in I). Mild mitochondrial swelling has been observed in hearts of 10-week-old conditional HIF-P4H-2 null mice three weeks after tamoxifen treatment (Minamishima et al. 2009). We found that the mean mitochondrial area was also increased in the HIF-P4H-2<sup>gt/gt</sup> hearts.

5.1.3 Several HIF target genes are upregulated in HIF-P4H-2<sup>gt/gt</sup> hearts

To analyze the consequences of the lack of HIF-P4H-2 and the subsequent stabilization of HIF-1α and HIF-2α in the HIF-P4H-2<sup>gt/gt</sup> hearts, qPCR analysis of mRNA levels of select known HIF targets and some other genes was performed. HIF-1α and HIF-2α mRNA levels were unchanged in HIF-P4H-2<sup>gt/gt</sup> hearts (Figure 6 in I), confirming that the increases in the respective protein levels were indeed due to protein stabilization. Significant increases were observed in the mRNA levels of several glucose metabolism genes including glucose transporter 1 (Glut-1), phosphofructokinase L (PfkL), triosephosphate isomerase (Tpi), and phosphoglycerate kinase 1 (Pgk-1), and a trend for an increase was found in the mRNA level of enolase 1 (Eno-1) (Figure 6 in I). The mRNA level of pyruvate dehydrogenase kinase 1 (Pdk-1) was also increased, and significant increases were additionally seen in the mRNA levels of angiopoietin 2 (Ang-2) and adrenomedullin (Adm). Apelin, the apoptosis-inducing protein Bnip-3 and Redd-1 all showed a trend for increased mRNA expression. The mRNA levels of several known HIF targets, including the genes encoding Cd73, the key enzyme for
extracellular adenosine generation, and A2b adenosine receptor (A2bar), which were markedly increased after acute stabilization of HIF-1α following left-ventricular HIF-P4H-2 siRNA (Eckle et al. 2008), and Pdk-4 and Ppara, which were increased in the skeletal muscle of HIF-P4H-1−/− mice (Aragones et al. 2008), were unchanged in HIF-P4H-2−/− hearts (Figure 6 in I). Microarray analysis identified no additional genes with significantly changed mRNA levels. By Western blotting we showed that also the protein levels of Glut-1 and Pdk-1 were increased in HIF-P4H-2−/− hearts relative to wild-type (Figure 6 in I).

5.1.4 HIF-P4H-2−/− mice show cardioprotection against acute ischemia-reperfusion injury

To study whether the chronic cardiac stabilization of HIF-1α and HIF-2α provides cardioprotection against ischemia-reperfusion (I/R) injury, isolated Langendorff-perfused hearts from HIF-P4H-2−/− and wild-type mice were subjected to a ischemia-reperfusion protocol including a stabilization perfusion of at least 30 min followed by a 20-min global ischemia and reperfusion of 45 min (Figure 7A in I). Prior to ischemia, no differences were observed in the hemodynamic parameters between HIF-P4H-2−/− and wild-type hearts (Figure 7B in I). Furthermore, the heart rates in HIF-P4H-2−/− and wild-type hearts were similar during the whole 45 min reperfusion period (Figure 7C in I). However, during reperfusion, the rate pressure product (RPP = heart rate x left ventricle developed pressure (LVDP)) and dP/dt max (the maximal rate of rise of the left ventricular pressure) returned to and even exceeded the pre-ischemic level in the HIF-P4H-2−/− hearts, while the wild-type hearts reached the pre-ischemic levels only momentarily in early reperfusion before falling to a level of about 65% of the pre-ischemic one (Figure 7D-E in I). The preservation and surpassing of the pre-ischemic RPP and dP/dt max levels following the ischemic insult clearly demonstrated a markedly improved mechanical recovery in HIF-P4H-2-deficient hearts (p < 0.01). Recovery of the coronary flow rate was also significantly improved in HIF-P4H-2−/− hearts (Figure 7F in I). Conversely, the cumulative release of lactate dehydrogenase during reperfusion – an indirect marker of infarct size – was significantly reduced in HIF-P4H-2−/− hearts (Figure 7G in I). Taken together, these data provide solid evidence that chronic cardiac HIF-P4H-2 deficiency provides protection against acute myocardial ischemia-reperfusion injury.
In order to understand in more detail the mechanism underlying cardioprotection in HIF-P4H-2^gt/gt hearts, we analyzed the level of HIF-1α stabilization at end-ischemia and after I/R. HIF-1α was stabilized to a similar extent in both wild-type and HIF-P4H-2^gt/gt hearts at the end of the 20-min global ischemia (end-ischemia), but the HIF-1α was almost completely degraded during the 45-min reperfusion in wild-type hearts, while its amount was unchanged and remained at a high level in HIF-P4H-2^gt/gt hearts (Figure 8A in I). After I/R, the mRNA levels of the genes that had been significantly induced transcriptionally in the pre-ischemic HIF-P4H-2^gt/gt hearts relative to wild type, were also increased in wild-type hearts in all cases except for the phosphofructokinase L and angiopoietin 2 genes (Figure 8B in I), demonstrating that the mRNA expression changes of genes contributing to the cardioprotection in the HIF-P4H-2^gt/gt hearts must precede the ischemic insult. We also determined the amounts of certain key metabolites in HIF-P4H-2^gt/gt and wild-type hearts in pre-ischemia and at end-ischemia. The amount of lactate was significantly increased in the pre-ischemic HIF-P4H-2^gt/gt hearts relative to wild-type (Figure 8C in I), while the lactate levels at end-ischemia were equal in both genotypes. End-ischemic ATP concentration in the HIF-P4H-2^gt/gt hearts was 69% of the pre-ischemic value, while being 60% in wild-type hearts (Figure 8C in I). No differences were seen in the amount of total ADP in HIF-P4H-2^gt/gt and wild-type hearts in pre-ischemia or at end-ischemia. Finally, the end-ischemic vs. pre-ischemic ratio of creatine phosphate to creatine (CrP/Cr) concentration was significantly higher in HIF-P4H-2^gt/gt than in wild-type hearts (Figure 8C in I). Collectively, these data indicate that the cellular energy state is retained at a higher level in HIF-P4H-2^gt/gt hearts during ischemia, which is likely to have substantially contributed to the cardioprotection observed in HIF-P4H-2^gt/gt hearts. Additionally, the transcriptional induction of several other genes encoding proteins that have been shown to mediate potentially cardioprotective effects most probably add to the cardioprotection observed in HIF-P4H-2^gt/gt hearts.
5.2 Cloning and characterization of the in vitro and in vivo roles of a novel ER transmembrane prolyl 4-hydroxylase, P4H-TM (II, III)

5.2.1 Cloning, expression and analysis of recombinant human P4H-TM and identification of its zebrafish homolog

A gene bank search with C-P4H α(I) as bait identified an EST for a novel P4H-like polypeptide of 502 residues that contained no signal peptide but a predicted transmembrane domain spanning residues 59–82 (Figure 1 in II), and the novel polypeptide was termed P4H-TM. While our work was in progress, a short paper reported the identification and initial characterization of the same enzyme (Oehme et al. 2002). Comparison of the full-length amino acid sequences revealed relatively low sequence identity between the human P4H-TM and the C-P4H α(I)- α(III) subunits and HIF-P4Hs (14–15% and 10–13%, respectively). In accordance, Oehme and coworkers reported 12–13.2% sequence similarity between P4H-TM and C-P4H α(I) and α(II) subunits, respectively (Oehme et al. 2002). The identity within the catalytically important C-terminal region was 26–28% compared to C-P4Hs, while being 13–15% compared to HIF-P4Hs (Figure 1 in II). Similar to the C-P4Hs, the residue binding the C-5 carboxyl group of 2-oxoglutarate in P4H-TM is a lysine, while it is an arginine in the HIF-P4Hs (Bruick & McKnight 2001, Epstein et al. 2001, Ivan et al. 2002, Myllyharju & Kivirikko 1997). However, P4H-TM lacks the distinct peptide-substrate-binding domain present in the C-P4Hs. The C-terminus of the human P4H-TM comprises a functional variant of an ER retention signal (-Arg-Val-Glu-Leu), and P4H-TM also contains two potential N-glycosylation sites.

Based on database searches, P4H-TM is also found in other vertebrates, the homology between human and mouse P4H-TMs being 91% (Figure 1 in III). Zebrafish, Danio rerio, encodes a 487-amino acid polypeptide with 51% identity to the human P4H-TM sequence (Figure 1 in III) (Acc. no. ENSDART00000012024.3), the highest degree of sequence identity being again found in the catalytic C-terminus (e.g. 76% sequence identity between residues 312–487) (Figure 1 in III) and the catalytically important amino acids being conserved (Figure 1 in III). The lowest degree of sequence identity is found in the short cytosolic N-terminal part (14%), while the transmembrane domain is 62% identical between human and zebrafish (Figure 1 in III). Invertebrates C. elegans and D. melanogaster, whose genomes have been fully sequenced, do not encode P4H-TM homologs, suggesting that P4H-TM may only be found in vertebrates.
Analysis of the recombinant human P4H-TM showed that in addition to the full-length polypeptide, P4H-TM exists as a cleaved isoform lacking the transmembrane domain. Insect and mammalian cells were used to express the recombinant P4H-TM, and in both cell types the cleaved as well as the full-length P4H-TM were found to be N-glycosylated and to form homodimers in solution (Figure 3 in II). Immunofluorescence analysis indicated that P4H-TM is a cytoplasmic protein and immunoelectron microscopy localized P4H-TM in the endoplasmic reticulum (ER) membranes in an orientation in which the catalytic site resides inside the ER lumen (Figure 5 in II). This location is in agreement with our data indicating N-glycosylation of the polypeptide and the presence of the ER retention signal variant in P4H-TM, as well as with the co-localization of P4H-TM with an ER-marker in immunohistochemistry as reported by Oehme et al. (Oehme et al. 2002).

5.2.2 P4H-TM is widely expressed and induced by hypoxia

To study the mRNA expression of P4H-TM, Northern Blot analysis, RT-PCR and PCR were performed. P4H-TM mRNA was found to be expressed in many human tissues, with adult tissues showing higher levels than fetal tissues (Figure 2A-C in II). Three mRNA species of 2.7, 2.3 and 1.8 kb were seen in several tissues, the 1.8 kb species being the main form in all tissues except the brain. P4H-TM mRNA was also expressed in epiphyseal cartilage and in fibroblasts and in all tumor samples studied (Figure 2D-E in II). In agreement with our results, Oehme et al. reported P4H-TM mRNA expression in all tissues studied, with the highest levels being found in the brain, adrenal gland and kidney (Oehme et al. 2002). The mRNA expression pattern of P4H-TM thus differs markedly from those of the C-P4H α(I)-(III) mRNAs, which, for instance, show very little expression in the adult brain, where P4H-TM mRNA was expressed at high levels. Western blot analysis showed that the endogenous P4H-TM is expressed in various human cell lines with the highest normoxic expression level seen in the fibrosarcoma (HT1080) cells (Figure 4 in II). Interestingly, the amount of P4H-TM was greatly increased by hypoxia in most of the cell lines studied (Figure 4 in II), and in accordance with this, analysis of the 5’ untranslated sequence of the gene encoding human P4H-TM revealed five HREs (5’-RCGTG-3’). Immunofluorescence staining of normoxic and hypoxic HEK293 cells further supported the hypoxia-inducibility of P4H-TM. In contrast to our results, Oehme
and others saw no change in P4H-TM mRNA levels in A549 human lung carcinoma cells after hypoxic exposure (Oehme et al. 2002).

To analyze the expression of P4H-TM in the developing zebrafish and adult zebrafish tissues, real-time quantitative PCR analysis (qPCR) was carried out. Based on qPCR, P4H-TM mRNA expression is low during the first two days post-fertilization (dpf) and increases to 5-fold at 3 dpf, reaching a comparably steady level at 5 dpf (about 20-fold compared to 1 and 2 dpf) (Figure 2A in III). In the adult zebrafish, highest mRNA expression was observed in the eye and brain, where the expression of P4H-TM was 14- and 10-fold higher than in any other tissue studied and 10- and 7-fold higher than in 7 dpf whole embryo, respectively (Figure 2B in III). P4H-TM mRNA expression was the third-highest in the muscle, while the next highest expression levels were seen in heart, kidney and liver, which were comparable to each other; the lowest mRNA expression of P4H-TM was observed in intestine and gonad (Figure 2B in III). In situ hybridization of 3 dpf wild-type zebrafish embryos showed intense staining indicative of strong P4H-TM expression in the head region and in the eye (Figure 2C in III).

5.2.3 P4H-TM hydroxylates HIF-1α in vitro and regulates its stability but may also have other substrates

Circular dichroism analysis and enzyme activity assays showed that the recombinant P4H-TM was correctly folded and catalyzed uncoupled decarboxylation of 2-oxoglutarate. Therefore, a wide range of synthetic peptides were tested as potential substrates for P4H-TM in vitro in an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (Kivirikko & Myllylä 1982). The non-purified full-length P4H-TM and the purified truncated P4H-TM lacking the transmembrane domain were used as sources of enzyme. The peptides studied included collagen-like peptides such as (Pro-Pro-Gly)10, bradykinin analogs that serve as in vitro substrates for the C-P4Hs (Kivirikko & Pihjalaniemi 1998), proline-rich peptides that act as in vitro substrates for viral and plant P4Hs (Hieta & Myllyharju 2002), and various 19–20 and 35-residue HIF-α-like peptides (Hirsilä et al. 2003, Koivunen et al. 2006). None of the tested peptides served as substrates, however.

As overexpression of P4H-TM was shown to repress HIF transactivation activity in cellular reporter assays and reduce the HIF-2α protein level (Oehme et al. 2002), we co-transfected cultured human neuroblastoma Kelly cells with a reporter plasmid for the N-terminal or C-terminal region of the HIF-1α or HIF-2α
ODDD and increasing amounts of a plasmid encoding either full-length P4H-TM or HIF-P4H-2. Both enzymes dose-dependently reduced the levels of all four ODDD polypeptides with about equal efficiency (Figure 6A in II). Furthermore, when Kelly cells were transfected with siRNA targeting P4H-TM or any of the three HIF-P4Hs, increases were observed in the HIF-1α protein level (Figure 6B in II). Our data thus indicate that overexpression and silencing of P4H-TM influence the stability of HIF-1α in a manner similar to those of the HIF-P4Hs.

It was recently shown that HIF-P4Hs require long substrates for efficient hydroxylation \textit{in vitro} and have much lower $K_m$ values for recombinant HIF-1α and HIF-2α ODDDs than for short synthetic peptides (Hirsilä \textit{et al.} 2003, Koivunen \textit{et al.} 2006). We therefore decided to study whether P4H-TM hydroxylates the full-length HIF-1α polypeptide and its wild-type and mutant ODDDs produced in rabbit reticulocyte lysates as L-[2,3,4,5-3H]proline-labeled polypeptides. The full-length HIF-1α polypeptide and its ODDD served as substrates, whereas type I procollagen α(I) chain was not hydroxylated by P4H-TM (Table 1 in II). However, the hydroxylation efficiency of P4H-TM was only 20–50% of those of the HIF-P4H-2 and HIF-P4H-3, respectively. Similar to HIF-P4H-3, P4H-TM demonstrated a strong preference for the C-terminal hydroxylation site of HIF-1α ODDD, as the Pro402Ala mutant was almost as good a substrate as the wild-type ODDD, while the Pro564Ala mutant ODDD, possessing only the Pro402 hydroxylation site, gave a markedly reduced amount of 4-hydroxy[3H]proline (10%) (Table 1 in II). A low amount of 4-hydroxy[3H]proline was formed even with the Pro402,564Ala double mutant HIF-1α ODDD (Table 1 in II), indicating that P4H-TM also hydroxylated some additional proline(s) present in the HIF-1α ODDD substrate that are not recognized by the three HIF-P4Hs.

5.2.4 Knockdown of zebrafish P4H-TM during embryogenesis leads to morphological alterations and pericardial edema and changes the mRNA expression levels of select genes

To study the role of P4H-TM \textit{in vivo} we exploited the antisense morpholino (MO) oligomer technology to knock down P4H-TM expression during zebrafish development. Fertilized zebrafish eggs were injected with three independent P4H-TM sequence specific MOs, TM1-TM3 (Figure 3A in III), a 5-basepair mismatch control for TM1 (MM) or a random control (RC). To avoid the unspecific p53-mediated effects on neuronal apoptosis during early development, a p53-targeting
morpholino was coinjected with the morpholino targeting P4H-TM. TM1 prevented the initiation of translation, leading to a total lack of the P4H-TM protein. Injection of embryos with TM2 resulted in deletion of exon 2, causing a shift in the reading frame and formation of a truncated protein of 143 residues encoded by the first exon followed by 12 scrambled amino acids. TM3 targeted the intron 8 - exon 9 boundary, leading to inclusion of the intron 8 and generation of a truncated polypeptide with 5 scrambled residues following the 445 residues encoded by exons 1–8. The truncated P4H-TM polypeptide lacks the catalytically critical amino acids H457 and K467 encoded by exon 9 and is thus catalytically impaired.

Analysis of the 4 dpf TM1-injected embryos revealed a reduction in the size of the head and eye, morphological alterations in the head and severe pericardial edema compared to the MM-injected control embryos (Figure 3C in III). The phenotype of the embryos co-injected with TM1 and p53 MOs was identical to that obtained with TM1 alone, except for the attenuation of the reduction in the head size (Figure 3C in III). The phenotype of the embryos injected with TM2 and TM3 MOs resembled that of TM1-injected embryos, although the morphological alterations in the head of the embryos were milder (Figure 3C in III). To determine whether the pericardial edema was of cardiac origin, the cardiac function of TM1 morphants was analyzed, but no differences were found between TM1 morphants and MM or RC control morphants (data not shown). The normal cardiac function of TM1 morphants suggests that as is usually the case at this stage of zebrafish development, the pericardial edema displayed by the embryos injected with any of the three P4H-TM-targeting MOs is caused by a dysfunction of the kidney, the primary function of which in zebrafish is osmoregulation (Drummond 2005).

As we have shown earlier in this thesis work that human P4H-TM is able to hydroxylate HIF-1α \textit{in vitro} and that it regulates HIF-1α protein levels \textit{in cellulo}, we used qPCR to study whether the knockdown of zebrafish P4H-TM alters the expression of select known HIF target genes, since we could not assess the stabilization of HIF-α due to the lack of antibodies. The qPCR data demonstrate that in 3 dpf wild-type embryos the expression of HIF-1α is significantly higher than that of HIF-2α, and that HIF-P4H-2 is the most abundantly expressed isoenzyme mRNA among the HIF-P4Hs, as is reported in human cells (Figure 4A in III) (Berra \textit{et al.} 2003). The mRNA expression of P4H-TM was lower than that of any of the three HIF-P4H mRNAs (Figure 4A in III). HIF-1α mRNA was statistically significantly induced in the whole 3 dpf TM1-injected embryos to
about 2.2-fold compared to controls, while no change was seen in the amount of HIF-2α mRNA (Figure 4B in III). The transcriptional induction of HIF-1α is in agreement with previous data on zebrafish, which are very hypoxia-tolerant animals and appear to respond to hypoxic stress differently from mammals (Ton et al. 2003), in which the mRNA levels of HIF-1α are usually unchanged by hypoxia. The mRNA expression level of P4H-TM was unchanged in the TM1 morphants (Figure 4B in III). The expression levels of well-characterized HIF-targets, HIF-P4H-3, VEGF-A, EPO and GLUT-1 were significantly induced in TM1-injected embryos compared to controls (MM) (Figure 4B in III). Despite the transcriptional induction of EPO, no signs of polycythemia, or anemia on the other hand, were observed in hemoglobin stainings of TM1 morphants compared to controls (data not shown). The extracellular matrix (ECM) enzyme Lox, which has been identified as a highly hypoxia inducible gene in many microarrays (Manalo et al. 2005), was surprisingly significantly downregulated to about 0.5-fold in TM1-morphants compared to MM-morphants (Figure 4B in III). Of the other analyzed ECM genes, the mRNA expression of type XVIII collagen α(1) chain was significantly induced to about 2.1-fold in TM1-morphants compared to controls, while no alterations were observed in the mRNA levels of type IV collagen α(3) chain or collagen P4H α(I) (Figure 4B in III).

5.2.5 Deficiency of P4H-TM disturbs the normal lens development and leads to alteration of the basement membrane structure in the eye

Given that our data indicated strong P4H-TM expression in the eye and the TM1 morphants displayed a reduction in the size of the eye (Figures 2B and 3C in III), we examined the morphology of the eye of the P4H-TM morphants in more detail. Histological analysis showed that the size of the eye and the opacity of the lens were markedly reduced in TM1-injected embryos relative to controls (Figure 5A in III). Moreover, blue spots, representing remnant nuclei, were present in the lens of the TM1-injected embryos but not in control embryos (Figure 5A in III). TEM confirmed the presence of numerous nuclei, accompanied by degeneration particles and deposits in the lens of the TM1-morphants (Figure 5B in III), suggesting a delay in the differentiation of the lens-forming epithelial cells in TM1-injected embryos. Furthermore, the retinal layers of the TM1-injected embryos were poorly organized compared to controls (Figure 5A in III). However, as the organization of the retinal layers was improved with coinjection of p53-MO,
this may represent a non-specific p53-mediated effect (Figure 5A in III). On the contrary, simultaneous knockdown of p53 and TM1 did not correct the lens phenotype (Figure 5A in III). TEM of the eye also revealed thinning of the basement membrane in TM1-morphants (Figure 5C in III), suggesting that the deficiency of P4H-TM may lead to generalized basement membrane defects.

5.2.6 P4H-TM is essential for the normal structure and function of the pronephric zebrafish kidney and its deficiency causes proteinuria

As we had observed that the injection of embryos with any of the three P4H-TM targeting MOs resulted in pericardial edema, which appeared not to be caused by cardiac dysfunction, we next analyzed the pronephric kidneys of the P4H-TM-deficient embryos in more detail. For histological analysis of the kidney, 4 dpf MO-injected embryos were processed into paraffin sections and stained with hematoxylin and eosin (Figure 6A in III). A clear reduction was observed in the number of podocytes, accompanied by a dilatation of Bowman’s capsule in the glomeruli of the TM1-injected embryos (Figure 6A in III). Interestingly, these changes were found to be independent of p53 but dependent on the catalytic activity of P4H-TM, as the phenotype was unchanged in embryos co-injected with TM1 and p53 MOs and in TM3-injected embryos (Figure 6A in III). Transmission electron microscopy revealed fragmentation of the glomerular basement membrane (GBM), swelling of podocytes and abnormal shaping and organization of the podocyte footprocesses in TM1-injected embryos when compared to controls (Figure 6B in III). Furthermore, endothelial cells were swollen and detached from the GBM in TM1-morphants (Figure 6B in III).

To assess the in vivo function of the pronephric zebrafish glomerulus, a functional assay to detect proteinuria, a symptom of disturbed glomerular filtration, was used. Different molecular weight fluorescent compounds were injected into the general circulation of 3 dpf embryos, followed by monitoring their passage through the glomerulus and the subsequent endocytic uptake into the epithelial cells of the tubuli and ducts (Drummond et al. 1998, Majumdar & Drummond, 2000). TM1-, MM- and RC-injected 3 dpf embryos were anaesthetized and intravenously injected with either 70 kDa or 500 kDa FITC-labeled dextran followed by fixation and processing into paraffin sections 5 h after injection. The 500 kDa dextran can be used as an indicator of the zebrafish pronephric glomerular filtration barrier discrimination, as it should be completely...
retained within the vascular system following intravenous injection, while the 70 kDa dextran readily passes into the glomerular filtrate and can thus be used as a positive control in the experiment (Kramer-Zucker et al. 2005). Confocal microscope analysis of serial paraffin sections surprisingly showed that no FITC-positive endosomes were seen in the tubuli of TM1 morphants after injection with either the 70 kDa or the 500 kDa dextran, while the RC-injected control embryos showed FITC-positive endosomes after injection with the 70 kDa, but not 500 kDa dextran (Figure 6C in III). To study whether the apparent lack of proteinuria in the TM1 morphants could in fact be an artefact caused by disturbed tubular endocytosis function, the dextran injections were repeated with only the 500 kDa FITC dextran. The water in which the larvae were maintained was collected for analysis based on the assumption that if the 500 kDa dextran passes through the glomerulus but is not reabsorbed into the tubules, it must be secreted into the fish water. We were able to show that the fluorescence emitted by the 500 kDa FITC-dextran molecules in the fish water was 9–14-fold higher in the TM1 morphants compared to RC-injected controls (Figure 6D in III). Importantly, this result demonstrates that deficiency of P4H-TM during zebrafish development leads to not only severe structural alterations in the pronephric zebrafish kidney, but also to proteinuria and defective tubular endocytosis, thus compromising kidney function.

5.3 Differences in hydroxylation and binding of Notch and HIF-1α demonstrate substrate selectivity for factor inhibiting HIF-1 (FIH-1) (IV)

5.3.1 Hydroxylation of Notch1 and HIF-1α substrates by FIH is differentially influenced by substrate length

FIH has been reported to require particularly long substrates for efficient hydroxylation of HIF-1α compared to the HIF-P4Hs. Furthermore, the substrate affinity of HIF-P4Hs and FIH has been shown to increase with substrate length. (Ehrismann et al. 2007, Hirsilä et al. 2003, Koivunen et al. 2004, 2006.) FIH-mediated hydroxylation of asparagines in ankyrin repeat domains (ARDs or ANKs) of several proteins, including Notch1, has recently been reported (Coleman et al. 2007, Zheng et al. 2008), and asparagine hydroxylation by FIH has been proposed to be a common modification among ARD-containing proteins.
The functional role of this modification remains unclear, but it has been suggested that ARD-substrates sequester FIH away from HIF-1α (Coleman et al. 2007). Given the large number of potential FIH-substrates within any cell, the relative affinity of FIH for each substrate and the hydroxylation efficiency will be crucial determinants of substrate selection and modification. We therefore set out to study the effects of substrate sequence composition and length and oxygen concentration on the hydroxylation of HIF-1α and Notch1-4 by FIH.

To characterize the FIH-mediated hydroxylation of Notch substrates, short (S1 and S2) and long (L1 and L2) peptides encompassing the two hydroxylation sites in Notch1, Site 1 and Site 2, asparagines were synthesized (Figure 1, Table 1 in IV). HIF-1α peptides of similar length were generated for comparison (Table 1 in IV). FIH was found to have a similar affinity for the short Notch1 and HIF-1α peptide substrates (Table 2 in IV). Increasing the substrate length reduced the K_m of FIH over 150-fold for the Notch1 Site 1 L1 peptide, while the K_m for Site 2 L2 decreased about 10-fold (Table 2 in IV). In comparison, a similar increase in length of the HIF-1α peptide only improved the binding efficiency 2-fold (Table 2 in IV). To take into account the influence of the tertiary structure, the hydroxylation of Notch1 was also studied using a recombinant (mNotch1 ANK1-7) substrate containing both acceptor asparagines. The K_m value of FIH for Notch1 ANK1-7 was found to be too low to be determined accurately with this assay (Table 3 in IV) and thus considerably lower than those for the long peptides (compare Tables 2 and 3 in IV). In contrast to the high affinity of FIH for Notch1 ANK1-7, the K_m of FIH for the recombinant human HIF-1α ODDD/CTAD was found to be identical to that for the HIF-1α long peptide (compare Tables 2 and 3 in IV). The V_max values for the Notch1 and HIF-1α polypeptides did not differ greatly from those obtained with the peptide substrates (compare Tables 2 and 3 in IV). Our results thus demonstrate that substrate length has a much greater influence on the FIH-dependent hydroxylation of Notch than on that of HIF-α, and this effect is predominantly mediated by substrate binding affinity. Furthermore, our results confirm and extend previous observations that FIH has a 250-fold higher affinity for Notch than for HIF-α (Coleman et al. 2007, Zheng et al. 2008).
5.3.2 FIH hydroxylates two asparagines in Notch2 and Notch3 and has a strong preference for Site 1 over Site 2 in Notch1-3

The Notch2 and Notch3 proteins, but not Notch4, have been reported to be hydroxylated by FIH (Coleman et al. 2007, Zheng et al. 2008), although their specific hydroxylation sites and kinetics have not been characterized. To identify the FIH hydroxylation sites in Notch2 and 3, wild-type and mutant ANK1-7 polypeptides with asparagine-to-alanine substitutions in equivalent positions to Sites 1 and 2 in Notch1 were expressed (Figure 2 A-C in IV). FIH-mediated hydroxylation of both sites in Notch2 and 3 was demonstrated in vitro (Figure 2 A-C in IV). MALDI-MS of tryptic peptides of Notch2 and 3 incubated in the presence of FIH demonstrated a mass change of +16 compared with untreated samples, consistent with asparaginyl hydroxylation at positions 1902 and 1969 of Notch2, and 1867 and 1934 of Notch3 (Figure 3 A-D in IV). The identity of the hydroxylated residues was subsequently confirmed by MALDI-MS-MS and LQT-Orbitrap analysis. Together these data confirm that both Notch2 and 3 become hydroxylated by FIH at positions equivalent to those of Sites 1 and 2 in Notch1.

The FIH-mediated hydroxylation of Notch1 Sites 1 and 2 in a single substrate was studied using mNotch1 ANK1-7 polypeptides with single alanine substitutions. The $K_m$ for the Site 2 mutant (N2012A), representing hydroxylation at Site 1 (N1945), was too low to be determined accurately with this assay, while the $K_m$ for the Site 1 mutant (N1945A), representing Site 2 hydroxylation of Notch1, was higher than for the wild-type polypeptide (Table 3 in IV), indicating that FIH has a higher affinity for Site 1 in vitro. The 20-fold difference observed between the $K_m$ values for the Notch1 L1 and L2 peptides further supports this data (Table 2 in IV). To study whether the preference of FIH for the Site 1 over Site 2 is common to all Notch substrates, long peptides corresponding to Sites 1 and 2 (L1 and L2) in Notch2 and 3 were synthesized (Figure 1, Table 1 in IV). The $K_m$ values for Site 1 and Site 2 in Notch2 and 3 were in the same order of magnitude as those for Site 1 and Site 2 in Notch1 (Tables 2 and 4 in IV), indicating at least 18-fold higher affinity of FIH for Site 1 than for Site 2 in all Notch substrates. Although the $V_{max}$ values for the Site 2 peptides of Notch1-3 were slightly higher than those for Site 1 (Tables 2 and 4 in IV), the relative activity obtained for Site 1 in the full-length mNotch1-3 ANK1-7 ankyrin substrate was clearly higher than that of Site 2 (Figure 2 A-C in IV). Notch2 and 3 peptides demonstrated higher $V_{max}$ values for both sites than the corresponding Notch1 peptides and the full-length Notch1 ANK domain (Tables 2–4 in IV),
suggesting that Notch2 and 3 are more efficiently hydroxylated than Notch1 at saturating substrate concentrations (Zheng et al. 2008).

To determine the relative FIH-binding affinities of Notch and HIF-1α recombinant polypeptides, we developed an assay in which polypeptide substrates were used to compete with a fluorescently-labeled Notch1 L1 peptide for FIH binding (Figure 4 A and B in IV). The mean $K_i$ values for Notch1-3 demonstrated a small, yet statistically significant difference in the ability of FIH to bind the Notch1-3 proteins, with Notch2 having the highest affinity. Consistent with the $K_m$ values, a markedly lower affinity was observed for the HIF-1αODDD/CTAD protein, with a mean $K_i$ at least 50-fold higher than those obtained for Notch1-3 (Table 5 in IV). Although Notch4 is not a FIH substrate, we demonstrated that it is able to bind to FIH, albeit with a $K_i$ implying an order of magnitude lower affinity than those for Notch1-3 (Table 5 in IV). The discrepancy with an earlier report that Notch4 was incapable of interacting with FIH (Coleman et al. 2007) is likely to be due to the higher sensitivity of the FP binding assay.

5.3.3 The $K_m$ of FIH for oxygen is lower with Notch1 than with HIF-1α as a substrate

The $K_m$ values of FIH for oxygen have not been reported with non-HIF-α substrates. To better understand the physiological role of FIH, including the range of oxygen concentrations at which it is active and whether its activity is influenced by the nature or the length of its substrate, we determined the $K_m$ value of FIH for oxygen using the mNotch1 ANK1-7 wild-type and mutant polypeptides and hHIF-1αODDD/CTAD as substrates. In marked contrast to the 90μM obtained for HIF-1αODDD/CTAD, which is identical to the value previously observed for the HIF-1α synthetic peptide (Koivunen et al. 2004), the $K_m$ of FIH for oxygen for Notch1 ANK1-7 was almost 8-fold lower, 12μM (Table 6, Figure 5 A and D in IV). This demonstrates that FIH has a significantly higher affinity for oxygen with Notch1 as a substrate than with HIF-1α. Interestingly, also the oxygen sensitivity of FIH for Notch1 appears to be predominantly dictated by Site 1, since the $K_m$ of FIH for oxygen obtained with the N2012A mutant polypeptide was essentially identical to that for the wild-type polypeptide (Table 6 in IV).

Finally, determination of the $K_m$ values of FIH for oxygen using the Notch2 and 3 long peptides as substrates demonstrated that the hydroxylation sites in Notch2 and 3 are similar in their oxygen affinity to those of Notch1, the $K_m$.
values for the Site 1 peptides being markedly lower than those for the Site 2 peptides (Table 6 in IV). Hence, in the context of \textit{in vitro} hydroxylation, FIH has a higher affinity for oxygen at Site 1 of all the Notch family substrates than at Site 2 or HIF-1α. The Site 1 positions in all Notch proteins are thus likely to become hydroxylated efficiently even under relatively severe hypoxic conditions, where HIF-α hydroxylation would be reduced.
6 Discussion

6.1 Chronic cardiac HIF-P4H-2 deficiency provides protection against acute myocardial ischemia-reperfusion injury

We have generated a novel viable HIF-P4H-2 hypomorphic mouse line that expresses varying levels of wild-type HIF-P4H-2 mRNA in different organs, the lowest level, only 8%, being found in the heart. These mice were generated from an ES cell line, in which the HIF-P4H-2 gene is disrupted by a Genetraps insertion cassette. Interestingly, the recognition of the splice acceptor site in the insertion cassette is not absolute, and depending on the tissue, the ratio of the mutant to wild-type transcript generated varies. In the heart HIF-P4H-2 deficiency leads to chronic stabilization of HIF-1α and HIF-2α with no obvious adverse effects and provides cardioprotection against acute ischemia-reperfusion injury. Deficiency of HIF-P4H-2 has been speculated to lead to deleterious effects, as conditional broad-spectrum inactivation (CKO, conditional knockout) of HIF-P4H-2 in mouse has been associated with development of congestive heart failure and premature death (Minamishima et al. 2008, 2009). However, given that our mice have a normal life span despite the high level of HIF-P4H-2 inactivation (92%) in the heart, the heart failure is likely to be largely an indirect consequence of the severe polycythemia and hyperviscosity observed in the CKO mice (Minamishima et al. 2008, 2009). Moreover, our mouse line appears to be an excellent model for studying the consequences of cardiac-specific HIF-P4H-2 deficiency, as the level of HIF-P4H-2 inactivation in other organs is not high enough to induce polycythemia or cause hyperactive angiogenesis.

During myocardial I/R, the ultimate fate of the cardiomyocyte is critically dependent on preserving the function of its mitochondria, as an event called mitochondrial permeability transition (MPT) has been shown to play a crucial role in determining the extent of irreversible injury following myocardial I/R. Oxidative stress, calcium overload, ATP depletion and elevated phosphate levels and the rapid correction of the intracellular pH upon reperfusion induce MPT with formation of nonspecific MPT pores (MPTP) in the inner mitochondrial membrane. MPTP opening uncouples oxidative phosphorylation and compromises intracellular ATP levels ultimately leading to mitochondrial dysfunction and cell death primarily through necrosis. Counteracting any of the above-mentioned factors is likely to reduce the extent of MPTP opening in the
reperfused heart, thereby protecting the myocardium from I/R injury, and importantly, IP has been postulated to elicit its cardioprotective effect via inhibiting the MPTP opening, although the molecular mechanism remains to be resolved. (For reviews, see Halestrap & Pasdois 2009, Halestrap 2009, Hausenloy et al. 2009, and Javadov et al. 2009.) Furthermore, pharmacological inhibition of MPTP opening at the onset of reperfusion has already been shown to reduce myocardial infarct size by 30–40% in patients presenting with acute myocardial infarction (Piot et al. 2008).

Our analysis revealed upregulation of several HIF-target genes at the mRNA level in the hearts of HIF-P4H-2 hypomorphic mice, indicative of increased transcriptional activity of HIF. The upregulation of the mRNAs for the glucose transporter Glut-1 and several enzymes of glycolysis, all of which are known HIF targets (Semenza 2007b), and the subsequent protein-level increase in Glut-1, suggest increased glycolysis in the HIF-P4H-2gt/gt hearts. This is supported by the higher amount of pre-ischemic lactate in the HIF-P4H-2gt/gt hearts relative to wild-type. Furthermore, activity of the pyruvate dehydrogenase complex may be reduced in HIF-P4H-2gt/gt hearts, given that Pdk-1 mRNA level was increased in HIF-P4H-2gt/gt hearts, resulting in shunting of pyruvate from the citric acid cycle to glycolysis. Very interestingly, it was recently shown that the hypoxia-inducible microRNA miR-210 suppresses mitochondrial respiration in hypoxia via direct repression of the iron-sulfur cluster assembly proteins (ISCU1/2), which in turn decreases the activity of prototypical iron-sulfur proteins that control mitochondrial metabolism. HIF-dependent induction of miR-210 thereby favors a shift in cellular metabolism towards glycolytic ATP production (Chan et al. 2009.) It can be speculated that the stabilization of HIF has led to the induction of miR-210, which in turn has played an important role in the metabolic shift observed in the HIF-P4H-2 hypomorph hearts. Furthermore, reprogramming of basal metabolism from oxidative to more anaerobic ATP production caused by HIF-P4H-1 deficiency has been shown to provide skeletal muscle ischemia tolerance in mice (Aragones et al. 2009), and the same phenomenon could have played a role in the cardioprotection observed in HIF-P4H-2 hypomorphic hearts.

We observed that the end-ischemic vs. pre-ischemic ATP concentration and the end-ischemic vs. pre-ischemic [CrP/Cr], which qualitatively reflects the cellular energy state and the ratio of free [ATP] to [ADP], were higher in the HIF-P4H-2gt/gt hearts compared to wild-type. The cellular energy state is thus retained at a higher level in HIF-P4H-2 hypomorphic hearts during the ischemic period, which is likely to have reduced the MPTP opening relative to wild-type in early
reperfusion. Moreover, as ATP generated by glycolysis rather than by oxidative phosphorylation in mitochondria has been shown to allow the heart to better withstand ischemia, through ultimately preventing the intracellular Ca\(^{2+}\) overload and thereby reducing MPTP opening (Opie & Sack 2002), the increased glycolysis observed in HIF-P4H-2 hypomorph hearts may have further reduced the MPTP opening in this respect.

It has been hypothesized that a delay in the correction of intracellular pH upon reperfusion from the acidification induced by ischemia may inhibit MPTP opening and thus reduce myocardial cell death following I/R (for reviews, see Halestrap 2009, Hausenloy et al. 2009). In the HIF-P4H-2\(^{2}\) hearts, the increased glycolysis resulted in the accumulation of a higher amount of pre-ischemic lactate than in wild-type hearts. At the end of the ischemic episode, the amount of lactate was found to be equal in both genotypes, most likely due to lactate accumulation reaching limiting conditions caused by the concomitant pH decrease. However, as HIF-1\(\alpha\) was almost completely degraded during reperfusion in wild-type hearts, but stayed unchanged and at high levels in the HIF-P4H-2\(^{2}\) hearts, we assume that as the oxygen supply returns to normal, the difference in the amount of intracellular lactate rapidly reappears. This results in the induction of a more acidic cellular environment in the HIF-P4H-2\(^{2}\) hearts relative to wild-type, which may improve the survival of the myocardium by reducing MPTP opening. Taken together, these data suggest that the HIF-P4H-2\(^{2}\) hearts are primed for preference towards glycolytic metabolism and therefore have an advantage in surviving an ischemic insult.

HIF target genes encoding adrenomedullin, apelin and Bnip-3 were also upregulated in the HIF-P4H-2\(^{2}\) hearts. Adrenomedullin is a vasodilatory peptide that increases the tolerance of cells to oxidative stress, while apelin is a peptide protecting the myocardium against I/R injury-induced infarction and apoptosis (Burley et al. 2007, Cormier-Regard et al. 1998, Kleinz & Baxter 2008, Ronkainen et al. 2007). A pro-apoptotic role has been proposed for Bnip-3, but several reports indicate that it contributes to a survival mechanism under hypoxic conditions (Bellot et al. 2009). Changes in the mRNA levels of these genes are likely to have conveyed at least a part of the cardioprotection observed in HIF-P4H-2\(^{2}\) hearts.

HIF-1\(\alpha\) is essential for both the acute and delayed phases of cardioprotection induced by IP (Cai et al. 2003, 2008, Eckle et al. 2008). Acute silencing of HIF-P4H-2 by left ventricular siRNA has been shown to lead to 10–15-fold induction of genes encoding Cd73, the enzyme generating extracellular adenosine, and the
adenosine receptor A2b, resulting in increased cardiac adenosine concentration and cardioprotection (Eckle et al. 2008). As the mRNAs for both Cd73 and A2bar were unchanged in the HIF-P4H-2<sup>gt</sup> hearts, the mechanism behind the cardioprotection appears to be different when silencing of HIF-P4H-2 is achieved by a germ-line genetic modification instead of siRNA treatment. Furthermore, all of the changes observed in the mRNA levels of various genes in HIF-P4H-2<sup>gt</sup> hearts were rather small, less than 1.5-fold. This suggests that it is the concerted action of an array of gene products involved in the regulation of glucose metabolism, cardiac function and blood pressure that provides the cardioprotection in the HIF-P4H-2 hypomorphic mice. Importantly, our data clearly indicate that the cardioprotection in the HIF-P4H-2<sup>gt</sup> hearts is due to HIF-mediated differences in gene expression preceding the ischemic insult.

6.2 The actions of P4H-TM resemble those of the HIF-P4Hs, but it may also have other substrates

The results of our study demonstrate that the novel transmembrane P4H, P4H-TM, more closely resembles the C-P4Hs in terms of sequence identity and subcellular localization, but its actions mimic those of the HIF-P4Hs; overexpression of P4H-TM in cultured human neuroblastoma Kelly cells dose-dependently reduced the recombinant HIF-α ODDD levels, while silencing of endogenous P4H-TM by siRNA increased HIF-1α levels in Kelly cells. Furthermore, purified recombinant human P4H-TM hydroxylated the two critical prolines in recombinant [<sup>3</sup>H]proline-labeled HIF-1α and its ODDD in vitro. In contrast, it failed to hydroxylate the recombinant procollagen I(α)1 polypeptide chain and lacks any sequences corresponding to the peptide-substrate-binding domain of the C-P4Hs (Myllyharju & Kivirikko 1999). Moreover, the expression pattern of the P4H-TM mRNA, a high level of expression being observed e.g. in the brain, is also in distinct disagreement with those of the C-P4Hs.

Given the several pieces of evidence demonstrating that P4H-TM resides in the ER membranes with its catalytic site inside the ER lumen, an important question arising is where and how does the interaction between P4H-TM and HIF-1α take place. Hydroxylation of HIF-1α is assumed to occur either in the cytoplasm or the nucleus, as the HIF-P4Hs are found in these cellular compartments (Metzen et al. 2003a, Steinhoff et al. 2009, Yasumoto et al. 2009). Numerous studies have demonstrated that HIF-α is located in the nucleus in hypoxia (Jiang et al. 1996, Kallio et al. 1998), but the low HIF-α levels in
normoxic cells make it difficult to reliably determine its subcellular localization. Localization of substantial amounts of HIF-α in the ER has been reported in normoxia (Liu et al. 2004), possibly due to the interaction with pVHL on the cytosolic surface of ER membranes. Interestingly, HIF-1α and HIF hydroxylases have been shown to be targeted to peroxisomes in hypoxic primary hepatocytes, indicating that these polypeptides can also be found in membrane cytoplasmic organelles (Khan et al. 2006). In addition to the major P4H-TM form located in the ER membranes, transfected cells were also found to contain an N-terminally truncated form of the polypeptide lacking the transmembrane domain. Although our immunofluorescence and immuno-EM data gave no evidence for at least any major presence of P4H-TM in the cytoplasm or nucleus, we cannot exclude the possibility that a fraction of the shed P4H-TM form is somehow transported to either of these locations.

P4H-TM was found to be greatly induced by hypoxia at the protein level in the majority but not all of the cell lines studied. This is in agreement with the observation that the gene encoding P4H-TM contains five sequences corresponding to the HRE element. Furthermore, both HIF-P4H-2 and HIF-P4H-3 have been shown to be direct HIF-target genes that are induced by hypoxia (Metzen et al. 2005, Pescador et al. 2005). However, in contrast to our results, Oehme and coworkers reported that the amount of P4H-TM was unchanged by hypoxia in human A549 lung carcinoma cells (Oehme et al. 2002). Given that we have not directly demonstrated that the hypoxia-induced increase in the amount of P4H-TM is dependent on HIF, it is also possible that hypoxia increases the stability of P4H-TM rather than inducing its transcription. Due to the high degree of substrate sequence redundancy of the HIF-P4Hs, it has been speculated that they may have additional physiological substrates besides the HIF-αs, and indeed, very recently the β-adrenergic receptor β2AR was identified as a novel substrate for HIF-P4H-3 (Xie et al. 2009). Moreover, indirect evidence suggests that the large RNA subunit of RNA polymerase II (Kuznetsova et al. 2003), iron regulatory protein IRP-2 (Hanson et al. 2003, Wang et al. 2004), Iκκ kinase-β (Cummins et al. 2006) and the activating transcription factor-4 (ATF-4) (Köditz et al. 2007) may be substrates of HIF-P4Hs. Interestingly, P4H-TM also acted at a low rate on some proline residue(s) other than the ones hydroxylated by the HIF-P4Hs in the HIF-1α polypeptides, giving rise to the possibility that P4H-TM may have additional physiological substrates.
6.3 Lack of P4H-TM in zebrafish during development results in basement membrane defects and kidney failure

A zebrafish homolog of P4H-TM with 51% overall amino acid sequence identity to the human P4H-TM was identified in a database search. Considering the advantages of using zebrafish as a model of vertebrate organogenesis, including the feasibility of applying forward genetic tools to define gene function and the ease of observation due to the transparency of the embryos, we decided to study the in vivo role of P4H-TM in zebrafish embryos. qPCR analysis and in situ hybridization demonstrated strong expression of P4H-TM mRNA in the brain and in the eye of developing 3 dpf wild-type embryos and in adult zebrafish. Expression of P4H-TM mRNA was observed in all adult organs studied by qPCR, including the kidney. Knockdown of P4H-TM was achieved by the means of specific antisense morpholino (MO) oligomers, and the effect of the lack of P4H-TM was studied in 1–4 dpf embryos. As zebrafish embryos develop from fertilized eggs to free-swimming larvae in 2.5 days, all stages of organ development can be observed during the time window of MO-mediated gene silencing. Of note, the formation of the zebrafish pronephric kidney is completed during the first 2 d of development and blood filtration by the pronephros begins at approximately 40 hpf. (For a review, see Drummond 2005.) Despite its simplicity in form, the functional larval pronephros of zebrafish contains cell types typical of higher vertebrate kidneys (Drummond et al. 1998).

The most striking and unifying of the phenotypic abnormalities observed in embryos injected with three independent P4H-TM-targeting MOs (TM1-TM3) was the development of severe pericardial edema by 3–4 dpf. Gross edema observed in 3–4 dpf embryos is generally a result of kidney dysfunction, as the primary function of the fish pronephros is osmoregulation (for a review, see Drummond 2005). Furthermore, a phenotype similar to that observed in TM1-morphants has been reported by others for the zebrafish morphants lacking the coxsackie and adenovirus receptor CAR, which was subsequently found to be required for renal epithelial differentiation in the zebrafish pronephric kidney (Raschperger et al. 2008). We were thus interested in studying in more detail the pronephric kidney of the embryos lacking P4H-TM.

Severe alterations were observed in the structure of the glomeruli of 4 dpf TM1-morphants already at the light microscopic level. Transmission electron microscopy revealed changes in the ultrastructure of both the endothelial and epithelial cells of the glomerulus, and fragmentation of GBM, suggesting that the
filtration function might consequently be altered. Surprisingly, we could not detect the uptake of low or high molecular weight fluorescent proteins into the pronephric tubule or duct cells in the P4H-TM-deficient embryos in the serial sections analyzed by confocal microscopy. In controls the low molecular weight fluorescent protein passed through the glomerular filter and was taken up in the tubuli, while the high molecular weight protein stayed in circulation as expected. Importantly, however, we were able to show that the apparent lack of proteinuria was in fact due to a defect in the tubular endocytosis function, and the 500 kDa FITC-dextran that had passed through the glomerular filtration barrier had not been reabsorbed in the tubules but was instead secreted into the fish water by the TM1 morphants. Lack of P4H-TM was thus shown to lead to a severe leakage of high molecular weight proteins, i.e. proteinuria, in 3 dpf zebrafish embryos, suggesting that P4H-TM is critical to the normal development and function of the pronephric zebrafish kidney.

Proteinuria is typical of many human nephropathies and also a common complication of diabetes (Cooper et al. 2002). Proteinuria can result from a failure of the formation of the slit diaphragm, an adherens junction formed between podocyte foot processes (Reiser et al. 2000), but TEM analysis revealed the presence of normal slit diaphragms in the glomeruli of embryos lacking P4H-TM. The kidneys of P4H-TM-deficient embryos bear some phenotypic similarities to the previously characterized pronephric mutant embryos (Drummond et al. 1998). However, flattening of the epithelial cells of the pronephric tubules or ducts, characteristic of cystic maldevelopment of zebrafish pronephros (Drummond et al. 1998), was not observed in embryos lacking P4H-TM.

Structural integration of glomerular podocytes and blood vessels is required for normal development of the pronephric zebrafish kidney (for a review, see Drummond 2005). Podocytes are believed to organize the vessel growth by expressing VEGF and thereby attracting and assembling the glomerular tuft (Majumdar & Drummond, 2000). However, zebrafish mutants with disrupted blood flow fail to form a proper glomerular capillary tuft, suggesting that vascular shear force is required to drive the capillary formation (Serluca et al. 2002). Degradation and remodeling of the GBM has been reported to be another key step in capillary tuft formation (Serluca et al. 2002). The phenotype observed in the pronephric kidneys of the TM1-morphants may therefore be a result of several dysregulated functions, i.e. compromised blood flow into the glomerulus caused by alterations in the endothelial cells and failure in GBM remodeling evidenced
by fragmented GBM. At the gene expression level, we observed significant alterations in the levels of two studied ECM components in the TM1-morphants compared to controls. Expression of type XVIII collagen α(1) chain was induced while that of Lox was reduced, which may have contributed to altered GBM modeling. Moreover, the observed induction in VEGF-A expression may be regarded as a sign of podocyte dysfunction. Interestingly, overexpression of VEGF in zebrafish during development has been reported to cause ectopic vasculature and blood cells as well as pericardial edema starting as early as at 30 hpf (Liang et al. 2001).

In addition to the kidney, the eye contains highly specialized basement membranes, i.e. the lens capsule, inner limiting membrane and Bruch’s membrane. It was of great interest to notice that abnormalities were also present in the lens capsule basement membrane in the developing eyes of the P4H-TM-deficient embryos. The lens phenotype started to become visible at 2 dpf, while at 4 dpf, when the lens should have been completely enucleated, numerous nuclei accompanied by degeneration particles and deposits were observed in the lenses of P4H-TM-deficient embryos. Because the gene knockdown by antisense morpholino oligonucleotides is transient, we were unable to follow the lens development longer to determine whether the development of the lens was only delayed or permanently disturbed due to the lack of P4H-TM. However, the morphological alterations of the lens capsule basement membrane may have contributed to the severely compromised epithelial cell differentiation. Interestingly, however, others have reported that laminin γ1-deficient zebrafish embryos have little to no lens capsule basement membrane at 3 dpf, but the enucleation process in their lenses is normal (Lee & Gross 2007).

Although no data are currently available on the in vivo role of any of the human C-P4H or HIF-P4H homologs in zebrafish, two zebrafish lines carrying germ-line mutations in the zebrafish vhl homolog have been found in a Hubrecht target-selected ENU-mutagenized zebrafish library (van Rooijen et al. 2009). The vhl mutant embryos display a hypoxic response and recapitulate key aspects of Chuvash polycythemia, which is caused by deficiency in binding of hydroxylated HIF-α to pVHL and its subsequent targeting for proteosomal degradation (van Rooijen et al. 2009). Knockdown of P4H-TM did not result in any signs of polycythemia that was observed in the vhl mutants and vice versa, no alterations in the kidney or lens morphology were reported in the vhl mutants (van Rooijen et al. 2009). Nevertheless, the P4H-TM-deficient morphants, like the vhl mutants, showed upregulation of several HIF-regulated genes. Therefore, if the phenotype
of the P4H-TM-deficient morphants was caused by deficiency in downregulating HIF-α in the eye and kidney, alternating routes for its degradation might exist in these tissues. Interestingly, zebrafish embryos injected with the pan 2-oxoglutarate inhibitor DMOG reproduced the phenotype of the vhl mutants, but also displayed additional alterations not seen in the vhl mutants. For example, in the proximal renal tubule, strong VEGF-A expression was seen in the DMOG-treated embryos, but not in the vhl mutants, suggesting that another 2-oxoglutarate requiring protein is responsible for the regulation of VEGF-A expression there (van Rooijen et al. 2009). Expression studies on zebrafish HIF-1α and 2α during development show that both forms are expressed in all tissues during early development (Rojas et al. 2007) and their dysregulation may therefore have contributed to the phenotype observed in the P4H-TM-deficient morphants. However, it seems unlikely that all of the phenotypic alterations observed in the P4H-TM-deficient embryos could be mediated solely by the HIF pathway.

Taken together, our data suggest that the P4H-TM enzyme is required for normal development of the pronephric kidney and lens in zebrafish. P4H-TM appears to play a role in the integrity of two highly specialized basement membranes, the lens capsule and the GBM. These functions may be regulated via HIF, but it cannot be excluded that P4H-TM has a yet another unidentified substrate, the reduced hydroxylation of which results in the phenotype observed in zebrafish embryos deficient in P4H-TM. To address this issue in the future, it would be interesting to carry out double-Morpholino injections and simultaneously knock down both P4H-TM and HIF-1α in zebrafish and compare the phenotype displayed by these morphants with the one obtained with P4H-TM targeting MOs alone.

6.4 FIH displays substrate selectivity in terms of hydroxylation and binding of Notch proteins and HIF-1α

Given the large number of potential FIH-substrates within any cell, the relative binding affinity and hydroxylation rate in addition to the subcellular co-localization of the substrate and enzyme are crucial determinants of substrate selection and modification by FIH in vivo. In this study we determined the contributions of substrate sequence composition and length and of oxygen concentration to the FIH-binding and/or hydroxylation of Notch1-4 and compared them with those for HIF-1α. Our data demonstrate that substrate length has a
much greater influence on FIH-dependent hydroxylation of Notch than of HIF-1α, predominantly through binding affinity rather than maximal reaction velocity. Furthermore, the $K_m$ value of FIH for Notch1, $<0.2 \mu M$, is at least 250-fold lower than that of 50 $\mu M$ for HIF-1α, indicating a 250-fold higher affinity of FIH for Notch than for HIF-1α. We also demonstrated hydroxylation of two asparagines in Notch2 and 3, corresponding to Sites 1 and 2 of Notch1, by mass spectrometry for the first time. Site 1 appeared to be the preferred site of FIH hydroxylation in Notch1-3. Interestingly, we observed binding of Notch4 to FIH with an affinity almost 10-fold lower than for Notch1-3, but detected no hydroxylation.

Importantly, our results demonstrate that the $K_m$ values of FIH for oxygen at the preferred Site 1 of Notch1-3 are an order of magnitude lower than those for Site 2 or HIF-1α. Despite this difference, however, it is clear from previous functional studies that FIH-dependent hydroxylation of endogenous HIF-α occurs and it modulates HIF-α activity in cells during normoxia and hypoxia (Dayan et al. 2006, Stolze et al. 2004). The variation in the $K_m$ values for oxygen between these two substrates supports the proposed order of binding to FIH and other 2-oxoglutarate-dependent dioxygenases (for reviews, see Myllyharju & Kivirikko 2004, Schofield & Ratcliffe 2005), with the peptide substrate preceding the binding of oxygen to the active site and thus influencing it through a conformational change. It is possible that a substrate that binds with a higher affinity ‘induces’ a conformation of FIH that displays a higher affinity for oxygen. This may have important implications for the cellular hypoxia response, as hydroxylation of Notch, specifically at its Site 1, is less sensitive to decreases in available oxygen than is the hydroxylation of HIF-α. Hence, as oxygen levels decrease, hydroxylation of the HIF-1α CTAD will be inhibited much earlier than that of Notch at Site 1, adding another level of complexity and a new possibility for fine-tuning the hypoxia response in physiological or pathological conditions.

Considering that the affinity of FIH for Notch decreases upon its hydroxylation (Coleman et al. 2007, Zheng et al. 2008), this may influence the ability of Notch to sequester FIH away from HIF-α and thus regulate transcriptional output from CTAD. Once oxygen concentrations have decreased to levels low enough to inhibit Notch hydroxylation and thus increase its affinity for FIH, the oxygen levels at which HIF-α can be efficiently hydroxylated will long be surpassed. Thus, the functional effect of FIH sequestration by Notch may be redundant in terms of hypoxic signalling, although it may be more significant in situations such as sudden changes in oxygen levels rather than gradual decreases, in chronic hypoxia (Coleman et al. 2007), or upon re-oxygenation. Cell-type-
dependent differences are also likely to affect the extent of FIH sequestration by Notch.

In conclusion, our data demonstrate that hydroxylation of the Notch proteins, and presumably of other ARD-containing proteins, by FIH differs considerably in terms of affinity and dependence on oxygen from that of the HIF substrates. *In cellulo* analysis is needed to demonstrate the importance of these *in vitro* differences in FIH hydroxylation and substrate preference under physiological hypoxia.
7 Conclusions and future prospects

This study has provided important novel information on the in vitro, in cellulo and in vivo roles of some of the key enzymes involved in the regulation of oxygen homeostasis. We showed that cardiac silencing of the most important HIF-α stability regulating HIF-P4H isoenzyme, HIF-P4H-2, by a genetic modification in mouse results in cardioprotection in an ex vivo I/R setting. In contrast to previous reports on CKO mice with broad-spectrum HIF-P4H-2 inactivation, we did not observe any adverse effects in the HIF-P4H-2-deficient mice. However, it must be born in mind that our mouse model considerably differs from the CKO models, as the level of HIF-P4H-2 inactivation greatly varies from one organ to another, the level of wild-type HIF-P4H-2 mRNA being as high as 85% in the liver. Development of HIF-P4H inhibitors as therapeutics in ischemic diseases such as myocardial infarction is under way but remains a challenging task. It has become clear from gene silencing studies that in order to achieve the desired beneficial effect while avoiding harmful side-effects, these enzyme inhibitors must exhibit high specificity to the isoform in question. Furthermore, the role of HIF-P4H-2 in malignant progression is ambiguous for the moment and needs to be elucidated by additional in-depth studies in order to proceed with the development of inhibitors.

The family of HIF-P4Hs is known to consist of three members. In this study we cloned and characterized a novel transmembrane prolyl 4-hydroxylase P4H-TM, which appears to be a fourth member of this enzyme family. Similarly to the HIF-P4Hs, P4H-TM regulates HIF-1α protein levels in cellulo and hydroxylates HIF-1α in vitro, but in terms of sequence identity and subcellular localization it more closely resembles the C-P4Hs. Very interestingly, P4H-TM was able to hydroxylate some prolines in the HIF-1α double mutant ODDD (Pro402Ala, Pro564Ala) that are not hydroxylated by HIF-P4Hs. Considering this and the discrepancy between the subcellular localization of P4H-TM and its only identified substrate HIF-1α, it is tempting to speculate that this novel transmembrane prolyl 4-hydroxylase might play an even more important role in regulating some other pathway by hydroxylating another molecule in addition to HIF-α. On this account, and given that expression of P4H-TM appears to be restricted to vertebrates, we exploited the available genetic tools to study the effects of P4H-TM knockdown during development in the most simple model organism available, the zebrafish. We observed that deficiency of P4H-TM leads to basement membrane defects and compromises the function of the zebrafish pronephric kidney. However, it is currently unknown whether these phenotypic
alterations result from stabilization of HIF-α. Further studies to identify novel candidate substrates for P4H-TM are in progress, and the analysis of P4H-TM-deficient mice is expected to provide important information on the physiological role of P4H-TM.

FIH has recently been shown to hydroxylate asparagine residues in many proteins containing ankyrin repeats, including Notch1-3. We observed that hydroxylation of Notch1-3 by FIH differs considerably in terms of affinity and dependence on oxygen from that of HIF-1α. We also observed that the $K_m$ of FIH for oxygen is markedly lower with Notch1-3 than with HIF-1α as a substrate, implying that FIH-mediated hydroxylation of Notch can continue in cells in oxygen concentrations where HIF-1α hydroxylation would be notably reduced. Given the high number of potential FIH-substrates within any cell, the affinity of FIH for each of these substrates will be of crucial importance in substrate selection and modification in vivo.
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CHARACTERIZATION OF THE IN VIVO ROLE OF HIF-P4H-2 IN MOUSE HEART, OF A NOVEL P4H IN HUMAN AND ZEBRAFISH AND OF THE CATALYTIC PROPERTIES OF FIH