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Ellinoora Aro

PROLYL 4-HYDROXYLASES, KEY ENZYMES REGULATING HYPOXIA RESPONSE AND COLLAGEN SYNTHESIS

THE ROLES OF SPECIFIC ISOENZYMES IN THE CONTROL OF ERYTHROPOIESIS AND SKELETOGENESIS

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF BIOMEDICINE,
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY;
BIOCENTER OULU;
CENTER FOR CELL-MATRIX RESEARCH

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The roles of specific isoenzymes in the control of erythropoiesis and skeletogenesis

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Abstract

Oxygen deprivation (hypoxia) is related to many disease conditions, such as anemia, but is also a critical regulatory signal during normal development. Cellular responses to hypoxia are largely mediated through alterations in gene regulation brought about by the transcription factor known as hypoxia inducible facor (HIF). One of the most extensively studied systemic consequences of hypoxia is the induction of red blood cell production, erythropoiesis, which occurs through a HIF-dependent increase in erythropoietin (*EPO*) gene expression. The amount of HIF in cells is regulated by three HIF prolyl 4-hydroxylases (HIF-P4Hs) while a fourth P4H possessing a transmembrane domain (P4H-TM) is able to act on HIF at least *in vitro*. The putative role of P4H-TM in regulating erythropoiesis is studied here by administering a HIF-P4H inhibitor, FG-4497, to *P4h-tm* null and wild-type mice. By comparing the observed effects with those seen in FG-4497 treated hypomorphic *Hif-p4h-2* and *Hif-p4h-3* null mice, it is demonstrated for the first time that P4H-TM is involved in the regulation of Epo production in the mammalian kidney, but not in the liver.

Long bones are formed via endochondral ossification, in which a cartilaginous template, the growth plate, is first laid down and then replaced with bone. The growth plate is rich in extracellular matrix (ECM) and contains a hypoxic central region in which HIF has been shown to regulate chondrocyte function. Importantly, growth plate chondrocytes are highly active in collagen synthesis. Collagen prolyl 4-hydroxylases (C-P4Hs I-III) provide collagen molecules with thermal stability and are thus necessary for the formation of a proper ECM. Through an *in vitro* approach it is demonstrated that hypoxia increases the amount and activity of C-P4H in primary mouse epiphyseal growth plate chondrocytes in a HIF-1-dependent manner. Lastly, it was set out to characterize mouse lines with complete inactivation of C-P4H-II with or without partial inactivation of C-P4H-I. A significant reduction in the total amount of C-P4H and its activity was found to result in mild chondrodysplasia and altered bone properties. The above mouse models provided new information on the specific *in vivo* roles of the C-P4H isoenzymes I and II.

Keywords: cell hypoxia, chondrocytes, erythropoietin, hypoxia-inducible factor, prolyl 4-hydroxylase

Aro, Ellinoora, Prolyyli-4-hydroksylaasit solujen hypoksiavasteessa sekä kollageenisynteesissä. Eri isoentsyymeiden roolien karakterisointi erytropoieesissa ja luuston muodostuksessa

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja molekyylibiologia; Biocenter Oulu; Center for Cell-Matrix Research, PL 5000, 90014 Oulun yliopisto

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Tiivistelmä

Kudosten alentunut happipitoisuus (hypoksia) liittyy osana moniin elimistön patologisiin tiloihin, kuten anemiaan. Lisäksi se on tärkeä säätelytekijä normaalin yksilönkehityksen aikana. Jotta solut havaitsisivat hypoksian ja reagoidakseen siihen, on niille kehittynyt säätelyjärjestelmä, jossa hypoksiassa indusoituva transkriptiotekijä, HIF, on tärkeässä asemassa. Yksi merkittävin HIF:n indusoima systeeminen vaikutus elimistössä on punasolujen tuotannon, erytropoieesin, kiihtyminen. Sitä tapahtuu erytropoietiinia koodittavan geenin (*EPO*) lisääntyneen ilmentymisen kautta. HIF-tekijän määrää soluissa säätelee kolme HIF-prolyyli-4-hydroksylaasientsyymiä (HIF-P4Ht 1-3). Transmembraanisen prolyyli-4-hydroksylaasin (P4H-TM) tiedetään myös vaikuttavan HIF-tekijän määrään soluissa *in vitro*, mutta sen vaikutusta nisäkkään erytropoieesiin ei ole aiemmin tutkittu. Käyttämällä hyväksi kolmea eri transgeenista hiirilinjaa (*P4h-tm*$^{-/-}$, *Hif-P4h-2*$^{gt/gt}$, *Hif-p4h-3*$^{-/-}$) ja HIF-P4H entsyymeitä inhiboivaa lääkeainetta, FG-4497, tässä työssä osoitettiin ensimmäistä kertaa, että P4H-TM osallistuu nisäkkään Epo-hormonin tuoton säätelyyn.

Pitkät luut muodostuvat endokondraalisen luutumisen kautta. Siinä ensin muodostuu rustoinen malli, kasvulevy, joka vähitellen korvaantuu luukudoksella. Kasvulevyn sisin kerros on sen soluille, kondrosyyteille, hypoksinen kasvuympäristö. HIF:illä on todettu olevan tärkeä rooli kondrosyyttien toiminnan säätelijänä. Kasvulevyn soluvälitila sisältää runsaasti kollageeneja. Kollageenin prolyyli-4-hydroksylaasit (C-P4Ht I-III) ovat avainasemassa kollageenien biosynteesissä ja siten niiden toiminta on välttämätöntä kestävän soluvälitilan muodostumiselle. Käyttämällä *in vitro* menetelmiä, tässä työssä osoitettiin, että hiiren epifyseaalisten kasvulevyjen kondrosyyteissä hypoksia lisää C-P4H:n määrää ja aktiivisuutta HIF-tekijästä riippuvalla mekanismilla. Eri C-P4H-isoentsyymeiden toiminnasta ja merkityksestä *in vivo* tiedetään vain vähän. Tässä työssä karakterisoitiin hiirilinja, jossa C-P4H-II on täysin inaktiivinen, ja hiirilinja, jossa lisäksi C-P4H-I on osittain inaktiivinen. Merkittävästi alentuneen C-P4H:n aktiivisuuden todettiin aiheuttavan hiirimallissa lievän kondrodysplasian sekä heikentyneet luun ominaisuudet.

Asiasanat: erytropoietiini, hypoksiaindusoituva tekijä, kondrosyytit, prolyyli-4-hydroksylaasi, soluhypoksia

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Kokkola, December 2012 Ellinoora Aro

Abbreviations

4Hyp	4-hydroxyproline
aa	amino acid
ARNT	aryl hydrocarbon nuclear translocator
bHLH	basic helix-loop-helix
BM	basement membrane
BS	Bruck syndrome
bp	base pair
CAD	C-terminal transactivation domain of HIF-α
cDNA	complementary DNA
C-P4H	collagen prolyl 4-hydroxylase
DFO	desferrioxamin
E	embryonic day
ECM	extracellular matrix
EDS	Ehlers-Danlos syndrome
EM	electron microscopy
EPO	erythropoietin
ER	endoplasmic reticulum
FACIT	fibril-associated collagens with interrupted helices
FIH	factor inhibiting HIF
GGT	hydroxylysyl glucosyltransferase
GT	hydroxylysyl galactosyltransferase
HB	hemoglobin
HCT	hematocrit
HRE	hypoxia responsive element
HIF	hypoxia inducible factor
HIF-P4H	HIF prolyl 4-hydroxylase
Hyp	hydroxyproline
IC_{50}	inhibitory constant
IPAS	inhibitory PAS
kDa	kilodalton(s)
K_i	inhibitory constant
K_m	Michaelis-Menten constant
LH	lysyl hydroxylase
MCDS	metaphyseal chondrodysplasia of the Schmid type
MED	multiple epiphysial dysplasia

mRNA	messenger RNA
miRNA	microRNA
NLS	nuclear localization signal
ODDD	oxygen-dependent degradation domain
OI	osteogenesis imperfecta
P4H	prolyl 4-hydroxylase
PAS	Per-Arnt-Sim
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PER	*Drosophila* periodic clock protein
PHY	*C. elegans* C-P4H α subunit
P4ha	mouse gene for the C-P4H α subunit
P4H-TM	P4H with a transmembrane domain
pVHL	von Hippel-Lindau tumor suppressor protein
Q-PCR	real-time quantitative PCR
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SIM	*Drosophila* single-minded protein
TAD	transactivation domain
T_m	midpoint of thermal transition
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
Vhl	mouse gene for pVHL
X, in -Gly-X-Y-	any amino acid
Y, in -Gly-X-Y-	any amino acid

List of original papers

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

I Laitala A, Aro E, Walkinshaw G, Mäki JM, Rossi M, Heikkilä M, Savolainen ER, Arend M, Kivirikko KI, Koivunen P* & Myllyharju J* (2012) Transmembrane prolyl 4-hydroxylase is a fourth prolyl 4-hydroxylase regulating EPO production and erythropoiesis. Blood 120: 3336–3344.

II Aro E, Khatri R, Gerard-O'Riley R, Mangiavini L, Myllyharju J* & Schipani E* (2012) Hypoxia-inducible factor-1 (HIF-1) but not HIF-2 is essential for hypoxic induction of collagen prolyl 4-hydroxylases in primary newborn mouse epiphyseal growth plate chondrocytes. J Biol Chem 287: 37137–37144.

III Aro E, Salo AM, Khatri R, Finnilä M, Miinalainen I, Sormunen R, Pakkanen O, Holster T, Soininen R, Tuukkanen J, Schipani E* & Myllyharju J* (2012) Mice lacking collagen prolyl 4-hydroxylase isoenzyme II in combination with a reduced amount of isoenzyme I display abnormalities in skeletogenesis. Manuscript.

*Equal contribution

Contents

1 Introduction

Although decreased tissue oxygenation is characteristic of many disease states such as anemia, myocardial infarction, stroke, pulmonary hypertension, cancer, and inflammation, lowered oxygen tension (hypoxia) is also an important regulatory signal during normal development. Hypoxia inducible factor, HIF, is a heterodimeric ($\alpha\beta$) transcription factor coordinating cellular adaptation to hypoxia. The HIF-α subunit has three isoforms in vertebrates. Under well oxygenated conditions (normoxia) the hydroxylation of specific proline residues within HIF-α and subsequent binding of von Hippel-Lindau tumor suppressor protein (pVHL) marks the subunit for proteasomal degradation. Under hypoxic conditions the amount of HIF-α protein rapidly increases, it dimerizes with its β subunit partner and induces the expression of a great number of genes. Among many others, HIF target genes include *Epo*, the protein product of which erythropoietin (Epo), is crucial for red blood cell formation (erythropoiesis), and many genes involved in the synthesis of extracellular matrix (ECM).

The oxygen-dependent hydroxylation of HIF-α in vertebrates is known to be catalyzed by three HIF prolyl 4-hydroxylases (HIF-P4Hs 1-3), which belong to a large group of 2-oxoglutarate dioxygenases and require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate for their activity. In addition, a fourth P4H possessing a transmembrane domain (P4H-TM) is able to act on HIF at least *in vitro* and in cultured cells but nothing is known about its role in the regulation of mammalian erythropoiesis. Pharmacological HIF stabilization appears to be a promising therapeutic strategy for treating diseases associated with acute or chronic oxygen deprivation, such as anemia, and many novel small-molecular compounds that inhibit HIF-P4Hs with respect to their co-substrate 2-oxoglutarate have been developed recently. In the present work the putative role of P4H-TM in regulating erythropoiesis was studied by administering a HIF-P4H inhibitor, FG-4497, to *P4h-tm* null and wild-type mice. The observed effects were then compared with those seen in FG-4497-treated hypomorphic *Hif-p4h-2* and *Hif-p4h-3* null mice versus wild-type mice.

Most bones in the skeleton are formed through endochondral ossification. It is a two-stage mechanism whereby chondrocytes first form a cartilaginous template, the growth plate, in which osteoblasts then differentiate to form bone. The fetal growth plate is an example of an avascular hypoxic structure of mesenchymal origin and HIF has been shown to play a key role in the survival, development and differentiation of growth plate chondrocytes. The cartilaginous

growth plate is rich in extracellular matrix (ECM) and its main components, collagens. Collagen prolyl 4-hydroxylases (C-P4Hs) play a crucial role in collagen biosynthesis, as the resulting 4-hydroxyprolines are necessary for the stability of all collagen molecules. Like HIF-P4Hs, the C-P4Hs belong to the 2-oxoglutarate dioxygenases and have a similar reaction mechanism but, as indicated above, they hydroxylate distinct substrates, as the HIF-P4Hs act on specific proline residues on HIF-α while the C-P4Hs hydroxylate proline residues in the -X-Pro-Gly- triplets found in collagen molecules and some other proteins. The vertebrate C-P4H family consists of three isoenzymes, C-P4H-I being the main form in most cell types and tissues studied, while C-P4H-II is regarded as the predominant form in chondrocytes and in certain other cell types. Complete inactivation of C-P4H-I in mice has been shown to result in embryonic lethality between E10.5 and E11.5 due to disruption of the basement membranes (BMs).

We postulated here that the post-translational modification of collagens, particularly proline hydroxylation, could be one of the modalities by which HIF regulates the adaptive responses of chondrocytes in early growth plates. Primary epiphyseal growth plate chondrocytes isolated from newborn mice with conditionally inactivated *Hif-1a*, *Hif-2a* or *Vhl* genes were used to address this hypothesis. Since *Vhl* gene encodes for pVHL, its inactivation results in constant stabilization of HIF-1α and HIF-2α even under normoxic conditions. Finally, the *in vivo* roles of different C-P4H isoenzymes were studied by characterizing mouse lines with complete inactivation of C-P4H-II with or without partial inactivation of C-P4H-I. Considering the predominant expression of C-P4H-II in chondrocytes reported earlier, we focused on analysing the skeletal phenotype of the above mouse lines.

2 Review of the literature

2.1 Hypoxia

All multicellular animals and most simple organisms require oxygen (O_2) for survival. O_2 serves as the final electron acceptor in oxidative phosphorylation, a highly efficient mechanism for generating energy in the form of ATP. While the oxygen content of the ambient air at sea level is 21% (150 mmHg) the physiologically normal oxygen content (normoxia) for embryonic or adult cells and tissues varies considerably, being largely in the range 2–9% (14.4-64.8 mmHg). Certain tissues, e.g. the bone marrow niches, growth plate cartilage and the kidney medulla are adapted to 1% O_2 (7.2 mmHg) (Semenza 2009, Simon & Keith 2008).

An acute or chronic decrease in cellular O_2 (hypoxia) will create significant stress in living cells. Such a situation may arise from a reduction in the environmental O_2 supply, e.g. an increase in altitude, or from localized tissue ischemia due to inadequate blood flow to a given area. Accordingly, hypoxia is related to many human disorders, such as severe anemia, inflammation, ischemia, atherosclerotic cardiovascular diseases, pulmonary hypertension and the growth of solid tumors. Nevertheless, hypoxia is a fundamental physiological stimulus that occurs during normal development in response to tissue growth (Majmundar *et al.* 2010, Semenza 2009, Simon & Keith 2008).

During hypoxia, cells activate multiple responses which serve to facilitate oxygen delivery and cellular adaptation in order to maintain oxygen homeostasis. Even moderate hypoxia triggers immediate responses that may be transient, such as changes in the carbohydrate metabolism of tissues, or more long-term and permanent, such as changes in the production of red blood cells and in local blood vessel networks. The molecular mechanisms underlying these events include the function of hypoxia inducible factors (HIFs) (Semenza 2009, Simon & Keith 2008).

2.2 Hypoxia inducible factor – HIF

The transcription factor known as hypoxia inducible factor HIF is the master regulator of oxygen homeostasis in mammalian cells. It was initially identified as a nuclear factor that binds to a *cis*-acting hypoxia response element (HRE; 5’-

RCGTG-3' R:A/G) in the 3' flanking region of the human *EPO* gene (Semenza & Wang 1992) and it has been increasingly recognized since for its key roles in a wide variety of cellular processes that facilitate adaptation to hypoxic conditions resulting from either pathological or physiological events. Over 100 HIF-responsive genes have emerged, and the list is constantly growing. In addition to erythropoiesis, this array includes genes encoding proteins that direct angiogenesis, glucose utilization, iron transport, cell proliferation, differentiation, survival and apoptosis, tumor progression and extracellular matrix production and several other genes whose protein products increase O_2 delivery or facilitate metabolic adaptation to hypoxia (Lendahl *et al.* 2009, Majmundar *et al.* 2010, Semenza 2009, Simon & Keith 2008).

HIF is an obligate heterodimer of 120 kDa composed of two subunits: a constitutively stable subunit HIF-β (also known as the aryl hydrocarbon nuclear translocator ARNT) and an oxygen-labile subunit HIF-α. Both contain basic-helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains that enable heterodimerization and DNA binding (Fig. 1) (Jiang *et al.* 1996, Wang *et al.* 1995). Since HIF-β is present in excess, the transcriptional activity of HIF is defined by the amount of the HIF-α subunit (Semenza *et al.* 1996). Three HIF-α isoforms exist in vertebrates, HIF-1α, HIF-2α and HIF-3α (also termed inhibitory PAS protein, IPAS), each encoded by a distinct gene locus. HIF-1α and HIF-2α are the most extensively studied and account for the vast majority of the HIF transcriptional responses. Similarly, three isoforms of HIF-β (ARNT1, ARNT2, and ARNT3) with partial redundancy and overlapping functions have been identified (Zagorska & Dulak 2004). HIF-3α is substantially different from HIF-1α and HIF-2α and its biological relevance remains somewhat obscure. In humans it exists in at least seven splicing variants, some of which lack a transactivation domain, and it is dominant-inhibitory with respect to HIF-dependent transcription (Heikkila *et al.* 2011, Makino *et al.* 2001, Makino *et al.* 2002, Makino *et al.* 2007, Maynard *et al.* 2007, Pasanen *et al.* 2010). Further details will be given for HIF-1α and HIF-2α.

Structure of HIF-α

HIF-1α contains 826 amino acids and is structurally closely related to HIF-2α, which is a polypeptide of 870 amino acids. They share 48% overall amino acid identity, the highest degree of sequence homology being in the bHLH (85%), PAS-A (68%) and PAS-B (73%) domains (Ema *et al.* 1997, Tian *et al.* 1997). As

shown in Figure 1, both HIF-1α and HIF-2α contain two conserved sites for prolyl 4-hydroxylation (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) within a central oxygen-dependent degradation domain (ODDD) (Patel & Simon 2008). The bHLH and PAS domains of HIF-1α and HIF-2α are located at the N termini, while the transactivation domain (TAD) resides at their C termini (Ema *et al.* 1997, Jiang *et al.* 1996, O'Rourke *et al.* 1999). The C termini are further subdivided into an N-terminal transactivation domain (N-TAD), an inhibitory domain and a C-terminal transactivation domain (C-TAD). N-TAD partially overlaps with ODDD, while C-TAD contains a site for asparagine hydroxylation (Asn803 in human HIF-1α; Asn851 in human HIF-2α) (Huang *et al.* 1998, Jiang *et al.* 1996, Loboda *et al.* 2010). Both TADs are required for optimal common HIF target gene induction; N-TAD is crucial for unique HIF-α activity, while C-TAD contributes to the transcriptional activity of most HIF target genes (Dayan *et al.* 2006, Hu *et al.* 2007, Pugh *et al.* 1997). Moreover, HIF-α contains N and C-terminal nuclear localization signals (N-NLS and C-NLS, respectively) and accordingly most of the protein is found in the nucleus (Kallio *et al.* 1998).

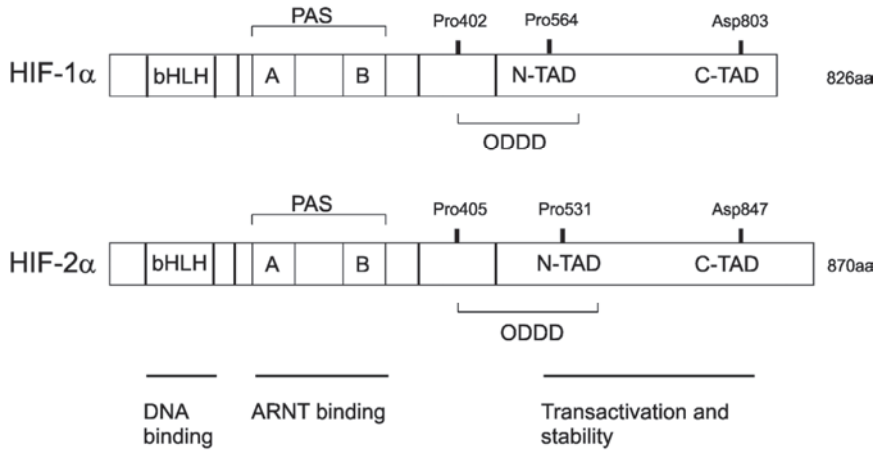

Fig. 1. The domain structure of human HIF-1α and HIF-2α. Both HIFs contain a basic-helix-loop-helix (bHLH) domain, two Per-Arnt-Sim subdomains (PAS A and B) and N and C-terminal transactivation domains (N-TAD and C-TAD, respectively), which are separated by an inhibitory domain (ID). The two prolines involved in the regulation of HIF-α stability (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) are located in the oxygen-dependent degradation domain (ODDD), while the asparagine crucial for binding of the co-activator p-300 (Asn803 in human HIF-1α; Asn851 in human HIF-2α) resides within the C-TAD. Modified from Loboda *et al*. 2010.

Regulation of HIF-α

Under conditions of hypoxia, nuclear levels of HIF-α rise considerably, but upon re-oxygenation the protein rapidly dissipates, with a half-life of only a few minutes in normoxic cells (Wang *et al.* 1995). The regulatory mechanism by which the oxygen content is transduced to changes in the HIF-α protein level involves post-translational modifications of the subunit, as depicted in Figure 2. Under well-oxygenated conditions HIF-α becomes hydroxylated within the ODDD at one or two prolyl residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) by the HIF-P4Hs (Bruick & McKnight 2001, Epstein *et al.* 2001, Huang *et al.* 1998, Ivan *et al.* 2001, Jaakkola *et al.* 2001, Pugh *et al.* 1997, Yu *et al.* 2001). This initiates the binding of von Hippel Lindau protein (pVHL), which further interacts with Elongin C and recruits a ubiquitin ligase complex. HIF-α is then targeted for proteasomal degradation (Maxwell *et al.* 1999). In a complementary mechanism, hydroxylation of a conserved asparagine residue (Asn803 in human HIF-1α and Asn851 in human HIF-2α) within the C-TAD prevents binding of the transcriptional co-activator p300 and thus impairs the transcriptional activity of HIF (Lando *et al.* 2002b). This reaction is catalyzed by a specific asparaginyl hydroxylase which is identical to a protein known as factor inhibiting HIF, FIH-1 (Hewitson *et al.* 2002, Lando *et al.* 2002a, Mahon *et al.* 2001).

In hypoxia oxygen becomes the limiting co-substrate for both HIF-P4Hs and FIH and their activity decreases. As a result, the HIF-α subunits are stabilized, translocate to the nucleus and form an active transcription factor complex with HIF-β and bind transcriptional co-factors such as p300 and CREB-binding protein (CBP). The gene activation then occurs through binding of the HIF-αβ heterodimer to a conserved G/ACGTG core sequence in the HREs of the HIF target genes (Kaelin & Ratcliffe 2008, Majmundar *et al.* 2010, Myllyharju 2008, Myllyharju & Schipani 2010, Semenza 2009).

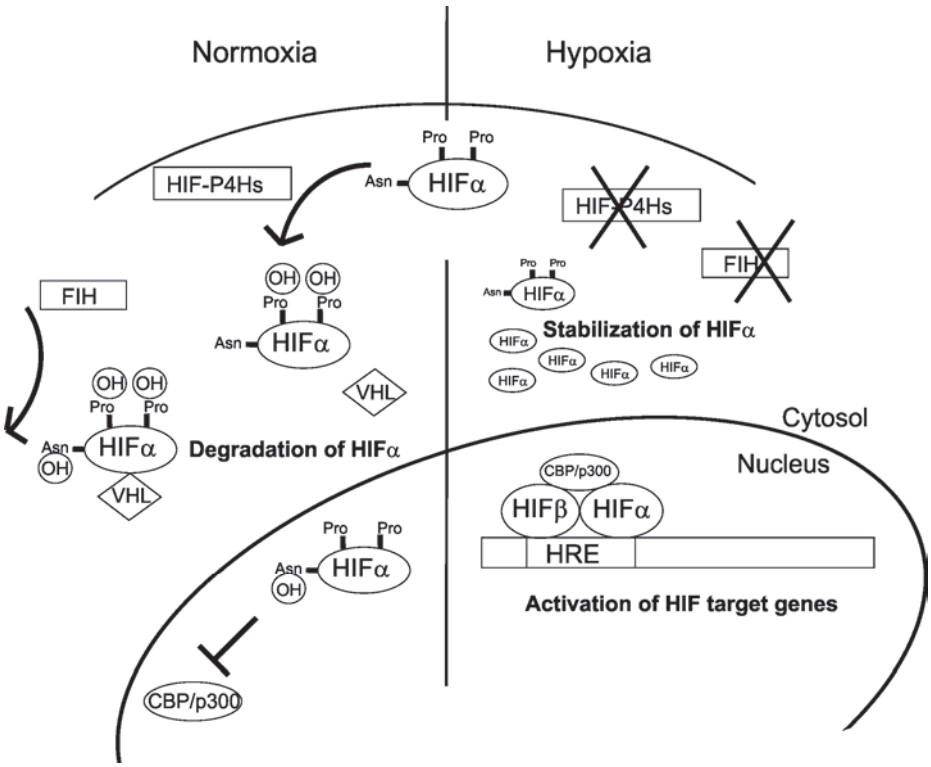

Fig. 2. Oxygen-dependent regulation of HIF by HIF-P4Hs and FIH. Under normoxic conditions two proline residues of HIF-α become hydroxylated by HIF-P4Hs. This event is required for binding of the VHL E3 ubiquitin ligase complex and for subsequent proteasomal degradation. In a complementary mechanism hydroxylation of a particular asparagine residue by FIH interferes with the ability of HIF-α to recruit the transcriptional co-activator p300/CBP. Hypoxia inhibits both HIF-P4Hs and FIH and HIF-α escapes ubiquitination and degradation and translocates into the nucleus where after dimerization with HIF-β and binding of p300/CBP, it is able to target genes that possess the hypoxia responsible element (HRE).

Although the principal pathway regulating HIF-α involves its oxygen-dependent hydroxylation followed by pVHL-mediated degradation, as described above, several O_2/HIF-P4H/pVHL-independent mechanisms controlling the amount of HIF-1α protein and its function have recently been described (Koh *et al.* 2008, Koh *et al.* 2011, Liu *et al.* 2007a, Liu *et al.* 2007b, Luo *et al.* 2010). Both HIF-1α and HIF-2α are subjected to sumoylation, which is another post-translational modification catalyzed by SUMO-specific ligases and reversed by

sentrin/SUMO-specific proteases (SENPs) (Carbia-Nagashima *et al.* 2007, Cheng *et al.* 2007). Sumoylation is likewise dependent on oxygen and involves pVHL-mediated degradation of HIFs, although this occurs independently of proline hydroxylation (Cheng *et al.* 2007, van Hagen *et al.* 2010).

Hypoxia-associated factor (HAF), a novel E3-ubiquitin ligase expressed by proliferating cells, has recently been shown to be a specific regulator of the stability of HIF-1α (Koh *et al.* 2008). The expression of HAF is decreased during acute hypoxia, but increased in response to prolonged hypoxia. In multiple experiments HAF caused HIF-1α ubiquitination and proteasomal degradation irrespective of the cellular oxygen content and without any involvement of pVHL (Koh *et al.* 2008). A subsequent study by the same group revealed that HAF influences both HIF-1α and HIF-2α-dependent gene regulation (Koh *et al.* 2011). Using cancer cell lines, they showed that HAF also binds to HIF-2α, although at a different site from HIF-1α, and increases HIF-2α transactivation independently of the E3 ligase activity of HAF. In so doing HAF switches the hypoxic response of cancer cells from HIF-1α-dependent to HIF-2α-dependent transcription, activates genes involved in invasion and promotes more aggressive growth of tumors under prolonged hypoxia (Koh *et al.* 2011).

Furthermore, nitric oxide (NO)-mediated S-nitrosylation enhances the stability and activity of HIF-1α under certain normoxic conditions (Li *et al.* 2007a) while an important chaperone, the extracellular heat shock protein 90 (Hsp90) regulates steady-state levels of HIF-1α through receptor-activated protein kinase C (RACK1) (Liu *et al.* 2007a, Liu *et al.* 2007b). By competing with Hsp90 RACK1 promotes HIF-1α ubiquitination and degradation (Liu *et al.* 2007a, Liu *et al.* 2007b). Moreover, Hsp70 and the carboxyl terminus of Hsp70-interacting protein (CHIP) were found to interact with HIF-1α, but not HIF-2α, and to control its regulation and degradation (Luo *et al.* 2010).

Cellular oxygen content has been shown to control the level of HIF-α mRNA (Uchida *et al.* 2004, Wiener *et al.* 1996). While acute hypoxia similarly induces the expression of both subunits, the mRNA levels of HIF-2α increase significantly during prolonged hypoxia, whereas the amount of HIF-1α is diminished. Thus, HIF-2α is likely to be more important for adaptation to chronic hypoxia (Holmquist-Mengelbier *et al.* 2006, Uchida *et al.* 2004, Wiesener *et al.* 2003).

Target genes and tissue distribution of HIF-1α versus HIF-2α

HIF-1α and HIF-2α regulate both shared and unique target genes (Fig. 3). HIF-1α is exclusively responsible for the hypoxia-dependent upregulation of glycolytic enzymes such as phosphoglycerate kinase 1 (*PGK1*), lactate dehydrogenase (*LDH-A*), aldolase A (*ALDA*) and carbonic anhydrase-9 (*CA IX*), and of the pro-apoptotic gene *BNIP3* (Grabmaier *et al.* 2004, Hu *et al.* 2003, Raval *et al.* 2005, Wang *et al.* 2005), whereas the genes for erythropoietin (*EPO*), divalent metal transporter 1 (*DMT1*), transforming growth factor-α (*TGFα*), *CYCLIND1*, *TWIST1* and the embryonic transcription factor OCT-4 (*POU5F1, OCT-3/4*) are preferential HIF-2α targets (Baba *et al.* 2003, Covello *et al.* 2006, Gort *et al.* 2008, Gruber *et al.* 2007, Gunaratnam *et al.* 2003, Mastrogiannaki *et al.* 2009, Raval *et al.* 2005, Warnecke *et al.* 2004). On the other hand, there are a great number of hypoxia-inducible genes, such as the classical HIF target vascular endothelial growth factor (*VEGF*), that are regulated by both isoforms (Hu *et al.* 2003, Raval *et al.* 2005)

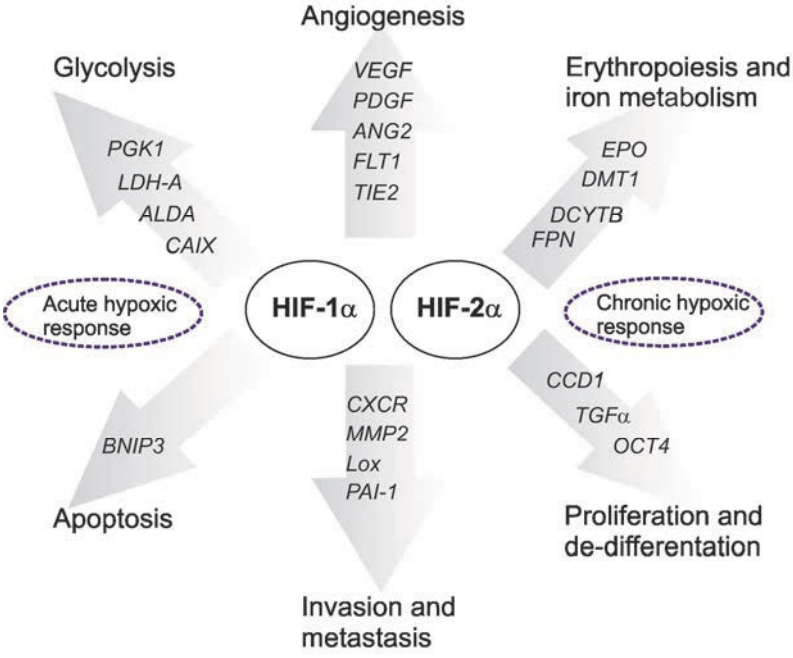

Fig. 3. Some examples of shared and distinct target genes and the corresponding functions of HIF-1α versus HIF-2α. Modified from Gordan & Simon 2007.

Similarly, the expression patterns of HIF-1α and HIF-2α in tissues can be either overlapping or unique. The first characterized isoform, HIF-1α, is expressed ubiquitously. HIF-2α instead was first identified as a hypoxia-inducible PAS domain-containing transcription factor selectively expressed in endothelial cells and accordingly termed endothelial PAS domain protein 1 (EPAS1) (Tian *et al.* 1997). It was not long, however, before HIF-2α was detected in various organs such as the kidney, lung, heart, small intestine, brain, pancreas and the organ of Zuckerkandl (Tian *et al.* 1998, Wiesener *et al.* 2003).

Moreover, the expression of HIF-2α versus HIF-1α can vary between cell populations within the same organ. In the ischemic rat kidney, for example, HIF-2α was localized mainly in the fibroblasts and endothelial cells, while the tubular cells appeared to express exclusively HIF-1α (Rosenberger *et al.* 2002). Likewise, marked stabilization of HIF-2α was detected in the type II pneumocytes and alveolar epithelial cells of the hypoxic lung, while HIF-1α was undetectable (Compernolle *et al.* 2002, Ema *et al.* 1997). An analysis of the HIF-α expression pattern in the rat ischemic myocardium after coronary artery occlusion revealed acute induction of both α subunits in the region surrounding the infarcted tissue, including cardiomyocytes, endothelial cells and macrophages, whereas a progressive increase in HIF-2α, but not HIF-1α, was detected in areas further away from the infarcted area, including the interventricular septum (Jurgensen *et al.* 2004).

Differential functions of HIF-1α and HIF-2α

The substantially different outcomes of germline *Hif-1a* or *Hif-2a* gene disruption in mice clearly illustrates the non-redundant roles of the two isoforms during development. *Hif-1a* knockout leads to embryonic lethality by E11 due to defects in the development of cardiovascular system (Iyer *et al.* 1998). Instead, deletion of *Hif-2a* results in divergent phenotypes depending on the genetic background of the targeted mice: embryonic lethality due to vascular defects and bradycardia, perinatal death due to impaired lung maturation, and finally both embryonic and post-natal death caused by multiorgan failure, metabolic abnormalities and mitochondrial dysfunction (Table 4) (Compernolle *et al.* 2002, Peng *et al.* 2000, Scortegagna *et al.* 2003, Tian *et al.* 1998).

Numerous studies have demonstrated that the HIF-α subunits are overexpressed in a number of primary and metastatic human cancers with distinct abilities to promote tumor growth (Qing & Simon 2009). HIF-2α is more

frequently induced in solid tumors and its expression is more closely associated with poor patient outcomes at least in the case of renal clear cell carcinoma (RCC), non-small cell lung cancer and neuroblastoma (Giatromanolaki *et al.* 2001, Holmquist-Mengelbier *et al.* 2006, Kondo *et al.* 2003, Raval *et al.* 2005). Embryonic stem cell-derived teratomas with a HIF-2α knock-in allele at the HIF-1α locus exhibited a 4-fold increase in mass and were more proliferative than the HIF-1α-expressing controls (Covello *et al.* 2005). HIF-2α has been of particular interest in the pathogenesis of RCC. Loss of *VHL* is associated with the hereditary cancer syndrome called von Hippel-Lindau disease that is characterized by the development of highly vascular tumors, including RCC, hemangioblastomas of the nervous system and pheochromocytomas (Kaelin 2002). Loss of VHL function resulting in the accumulation of HIF-α proteins is also present in the majority (80–90%) of sporadic RCCs (Kim & Kaelin 2004). Examination of the kidneys from patients with VHL renal disease showed strong HIF-2α staining in overt carcinomas and cell lines derived from renal tumors (Raval *et al.* 2005). Restoration of VHL protein to *VHL*-deficient RCC cell lines in xenograft models inhibited tumor growth, and surprisingly, the expression of normoxically stable HIF-1α further enhanced this inhibitory effect, while the expression of stabilized HIF-2α promoted xenograft growth (Kondo *et al.* 2002, Kondo *et al.* 2003, Maranchie *et al.* 2002).

Importantly, both HIF-1α and HIF-2α possess transcription-independent activity that is likely to contribute to their distinct functions (Gordan *et al.* 2007, Gustafsson *et al.* 2005, Koshiji *et al.* 2004). The Myc family of oncoproteins is frequently expressed in human cancer types possessing genetic rearrangements (Lendahl *et al.* 2009) and HIF-1α and HIF-2α display opposite effects in modulating the activity of the Myc transcription factor. Under hypoxia HIF-1α inhibits Myc function and causes cell cycle arrest (Gordan *et al.* 2007, Koshiji *et al.* 2004) but the effect of HIF-1α on Myc is complex and several mechanisms have been suggested. For example, HIF-1α may antagonize Myc functionally by displacing Myc binding from its promoters, such as p21 (Koshiji *et al.* 2004). Moreover, hypoxia-dependent activation of *MXI1,* a gene coding for a potential tumor suppressor, Max interactor 1, could probably regulate Myc in negative manner (Lofstedt *et al.* 2009). A Myc antagonist, the MNT protein, has recently been shown to be targeted in part by miR-210, which is a master microRNA (miRNA) responsible for HIF-1α regulation (Zhang *et al.* 2009). By contrast, it has been proposed that HIF-2α may potentiate Myc transcriptional activity and

subsequently enhance cell proliferation (Gordan & Simon 2007, Gordan *et al.* 2008).

Moreover, several interactions have been established between the HIF pathway and Notch signalling. Hypoxia results in increased Notch signalling through transcriptional upregulation of the Notch ligand delta-like1 (DLL1) and DLL4 (Diez *et al.* 2007, Li *et al.* 2007b, Sahlgren *et al.* 2008). HIF-1α has been reported to increase the half-life and transcriptional activity of Notch1 by direct interactions with its intracellular domain (Notch ICD) (Gustafsson *et al.* 2005) while FIH binds Notch ICD and negatively affects Notch signaling (Zheng *et al.* 2008). The strong interaction between FIH and Notch ICD could potentially relieve HIF-1α from FIH-mediated transcriptional repression and result in increased expression of hypoxia-inducible genes (Zheng *et al.* 2008).

Even though the specific *in vivo* functions of the multiple interactions of the Myc and Notch signalling pathways with the hypoxia pathway are not fully understood they are likely to be important in many cellular contexts. Given the multiple roles of Myc and Notch in tumorigenesis and development, the intersection of the pathways with HIF may contribute to hypoxia-dependent events such as stem and progenitor cell maintenance, tumor invasion, de-differentiation and epithelial-to-mesenchymal transition (Lendahl *et al.* 2009).

MiRNAs are short, non-coding RNAs that provide an important mechanism for post-transcriptional gene regulation. They repress translation and/or cause RNA degradation by binding to 3'UTRs in their target transcripts (Lendahl *et al.* 2009). The link between a specific group of miRNAs and hypoxia has been highlighted in many studies. One member of this group, miR-210, is the most consistently hypoxia-responsive miRNA and is overexpressed in many cancer types (Huang *et al.* 2010, Lendahl *et al.* 2009). Both HIF-1α and HIF-2α have been shown to regulate miR-210, and such regulation is likely to be cell type-specific (Camps *et al.* 2008, Zhang *et al.* 2009). Some miRNAs, including miR-424, miR-20b, and miR-199a, have been reported to target HIF-1α mRNA and thus directly regulate its expression (Cascio *et al.* 2010, Ghosh *et al.* 2010, Lei *et al.* 2009). So far little is known about HIF isoform-specific interactions with unique miRNAs.

2.2.1 HIF-hydroxylating enzymes

A family of cytoplasmic and nuclear P4Hs, as distinct from the endoplasmic reticulum (ER) luminal collagen P4Hs, is mostly responsible for regulation of the

stability of HIF-α. The mammalian genome encodes three HIF-P4Hs 1, 2 and 3, also known as prolyl hydroxylase domain enzymes (PHDs) 1, 2 and 3, Egl-nine (EGLNs) 2, 1 and 3, or HIF prolyl hydroxylases (HPHs) 3, 2 and 1, respectively (Bruick & McKnight 2001, Epstein *et al.* 2001, Ivan *et al.* 2001). *Drosophila melanogaster* and *Caenorhabditis elegans* possess a single protein with a corresponding function named *Fatiga* and *EGL9*, respectively (Centanin *et al.* 2005, Epstein *et al.* 2001). Mammals have a single HIF asparaginyl hydroxylase (factor inhibiting HIF, FIH), which is not present in *D. melanogaster* or *C. elegans* (Hewitson *et al.* 2002, Lando *et al.* 2002a, Lando *et al.* 2002b). In addition, a vertebrate ER transmembrane prolyl 4-hydroxylase (P4H-TM) has been shown to influence HIF activity at least *in vitro* and in cultured cells (P4H-TM, see 2.2.2) (Koivunen *et al.* 2007, Oehme *et al.* 2002).

The HIF-P4H isoenzymes share 42–59% amino acid sequence, HIF-P4H-2 being the most closely related to the single HIF-P4H in *D. melanogaster* and *C. elegans* (Taylor 2001). The human HIF-P4H-1 and 2 polypeptides consist of 407 and 426 residues, respectively, whereas HIF-P4H-3 is substantially smaller, consisting of 239 residues (Fig. 4) (Bruick & McKnight 2001, Epstein *et al.* 2001, Ivan *et al.* 2001). Like other those of Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases the catalytic site of HIF-P4H-2 is composed of a common secondary structure of eight β-strands folded into a ‘‘jelly-roll’’ motif, or double stranded β-helix (Schofield & Ratcliffe 2004). The crystal structure of the catalytic domain of HIF-P4H-2 has been decribed and sequence comparisons and modelling studies have shown that it is likely to be highly conserved among the three human HIF-P4H isoenzymes (Chowdhury *et al.* 2009, McDonough *et al.* 2006). HIF-P4H-2 crystallizes as a homotrimer, however, it is most likely to exist as a monomer in solution (McDonough *et al.* 2006). The catalytically critical motifs of the HIF-P4Hs contain three conserved Fe^{2+}-binding residues, two histidines and one aspartate, and an arginine that binds the 2-oxoglutarate at position +9 from the second iron-binding histidine (Bruick & McKnight 2001, Epstein *et al.* 2001, Ivan *et al.* 2001). The opening of the HIF-P4H active site is sterically narrow, which may explain the tight binding constants for Fe^{2+} and 2-oxoglutarate (McDonough *et al.* 2006, McNeill *et al.* 2002). The N-terminal region of HIF-P4H-2 contains a zinc finger domain that may inhibit the catalytic activity of the isoenzyme (Choi *et al.* 2005).

HIF-P4H-2 and HIF-P4H-3 mRNAs are subject to alternative splicing as two inactive variants of HIF-P4H-2 and one of HIF-P4H-3 have been identified (Cervera *et al.* 2006, Hirsila *et al.* 2003). These splicing forms are widely

expressed in all tissues studied, while at least a partially active second splicing variant of HIF-P4H-3 is restricted to primary cancer tissues (Cervera *et al.* 2006, Hirsila *et al.* 2003). Moreover, two variants of HIF-P4H-1 are generated by alternative translational initiation, both forms being biologically active with similar HIF-P4H activity (Tian *et al.* 2006).

FIH is composed of 349 amino acid residues (Fig. 4) (Mahon *et al.* 2001). Although it contains a β-sheet core similar to that of the HIF-P4Hs and other 2-oxoglutarate-dependent dioxygenases, the key residues or their locations involved in co-substrate binding are different. The iron binding site includes a conserved two-histidine, and one-aspartate motif, but the basic residue that binds the C-5 carboxyl group of 2-oxoglutarate is a lysine in position 214 (Dann *et al.* 2002, Elkins *et al.* 2003, Lee *et al.* 2003). In solution, FIH forms a homodimer by means of its C-terminal dimerization domain (Dann *et al.* 2002, Lee *et al.* 2003). This dimerization is likely to be crucial for the substrate recognition and activity of FIH (Dann *et al.* 2002, Lee *et al.* 2003). The FIH structure contains a prominent groove at the centre of the molecule that extends from the active site towards the dimerization domain and most probably serves as the binding site for HIF-α (Lee *et al.* 2003). The dimeric structure also contains a putative pVHL binding site, suggesting the formation of ternary complexes by FIH, HIF and pVHL (Lee *et al.* 2003).

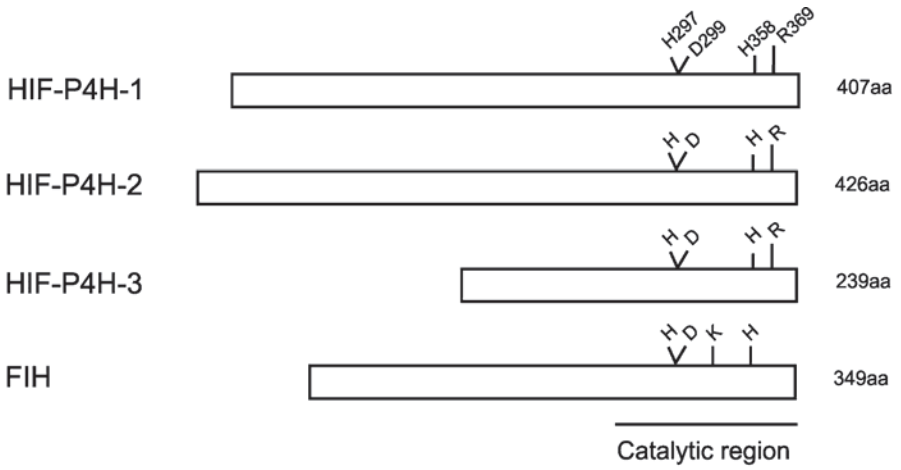

Fig. 4. Schematic representation of the human HIF-P4Hs and FIH, showing the lengths of the polypeptides on the right and the catalytically critical residues above the polypeptides.

Reaction mechanism and catalytic properties

Together with the relatively extensively characterized C-P4Hs, the HIF-P4Hs and FIH belong to the large evolutionally conserved family of iron and 2-oxoglutarate-dependent dioxygenases whose activity is dependent on molecular oxygen (Kaelin & Ratcliffe 2008, Myllyharju 2008). During the hydroxylation reaction one oxygen atom is incorporated directly into a peptidyl proline of the HIF-α subunit to form 4-hydroxyproline and the other oxygen atom is used in the oxidative decarboxylation of 2-oxoglutarate in a reaction that yields succinate and CO_2. Fe^{2+} is crucial for activating O_2 and as a template for orderly binding of the reactants, while ascorbate serves as an alternative oxygen acceptor in uncoupled decarboxylation cycles (Fig. 9) (Hewitson *et al.* 2002, McNeill *et al.* 2002, Myllyharju 2008). Since the reaction mechanism is similar to that of the C-P4Hs it will be discussed in more detail in section 2.3.3.

The oxygen dependence of the HIF-P4Hs is reflected in their relatively high K_m values for O_2 somewhat different values in the range 85–250 μM having been published depending on the recombinant enzyme, substrate length, and assay conditions (Ehrismann *et al.* 2007, Hirsila *et al.* 2003, Koivunen *et al.* 2006). In all cases, however, the $K_m(O_2)$ is significantly higher than the ambient oxygen

level in cells (typically in the range 10–39 μM) making the HIF-P4Hs efficient oxygen sensors (Table 1). The K_m of FIH for O_2 is about one-third of those of the HIF-P4Hs, which theoretically allows it to function still on any HIF-α chain that survives the degradative pathway under conditions of moderate hypoxia (Koivunen *et al.* 2004, Koivunen *et al.* 2006). The K_m values of HIF-P4H-1 and 2 for Fe^{2+} (0.03μM) and 2-oxoglutarate (2 μM and 1 μM, respectively) are very similar, while the corresponding values of HIF-P4H-3 are 3–10 times higher (Hirsila *et al.* 2005, Koivunen *et al.* 2007, McNeill *et al.* 2002). The K_m values of all three isoenzymes for ascorbate are in a range 140–180 μM (Hirsila *et al.* 2003). The K_m value of FIH for 2-oxoglutarate (25 μM) is similar to that of the C-P4Hs and much higher than that of the HIF-P4Hs (Hewitson *et al.* 2002, Kivirikko & Pihlajaniemi 1998, Koivunen *et al.* 2004), while the K_m of FIH for Fe^{2+} is 0.5 μM and that for ascorbate 260 μM (Table 1) (Koivunen *et al.* 2004).

Table 1. K_m(μM) values of recombinant human HIF-P4Hs and FIH for the reaction co-substrates and substrates.

Co-substrate	HIF-P4H-1	HIF-P4H-2	HIF-P4H-3	FIH
Fe^{2+}	0.03[a]	0.03[a]	0.1[a]	0.5[e]
2-Oxoglutarate	2[b]	1[b]	12[b]	25[e]
Ascorbate	170[c]	180[c]	140[c]	260[e]
O_2	230[c]	100-250[d, c]	230[c]	90-237[e,f]
HIF-1αODDD	0.01-0.02[d]	0.14[d]	0.07[d]	NS
HIF-2αODDD	0.01-0.02[d]	0.06[d]	0.1[d]	NS
HIF-1αCTAD	NS	NS	NS	100-222[e,f]
HIF-2αCTAD	NS	NS	NS	160-229[e,f]

[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]Ehrishman *et al.* 2007; NS, not a substrate

The HIF-P4Hs hydroxylate only peptidyl prolines that are present in HIF-α in Leu-X-X-Leu-Ala-Pro sequences. There is no absolute sequence requirement in addition to proline itself in this conserved core motif with respect to substrate specificity (Hirsila *et al.* 2003, Huang *et al.* 2002, Li *et al.* 2004). The two prolines in both HIF-1α and HIF-2α are differentially hydroxylated by the HIF-P4H isoenzymes, the N-terminal proline being hydroxylated less efficiently than the C-terminal one, especially in the case of HIF-P4H-3 (Ehrismann *et al.* 2007, Flashman *et al.* 2008, Hirsila *et al.* 2003, Koivunen *et al.* 2006). By comparison with the HIF-P4Hs, FIH requires a relatively long substrate for efficient hydroxylation (Koivunen *et al.* 2004, Koivunen *et al.* 2006). Studies with cultured

cells have suggested that HIF-P4H-2 may act more efficiently on HIF-1α than on HIF-2α, while HIF-P4H-3 has the opposite effect (Appelhoff *et al.* 2004). Regarding FIH, peptides representing HIF-2α were less efficiently hydroxylated than those representing HIF-1α (Koivunen *et al.* 2004). It is most likely, however, that the relative contributions of the HIF-P4H isoenzymes and FIH to the regulation of the two HIF-α subunits are determined by multiple factors, including cellular location and the abundance of the enzymes and physiological status of the cell in addition to differences in their K_m values (Hirsila *et al.* 2003, Koivunen *et al.* 2006).

The non-HIF hydroxylation targets of HIF-P4Hs and FIH have been under extensive research in the past few years. Several ankyrin repeat domain (ARD)-containing proteins including IKKβ and Notch receptor family members have been identified as alternative (non-HIF) FIH substrates, but the functional significance of the FIH-mediated hydroxylation of IKKβs and Notch receptors remains unclear (Cockman *et al.* 2006, Coleman *et al.* 2007, Zheng *et al.* 2008). Interestingly, IKKβ, which is an inhibitor of NF-κB signaling, is a putative HIF-P4H-1 and HIF-P4H-2 target, suggesting that several HIF hydroxylases may regulate this pathway (Cummins *et al.* 2006) Like HIF, ARD hydroxylation is suppressed by hypoxia, and it has been speculated that these novel substrates may be involved in hypoxia signalling via other, as yet uncharacterized systems (Coleman *et al.* 2007, Zheng *et al.* 2008). HIF-P4H-3 has been shown to target ATF-4 transcription factor and myogenin in a hydroxylation-dependent manner, although the actual hydroxylation event has not been demonstrated (Fu *et al.* 2007, Koditz *et al.* 2007, Wottawa *et al.* 2010). In addition, HIF-P4H-3 appears to hydroxylate HCLK2 (the human homologue of the *Caenorhabditis elegans* biological clock protein CLK-2) and the β2 adrenergic receptor (Xie *et al.* 2009, Xie *et al.* 2012). More recently, however, HIF-P4H-2 has been shown to hydroxylate β arrestin 2, and in so doing to regulate internalization of the β2 adrenergic receptor (Yan *et al.* 2011). Furthermore, degradation of RNA polymerase II and iron regulatory protein 1 (IRP1) occurs in a hydroxylation-dependent manner, although the dioxygenase catalyzing this reaction has not yet been identified (Hanson *et al.* 2003, Kuznetsova *et al.* 2003, Mikhaylova *et al.* 2008, Wang *et al.* 2004)

Numerous compounds are known to inhibit HIF-P4Hs and FIH competitively with respect to their co-substrates or substrate (see Table 2). Bivalent cations, such as Zn^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} inhibit HIF-P4Hs, FIH and C-P4Hs competitively with respect to Fe^{2+} (Bruick & McKnight 2001, Epstein *et al.* 2001,

Hirsila *et al.* 2005) and Co^{2+} and Ni^{2+} have been used as hypoxia mimetics to stabilize HIF-1α in cultured cells (Ivan *et al.* 2001, Jaakkola *et al.* 2001). Competitive inhibition of HIF-P4Hs by these metals is not particularly strong, however, and it is therefore likely that other mechanisms may be involved in metal-induced HIF-α stabilization (Hirsila *et al.* 2005, Salnikow *et al.* 2004, Yuan *et al.* 2003). Iron chelators such as α,α'-dipyridyl, desferrioxamine, and cipropiroxolamine, inhibit all the P4Hs and FIH, although the HIF-P4Hs are inhibited much less effectively than FIH and the C-P4Hs, presumably due to their tighter binding of Fe^{2+} (Bruick & McKnight 2001, Epstein *et al.* 2001, Hirsila *et al.* 2005, Hirsila *et al.* 2005). Several structural analogues of 2-oxoglutarate such as oxalylglycine block the binding of 2-oxoglutarate and subsequently inhibit HIF-P4Hs and FIH along with the C-P4Hs (Bruick & McKnight 2001, Epstein *et al.* 2001, Hirsila *et al.* 2003, Ivan *et al.* 2001, Muchnik & Kaplan 2011, Myllyharju 2009). There seem to be distinct differences between the HIF-P4Hs, FIH and the C-P4Hs in their inhibitory properties, indicating that their catalytic sites differ sufficiently to allow the development of selective pharmacological agents for therapeutics (Myllyharju 2009). Novel HIF-P4H inhibitors and their therapeutic advantages will be discussed in section 2.2.4.

Table 2. Inhibition of recombinant human HIF-P4Hs and FIH by certain metals and 2-oxoglutarate analogues.

Inhibitor	HIF-P4H-1	HIF-P4H-2	HIF-P4H-3	FIH
Zn^{2+} (IC_{50})	28[a]	130[a]	4[a]	0.5[a]
Co^{2+} (IC_{50})	38[a]	100[a]	9[a]	1[a]
Ni^{2+} (IC_{50})	130[a]	>1000[a]	120[a]	10[a]
Pyridine 2,4-dicarboxylate (K_i)	40[b]	7[b]	8[b]	30[c]
Pyridine 2,5-dicarboxylate (K_i)	>300[b]	>300[b]	>300[b]	50[c]
3-Hydroxypyridine-2carbonyl-glycine	15[b]	2[b]	1[b]	>300[c]
Oxalylglycine (K_i)	50[b]	8[b]	10[b]	2[c]
3,4-Dihydroxybenzoic acid (K_i)	>300[b]	>300[b]	>300[b]	10[c]
N-((3-Hydroxy-6-chloroquinolin-2-yl)carbonyl)glycine (K_i)	0.8[b]	0.2[b]	0.2[b]	>300[c]

[a]Hirsilä *et al.* 2005, [b]Hirsilä *et al*. 2003, [c]Koivunen *et al.* 2004

Tissue distribution and subcellular localization of HIF hydroxylases

The HIF-P4Hs are ubiquitously expressed in various vertebrate tissues and cell lines that differ in the relative abundance of their mRNA (Appelhoff *et al.* 2004,

Hirsila *et al.* 2003). The primary HIF-α-hydroxylating enzyme, HIF-P4H-2, is widely expressed in most tissues, with relatively uniform expression patterns, although showing particularly abundant expression in adipose tissue (Lieb *et al.* 2002, Oehme *et al.* 2002, Willam *et al.* 2006). The level of HIF-P4H-1 expression is highest in the testis and placenta and slightly lower in the brain, liver, heart and adipose tissue (Cioffi *et al.* 2003, Hirsila *et al.* 2003, Lieb *et al.* 2002, Willam *et al.* 2006). HIF-P4H-3 mRNA is expressed at a low level in many tissues the highest expression being detected in the heart and placenta and slightly lower expression in the skeletal muscle and adipose tissue (Cioffi *et al.* 2003, Lieb *et al.* 2002, Willam *et al.* 2006). The mRNA expression of FIH has not yet been studied in detail.

Studies of HIF-P4H protein expression do not show complete correlation with the mRNA expression patterns. Immunohistochemical analyses have revealed detectable levels of HIF-P4H-1 protein in the epithelium of many human tissues, with the greatest intensity in the pancreatic and salivary gland ducts, gallbladder and renal tubules (Soilleux *et al.* 2005). HIF-P4H-2 showed a rather similar distribution to HIF-P4H-1, but was also significantly present in tracheal respiratory epithelial cells, endothelial cells of the skin and the basal proliferating layer of the epidermis (Jokilehto *et al.* 2006, Soilleux *et al.* 2005). The predominant staining for HIF-P4H-3 was seen in the epithelium of several gastrointestinal organs but it was also detected in the renal tubules, endometrium, breast myoepithelial and luminal cells, respiratory epithelium, pneumocytes, thymic epithelium, testicular cells and lymphocytes (Soilleux *et al.* 2005). Interestingly, age-dependent increases in HIF-P4H-3 protein level correlating with decreased expression of HIF-1α have been reported in the human and mouse heart (Rohrbach *et al.* 2005). FIH has been detected in a wide range of epithelial cells, particularly in the oesophagus, stomach, enterocytes, liver, gallbladder, pancreatic ducts, salivary gland ducts, renal tubules, tracheal and bronchial respiratory epithelium and testicular cells (Soilleux *et al.* 2005).

Subcellular localization studies have revealed distinct intracellular allocation of the HIF-hydroxylating enzymes (Jokilehto *et al.* 2006, Metzen *et al.* 2003a, Soilleux *et al.* 2005, Steinhoff *et al.* 2009). In a study with human osteosarcoma cells and overexpressed HIF-P4Hs fused to green fluorescent protein (GFP), HIF-P4H-1 was detected exclusively in the nucleus, while HIF-P4H-2 was mainly localized in the cytoplasm and HIF-P4H-3 in both cell compartments (Metzen *et al.* 2003a). Endogenous HIF-P4Hs and FIH were mostly found in the cytoplasm, however (Soilleux *et al.* 2005), while increased expression of HIF-P4H-2 with

nuclear accumulation was observed in head and neck squamous cell carcinomas (HNCCs) (Jokilehto *et al.* 2006). Later, HIF-P4H-2 was shown to shuttle between the nucleus and cytoplasm by means of its putative subcellular localization signals in the N terminus (Steinhoff *et al.* 2009). It should also be noted that although overall expression of the HIF-P4Hs is dependent on cellular oxygen content, hypoxia does not influence their subcellular localization (Metzen *et al.* 2003a).

Regulation of the HIF-P4Hs

The expression of HIF-P4H-2 and HIF-P4H-3 mRNAs is upregulated by hypoxia, while HIF-P4H-1 levels remain stable regardless of the oxygen content or may even be reduced by hypoxia (Appelhoff *et al.* 2004, Berra *et al.* 2003, Cioffi *et al.* 2003, D'Angelo *et al.* 2003, Epstein *et al.* 2001, Erez *et al.* 2004, Marxsen *et al.* 2004). The hypoxic induction of HIF-P4H-2 and 3 expression is thought to generate a negative feedback loop that attenuates excessive HIF activity under conditions of lowered oxygen tension and effectively turns down HIF-mediated gene regulation during the reoxygenation of hypoxic cells (Berra *et al.* 2003, Ginouves *et al.* 2008). During long-term hypoxia not only does the pool of all three HIF-P4Hs increase but the activity of the enzymes also increases and triggers HIF-α desensitization. This feedback mechanism is probably required to protect cells against necrotic cell death and thus to adapt them to chronic hypoxia (Ginouves *et al.* 2008). Besides hypoxia, certain distinct growth factors and hormones are known to regulate the mRNA levels of HIF-P4Hs. Oestrogen is a well characterized upregulator of HIF-P4H-1, while among the growth factors, TGF-β1 has been shown to selectively inhibit HIF-P4H-2 expression (Appelhoff *et al.* 2004, McMahon *et al.* 2006, Seth *et al.* 2002).

In contrast to 2-oxoglutarate, which is an obligate requirement for the HIF-P4Hs, several other intermediates of the tricarboxylic acid (TCA) cycle and glycolysis such as citrate, isocitrate, succinate, fumarate, malate, oxaloacetate, and puryvate have all been reported to inhibit HIF-P4Hs with varying degrees of potency (Dalgard *et al.* 2004, Hewitson *et al.* 2007, Isaacs *et al.* 2005, Koivunen *et al.* 2007, Selak *et al.* 2005). The most consistent results have been obtained with fumarate and succinate, which inhibit all the HIF-P4Hs competitively with respect to 2-oxoglutarate (Koivunen *et al.* 2007). Neither of these molecules has a significant effect on FIH, which appears to be inhibited most effectively by citrate and oxaloacetate (Hewitson *et al.* 2007, Koivunen *et al.* 2007). TCA cycle

intermediates and their effects on HIF-P4Hs have been of particular interest in studies of cancer metabolism. Many neoplasias possess mutations that impair the activity of TCA cycle enzymes, resulting in the accumulation of intermediates and increased levels of the HIF-1α and HIF-2α proteins (Jokilehto & Jaakkola 2010, Kaelin & Ratcliffe 2008).

The mitochondrial electron transport chain is known to generate ROS, a superoxide, at low levels at complex I, II, and III. A series of papers have reported the requirement of ROS produced by complex III for stabilization of hypoxia-dependent HIF-1α (Bell *et al.* 2007, Brunelle *et al.* 2005, Guzy *et al.* 2005, Mansfield *et al.* 2005). ROS generated in response to hypoxia has been thought to inhibit the activity of HIF-P4Hs, probably through oxidation of Fe^{2+}, which is an essential co-factor for the HIF-P4Hs and thus results in the accumulation of unhydroxylated HIF-1α (Bell *et al.* 2007, Mansfield *et al.* 2005). This conclusion was reached by means of a set of experiments in which the electron transport chain was blocked by genetic or pharmacological interventions, thus eliminating the ROS production in the respiratory complex III (Bell *et al.* 2007, Brunelle *et al.* 2005, Guzy *et al.* 2005, Mansfield *et al.* 2005). A number of contradictory results with no correlation between mitochondrial generation of ROS and hypoxic stabilization of HIF have nevertheless been reported (Chua *et al.* 2010, Naranjo-Suarez *et al.* 2012, Srinivas *et al.* 2001, Vaux *et al.* 2001).

In addition, the physiological effector molecule NO modifies the signalling consequences of hypoxia. In normoxia it has a tendency to stabilize the HIF protein, probably by directly inhibiting HIF-P4H function (Metzen *et al.* 2003b, Palmer *et al.* 2000, Sandau *et al.* 2001). The most reasonable molecular explanation for this inhibition could be the ability of NO to block the interaction of Fe-containing enzymes with molecular oxygen (Kaelin 2005, Metzen *et al.* 2003b) but paradoxically, NO has been reported to attenuate the induction of the HIF pathway under hypoxic conditions (Hagen *et al.* 2003, Mateo *et al.* 2003). NO may increase intracellular oxygen availability by inhibiting mitochondrial respiration and redistribute oxygen towards the HIF-P4Hs, so that they do not recognize the state of hypoxia (Hagen *et al.* 2003). This hypothesis has not been fully supported by other studies, however (Doege *et al.* 2005).

Finally, HIF-P4H activity can be modulated by post-translational mechanisms and protein associations. Siah ubiquitin ligases, in particular Siah1a/2, were shown to regulate the abundance of HIF-P4H-1 and HIF-P4H-3 by targeting them for proteasomal degradation under hypoxic conditions (Nakayama *et al.* 2004, Nakayama *et al.* 2007). Interestingly, hypoxia-induced HIF-1α stabilization was

completely abolished in Siah1a/2 null cells, even though the level of HIF-P4H-2 remained unaffected (Nakayama *et al.* 2004). It was then demonstrated that HIF-P4H-3 is able to form complexes that include homodimers and heterodimers/multimers that probably contain HIF-P4Hs and other proteins that influence their localization and activity and that complex formation affects its activity towards HIF-1α and its susceptibility to degradation by Siah2 (Nakayama *et al.* 2007). On the other hand, the regulation of HIF-P4H-2 protein abundance appears to be dependent on FK506-binding protein (FKBP) 38 (Barth *et al.* 2007, Barth *et al.* 2009). FKBP38-bound HIF-P4H-2 is probably constantly degraded by a ubiquitin-independent proteasomal pathway, whereas cytosolic HIF-P4H-2 is stable and able to mediate its functions as an active P4H (Barth *et al.* 2007, Barth *et al.* 2009). Recruitment of a tumor suppressor protein, IGN4, allows HIF-P4H-2 to modulate HIF activity, however, without affecting the hydroxylase activity of HIF-P4H-2 or the stability of HIF (Ozer *et al.* 2005). Moreover, the protein OS-9 was shown to promote HIF-P4H activity towards HIF-1α in a hydroxylation-independent manner via the formation of ternary complexes composed of OS-9, HIF-1α and HIF-P4H-2 or HIF-P4H-3 (Baek *et al.* 2005).

Mouse models

Mouse models with germline disruptions of the HIF-P4Hs have emphasized the importance of HIF-P4H-2 as the main regulator of HIF signalling under basal conditions. Inactivation of the *Hif-p4h-2* gene resulted in severe placental and heart defects, significant increases in HIF-1α and HIF-2α expression in the placenta, but not in the heart, and embryonic lethality between E12.5 and E14.5, while mice with homozygous disruptions of the *Hif-p4h-1* or *Hif-p4h-3* gene were viable with no appreciable phenotypic abnormalities (Takeda *et al.* 2006, Takeda & Fong 2007, Takeda *et al.* 2008). Moreover, mice with broad-spectrum conditional inactivation of *Hif-p4h-2* developed severe defects in both the vascular system and blood homeostasis since hyperactive angiogenesis, angiectasia, and profound polycythemia associated with venous congestion, dilated cardiomyopathy and premature mortality have been reported (Minamishima *et al.* 2008, Takeda & Fong 2007, Takeda *et al.* 2008). Although single mutants for *Hif-p4h-1* or *Hif-p4h-3* displayed no signs of induced blood cell production or vascular abnormalities, *Hif-p4h-1*$^{-/-}$*;Hif-p4h-3*$^{-/-}$ double knockout mice developed moderate erythrocytosis, partly by activating the hepatic HIF-2α/Epo pathway (Takeda *et al.* 2008).

Hif-p4h-1 null mice were nevertheless reported to have lowered oxygen consumption caused by reprogramming of glucose metabolism from oxidative to more anaerobic energy production, which provided acute protection against lethal ischemia in the skeletal muscle (Aragones *et al.* 2008). *Hif-p4h-3* null mice had altered sympathoadrenal development and function with reduced catecholamine secretion and systemic hypotension, establishing the importance of functional HIF-P4H-3 for proper anatomical and physiological integrity of the system (Bishop *et al.* 2008). Unexpectedly, heterozygous *Hif-p4h-2* deficiency restored tumor oxygenation and suppressed tumor invasion, intravasation, and metastasis via endothelial normalization (Mazzone *et al.* 2009).

Mice with the inactivated *Fih* gene had no significant changes in vascularization, erythropoiesis or embryonic development (Zhang *et al.* 2010). Rather, FIH was found to play a role in regulating energy metabolism, as these FIH knockout mice displayed decreased body weight, increased energy expenditure and improved insulin sensitivity, although with normal glucose tolerance (Zhang *et al.* 2010). The accelerated metabolic rate was not associated with any increase in glycolysis and therefore it was unlikely to have been a straightforward consequence of HIF activation (Zhang *et al.* 2010). Mice with neuron-specific deletion of FIH displayed a phenocopy of global FIH inactivation, while animals with hepatic deletion did not have any metabolic phenotype arguing for a predominant role of FIH in the nervous system (Zhang *et al.* 2010). Table 3 summarises the phenotypic outcomes of germ-line genetic manipulation of the hypoxic signaling pathway in mice.

Table 3. Summary of the phenotypes generated by germ-line knock-out of genes encoding for HIF-1α, HIF-2α, HIF-P4Hs or FIH in mice.

Hypoxic signalling factor	Phenotype
HIF-1α	Embryonic lethality by E11 due to impaired development of cardiovascular system[1]
HIF-2α	Bradycardia, reduced catecholamine levels and eventually embryonic lethality at midgestation[2]
	Embryonic lethality by E13.5 due to vascular defects typically in yolk sac[3]
	Embryonic lethality due to cardiac failure and neonatal lethality due to reduced surfactant production and respiratory distress syndrome[4]
	Pancytopenia, hepatic steatosis, retinopathy, cardiac hypertrophy, increased oxidative stress[5]
HIF-P4H-1	Lowered tissue oxygen comsumption providing protection against acute ischemia in skeletal muscle[6]
HIF-P4H-2	Embryonic lethality between E12.5 and E14.5 due to defects in placenta and heart[7],
HIF-P4H-3	Altered sympathoadrenal development[8]
HIF-P4H-1/HIF-P4H-3	Moderate polycythemia[9]
FIH	Lowered body weight with increased energy expenditure, and improved insulin sensitivity with normal glucose tolerance[10]

[1]Iyer *et al.* 1998, [2]Tian *et al.* 1998, [3]Peng *et al.* 2000, [4]Compernolle *et al.* 2002, [5]Scortegagna *et al.* 2003, [6]Aragones *et al*. 2008, [7]Takeda *et al.* 2006, [8]Bishop et al. 2008, [9]Takeda *et al.* 2008, [10]Zhang *et al*. 2010

2.2.2 Transmembrane prolyl 4-hydroxylase

Besides the relatively extensively studied HIF-P4Hs and C-P4Hs, a third type of P4H exists (Koivunen *et al.* 2007, Oehme *et al.* 2002). Known as transmembrane P4H (P4H-TM), this possesses a transmembrane domain and is located in the ER in an orientation in which the catalytic site is inside the lumen (Koivunen *et al.* 2007, Oehme *et al.* 2002). P4H-TM is found only in vertebrates, including zebrafish, and is absent in flies and nematodes (Koivunen *et al.* 2007). It is expressed in many human tissues, with the highest mRNA levels detected in the adult pancreas, heart, skeletal muscle, brain, placenta, adrenal gland and kidney, as well as in various cell lines and tumor types (Koivunen *et al.* 2007, Oehme *et al.* 2002). Zebrafish *P4h-tm* mRNA is expressed at its highest levels in the eye and brain, lower levels being observed in the skeletal muscle, kidney and heart (Hyvarinen *et al.* 2010b).

The human P4H-TM is a homodimer which consists of 502 amino acid residues (Fig. 5) (Koivunen *et al.* 2007, Oehme *et al.* 2002). The corresponding 503 and 487 amino acid mouse and zebrafish polypeptides, respectively, revealed 91 and 51% overall amino acid sequence identity with human P4H-TMs (Hyvarinen *et al.* 2010b). P4H-TM contains a transmembrane domain located between residues 59-82 in the human polypeptide and two N-glycosylation sites (Asn-Val-Thr residues 368-370 and Asn-Arg-Thr resudues 382-384 in human P4H-TM) (Koivunen *et al.* 2007, Oehme *et al.* 2002). In addition to the major P4H-TM form which resides in the ER membranes, an N-terminally truncated polypeptide has been detected in cultured human cell lines (Koivunen *et al.* 2007). Hypoxia has been shown to increase the P4H-TM level but does not affect its location (Koivunen *et al.* 2007).

Although the C-terminal catalytic region of P4H-TM is more closely related to the C-P4Hs than to the HIF-P4Hs P4H-TM lacks the sequences corresponding to the peptide substrate binding domain of the C-P4Hs and fails to hydroxylate procollagen polypeptides *in vitro* (Koivunen *et al.* 2007, Myllyharju 2008, Oehme *et al.* 2002). The actions of P4H-TM resemble more closely those of the three HIF-P4Hs, as it was shown to hydroxylate the two critical proline residues of HIF-1α ODDD *in vitro*. Moreover overexpression of P4H-TM *in cellulo* reduced the HIF-α ODDD polypeptide levels and silencing of the endogenous P4H-TM by siRNA increased the cellular HIF-1α level in a very similar manner to that observed with the HIF-P4Hs (Koivunen *et al.* 2007, Oehme *et al.* 2002). The cellular location of P4H-TM is nevertheless significantly different from those of the nuclear and cytosolic HIF-P4Hs. Furthermore, under hypoxic conditions HIF-α is found only in the nucleus, while its half life in normoxia is very short. It therefore remains unclear how the transfected P4H-TM actually acted on HIF-1α ODDD and how the silenced endogenous P4H-TM was able to affect the cellular HIF-1α protein level (Koivunen *et al.* 2007, Myllyharju 2008).

The *in vivo* roles of P4H-TM have recently been studied in a zebrafish model using morpholino knockdown technology (Hyvarinen *et al.* 2010b). The most prominent phenotypic alteration detected in the developing zebrafish was pericardial oedema, which became visible at 3–4 dpf, a stage at which it most likely resulted from dysfunction of the pronephric kidney (Hyvarinen *et al.* 2010b). Structural and functional analysis of the pronephric kidneys revealed severe defects in the glomerular BMs, in the podocyte foot processes and in pronephric kidney function that resulted in proteinuria (Hyvarinen *et al.* 2010b). Moreover, the development of the lens capsule was compromised, as it contained

extra nuclei and deposits and altered BMs (Hyvarinen *et al.* 2010b). P4H-TM-deficient morphants did not show any signs of polycythemia although many HIF target genes, including *Epo*, were upregulated (Hyvarinen *et al.* 2010b). The reported phenotypic alterations were unlikely to have been mediated by changes in HIF activity. Rather, additional, as yet uncharacterized P4H-TM substrates may have contributed to the phenotype observed in the zebrafish (Hyvarinen *et al.* 2010b).

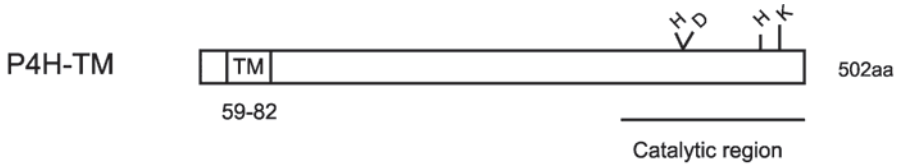

Fig. 5. Schematic representation of human P4H-TM. The transmembrane domain (residues 59-82) and the catalytically critical residues are shown. The polypeptide has a length of 502 amino acid residues.

2.2.3 The HIF pathway in the regulation of erythropoiesis

One of the most extensively studied systemic consequences of hypoxia is the stimulation of red blood cell production, erythropoiesis. Red blood cells, erythrocytes, function as essential oxygen transporters throughout the body constituting approximately one-half of the blood volume. The glycoprotein hormone erythropoietin (Epo) is a crucial regulator of erythropoiesis that links decreased tissue oxygenation to an appropriate response in red blood cell production by stimulating erythroid precursor cell viability, proliferation and differentiation (Haase 2010, Jelkmann 2007, Jelkmann 2011). Under physiological conditions Epo is present in the plasma at a concentration of 10^{-11} M, maintaining a hematocrit (HCT) of approximately 0.45 at sea level. Factors impairing tissue oxygenation, such as anemia, decreased ambient oxygen tension, or defective cardio-pulmonary function that perturbs normal renal perfusion, can induce serum Epo levels up to 1000-fold (Haase 2010, Jelkmann 2007, Jelkmann 2011).

Cells and tissues producing Epo

During embryonic development most of the Epo is produced in the liver by hepatocytes but after birth the site of the Epo production switches to the kidney where the fibroblast-like peritubular interstitial cells become the main physiological Epo source accounting for approximately 90% of the Epo plasma level (Bachmann *et al.* 1993, Maxwell *et al.* 1993, Weidemann & Johnson 2009). Although the liver maintains the ability to produce Epo in response to hypoxia, it does not make a significant contribution to the serum Epo pool under conditions of normoxia or moderate hypoxia. Further extra-renal sites capable of synthesizing Epo include the spleen, lung, testis, brain and erythroid progenitor cells. It was firts suggested that Epo produced by these mostly acts locally by affecting regional cellular responses (Haase 2010, Weidemann & Johnson 2009) but the systemic roles of extra-renal Epo production and the question of how far renal Epo synthesis is actually influenced by extra-renal sites under hypoxic conditions still remain to be clarified (Boutin *et al.* 2008, Rankin *et al.* 2012, von Wussow *et al.* 2005, Weidemann *et al.* 2009).

Local hypoxia of the brain stem has been found in rat experiments to be associated with an increase in renal Epo production (von Wussow *et al.* 2005) while the glial cells of the central nervous system have been shown to contribute to the systemic Epo level following the induction of hypoxia in a mouse line carrying astrocyte-specific deletions of genes encoding HIF-1α, HIF-2α and VHL (Weidemann *et al.* 2009). Additional findings suggest that the skin may modulate systemic Epo levels indirectly through NO-mediated effects on the cutaneous vascular flow (Boutin *et al.* 2008). Moreover, Rankin *et al.* (2012) discovered recently that osteoblasts have a capacity for regulating Epo expression in bone and by doing so directly modulate erythropoiesis. In that study conditional inactivation of *Vhl* in an osteoblast lineage with constitutively activated HIF signaling resulted in enhanced erythropoiesis and development of polycythemia in mutant mice by 8 weeks of age. Polycythemia was associated with increased *Epo* expression in the bone and decreased *Epo* expression in the kidney (Rankin *et al.* 2012).

Although *in vitro* Epo studies using a hepatoma cell line (Hep3B) first led to the discovery of HIF-1, later investigations have identified HIF-2 as the primary transcription factor regulating *Epo* expression (Chavez *et al.* 2006, Haase 2010, Jelkmann 2011, Semenza & Wang 1992, Warnecke *et al.* 2004). Experiments using a siRNA knockdown approach have shown that hypoxic induction of

endogenous Epo in Hep3B and Kelly cells and many other cell lines occurs largely in a HIF-2-dependent manner (Chavez *et al.* 2006, Warnecke *et al.* 2004) and consistent with this, histological studies of ischemic rat kidneys localized HIF-2α in the Epo-producing peritubular interstitial fibroblasts while HIF-1α was mainly detected in the tubular cells, connecting tubules and collecting ducts (Rosenberger *et al.* 2002).

HIF pathway-modified mouse lines and Epo production

Targeted disruption of HIF members has further provided important insights into their *in vivo* functions with respect to hematopoiesis. Analysis of *Hif-1a* null embryos at E9.5 revealed reduced myeloid multilineage and committed erythroid progenitors and a decreased hemoglobin content of erythroid colonies in yolk sacs (Yoon *et al.* 2006). A significant decrease was observed in levels of Epo receptor (*EpoR*) mRNA in *Hif-1a* null yolk sacs and a decrease in both *Epo* and *EpoR* mRNA in the embryo proper, reflecting dysregulation of Epo signalling (Yoon *et al.* 2006). Adult mice partially deficient in *Hif-1a* that were exposed to chronic continuous hypoxia showed a minor impairment of erythrocytosis, while a brief period of intermittent or continuous hypoxia treatment revealed a lack of *Epo* mRNA induction in the kidney (Cai *et al.* 2003, Yu *et al.* 1999). Adult mice with germline *Hif-2a* (*EPAS1*) deletion displayed moderate-to-severe anemia and hematopoietic deficiency associated with altered function of the bone marrow microenvironment (Scortegagna *et al.* 2003). Erythrocytosis was increased following treatment with exogenous recombinant Epo and renal Epo synthesis decreased, indicating inadequate kidney Epo production at baseline rathen than autonomous cell defects in erythroid precursor maturation in these mice (Scortegagna *et al.* 2005).

Widespread post-natal ablation of *Hif-2a* but not *Hif-1a* resulted in anemia that could be reversed by the administration of exogenous Epo (Gruber *et al.* 2007). *Hif-2a* deficient mice did not show any increase in renal Epo synthesis after treatment with phenylhydrazine (PHZ), an agent that induces severe hemolysis, while mice with *Hif-1a* deletion displayed conventional renal Epo induction (Gruber *et al.* 2007). Studies with both *Vhl*-deficient and anemic mice demonstrated the primary role of HIF-2 in regulating hepatic Epo expression, for in contrast to an earlier study with *Hif-1a* null embryos, it was now shown that during early post-natal development, before the switch in the primary source of systemic Epo from the liver to the kidney had occurred, hepatic Epo production

was dependent on HIF-2α rather than HIF-1α (Rankin *et al.* 2007). In the absence of renal HIF-2α hepatocyte-derived HIF-2α was shown to take over, contributing up to 70% of the serum Epo pool in adult mice (Kapitsinou *et al.* 2010). In line with this, Epo expression was shown to occur in a HIF-2α-dependent manner in bone osteoblasts and in osteoblast cultures obtained from neonatal mice (Rankin *et al.* 2012).

Acute global deletion of *Hif-p4h-2* in mice with subsequent HIF accumulation and activation of HIF target genes resulted in profound polycythemia accompanied by venous congestion, massive splenomegaly and premature death (Minamishima *et al.* 2008, Takeda *et al.* 2008). The phenotype was observed in both ageing and young animals upon acute inactivation of *Hif-p4h-2*. Epo levels were markedly increased in the serum together with induced *Epo* mRNA expression in the kidney but not in the liver, indicating that the former primarily controls circulating Epo (Minamishima *et al.* 2008, Takeda *et al.* 2008). Neither *Hif-p4h-1* nor *Hif-p4h-3* null mice displayed any signs of erythrocytosis, but their combined loss resulted in moderate polycythemia probably due to the induction of hepatic Epo production (Takeda *et al.* 2008). While deletion of *Hif-p4h-2* alone was sufficient to induce near maximal renal Epo production, deletion of all three HIF-P4H isoenzymes was required for maximal hepatic Epo production, with a subsequent dramatic increase in the hematocrit value (Minamishima & Kaelin 2010).

HIF in the regulation of hepcidin

The role of the hypoxia pathway in regulating erythropoiesis extends beyond the transcriptional control of Epo as hypoxia promotes the maturation and proliferation of erythroid progenitor cells, stimulates Epo receptor expression and regulates the homeostasis of components involved in the hemoglobin synthesis pathway (Haase 2010). Given that the availability of sufficient amounts of iron is crucial for erythropoiesis, it is not surprising that the expression of genes coding for duodenal cytochrome b (DCYTB), divalent metal transporter 1 (DMT1), and ferroportin (FPN), all of which are proteins increasing iron availability, is induced by hypoxia, apparently in a HIF-2-dependent manner (Anderson *et al.* 2011, Kapitsinou *et al.* 2010, Mastrogiannaki *et al.* 2009, Mastrogiannaki *et al.* 2012, Shah *et al.* 2009).

A key regulatory molecule in iron homeostasis is hepcidin, which is primarily produced by the liver. This downregulates iron uptake from the intestinal

epithelial cells by binding to FPN and reduces the release of iron by macrophages (Ganz & Nemeth 2012). Hepcidin affects anemia in cases of chronic disease and is also likely to play a role in the innate immune system by reducing the availability of iron, which could facilitate the growth of microscopic organisms. Iron loading increases hepcidin expression, while hypoxia, iron deficiency and increased erythropoiesis have the opposite effect (Ganz & Nemeth 2012, Nemeth & Ganz 2009).

There have been several suggestions for the mechanism that underlies the hypoxia-induced decrease in hepcidin expression. The bone morphogenic protein 6 (BMP6)/hemojuvelin (HJV) signalling cascade plays a certain role (Andriopoulos *et al.* 2009, Babitt *et al.* 2006, Meynard *et al.* 2009). Inactivation of HJV results in severe iron overload in both humans and mouse models (Huang *et al.* 2005, Niederkofler *et al.* 2005). Membrane-bound HJV serves as a co-receptor for BMPs, while soluble HJV appears to downregulate hepcidin competitively by interfering with BMP signalling (Lin *et al.* 2005). HIF has been implicated in part in the induction of a proprotein convertase, furin, which is responsible for the formation of soluble HJV (McMahon *et al.* 2005, Silvestri *et al.* 2008a). Similarly, transmembrane protease serine 6 (TMPRSS6) is regulated by HIF and is thought to modulate BMP6/HJV signalling in hypoxia (Du *et al.* 2008, Lakhal *et al.* 2011, Silvestri *et al.* 2008b). Moreover, studies with primary hepatocytes and HepG2 cells have indicated that Epo is capable of regulating hepcidin transcription directly via EpoR and the activation of CCAAT/enhancer-binding protein (C/EBP) α (Pinto *et al.* 2008). Furthermore, growth differentiation factor 15 (GDF15), which is a member of the TGF-β superfamily and is regulated by iron and hypoxia in a HIF-independent fashion, suppresses hepcidin in human hepatocytes and hepatoma cells (Lakhal *et al.* 2009, Tanno *et al.* 2007). GDF15 is expressed and secreted by maturing hematoblasts at low levels, while ineffective erythropoiesis markedly increases its expression (Tanno *et al.* 2010).

In vitro experiments using the HIF stabilizer desferrioxamine indicated direct downregulation of hepcidin by HIF-1 although this finding was not supported by other experiments involving HepG2 cells, which rather suggested indirect pathways between HIF and hepcidin crosstalk (Braliou *et al.* 2008, Choi *et al.* 2007, Peyssonnaux *et al.* 2007, Volke *et al.* 2009). Substantial repression of hepcidin was observed in a mouse model following conditional knockout of *Vhl* in the liver, while deletion of *Hif-1a* alone in the liver did not have a significant effect, thus indicating that HIF-2 may have contributed to hepcidin

downregulation (Peyssonnaux *et al.* 2007). The issue was further assessed using a combination of three experimental models: a model of hepatocyte-specific knockout mice harbouring either HIF-2α deficiency or constitutive HIF-2α stabilization, a dietary iron deficiency model and a set of *ex vivo* experiments with primary mouse hepatocytes (Mastrogiannaki *et al.* 2012). HIF-2 was shown to be dispensable with regard to *Hepcidin* gene regulation during the adaptive response to iron deficiency anemia, while its hepatocyte-specific overexpression indirectly downregulated *Hepcidin* expression through increased erythropoiesis and Epo production. Moreover, the *in vitro* data further supported the concept of a non-autonomous function of HIF-2 in hepcidin regulation (Mastrogiannaki *et al.* 2012).

A very recent study clarifying the crosstalk between the HIF pathway and Epo in regulating the hypoxic suppression of *Hepcidin* expression used an approach by which HIF activation was uncoupled from Epo synthesis (Liu *et al.* 2012). For this purpose a mouse model was generated in which HIF-1 and HIF-2 were activated through inactivation of *Vhl* with simultaneous inactivation of *Epo*. Hypoxia/HIF-mediated suppression of *Hepcidin* appeared to require Epo induction and was associated wih elevated serum GDF15 levels (Liu *et al.* 2012). It was also demonstrated that under conditions of hepatic HIF activation hepcidin suppression required increased erythropoiesis irrespective of the serum Epo level (Liu *et al.* 2012). Altogether it was established that activation of HIF in hepatocytes suppresses hepcidin indirectly via Epo-mediated stimulation of erythropoiesis (Liu *et al.* 2012).

Human mutations in the HIF pathway and eryhrocytosis

Clinically, mutations in the genes of the HIF pathway are potentially able to increase the erythropoietin level and result in erythrocytosis. Chuvash polycythemia is a rare recessive form of inherited erythrocytosis that was first reported in patients from the Chuvash region of Russia (Sergeyeva *et al.* 1997). In this example homozygous mutation in the *Vhl* tumor suppressor causes erythrocytosis that is associated with normal Epo levels and thrombotic complications (Sergeyeva *et al.* 1997). Later, families with mutations in *Hif-2a* or *Hif-P4h-2* were discovered, but no mutations in human *Hif-1a* causing erythrocytosis have been characterized to date, underscoring the importance of HIF-2α in the regulation of Epo (Lee & Percy 2011). A heterozygous missense mutation converts a HIF-2α glycine at position 537 to either tryptophan or

arginine close to the HIF-P4H target proline at position 531 (Percy *et al.* 2008b). The mutation produces a partial gain of function, as both the HIF-P4H-mediated hydroxylation and recognition of the hydroxylated HIF-2α by VHL are impaired (Percy *et al.* 2008b). Additional erythrocytosis-associated *Hif-2a* mutations have subsequently been described, all of them heterozygous, concentrated in a small region located C-terminally relative to the Pro-531, and typically resulting in elevated Epo levels (Gale *et al.* 2008, Martini *et al.* 2008, Percy *et al.* 2008a). Several types of heterozygous *Hif-p4h-2* mutation have been identified, almost all of them resulting in erythrocytosis characterized by a normal Epo level (Lee & Percy 2011).

2.2.4 Therapeutic implications: HIF-P4H inhibitors for anemia

Pharmacological inhibition of the HIF hydroxylases is considered a promising basis for drug development in the treatment of a variety of pathological states associated with hypoxia, including anemia, ischemic heart disease, stroke, cancer and pulmonary hypertension (Fraisl *et al.* 2009, Haase 2010, Muchnik & Kaplan 2011, Myllyharju 2008, Myllyharju 2009, Yan *et al.* 2010). While inhibition of the HIF-P4Hs is able to upregulate beneficial HIF pathway-dependent processes in anemia, stroke and ischemic heart disease, carefully focused activation of these enzymes could conversely moderate HIF-dependent processes in the growth of solid tumors and in certain forms of pulmonary hypertension. Moreover, HIF-P4H inhibition has shown a potential for neuroprotective applications (Harten *et al.* 2010, Myllyharju 2009).

Anemia is a severe consequence of chronic kidney diseases, and is also present in patients with many chronic inflammatory conditions or with cancer. Recombinant Epo has been in clinical use as a standard therapy for patients with renal and cancer-associated anemia, but this treatment is costly, requires parental administration and has been associated with an increased risk of cardiovascular complications and increased mortality (Bohlius *et al.* 2009, Haase 2010, Muchnik & Kaplan 2011). Moreover, a significant proportion of the patients manifest Epo resistance (Muchnik & Kaplan 2011).

One disadvantage of conventional HIF-P4H inhibitors such as oxalylglycine, is their lack of specificity and the subsequent inhibition of a wide range of biological reactions that are catalyzed by about 60 currently known 2-oxoglutarate-dependent dioxygenases (Myllyharju 2009, Rose *et al.* 2011). Efforts towards the development of selective HIF-P4H inhibitors have led to the

engineering of several novel pharmaceutical 2-oxoglutarate analogues (Rose *et al.* 2011, Smith & Talbot 2010) which have been shown to increase serum Epo in animal models, and some of these are already undergoing clinical trials (Barrett *et al.* 2011, Hsieh *et al.* 2007, Muchnik & Kaplan 2011, Myllyharju 2009, Yan *et al.* 2010). One such compound, orally administered FG-2216 (FibroGen), was first to be tested in a primate model (rhesus macaques) and was shown to significantly increase erythropoiesis and prevent phlebotomy-induced anemia with no apparent side effects (Hsieh *et al.* 2007). In another study, HIF-P4H selective 2-oxoglutarate competitive agent 1-(5-chloro-6-(trifluoromethoxy)-1*H*-benzo[d]imidazol-2-yl)-1*H*-pyrazole-4-carboxylic acid (JNJ-42041935) appeared to be effective in reversing inflammation-induced anemia in a rat model, whereas exogenous Epo had no effect (Barrett *et al.* 2011).

In two phase 2 studies, FG-2216 and FG-4592 (FibroGen) induced effective erythropoiesis with a subsequent increase in hemoglobin concentrations in anemic human patients with chronic kidney disease (Muchnik & Kaplan 2011, Myllyharju 2009). Furthermore, stimulation of Epo production by a HIF-P4H inhibitor was detected in anephric hemodialysis patients as strongly as in healthy patients suggesting that it caused effective Epo production in the liver (Muchnik & Kaplan 2011, Myllyharju 2009). Some other HIF-P4H inhibitors, notably AKB-6548 (Akebia) and GSK1278863A (GlaxoSmithKline) are also currently undergoing clinical trials (Muchnik & Kaplan 2011).

The vast majority of the Epo resistance found in patients with kidney disease is a result of a chronic inflammatory state causing functional iron deficiency that cannot be remedied with inravenous iron. Hepcidin, which has been associated with innate immune responses and anemia resulting from chronic diseases, is regarded as a key factor in this functional iron deficiency (Haase 2010, Muchnik & Kaplan 2011, Nemeth & Ganz 2009). As mentioned in section 2.2.3 the HIF pathway plays a role in iron handling and hepcidin regulation although the actual mechanisms remain to be resolved. HIF-P4H inhibitors might therefore be able to increase hemoglobin even in states of inflammation, presumably through effects on hepcidin and other proteins involved in iron homeostasis (Haase 2010, Muchnik & Kaplan 2011).

Since pharmacological stabilization of HIF results in increased hemoglobin as a downstream effect of increased Epo and increased iron availability, it could be a more beneficial and safer treatment for patients suffering from chronic kidney disease and inflammation-induced anemia than the current therapies with recombinant Epo (Muchnik & Kaplan 2011, Rose *et al.* 2011). On the other hand,

any therapeutic application involving manipulation of the HIF pathway should take into account the numerous other genes that are also regulated by HIF, affecting a multitude of biological processes. Such considerations are particularly important when treating chronic diseases such as anemia, in which medication over prolonged periods of time is commonly required (Muchnik & Kaplan 2011, Myllyharju 2009, Rose *et al.* 2011).

2.3 Collagens

The collagens are a large superfamily of extracellular matrix (ECM) proteins that are widespread throughout the body and collectively represent the most abundant protein to be found in animals. They are best recognized for their remarkable role in maintaining the structural integrity of organs and in providing tissues with tensile and mechanical strength. They are also well-established components of BMs, a role in which they are for instance crucial for molecular filtration in the kidney glomerulus, for instance. It is now being increasingly acknowledged, however, that collagens display a broad range of divergent biological functions in processes such as cell adhesion, proliferation, specification, migration, survival and differentiation, together with related events that include morphogenesis, tissue repair and cancer. The identification of transmembrane collagens on the surfaces of a variety of cells and of collagens with paracrine functions has recently added further interest and complexity to the collagen protein family (Gelse *et al.* 2008, Kadler *et al.* 2007, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004).

All the members of the collagen family share characteristic structural features. They consist of three individual polypeptide α chains, each in a left-handed helical conformation, that finally form a rope-like rod structure in which the individual α chains are wrapped together into a right-handed triple helix. The tight packing of the α chains within the triple helix requires every third residue to be the smallest amino acid, glycine, resulting in $(Gly\text{-}X\text{-}Y)_n$ repeats in each of the constituent chains. X and Y in this sequence can be any amino acid, but they are most frequently proline (28%) and 4-hydroxyproline (38%), respectively. Collagen molecules can be heterotrimeric, i.e. containing different α chains (e.g. type I collagen, which consists of two α1(I) chains and one α2(I) chain), or homotrimeric, with three identical α chains (e.g. type II collagen) (Kadler *et al.* 2007, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004, Shoulders & Raines 2009).

Although the triple helix is a key feature of collagens, noncollagenous domains flanking or interrupting the central helical part of the molecule also play important roles. In the fibrillar collagens, the C- and N-propeptides at the ends of the molecule participate in the initiation of triple helix formation and in the regulation of the primary fibril diameter, respectively, while short non-helical telopeptides are involved in the formation of crosslinks between collagens and other surrounding molecular structures (Gelse *et al.* 2003, Myllyharju & Kivirikko 2004).

The multiple critical roles of collagens have been clearly demonstrated by the outcomes of more than 1000 mutations that have been identified in these proteins and the enzymes that modify them. The resulting diseases, though relatively rare, include osteogenesis imperfecta (OI), Alport syndrome, Bethlem myopathy and Ullrich muscular dystrophy, some subtypes of Ehlers-Danlos (EDS) syndrome, Knobloch syndrome, specific subtypes of epidermolysis bullosa and chondrodysplasias, which are a genetically heterogeneous group of disorders with abnormalities in the development of cartilage and cartilaginous bone (Myllyharju & Kivirikko 2001, Rimoin *et al.* 2007). Moreover, collagen degradation and disrupted metabolism may contribute to the pathophysiology of common diseases such as osteoarthritis, osteoporosis, intervertebral disc disease and the formation of arterial aneurysms. Furthermore, disadvantageous collagen accumulation is characteristic of fibrosis, cirrhosis, cardiovascular diseases and abnormal scarring (Kadler *et al.* 2007, Kadler *et al.* 2007, Myllyharju & Kivirikko 2004).

2.3.1 Collagen types in vertebrates

The collagen superfamily as represented in vertebrates includes 28 different collagen types (numbered I-XXVIII; some with common names) that are made up of at least 46 distinct α chains. Even though there is no clear definition of a collagen, all the members of the family have a role in tissue assembly and maintenance and are characterized by containing triple-helical domain structures with Gly-X-Y repetitions (Kadler *et al.* 2007, Myllyharju & Kivirikko 2001, Veit *et al.* 2006).

The collagen superfamily can be divided into several subfamilies based on the function and domain homology of the molecules. As most collagens form polymeric assemblies such as fibrils, filaments and networks, the following classification has been established: (A) fibril-forming collagens, (B) fibril-associated collagens with interrupted triple helices (FACITs), (C)

network-forming collagens, (D) transmembrane collagens, (E) endostatin-producing collagens, (F) anchoring fibrils, and (G) beaded filament-forming collagens (Kadler *et al.* 2007). A further large group possessing triple-helical collagen-like motifs but lying outside the actual collagen superfamily, includes proteins such as the tail structure of acetyl cholinesterase, adiponectin, the subcomponent C1q of complement, type I and II macrophage scavenger receptors, pulmonary surfactant protein and ficolins (Kadler *et al.* 2007, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004).

The collagen fibrils in tissues are often composed of more than one collagen type. Type I collagen fibrils contain small amounts of collagens III, V and XII, while type II collagen fibrils, typical cartilage components, have a core of type XI collagen and a surface of type IX collagen (Blaschke *et al.* 2000, Cremer *et al.* 1998, Myllyharju & Kivirikko 2004). Most notably many of the primary transcripts for collagen polypeptide chains undergo alternative splicing and some of the chains use alternative transcription initiation sites. Alternative splicing was first described for type II collagen, the longer form of which, type IIA procollagen as expressed by chondroprogenitor cells, differs from the shorter form, type IIB procollagen which is the main product of mature articular chondrocytes. Since these instances came to light a great number of different transcripts have been reported for several collagens such as types VI, XI, XII and XIII (Myllyharju & Kivirikko 2004).

2.3.2 Biosynthesis of collagens

Collagen biosynthesis is a complex multi-step process characterized by a large number of intracellular and extracellular events that require assistance from at least 12 different enzymes. Since studies on fibril-forming collagens (mainly type I) have provided the most information on these mechanisms, they are described below as a model for collagen biosynthesis in general (Figure 6) (Gelse *et al.* 2003, Myllyharju & Kivirikko 2001, Prockop & Kivirikko 1995).

The fibril-forming collagens are first synthesized on the ribosomes of the rough ER as prepro-α chains that contain propeptide extensions at both the N and C-terminal end and a signal peptide which targets the newly synthesized polypeptides to the ER lumen. After translocation, the signal peptide is cleaved off by signal peptidase to yield pro-α chains. The co-translational and post-translational modifications of the nascent polypeptides begin during the translocation and include hydroxylation of certain proline residues to 4-

hydroxyprolines and 3-hydroxyprolines and of lysine residues to hydroxylysines by C-P4Hs, prolyl 3-hydroxylases, and lysyl hydroxylases, respectively. All these collagen hydroxylases belong to the group of 2-oxoglutarate-dependent dioxygenases, which require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate, reside within the ER lumen and possess at least three isoenzymes each (Gelse *et al.* 2003, Kivirikko & Pihlajaniemi 1998, Myllyharju 2003).

The presence of 4-hydroxyproline residues is crucial for the folding of the newly synthesized collagen polypeptide chains into thermally stable triple-helical molecules. Their amounts are relatively similar in the various collagen types and tissues; approximately 100 residues per 1000 amino acids (Myllyharju 2003, Myllyharju & Kivirikko 2004).

Hydroxylysine residues have at least two important functions. First, they are necessary for the formation of stable crosslinks between the collagen molecules. Collagen crosslink formation, which is eventually completed in the ECM by lysyl oxidase, provides the collagen fibrils with tensile strength and mechanical stability. Second, hydroxylysine residues serve as attachment sites for carbohydrate units, which are transferred to the hydroxyl groups by the enzymes hydroxylysyl galactosyltransferase and galactosylhydroxylysyl-glucosyltransferase. Interestingly, lysyl hydroxylase 3 (LH3) has been shown to possess glucosyltransferase and galactosyltransferase activity in addition to LH activity (Heikkinen *et al.* 2000, Risteli *et al.* 2009, Wang *et al.* 2002). The functions of the hydroxylysine-linked carbohydrate units are not completely understood but they are known to contribute to collagen fibril formation and morphology (Notbohm *et al.* 1999, Takaluoma *et al.* 2007) and are critical for the assembly of collagen IV networks (Rautavuoma *et al.* 2004, Ruotsalainen *et al.* 2006). The extent of lysine hydroxylation appears to vary largely depending on the tissue and collagen type (Myllyharju & Kivirikko 2004).

Mutations in the *PLOD1* gene for LH1 cause a kyphoscoliotic subtype of EDS in humans. EDS is a heterogenous group of connective tissue disorders characterized by hyperextensibility of the skin, hypermobility of the joints and generalized connective tissue fragility (Myllyharju & Kivirikko 2004). Mutations in the *PLOD2* gene encoding LH2 have been identified in patients with Bruck syndrome (BS), an autosomally recessively inherited disease with typical findings of fragile bones, congenital joint contractures, scoliosis and osteoporosis (Ha-Vinh *et al.* 2004, van der Slot *et al.* 2003).

The *in vivo* functions of 3-hydroxyproline in fibrillar collagens are not fully established and efforts to clarify the impact of 3-hydroxyproline residues on the

stability of the triple helix have led to controversial results (Hodges & Raines 2005, Jenkins *et al.* 2003, Mizuno *et al.* 2008). The proportion of 3-hydroxylation varies markedly between collagen types, being relatively extensive in the case of type IV, which contains 10-15 residues of 3-hydroxyproline per 1000 amino acids, whereas 3-hydroxyproline occurs at only a single site in the α chains of type I collagen, where its formation is catalyzed by a protein complex including prolyl 3-hydroxylase 1 (P3H1), cartilage-associated protein (CRTAP) and cyclophilin B (Vranka *et al.* 2004). Mutations in the genes encoding the three components of this enzyme complex have been shown to cause severe recessive forms of OI (Baldridge *et al.* 2008, Cabral *et al.* 2007, Morello *et al.* 2006, van Dijk *et al.* 2009).

Folding of the fibril-forming pro-α chains into procollagen molecules occurs in the ER lumen. The process is initiated by an association between the C-terminal regions of three pro-α chains in order to form a nucleation point from which triple helix formation propagates in a C to N direction in a zipper-like manner (Gelse *et al.* 2003, Myllyharju & Kivirikko 2001, Prockop & Kivirikko 1995). Proper folding requires the presence of several enzymes resident in the ER, such as peptidyl-prolyl *cis-trans* isomerase (PPI) and a specific heat shock-inducible chaperone, Hsp47 (Lamande & Bateman 1999, Nagata 2003). Moreover, protein disulphide isomerase (PDI), which also acts as the β subunit in C-P4Hs, catalyzes the formation of intrachain and interchain disulphide bonds and acts as a chaperone preventing the aggregation of nascent collagen chains (Myllyharju & Kivirikko 2004).

After the procollagen molecules have been completely modified and assembled they are packed into secretory vesicles in the Golgi compartment and secreted into the extracellular space. The N and C-terminal propeptides are subsequently cleaved by N and C proteinases, respectively, both of which are Zn^{2+}-dependent metalloproteinases, resulting in mature collagen molecules that are able to assemble spontaneously into fibrils (Prockop *et al.* 1998). The formation of lysine and hydroxylysine-derived intramolecular and intermolecular covalent crosslinks stabilizes the fibrils and provides them with mechanical resilience. The copper-dependent enzyme lysyl oxidase plays an important role in crosslink formation, as it catalyzes the oxidative deamination of specific lysine and hydroxylysine residues, leading to the allysine and hydroxyallysine crosslinking pathways (Kagan & Li 2003).

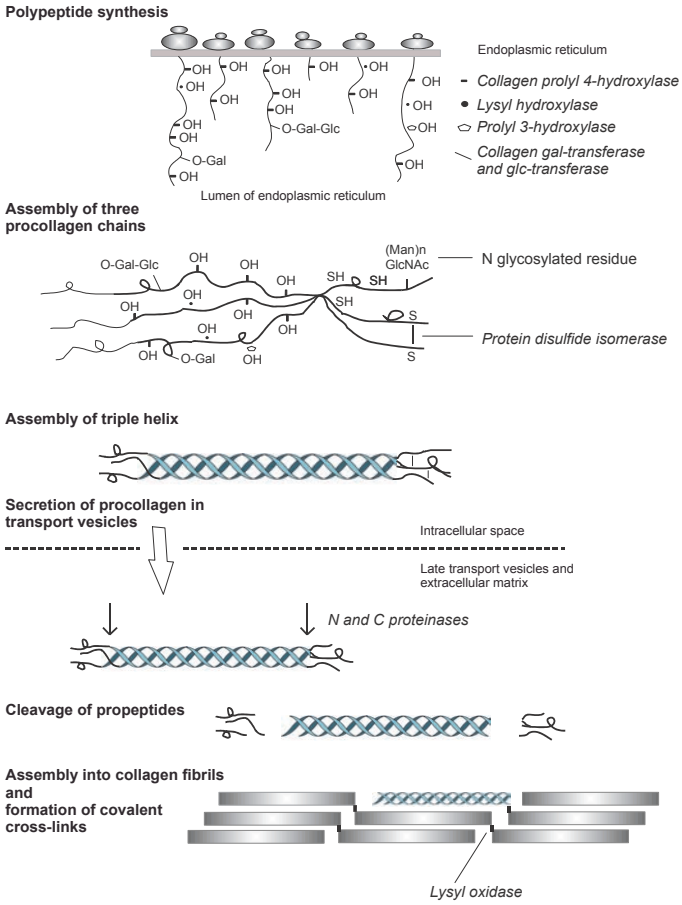

Fig. 6. Collagen biosynthesis. The collagen polypeptide chains are synthesized within the ribosomes of the rough ER. After cleavage of the signal peptide (not shown), the main steps in the biosynthesis occurring in the ER lumen include I) hydroxylation of certain proline and lysine residues to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine, II) glycosylation of some of the hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine, III) glycosylation of certain asparagine residues, IV) assembly of three C propeptides, and V) formation of intramolecular and intermolecular disulphide bonds. The triple helix is then formed in a zipper-like manner, propagating from the C-terminal nucleus towards the N terminus. The procollagen molecules are transported from the ER lumen through the Golgi stacks to the ECM in secretory vesicles. Subsequent steps take place in late transport vesicles and in the ECM, including cleavage of the N and C propeptides, spontaneous self-assembly of the resulting collagen molecules into fibrils, and formation of intramolecular and intermolecular covalent crosslinks. Modified from Myllyharju 2004.

2.4 Collagen prolyl 4-hydroxylases

The collagen prolyl 4-hydroxylases (C-P4Hs) are crucial enzymes in collagen biosynthesis. They reside within the ER lumen, where their most notable function is to catalyze the formation of 4-hydroxyproline residues in -X-Pro-Gly- triplets in collagen molecules and other proteins containing collagen-like sequences. They are also responsible, however, for the hydroxylation of the proline residue in an -X-Pro-Ala- motif found in the α3 chain of type IV collagen and in two of the polypeptide chains of the complement protein C1q and that of the $(Val–Pro–Gly–Val–Gly)_n$ repeats in elastin (Myllyharju & Kivirikko 2004, Myllyharju 2008). Recently another interesting function for C-P4H was discovered as it was found to regulate the stability of human Argonaute 2 (Ago2), an essential component of the RNA-induced silencing complexes, by hydroxylating Pro700 in an -X-Pro-Gly- triplet (Qi *et al.* 2008).

Prolyl 4-hydroxylation of collagens is critical for their correct folding and thermal stability (Myllyharju & Kivirikko 2004). Non-hydroxylated type I collagen is denatured at 24 ºC, while the fully hydroxylated molecules are stable at temperatures up to 39 ºC. Almost complete hydroxylation is required to generate stable triple-helical collagen molecules at body temperature (Jimenez *et al.* 1973). C-P4Hs also play an important role in collagen quality control as the enzyme associates with non-helical procollagen chains and retains them within the ER until the folding is properly completed (Walmsley *et al.* 1999).

The vertebrate C-P4Hs are $\alpha_2\beta_2$ tetramers in which the α subunits possess the catalytic activity and the β subunits are identical to PDI. Since the β subunit is invariant, it is the α subunits that define the C-P4H isoenzymes. Three isoforms of the α subunit, α(I), α(II) and α(III), have been identified to date and shown to form $[\alpha(I)]_2\beta_2$, $[\alpha(II)]_2\beta_2$ and $[\alpha(III)]_2\beta_2$ tetramers with PDI, corresponding to type I, II and III C-P4Hs, respectively (Myllyharju 2003, Myllyharju 2008). Data on coexpression studies in insect cells have shown that $\alpha(I)\alpha(II)\beta_2$ tetramers are not formed indicating that mixed tetramers are not likely to exist in vertebrates, although they are found in *C. elegans* (Annunen *et al.* 1997, Myllyharju *et al.* 2002). The molecular weight of the C-P4H tetramer is about 240 kDa, comprising 63 kDa and 58 kDa from the individual α and β subunits, respectively (Myllyharju & Kivirikko 2004).

2.4.1 Structure

The α subunit

The genes encoding the human α(I), α(II) and α(III) subunits are located on chromosomes 10q21.3-23.1, 5q31 and 11q12, respectively (Kukkola *et al.* 2003, Nokelainen *et al.* 2001, Pajunen *et al.* 1989). The exon-intron organizations are very similar, although the α(I) and α(II) mRNAs are subject to alternative splicing that affects the homologous exons 9 and 10 in the α(I) gene and 12a and 12b in the α(II) gene (Helaakoski *et al.* 1989, Kukkola *et al.* 2003, Nokelainen *et al.* 2001). The two splicing variants of the α(I) and α(II) subunits associate with PDI to form active tetramers and exhibit a conventional tissue distribution, with very similar expression patterns. No alternative splicing has been detected in the case of the α(III) transcript (Helaakoski *et al.* 1994, Kukkola *et al.* 2003, Nokelainen *et al.* 2001).

The human, mouse and rat α(I) subunits consist of 517 residues, and chicken α(I) of 516 residues, whereas the human and mouse α(II) subunits consist of 514 and 518 residues, respectively. The α(III) subunit is slightly longer, consisting of 525 residues in the human and rat and 520 residues in the mouse. All three newly synthesized α subunits contain an additional signal peptide of 16-21 amino acids that is later removed during processing (Annunen *et al.* 1997, Bassuk *et al.* 1989, Helaakoski *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). The overall amino acid sequence identity between the mature human α(I) and α(II) subunits is 65%, while human α(III) is 35–37% identical to the α(I) and α(II) subunits (Annunen *et al.* 1997, Helaakoski *et al.* 1995, Kukkola *et al.* 2003). The catalytically important C-terminal regions represent the highest degree of identity, 80% between the human α(I) and α(II) subunits and 56–57% between α(I) and α(III) and between α(II) and α(III) (Annunen *et al.* 1997, Kivirikko & Pihlajaniemi 1998, Kukkola *et al.* 2003, Myllyharju 2003). All four catalytically critical amino acids, the two histidines and one aspartate binding the iron and the one lysine binding the 2-oxoglutarate, are conserved among the three C-P4H α subunits (Fig. 7) (Myllyharju 2003).

The peptide substrate binding domain, which is separate from the catalytic domain, mostly defines the peptide binding properties of the C-P4Hs (Fig. 7) (Hieta *et al.* 2003). The domain is located in the N-terminal half of the polypeptide, residing between residues Gly138 and Ser244 in the human C-P4H

α(I) subunit (Myllyharju & Kivirikko 1999). Nuclear magnetic resonance (NMR) and crystal structure analysis of the recombinant human α(I) peptide substrate binding domain have shown that it is entirely helical, consisting of five antiparallel α-helices. It belongs to a family of tetratricopeptide repeat (TPR) domains that are involved in many protein-protein interactions, as it possesses two TPR motifs plus a solvating helix (Hieta *et al.* 2003, Pekkala *et al.* 2004). Based on mutagenesis data and the structure of the domain, the proline-rich peptide substrates very probably become bound to a deep aromatic groove that exists on the concave surface of the domain (Pekkala *et al.* 2004). It should be noted that the full-length catalytic α subunit is totally insoluble and non-functional unless assembled with PDI, and thus it is not amenable to structional studies as such (Kivirikko & Myllyharju 1998, Kivirikko & Pihlajaniemi 1998, Myllyharju 2003).

Although all human α subunits possess five conserved cysteine residues, α(II) and α(III) each having an additional cysteine between the conserved cysteines 4 and 5, and 1 and 2, respectively (Annunen *et al.* 1997, Helaakoski *et al.* 1989, Kukkola *et al.* 2003) there are no disulphide interchain bonds between the individual subunits in the C-P4H tetramer (Nietfeld *et al.* 1981). Site-directed mutagenesis assays indicate that intrachain disulphide bonds, which are important for assembly of the enzyme tetramer, are formed between the second and third and the fourth and fifth conserved cysteine residues (Lamberg *et al.* 1995). Moreover, the human α(I), α(II) and α(III) subunits each contain two N-glycosylation sites, although these do not have a role in the assembly of the enzyme tetramer or in its catalytic activity (Kukkola *et al.* 2003, Lamberg *et al.* 1995).

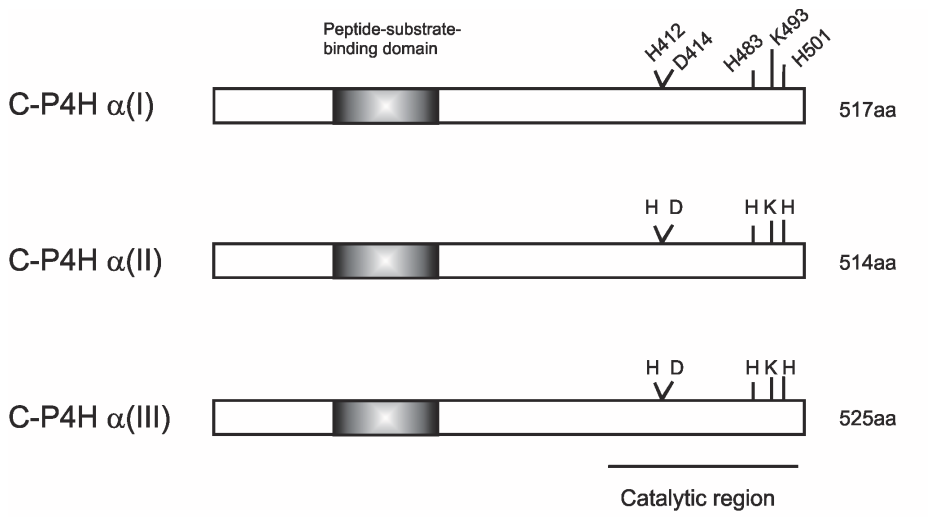

Fig. 7. Schematic representation of the human C-P4H α(I), α(II) and α(III) subunits. The lengths of the polypeptides are indicated on the right, and the peptide substrate binding domains are indicated as grey boxes. The catalytically critical amino acids are shown above the polypeptides. Modified from Myllyharju 2008.

PDI/β

The β subunit of the C-P4Hs is identical to the enzyme and chaperone PDI, which is an abundant multifunctional protein primarily located in the lumen of the ER (Koivu *et al.* 1987, Pihlajaniemi *et al.* 1987). Among its multiple functions, PDI catalyzes the formation, breakage and rearrangement of disulphide bonds in various ER-associated secretory and cell surface proteins and serves as a β subunit not only in all three vertebrate C-P4Hs but also in microsomal triglyceride transfer protein (Hatahet & Ruddock 2009). In addition, within its non-ER locations PDI has been associated with platelet aggregation during the blood clotting cascade and with activation of the fusion of the HIV virus, for example (Fenouillet *et al.* 2007, Turano *et al.* 2002).

PDI has at least two important functions in vertebrate C-P4H tetramers. First, by means of its C-terminal retention signal it retains the enzyme tetramer within the lumen of the ER (Vuori *et al.* 1992b) and second, it is required for keeping the α subunits in a soluble, catalytically active form (John *et al.* 1993, Vuori *et al.* 1992a). Another chaperone, BiP, also associates with the α subunit and forms

both soluble and insoluble complexes with it, but the soluble α-BiP complex does not possess C-P4H activity (John & Bulleid 1996, Veijola *et al.* 1996b). Even when serving as the β subunit of C-P4H, PDI shows disulphide activity, although this does not play a role in C-P4H assembly or in the hydroxylation reaction (Koivu *et al.* 1987, Vuori *et al.* 1992b). Since PDI is constantly produced in a large excess over the α subunits, the amount of active C-P4H tetramer is regulated through the latter (Kivirikko *et al.* 1990).

The human PDI consists of 491 amino acids with an additional 17-residue signal peptide (Pihlajaniemi *et al.* 1987). It has a modular structure composed of four domains, a, b, a' and b', in this order, followed by a highly acidic C-terminal extension c that harbours the ER retention signal KDEL (Fig. 8) (Ellgaard & Ruddock 2005, Freedman *et al.* 2002, Pihlajaniemi *et al.* 1987, Vuori *et al.* 1992a) The a and b domains are connected with a short linker region, designated as x, that consists of only 19 amino acids (Pirneskoski *et al.* 2004). Coexpression experiments in insect cells have demonstrated that the a' and b' domains fulfil the minimum requirements for the assembly of an active C-P4H $\alpha_2\beta_2$ tetramer, but addition of the a and b domains significantly increases the level of C-P4H activity obtained (Pirneskoski *et al.* 2001). The a and a' domains each contain the -Cys-Gly-His-Cys- motifs required for catalytic activity in PDI and are homologous to the ubiquitous redox protein thioredoxin (TRX), while the b and b' domains are inactive and, though not possessing sequences homologous to TRX, they adopt a similar secondary structure and global fold (Ellgaard & Ruddock 2005, Kemmink *et al.* 1997, Kemmink *et al.* 1999). The primary substrate binding site of PDI is located in the b' domain, but all four domains are involved in the binding of larger substrates or partner proteins (Klappa *et al.* 1998).

Although no published structure exists so far for full-length mammalian PDI, the complete crystal structure of full-length yeast PDI has been described by Tian and coworkers (2006), who showed that the four TRX-like domains assemble in the shape of a twisted U with the a and a′ domains representing the two mobile arms of the U and the b and b′ domains forming the rigid base (Tian *et al.* 2006). The inner surfaces of the b and b' domains make up a hydrophobic region that is thought to be critical for substrate binding. According to biochemical studies, all four domains together with the C-terminal tail appear to be required for the full catalytic activity of the yeast PDI (Tian *et al.* 2006). Mammalian and yeast PDIs most probably share the same architecture, given the similarity of their primary structures and domain boundaries (Tian *et al.* 2006), but the C-terminal extension

of mammalian PDI is not likely to play a role in its activity (Darby *et al.* 1998, Koivunen *et al.* 1999).

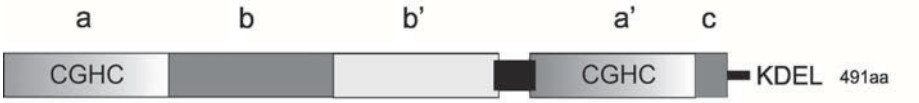

Fig. 8. Schematic representation of the domain structure of human PDI. The mature polypeptide consists of 491 aa. The catalytic motifs -Cys-Gly-His-Cys- (CGHC) reside within the a and a' domains. The black box indicates the linker domain connecting the b' and a' domains. C represents the C-terminal tail followed by KDEL that functions as the ER retention signal for the PDI. Modified from Kivirikko & Myllyharju 1998.

2.4.2 C-P4H isoenzymes and tissue distribution

The vertebrate C-P4H α(I) subunit was first cloned from human and chicken sources in 1989 and subsequently from the rat and mouse (Bassuk *et al.* 1989, Helaakoski *et al.* 1989, Helaakoski *et al.* 1995, Hopkinson *et al.* 1994). Later two additional α subunit isoforms were cloned and characterized: the α(II) subunit from mouse and human sources and the α(III) subunit from human, mouse and rat sources (Annunen *et al.* 1997, Helaakoski *et al.* 1995, Kukkola *et al.* 2003, Van Den Diepstraten *et al.* 2003).

The human α(I), α(II) and α(III) mRNAs are expressed in a variety of adult and fetal tissues, including the heart, liver, skeletal muscle, kidney, pancreas and placenta, but with distinct differences in their relative abundance (Annunen *et al.* 1997, Helaakoski *et al.* 1994, Helaakoski *et al.* 1995, Kukkola *et al.* 2003). Expression of the α(III) subunit mRNA is generally much lower than that of the α(I) or α(II) mRNA, the highest α(III) mRNA levels being reported in the placenta, adult liver, fetal skin and the fibrous cap of atherosclerotic human carotid arteries (Kukkola *et al.* 2003, Van Den Diepstraten *et al.* 2003).

The proportions of the C-P4H-I and II isoenzymes have been studied in certain cell types and tissues by a poly(L-proline) column method with subsequent enzyme activity analysis (Annunen *et al.* 1997, Annunen *et al.* 1998b). Due to differences in their peptide substrate binding properties, C-P4H-I becomes selectively bound to the poly(L-proline) column, while C-P4H-II is leached into the flow-through fraction. C-P4H-I was shown to be the major C-P4H isoform in many cultured cell lines and its contribution to the total C-P4H activity increased in confluence (Annunen *et al.* 1997, Annunen *et al.* 1998a). In mouse

chondrocytes, however, the contribution of C-P4H-II to the total activity was as high as 70%. In agreement with the results obtained with cultured cells, C-P4H-I represented approximately 90% of the total C-P4H activity in several tissues, such as the mouse kidney, heart, liver, skeletal muscle, and skin, while C-P4H-II represented 80% of the total C-P4H activity in cartilage, the corresponding percentage in mouse bone being about 45% (Annunen *et al.* 1998b). Immunofluorescence staining of the fetal human foot confirmed the presence of C-P4H-II in chondrocytes, but also in osteoblasts and capillary endothelial cells, whereas dermal fibroblasts and undifferentiated mesenchymal cells gave strong immunofluorescence with an antibody against the α(I) subunit (Annunen *et al.* 1998b).

Further immunohistochemical analyses of several fetal and adult human tissues, including some malignant tissues, have not only verified the spatial tissue distribution of the two isoenzymes but have also pointed to temporal differences in their location and abundance (Nissi *et al.* 2001, Nissi *et al.* 2004). C-P4H-I appeared to be the predominant form not only in cells of mesenchymal origin, but also in developing and malignant tissues, whereas C-P4H-II was expressed in more differentiated cells (Nissi *et al.* 2001, Nissi *et al.* 2004). Fibroblasts, skeletal myocytes, and smooth muscle cells gave a strong signal with an antibody against the α(I) subunit, while capillary endothelial cells stained with an α(II) antibody. Interestingly, the fetal calvaria, a bone that develops through intramembranous ossification, stained strongly with antibodies against both isoenzymes (Nissi *et al.* 2001). Moreover, C-P4H-I appeared to be the major isoenzyme in chondrosarcomas and osteosarcomas, whereas C-P4H-II was more readily expressed in benign bone tumors (Nissi *et al.* 2004).

So far no human diseases associated with mutations in any of the C-P4H α subunit genes have been identified. Mice with genetic ablation of the C-P4H α(I) subunit die early during embryogenesis, between E10.5 and E11.5, due to impaired assembly of collagen IV, leading to rupture of the BMs (Holster *et al.* 2007). Suprisingly, no obvious differences in the assembly of collagen fibrils were detected in the null embryos. The amount of total C-P4H activity in homozygous mutant embryos and in cultured embryonic fibroblasts was found to be reduced to about 20% of that in the wild-type embryos and cells (Holster *et al.* 2007).

2.4.3 Invertebrate C-P4Hs

Animal C-P4Hs have also been identified and characterized from the fly *D. melanogaster* and certain nematodes, including *C. elegans* (Myllyharju *et al.* 2002, Myllyharju & Kivirikko 2004). In addition, many higher plants, the green algae *Chlamydomonas reinhardtii* and *Volvox Carteri*, and *Paramecium bursaria Chlorella* virus-1 (PBCV-1) possess P4Hs which hydroxylate proline-rich substrates but, in contrast to the animal C-P4Hs, appear to exist in soluble monomers (Myllyharju 2003).

The *D. melanogaster* genome contains over 20 genes encoding C-P4H α subunit-like polypeptides, but only three genes coding for collagens, all of which are found in its BMs (Abrams & Andrew 2002, Myllyharju & Kivirikko 2004). The single *D. melanogaster* C-P4H α subunit that has been characterized so far forms a catalytically active $\alpha_2\beta_2$ tetramer with PDI (Abrams & Andrew 2002). Two *D. melanogaster* C-P4H α subunit polypeptides, SG1 and SG2, have been shown to be expressed specifically in the salivary gland, where they seem to play a role in the maintenance of an open salivary gland lumen and its proper secretory function (Abrams *et al.* 2006). Since the salivary gland does not produce collagens, and bearing in mind the small number of collagen types in this fly, it has been speculated that *D. melanogaster* C-P4Hs may hydroxylate substrates other than collagen polypeptides.

C. elegans has a large collagen family that is further divided into cuticle collagens, with over 170 different polypeptides, and BM collagens, with three different polypeptides (Myllyharju & Kivirikko 2004). It has at least four genes encoding the catalytic α subunit of C-P4H, namely *phy-1* (also known as *dpy-18*), *phy-2*, *phy-3* and *phy-4.1* (Friedman *et al.* 2000, Hill *et al.* 2000, Keskiaho *et al.* 2008, Myllyharju *et al.* 2002, Riihimaa *et al.* 2002, Winter & Page 2000) and three genes encoding PDI, termed *pdi-1*, *pdi-2*, and *pdi-3* (Eschenlauer & Page 2003, Veijola *et al.* 1996a, Winter & Page 2000). The PHY-1 and PHY-2 polypeptides are expressed in the cuticle hypodermal cells that produce collagen at all developmental stages (Winter & Page 2000), while PHY-3 is detected in embryos and late larval stages but its expression in adult nematodes is restricted to the spermatheca (Riihimaa *et al.* 2002). The expression of PHY-4.1 appears to be limited to the pharyngeal glands, the lumen of the pharynx, and the excretory duct (Keskiaho *et al.* 2008).

The properties of *C. elegans* PHY polypeptides and PDI with respect to their assembly into active C-P4H are versatile, the major form being a unique mixed

PHY-1/PHY-2 /$(PDI-2)_2$ tetramer (Myllyharju *et al.* 2002). PHY-1 and PHY-2 can also assemble into dimers with PDI-2, although PHY-2 does this very ineffectively (Myllyharju *et al.* 2002). PHY-4.1 assembles into $(PHY-4.1)_2$/$(PDI-2)_2$ tetramers and PHY-4.1/PDI-2 dimers (Keskiaho *et al.* 2008), whereas recombinant PHY-3 requires the presence of PDI-1 rather than PDI-2 for its catalytic activity (Riihimaa *et al.* 2002, Veijola *et al.* 1996a). It is not yet known, however, whether PHY-3 forms a tetramer or dimer with PDI-1, or whether PDI-1 only acts to assist in its correct folding (Riihimaa *et al.* 2002).

Homozygous inactivation of either the *C. elegans phy-1* or *phy-2* gene prevents formation of the mixed C-P4H tetramer, this being compensated for, at least in part, by increased assembly of the corresponding PHY-PDI-2 dimer (Myllyharju *et al.* 2002). The *phy-1* null mutation causes a dumpy (short and thick) phenotype, while the *phy-2* null nematode has no phenotypic abnormalities (Friedman *et al.* 2000, Myllyharju *et al.* 2002, Winter & Page 2000). Simultaneous inactivation of the *phy-1* and *phy-2* genes or inactivation of the *pdi-2* gene alone will result in embryonic lethality (Winter & Page 2000). Disruption of *phy-3* or *phy-4.1* does not produce morphological defects in excess of those detected in the *phy-1* and *phy-2* null nematodes (Keskiaho *et al.* 2008, Riihimaa *et al.* 2002).

2.4.4 Reaction mechanism and catalytic properties

Like the HIF-P4Hs, the C-P4Hs belong to the family of iron and 2-oxoglutarate-dependent dioxygenases that require Fe^{2+}, 2-oxoglutarate, molecular oxygen and ascorbate for their catalytic activity. The hydroxylation reaction that they catalyze involves an oxidative decarboxylation of 2-oxoglutarate in which the the 2-oxoglutarate is stoichiometrically decarboxylated, with one oxygen atom incorporated into the succinate and the other into the resulting hydroxy group formed on the proline residue (Fig. 9) (Kivirikko & Myllyharju 1998, Kivirikko & Pihlajaniemi 1998, Myllyharju 2003, Myllyharju 2008). Ascorbate is not consumed stoichiometrically, and its presence is not necessary for the hydroxylation reaction to occur, given that the P4Hs can catalyze a number of reaction cycles at essentially a maximal rate in its absence. The P4Hs also catalyze uncoupled decarboxylation cycles of 2-oxoglutarate, in which ascorbate acts as an alternative oxygen acceptor and is consumed stoichiometrically (Myllyla *et al.* 1984). In these cycles the reactive iron-oxo complex is probably converted to $Fe^{3+}{\cdot}O^{\cdot-}$ rendering the enzymes unavailable if not reduced by

ascorbate. Thus, if there is a prolonged shortage of ascorbate, hydroxylation will cease and ascorbate will come to be required for reactivation of the enzyme (Myllyla *et al.* 1984). Uncoupled decarboxylation of 2-oxoglutarate occurs along with the proline hydroxylation-coupled decarboxylation, even in the presence of saturating concentrations of the peptide substrates (Myllyla *et al.* 1984, Tuderman *et al.* 1977) An ascorbate deficiency, will lead to collagen instability, which in humans is manifested in scurvy (Smith & Talbot 2010).

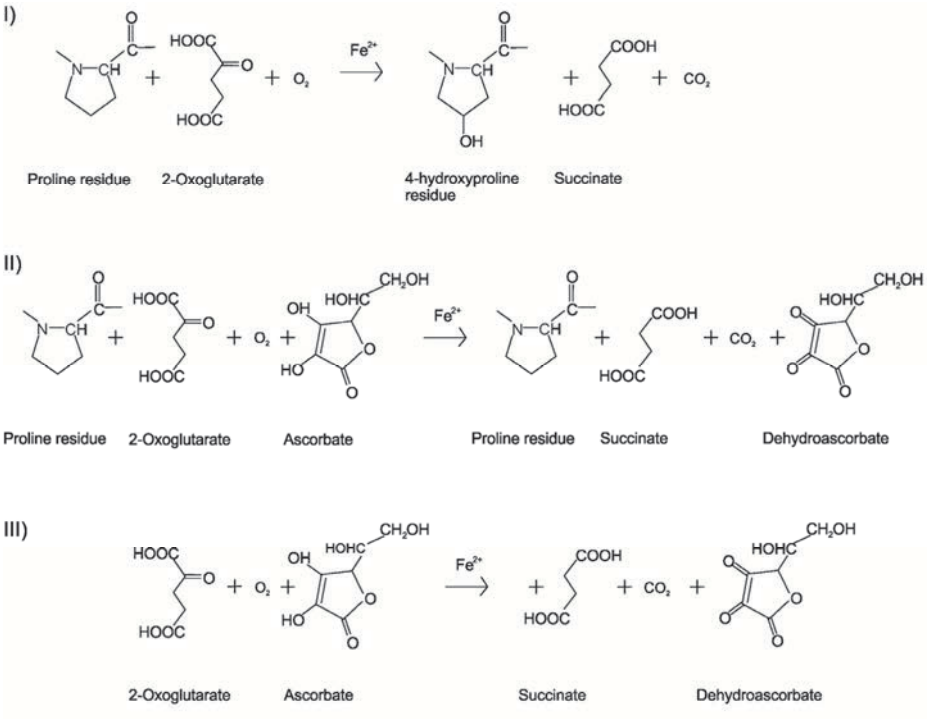

Fig. 9. Schematic representation of the reactions catalyzed by P4Hs. 2-Oxoglutarate is stoichiometrically decarboxylated during the hydroxylation reaction, which does not consume ascorbate (I). The enzymes also catalyze uncoupled decarboxylation of 2-oxoglutarate, which may occur with (II) or without (III) the peptide substrate, and during which ascorbate is consumed stoichiometrically.

The K_m values of type I, II and III C-P4Hs for Fe^{2+}, 2-oxoglutarate and ascorbate are very similar, being in the ranges 0.5–2 μM, 20–22 μM and 300–370 μM, respectively (see Table 4) (Kukkola *et al.* 2003, Myllyharju & Kivirikko 1997, Myllyharju 2008, Nokelainen *et al.* 2001). The C-P4Hs bind O_2 much more

efficiently than do the HIF-P4Hs (the K_m of C-P4H-I for oxygen being 40 μM), indicating that they can function effectively at low O_2 levels (Myllyharju 2008). The binding sites for Fe^{2+}, 2-oxoglutarate and ascorbate are located in the catalytically critical C-terminal domain of the α subunit and involve five conserved amino acid residues (Annunen *et al.* 1997, Helaakoski *et al.* 1989, Kukkola *et al.* 2003, Lamberg *et al.* 1995, Myllyharju & Kivirikko 1997). Two histidines and one aspartate bind the Fe^{2+} atom and a lysine binds the C-5 carboxyl group of the 2-oxoglutarate in position +10 with respect to the second iron-binding histidine. A fifth critical residue, a histidine that is probably involved in the binding of the C1 carboxyl group of the 2-oxoglutarate to the Fe^{2+} atom and the decarboxylation of this co-substrate, is likewise conserved in all three C-P4H α subunits (Annunen *et al.* 1997, Kukkola *et al.* 2003, Myllyharju & Kivirikko 1997).

The C-P4Hs mostly act on prolyl residues in individual protocollagen chains, but not on triple helices or free proline amino acids. The minimum requirement for hydroxylation is a tripeptide X-Pro-Gly in which the residue before the proline cannot be glycine (Myllyharju & Kivirikko 2004, Myllyharju 2008). $(Pro\text{-}Pro\text{-}Gly)_n$ polytripeptides are good substrates, with only the prolines preceding glycine being hydroxylated. C-P4Hs are also able to act on the tetrapeptides Pro-Pro-Ala-Pro and Pro-Pro-Glu-Pro, although to a lesser extent, which is consistent with the presence of a few -X-4Hyp-Ala- sequences in some collagen polypeptide chains and in the subcomponent of the complement protein C1q. The peptide substrate chain length has a major effect on the hydroxylation efficiency of the C-P4Hs, in that the K_m decreases with an increasing number of -X-Pro-Gly- triplets (Kivirikko & Myllyharju 1998, Kivirikko & Pihlajaniemi 1998, Myllyharju 2008). The K_m of C-P4H-I for the tripeptide Pro-Pro-Gly, for example, is about 20 mM, whereas that for $(Pro\text{-}Pro\text{-}Gly)_{20}$ is 50 μM and that for the non-hydroxylated 150 kDa type I procollagen polypeptide chain only about 0.2 μM (Kivirikko & Myllyharju 1998).

Although the catalytic properties of the three C-P4Hs are essentially equal, the isoenzymes have certain differences in their peptide substrate binding properties. The K_m values of the type I and III C-P4Hs for the peptide substrate $(Pro\text{-}Pro\text{-}Gly)_{10}$ are very similar, while that of the type II isoenzyme is about 5 times higher (Kivirikko & Myllyharju 1998, Kukkola *et al.* 2003).

Like the HIF-P4Hs, the C-P4Hs are inhibited by iron chelators and many other compounds with respect to their peptide substrates and all co-substrates. Many bivalent cations, such as Zn^{2+}, compete with Fe^{2+}, while several 2-

oxoglutarate analogues, such as pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate, act by competing with 2-oxoglutarate (Myllyharju 2008). Poly(L-proline) is a highly efficient competitive inhibitor of C-P4H-I, whereas C-P4H-II is inhibited only at much higher concentrations and C-P4H-III is inhibited by poly(L-proline) with an intermediate efficiency (Annunen *et al.* 1997, Helaakoski *et al.* 1995, Kukkola *et al.* 2003) The differences in peptide substrate binding properties between the type I and type II C-P4Hs may be caused by the presence of a glutamate and glutamine in the α(II) subunit in positions corresponding to Ile182 and Tyr233 in the α(I) subunit (Myllyharju & Kivirikko 1999).

Table 4. K_m values of recombinant human C-P4Hs I, II and III for reaction co-substrates and a peptide substrate. K_i and IC_{50} values for pyridine 2,4-dicarboxylate and poly(L-proline) are also given.

Co-substrate, substrate or inhibitor	Constant	C-P4H-I (μM)	C-P4H-II (μM)	C-P4H-III (μM)
Fe^{2+}	K_m	2 [a]	4 [a]	0.5 [a]
2-Oxoglutarate	K_m	22 [a]	22 [a]	20 [a]
Ascorbate	K_m	340 [a]	330 [a]	370 [a]
O_2	K_m	40 [a]	ND	ND
$(Pro\text{-}Pro\text{-}Gly)_{10}$	K_m	18 [a]	95 [a]	20 [a]
Pyridine 2,4-dicarboxylate	K_i	8 [a]	9 [b]	11 [c]
Poly(L-Pro), M_r5000-7000	IC_{50}	6 [c]	300 [c]	30 [c]

[a]Myllyharju 2005, [b]Helaakoski *et al.* 1995, [c]Kukkola *et al.* 2003; ND, not determined

2.4.5 Hypoxia inducibility of C-P4Hs

Many studies have demonstrated that hypoxia increases the deposition of ECM, including the accumulation of its main component collagens. Accordingly, several genes involved in the synthesis, maintenance and degradation of ECM have emerged as HIF targets (Myllyharju & Schipani 2010). The mRNAs of LHs 1 and 2, for example, are upregulated by hypoxia in various cell types, while the gene coding for lysyl oxidase is one of the most strongly hypoxia-induced genes (Denko *et al.* 2003, Elvidge *et al.* 2006, Hofbauer *et al.* 2003, Wang *et al.* 2005).

Peripheral lung parenchyma cells isolated from rats exposed to hypoxia showed an increase in the mRNA levels of proα1(I), proα1(III) and α2(IV) collagen chains (Berg et al. 1998), while hypoxia-stressed fibroblasts originating from various tissues have been shown to induce the mRNA expression of proα1(I) chains (Falanga *et al.* 1993, Norman *et al.* 2000, Tamamori *et al.* 1997, Tamamori

et al. 1998). In certain experiments with cultured cells increased deposition of type I and IV collagens was detected following exposure to hypoxia, but without any corresponding increase in the polypeptide mRNAs (Horino *et al.* 2002, Tajima *et al.* 2001). In these cases increased mRNA and protein levels of C-P4H-I were detected instead (Horino *et al.* 2002, Tajima *et al.* 2001). Later, several additional studies reported elevated mRNA levels of the C-P4H α(I) and α(II) subunits in response to lowered oxygen tension (Elvidge *et al.* 2006, Fahling *et al.* 2006, Hofbauer *et al.* 2003, Pollard *et al.* 2008). Rat vascular smooth muscle cells cultured in a hypoxic atmosphere caused a (5–12-fold) time-dependent upregulation of the mRNAs of the two P4H α subunits, with concomitant (2–4-fold) increases in the accumulation of the corresponding α subunit polypeptides (Hofbauer *et al.* 2003). In juxtraglomerular cell lines the hypoxic induction was more robust, the increases in the α(I) and α(II) mRNAs being 5–8-fold and 25–33-fold, respectively (Hofbauer *et al.* 2003), while the hypoxia-induced increases in the α(I) and α(II) mRNAs in the breast cancer cell line MCF7 were 5-fold and 7.5-fold, respectively, with no induction in the expression of the α(III) subunit mRNA to be detected (Elvidge *et al.* 2006, Pollard *et al.* 2008).

In a mouse hepatoma cell line and embryonic fibroblasts the hypoxia-induced upregulation of α(I) and α(II) mRNAs occurred in a HIF-1–dependent manner (Hofbauer *et al.* 2003). Moreover, under long-term hypoxia the increased synthesis of the α(I) subunit was shown to be controlled by interaction with the RNA binding protein nucleolin in the untranslated region of the mRNA (Fahling *et al.* 2006). An HRE has been identified in the mouse *P4ha1* gene encoding C-P4H α(I), residing approximately 120 bp upstream of the transcription start site (Takahashi *et al.* 2000). HIF has been shown to bind to this site, and accordingly, no hypoxic induction of either *P4ha1* or *P4ha2* mRNA was detected in a mouse hepatoma cell line lacking HIF-1 (Hofbauer *et al.* 2003, Takahashi *et al.* 2000).

2.5 Endochondral bone formation

The vertebrate skeleton is derived from three embryonic lineages: the cranial neural crest, the paraxial mesoderm and the lateral plate mesoderm, which give rise to the craniofacial, axial and limb skeleton, respectively (Olsen *et al.* 2000). During skeletal morphogenesis, cells from these lineages migrate to the locations in the embryo where the future skeleton will later develop, form characteristic mesenchymal condensations and differentiate into chondrocytes or osteoblasts (Hall & Miyake 1992, Olsen *et al.* 2000). In the case of a few bones, most notably

the flat bones of the skull and the medial clavicle, the mesenchymal cells differentiate directly into bone-forming osteoblasts through a process called intramembranous ossification. Most of the skeleton, however, is formed through endochondral ossification, a two-stage process whereby the mesenchymal cells first differentiate into chondrocytes to provide a cartilaginous template, the growth plate, which is subsequently replaced by bone and bone marrow (Burdan *et al.* 2009, Fosang & Beier 2011).

During endochondral ossification in the limb, chondrocytes undergo a sequence of events that are reflected in their morphology and visible in four well defined layers within the growth plate: the reserve, proliferative and hypertrophic zones followed by the primary spongiosa (see Fig. 10). In addition, the perichondrium at the periphery of the growth plate is formed by a thin layer of cells that express type I collagen. The cells residing within the reserve zone are round, small, uniform and compactly located, occurring singly or in pairs in a lacuna within the cartilage ECM, whereas in the proliferative zone the chondrocytes adopt a flat shape and pile up in columns parallel to the long axis of the bone. Both resting and proliferating chondrocytes have a high secretory activity, as they produce the typical cartilage ECM that is mainly composed of type II and IX collagens and aggrecan (Burdan *et al.* 2009, Fosang & Beier 2011).

Next, the chondrocytes stop proliferating and establish the hypertrophic zone. They enlarge and become prehypertrophic chondrocytes, which differentiate further into the post-mitotic hypertrophic chondrocytes that exit the cell cycle. Hypertrophic chondrocytes express predominantly type X collagen, which is exclusively synthesized by this chondrocyte subpopulation and serves to direct the mineralization of the surrounding matrix. Through their secretion of angiogenic factors such as vascular endothelial growth factor VEGF, the hypertrophic chondrocytes induce blood vessel invasion from the bone collar. They also attract chondroclasts, cells of the macrophage lineage that digest the cartilaginous matrix, and direct the adjacent perichondrial cells and other cells of the osteoblast lineage brought to the site by the invading blood vessels to become osteoblasts. The hypertrophic chondrocytes eventually undergo programmed cell death and the cartilage left behind is replaced by primary spongiosa (trabecular bone). With chondrocyte proliferation and continuing resorption of the primary spongiosa, the primary centre splits into two opposite growth plates and secondary ossification centres are formed (Burdan *et al.* 2009, Fosang & Beier 2011, Wuelling & Vortkamp 2011). A schematic representation of endochondral bone formation is presented in Figure 10.

The rapid longitudinal bone growth that takes place during fetal life is a combined result of chondrocyte proliferation, ECM secretion and hypertrophy. It has been roughly estimated that 60% of this bone growth is due to chondrocyte hypertrophy, 30% to ECM production and the remaining 10% to cell proliferation (Burdan *et al.* 2009) but the relative contributions of these parameters eventually depend on the developmental stage and anatomical location of the bone and vary between species (Wilsman *et al.* 1996). Given the fundamental role of endochondral ossification in bone growth, it is evident that the functions of the growth plate chondrocytes are tightly regulated. Several locally produced factors interact with circulating hormones and ECM components at multiple levels to regulate bone growth (Wuelling & Vortkamp 2011).

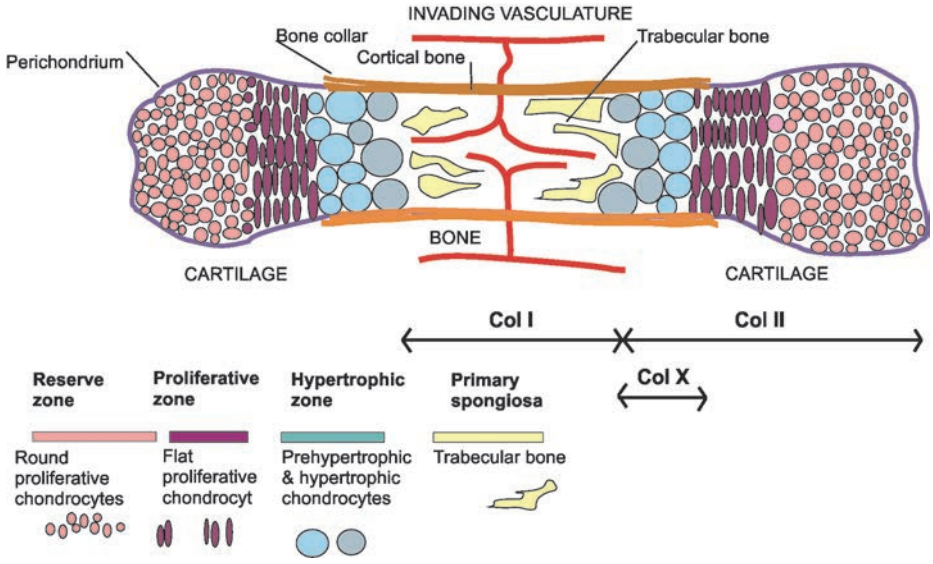

Fig. 10. Schematic representation of endochondral bone formation; a mouse tibia at a late stage in fetal development. Four characteristic layers and the major collagen types during the process are depicted. Modified from Provot & Schipani 2005.

2.5.1 Molecular mechanism of endochondral bone formation

The Sox and Runx transcription factors contribute substantially to skeletal patterning. Sox9 is required at every stage in chondrocyte differentiation: it directs the commitment of mesenchymal cells towards chondrocytes and

upregulates the expression of cartilaginous ECM genes. Sox9 is expressed in the proliferative chondrocytes of the growth plate but is switched off in the hypertrophic chondrocytes. Two other members of the Sox family, Sox5 and Sox6, are also involved in the control of chondrocyte differentiation, but their expression is dependent on the presence of Sox9 (Akiyama *et al.* 2002). Runx2 (previously called Cbfa1) is the major determinant of osteoblast differentiation, but it plays certain roles in chondrocytes as well. Runx2 is expressed by perichondrial cells and inhibits chondrocyte proliferation and hypertrophy (Karsenty *et al.* 2009, Provot & Schipani 2005). Besides these two major transcription determinants, additional transcription factors such as the HIFs have been shown to play a role in the regulation of endochondral bone formation (Maes *et al.* 2012, Schipani *et al.* 2001).

The balance in signalling between bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs) determines the rate of chondrocyte proliferation throughout chondrogenesis. The transition from chondroblasts to proliferating chondrocytes and on to hypertrophic chondrocytes involves crosstalk between BMPs and the Indian hedgehog (Ihh) signalling pathway and the BMP and Ihh signalling systems act in parallel to increase chondrocyte proliferation and the lengths of the proliferating columns in the growth plate (Wu *et al.* 2007). BMP and FGF signals regulate the same stages in chondrocyte proliferation and hypertrophy but mediate the opposite effects (Minina *et al.* 2001, Minina *et al.* 2002). Ihh, which is expressed by the prehypertrophic and early hypertrophic chondrocytes, inhibits differentiation of the hypertrophic chondrocytes and delays mineralization of the cartilaginous matrix (Provot & Schipani 2005, Wuelling & Vortkamp 2011). Parathyreoid hormone-related protein (PTHrP) is an autocrine/paracrine factor which is expressed in growth plates by the perichondrial cells and early proliferative chondrocytes. Ihh and PTHrP signalling builds up a negative feedback loop, with Ihh stimulating PTHrP expression and PTHrP serving in part to keep the proliferating chondrocytes in the proliferative pool, thus delaying Ihh synthesis (Provot & Schipani 2005, Wuelling & Vortkamp 2011). Moreover, BMP and FGF signalling has been shown to interact with the Ihh/PTHrP pathway (Provot & Schipani 2005, Wuelling & Vortkamp 2011).

MiRNAs have recently been shown to play a role in bone and cartilage homeostasis and to modulate both endochondral and intramembranous ossification (Dong *et al.* 2012, Wienholds *et al.* 2005). Comparison of miRNA expression profiles in both mouse and human bone marrow stromal cells (MSCs) has demonstrated both up and down-regulation of several miRNAs, such as miR-

199a, miR-124a and miR-96, during osteo- and/or chondrogenesis (Laine *et al.* 2012, Suomi *et al.* 2008). Importantly, evidence suggesting some interplay of miRNA target genes with transcription factors such as HIF-1α has been obtained (Suomi *et al.* 2008). Dicer is a RNaseIII-containing enzyme required for processing most miRNAs, and its conditional removal from the early limb bud mesenchyme in mice has been found to result in massive cell death in the growth plate and consequently in the development of much smaller limbs (Harfe *et al.* 2005). Specific deletion of *Dicer* in mouse chondrocytes partly impaired chondrocyte proliferation, but accelerated their differentiation (Kobayashi *et al.* 2008). Similarly, universal deletion of miR-140, a miRNA prevalently expressed in cartilaginous elements of developing bones in the mouse embryo and the zebrafish (Tuddenham *et al.* 2006, Wienholds *et al.* 2005) caused moderate but significant dwarfism, probably due to impaired chondrocyte proliferation (Miyaki *et al.* 2010). In addition to the examples mentioned above, several other miRNAs, such as miR-145, miR-675 and miR-221, have been linked to the regulation of osteo- and chondrogenesis (Dong *et al.* 2012, Dudek *et al.* 2010, Kim *et al.* 2010, Yang *et al.* 2011a, Yang *et al.* 2011b).

2.5.2 Growth plate ECM

Skeletal development involves the synthesis of a specialized ECM which in the cartilaginous growth plate is mostly composed of type II collagen and glycoproteins such as hyaluronan and proteoglycans. This ECM provides the tissue with the mechanical stability required for the integrity and shape of the growing skeleton. The collagen fibrils are responsible for tensile strength, while the proteoglycans, mainly aggrecan, bring about osmotic swelling and give the tissue its elastic properties. In addition to providing structural support, however, the cartilaginous ECM also serves as a dynamic network of molecules secreted by the cells and modulates their behaviour by affecting their proliferation and differentiation. The importance of the complex interactions between the components of the growth plate ECM and the cells surrounded by it for bone growth is illustrated by the dwarfing effect caused by mutations in the genes encoding these proteins (Bateman *et al.* 2009, Kadler *et al.* 2007, Olsen 1996, Olsen *et al.* 2000).

Collagens

Type II collagen, the major structural collagen component of cartilage, is synthesized in the growth plate by the proliferating and upper hypertrophic chondrocytes. The relative abundances of the minor growth plate collagens (types VI, IX, X, XI, XII and XIV) vary between the growth plate zones. The collagen fibrils in cartilage are heteropolymers, mostly composed of collagen types II, IX and XI. Type II collagen constitutes the bulk of the fibril, with type IX collagen covalently linked to the fibril surface and type XI forming a filamentous template at the core. The fibrils are arranged in staggered arrays that enable the formation of three-dimensional scaffolds through interactions with neighbouring collagen molecules and with a variety of glycoproteins and proteoglycans (Olsen 1996, Olsen *et al.* 2000).

Nascent type II collagen, a fibrillar homotrimer encoded by the gene *COL2A1*, is present in the nucleus pulposus of the intervertebral discs, the vitrous of the eye and the tectorial membrane of the ear in addition to cartilage. Mutations in *COL2A1* cause a spectrum of skeletal dysplasias that are mostly inherited autosomally dominantly (Alman 2008, Olsen *et al.* 2000, Spranger *et al.* 1994). Disorders in this group include congenital spondyloepiphyseal dysplasia, Kniest dysplasia, Stickler syndrome, and achondrogenesis, and other organs in which collagen II is present, such as the ear and eye, can also manifest these diseases. The clinical phenotypes range from lethality (achondrogenesis type II), to serious dwarfism (Kniest dysplasia) or relatively normal stature with early-onset osteoarthritis (Stickler syndrome) (Spranger *et al.* 1994). Several *COL2A1* mutant mouse lines have been generated and have been shown to reproduce corresponding phenotypes to those observed in humans. The growth plate in these mutants is generally disorganized, and the collagen fibrils are sparsely distributed in the cartilage matrix (Olsen *et al.* 2000).

Collagen type IX, which always co-exists with type II, belongs to the FACIT subgroup of collagens and is a heterotrimeric molecule composed of the products of three distinct genes, *COL9A1*, *COL9A2* and *COL9A3*. Collagen IX molecules are located on the surface of cartilage fibrils, so that the long triple-helical arm runs along the fibril and the short triple-helical arm protrudes from the fibril surface, allowing interactions with other matrix particles. Several covalent crosslinks formed through lysyl oxidase occur within the molecule and between collagens IX and II (Olsen 1996, Olsen *et al.* 2000). Mutations in all three collagen IX genes have been linked to multiple epiphysial dysplasia (MED)

which is a heterogenous disorder characterized by mildly short stature and early-onset osteoarthritis (Muragaki *et al.* 1996, Spayde *et al.* 2000).

Type X collagen, a member of a non-fibrillar short chain collagen family, is a homotrimeric molecule produced exclusively by growth plate hypertrophic chondrocytes. A variety of mutations in the human *COL10A1* gene can cause autosomal dominant metaphyseal chondrodysplasia of the Schmid type (MCDS) (Warman *et al.* 1993). MCDS is characterized clinically by mildly shortened limbs, bowing of the legs and coxa vara. Histological studies with transgenic mice carrying the corresponding *COL10A1* mutations show an elongated and disorganized hypertrophic zone in the growth plate (Ho *et al.* 2007, Nielsen *et al.* 2000, Rajpar *et al.* 2009). When the mutation results in the production of truncated collagen X, the protein is retained within the ER where it appears to trigger UPR which in part results in abnormal architecture and function of the growth plate (Ho *et al.* 2007, Rajpar *et al.* 2009).

Type XI collagen represents a minor collagen in cartilage. It is a heterotrimer composed of three polypeptide chains derived from the genes *COL11A1*, *COL11A2* and *COL2A1*. The ratio between these within the cartilage fibril appears to regulate the fibril diameter. Moreover, it has been suggested that collagens IX and XI may interact with proteoglygans and modulate the effect of extracellular signalling molecules (Olsen 1996, Olsen *et al.* 2000). Mutations in genes encoding collagen XI are associated with Stickler and Marshall syndromes, which are dominantly inherited chondrodysplasias characterized by high myopia, sensorineural hearing deficit and midfacial hypoplasia (Griffith *et al.* 1998, Richards *et al.* 1996). Mice with recessive chondrodysplasia (*cho*) harbour a single nucleotide deletion that results in a premature stop codon in the α1(XI) collagen chain (Li *et al.* 1995) and those that are homozygous for the mutation die at birth with cartilage abnormalities in the limbs, ribs, mandible and trachea (Li *et al.* 1995). The limbs are shorter, with wide metaphyseal areas, and the growth plate chondrocytes are disorganized and hypertrophy delayed. Moreover, the growth plate ECM is soft, lacks cohesion and contains thick collagen fibrils and soluble proteoglycan aggregates (Li *et al.* 1995).

Proteoglycans and other non-collagenous molecules

The predominant cartilage proteoglycan is aggrecan, which is a large chondroitin sulphate proteoglycan. Aggrecan exists in the form of proteoglycan aggregates with two other components, hyaluronan and a link protein. Hyaluronan forms a

central filament with over 100 aggrecan molecules radiating from it, each interaction being stabilized by the link protein (Watanabe *et al.* 1998). While aggrecan binds the link protein via its N-terminal globular domain (G1), a globular domain (G3) at the C-terminal site of the molecule has been shown to interact with certain matrix proteins containing EGF repeats (including the fibrillins, fibulins and tenascins) (Day *et al.* 2004). Aggrecan possesses high anionic charge density, mostly within the chondroitin sulphate chains of the protein, which creates a high osmotic environment and retains water in the tissue. Mice with an aggrecan frameshift mutation display short limbs, tails and snout, a cleft palate, and defects in the intervertebral disc (Watanabe *et al.* 1994, Watanabe *et al.* 1997).

Additional cartilage proteoglycans include small leucine-rich repeat proteoglycans (SLRPs) such as decorin, biglycan and fibromodulin and also the large proteoglycan perlecan, which has been shown to promote chondrogenic differentiation *in vitro* (French et al. 1999). Moreover, *in vivo* studies have shown histological abnormalities in the growth plate cartilage of perlecan null mice (Arikawa-Hirasawa *et al.* 1999, Costell *et al.* 1999, French *et al.* 1999). Other less abundant non-collagenous molecules include cartilage oligomeric protein (COMP), also known as thrombospondin-5, and the matrilins. Pseudoachondroplasia in humans is caused by mutations in COMP (Briggs & Chapman 2002) while MED can be caused by mutations in the genes encoding COMP or matrilin-3 in addition to those encoding collagen type IX (Briggs & Chapman 2002).

2.5.3 Hypoxia in the regulation of growth plate chondrocytes

Fetal growth plate is an avascular tissue that is hypoxic for most of its length (Rajpurohit *et al.* 1996, Schipani *et al.* 2001) but there is extensive vascularization in the surrounding tissue and primary spongiosa, and thus growth plate has a typical outside-inside oxygenation gradient (Schipani *et al.* 2001). The most hypoxic chondrocytes are located in the proliferative zone, in the centre of the columnar proliferative zone and in the upper portion of the hypertrophic zone, whereas the late hypertrophic chondrocytes at the border of the primary spongiosa are not hypoxic (Schipani *et al.* 2001). HIF transcription factors have been implicated in regulating a variety of processes during endochondral bone formation, including induction of VEGF expression, modulation of metabolic

pathways, autophagy and regulation of ECM formation and turnover (Araldi & Schipani 2010, Myllyharju & Schipani 2010, Schipani 2010).

A study with conditional knockout mice demonstrated that chondrocytes lacking *Hif-1a* undergo massive apoptotic cell death in the central parts of the growth plate (Amarilio *et al.* 2007, Schipani *et al.* 2001) and later HIF-1α turned out to be dispensable for mesenchymal condensations, but was required for the early and late stages of chondrogenesis and joint formation (Provot *et al.* 2007). It has been suggested that HIF-1α may promote chondrocyte function directly by inducing the expression of Sox9, which is one of the master regulators of chondrogenesis (Robins *et al.* 2005). In support of this hypothesis, mouse bone marrow stromal stem cells (ST2) cultured under hypoxic conditions differentiated along the chondrocyte pathway, expressing high levels of Sox9 via a HIF-1α-dependent mechanism (Robins *et al.* 2005).

Low oxygen tension and high lactic acid concentrations within the epiphyseal growth plate push chondrocytes into generating most of their energy through anaerobic glycolysis. Excessive hypoxia and failure to upregulate phosphoglycerate kinase (PGK-1), a key enzyme in anerobic glycolysis, were among the earliest manifestations in *Hif-1a* null growth plates, indicating affected anaerobic glycolysis in the mutant chondrocytes (Schipani *et al.* 2001). Moreover, murine primary growth plate chondrocytes lacking HIF-1α showed a significant reduction in free ATP and lactic acid when cultured under hypoxia, whereas control cells displayed a significant increase in lactic acid, paralleling the rising level of free ATP (Pfander *et al.* 2003). Hypoxia strongly induced the expression of PGK-1 and glucose transporter 1 (*Glut1*) mRNA in wild-type chondrocytes whereas *Hif-1a* null chondrocytes completely lacked the induced increase in the PGK-1 and Glut-1 message (Pfander *et al.* 2003).

VEGF-A has several functions during endochondral bone formation. Best known for its activity as an angiogenic factor, VEGF-A is required for blood vessel invasion into the primary ossification centre and for the maintenance of blood vessel growth in developing bones (Gerber *et al.* 1999a, Gerber *et al.* 1999b, Maes *et al.* 2002, Zelzer *et al.* 2002). Inhibition of VEGF-A in 24-day-old mice inhibited blood vessel invasion into the hypertrophic zone of the growth plate, resulting in impaired trabecular bone formation and expansion of the hypertrophic zone (Gerber *et al.* 1999b). Activation of the HIF pathway promoted angiogenesis and osteogenesis by inducing VEGF production in osteoblasts (Wang *et al.* 2007). Mice overexpressing HIF-1α in osteoblasts developed robust, heavily vascularized long bones but a normal skull, while loss of *Hif-1a* resulted in

narrow, less vascularized long bones (Wang *et al.* 2007). VEGF-A is expressed not only by hypertrophic chondrocytes and osteoblasts but also at a moderate level by proliferative chondrocytes in the central, hypoxic parts of growth plates (Pfander *et al.* 2003, Pfander *et al.* 2004). Interestingly, abolition of VEGF-A expression in murine growth plates resulted in extensive cell death in the epiphyseal and joint regions, i.e. a phenotype that closely resembled that detected in *Hif-1a* knockout growth plates (Zelzer *et al.* 2004). The severity of hypoxia in the *Hif-1a* deficient growth plate was only modestly corrected by increased vascularization, however. Hence VEGF is not likely to be the sole critical downstream effector of HIF-1α survival (Maes *et al.* 2011).

Hypoxia together with both HIF-1α and HIF-2α has been reported to support cartilaginous matrix formation, which in part contributes to chondrocyte survival and differentiation (Lafont *et al.* 2007, Lafont *et al.* 2007, Pfander *et al.* 2003, Pfander *et al.* 2004). Hypoxia led to the increased accumulation of cartilaginous matrix components such as collagen II in murine primary chondrocytes in a HIF-1α-dependent manner (Pfander *et al.* 2003) although the increased matrix formation did not coincide with upregulation of Sox9, indicating that there were also other mechanisms behind this matrix accumulation (Pfander *et al.* 2003, Provot *et al.* 2007). Enhanced cartilaginous matrix formation has nevertheless been reported to occur in part through the activation of Sox9 by a HIF pathway-dependent mechanism in hypoxic ST2 cells, micromass cultures and human articular chondrocytes (HACs) (Amarilio *et al.* 2007, Lafont *et al.* 2007, Robins *et al.* 2005). Moreover, in a study with HACs, hypoxia-driven upregulation of Sox9 with subsequent upregulation of cartilage matrix genes appeared to occur specifically through HIF-2α (Lafont *et al.* 2008). Using an RNAi-mediated approach, HIF-P4H-2 was identified as the key hydroxylase responsible for regulating HIF-2α in these cells (Thoms & Murphy 2010). Since the genes for the C-P4H α(I) and α(II) subunits are hypoxia-inducible in chondrocytes and other cell lines, it is possible that HIF-1α may promote chondrocyte function in hypoxic growth plates by enhancing the formation of a proper ECM (Hofbauer *et al.* 2003, Provot *et al.* 2007, Takahashi *et al.* 2000). Since there is no evidence that hypoxia could directly regulate the expression of collagen mRNAs in growth plate chondrocytes, HIF could mediate its functions by improving the post-translational modification of collagens, particularly the hydroxylation of their proline residues.

3 Aims of the present research

HIFs regulate genes whose protein products are involved in erythropoiesis (Epo) and ECM synthesis, for example. P4H-TM is also able to hydroxylate HIF-α, at least *in vitro* and *in cellulo*, but little is known about its *in vivo* roles. Many small-molecular compounds that inhibit HIF-P4Hs with respect to its co-substrate 2-oxoglutarate have been developed recently and have been shown to benefit patients suffering from anaemia brought about by inadequate Epo production. The hypothesis here was thus that P4H-TM is involved in the regulation of mammalian erythropoiesis. The specific aims were:

- to study the erythropoietic response of FG-4497, in *P4h-tm*$^{-/-}$ mice and also in *Hif-p4h-2*$^{gt/gt}$ (HIF-P4H-2 hypomorphs) and *Hif-p4h-3*$^{-/-}$ mice,
- to characterize the hematological phenotype of *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ double gene-modified mice.

The significance of hypoxia pathway on mouse epiphyseal growth plate chondrocytes was likewise addressed. Cartilaginous growth plate is a hypoxic structure of mesenchymal origin, and HIF has been shown to play a role in the survival, development and differentiation of its chondrocytes. Growth plate is rich in ECM and its main component, collagens, and the C-P4Hs are key enzymes in collagen biosynthesis, as the resulting 4-hydroxyprolines are necessary for the stability of all collagen molecules. While C-P4H-I is the main isoenzyme in most cell types and tissues, C-P4H-II predominates in chondrocytes. In the second study we used chondrocytes isolated from newborn mice with conditionally inactivated *Hif-1a*, *Hif-2a* or *Vhl* genes and in the third study a mouse model with complete inactivation of C-P4H-II with or without partial inactivation of C-P4H-I. The specific aims were:

- to assess whether the post-translational hydroxylation of collagens, in particular hydroxylation of proline residues, could be one of the modalities by which HIF regulates the adaptive responses of chondrocytes in early growth plates,
- to clarify the specific *in vivo* roles of C-P4H isoenzymes I and II in mammalian skeletogenesis.

4 Materials and methods

The genetically modified mouse lines used and characterized in this thesis are summarized in Tables 5 and 6 and the methods used are listed in Table 7. Figures 11 and 12 illustrate knockout strategies for targeting *P4h-tm*, *Hif-P4h-3*, and *P4ha1* and *P4ha2* genes, respectively. Detailed descriptions of the experimental procedures can be found in original papers I-III.

Table 5. Materials.

Knockout (KO)	Knockout strategy	Phenotype (if previously published)	Used in
P4h-tm$^{-/-}$	Conventional KO	Unpublished	I
Hif-p4h-2$^{gt/gt}$ *	Genetrap	Hearts show protection against acute ischemia-reperfusion injury[1]	I
Hif-p4h-3$^{-/-}$	Conventional KO	Altered sympathoadrenal development[2]	I
Hif-p4h-2$^{gt/gt}$/*P4h-tm*$^{-/-}$	Genetrap/Conventional KO	Unpublished	I
P4ha1$^{-/-}$	Conventional KO	Embryonic lethality (E10.5-11.5)[3]	III
P4ha2$^{-/-}$	Conventional KO	Unpublished	III
P4ha1$^{+/-}$/*P4ha2*$^{-/-}$	Conventional KO	Unpublished	III

*Wild-type *Hif-P4h-2* mRNA is expressed at various levels in *Hif-p4h-2*$^{gt/gt}$ tissues: 8% in the heart; 15% in the skeletal muscle; 34–47% in the kidney, spleen, lung and bladder; 60% in the brain and 85% in the liver[1]

[1]Hyvärinen *et al*. 2010, [2]Bishop *et al*. 2008, [3]Holster *et al*. 2007

Table 6. Genetically modified mouse lines used as sources of newborn primary mouse epiphyseal growth plate chondrocytes.

Knock-out	Knock-out strategy	Used in
Hif-1a$^{f/f}$	Conditional KO	II
Hf-2a$^{f/f}$	Conditional KO	II
Vhl$^{f/f}$	Conditional KO	II
P4ha1$^{+/-}$/*P4ha2*$^{-/-}$	Conventional KO	III

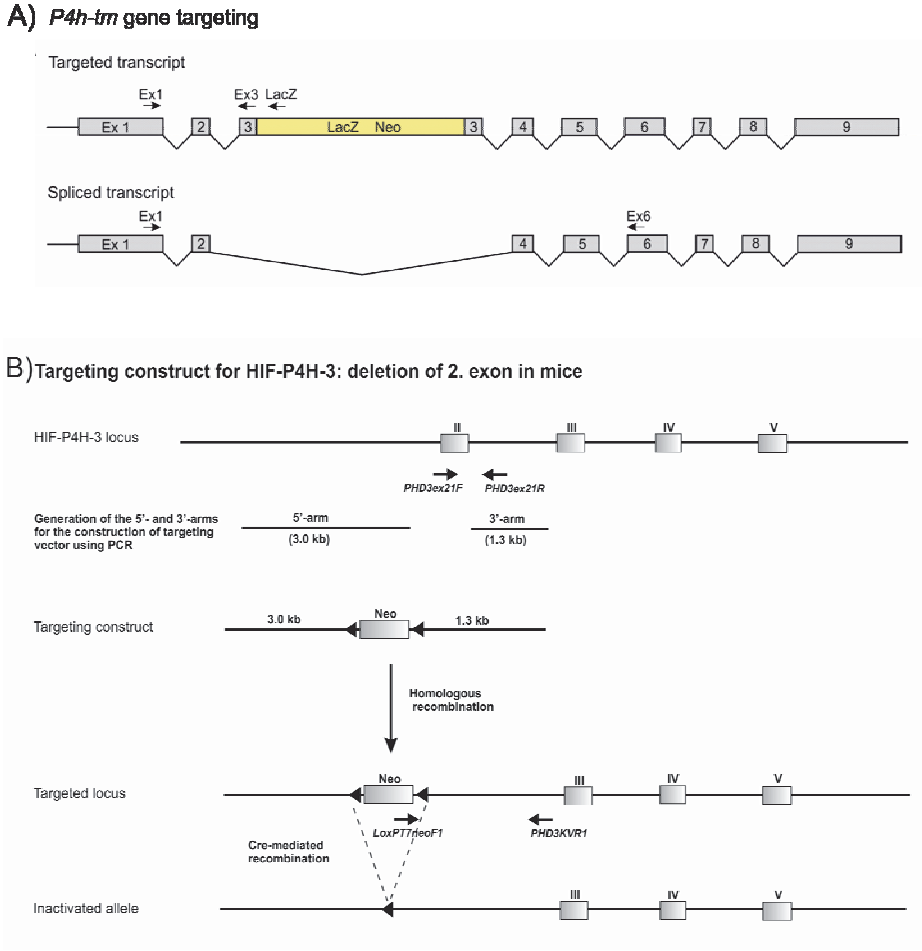

Fig. 11. Targeting constructs for A) *P4h-tm* and B) *Hif-p4h-3* have been prepared earlier by colleagues in Johanna Myllyharju group and will be published in future works. The figure is presented by courtesy of Peppi Koivunen (A) and Joni Mäki (B). Targeted disruption of *Hif-p4h-2* gene by gene-trap strategy has been described in Hyvärinen *et al.* 2010. *P4h-tm*$^{-/-}$, *Hif-p4h-3*$^{-/-}$, *Hif-p4h-2*$^{gt/gt}$ mice were used in paper I.

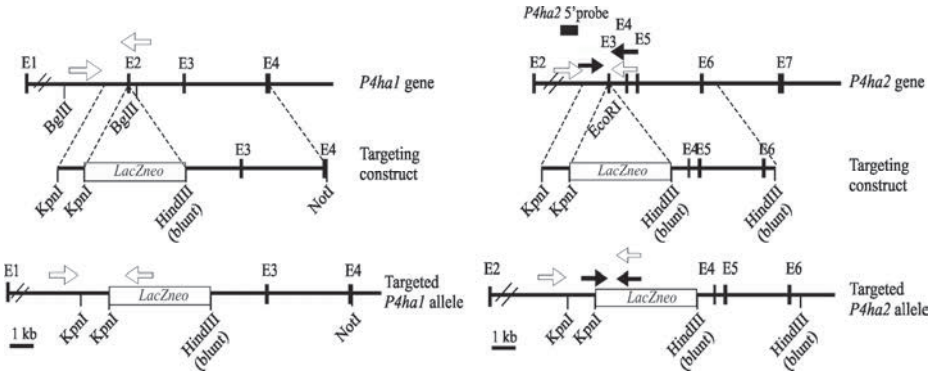

Fig. 12. Targeting the *P4ha1* and *P4ha2* genes for the mouse C-P4H α(I) and α(II) subunits, respectively. In both targeting constructs the *lacZ-PKG-neo* cassette was inserted in-frame into exon 2 in the case of *P4ha1* and exon 3 in the case of *P4ha2* after the translation initiation codon that the *neo* gene is in the opposite orientation. The locations of the PCR primers used in genotyping *P4ha2* mutants are indicated by black arrows while open arrows indicate the locations of PCR primers used for genotyping the *P4ha1;P4ha2* double mutants. *P4ha1*$^{-/-}$ mice were characterized by Holster *et al.* 2007. *P4ha2*$^{-/-}$ and *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice were characterized in article III.

Table 7. Methods.

Level	Method	Used in
DNA	Cloning techniques	II, III
	PCR	I, II, III
RNA	RNA isolation	I, II, III
	RT-PCR	I, II, III
	Quantitative real-time PCR	I, II, III
Protein	SDS-PAGE and Western blotting	I, II, III
	ELISA	I, II
	P4H activity assay using 2-oxo[1-^{14}C]glutarate	I, II, III
	C-P4H activity assay with 4-hydroxy[^{14}C]proline	II, III
	Amino acid analysis	III
Cells and tissues	Analysis of hematological parameters	I
	Isolation of primary mouse epiphyseal growth plate chondrocytes	II, III
	Cell culture	II, III
	Skeletal preparation and staining	III
	Preparation and staining of paraffin sections	III
	Immunohistochemical stainings	III
	In situ hybridization	III
	Transmission electron microscopy	III
	Bone mechanical testing	III
	Analysis of bone and skeleton with μCT	III
Other	Statistical analysis	I, II, III

5 Results

5.1 Role of P4H-TM in erythropoiesis (I)

5.1.1 FG-4497 is a 2-oxoglutarate analogue that inhibits both P4H-TM and HIF-P4Hs

The FG-4497 concentration required to inhibit purified recombinant human HIF-P4Hs by 50% (IC_{50}) was determined by measuring the hydroxylation-coupled release of CO_2 from 2-oxo[1-^{14}C]glutarate with a synthetic HIF-1α peptide as a substrate. IC_{50} values between 0.2–0.3 μM were obtained for all three HIF-P4Hs. The IC_{50} value for P4H-TM was determined by studying the effect of FG-4497 on the uncoupled decarboxylation of 2-oxoglutarate (decarboxylation without added substrate), since there is no synthetic substrate available for P4H-TM. The value obtained was as high as 40 μM. The IC_{50} value was next assayed for HIF-P4H-2 by measuring the effect of FG-4497 on its uncoupled decarboxylation, and the resulting value (0.2 μM) was identical to that obtained in the presence of the substrate. FG-4497 was thus shown to inhibit all three HIF-P4Hs and P4H-TM *in vitro*, although it appeared to be much less effective as a P4H-TM inhibitor.

To study the *in vivo* effect of FG-4497, increasing amounts of the compound were administered orally to wild-type mice on days 1, 3, 5 and 8, after which the mice were sacrificed. HIF-1α stabilization was detectable in the kidneys of the mice receiving repeated doses of 6 mg/kg, while stabilization of HIF-1α in the liver required a higher dose, of 60 mg/kg (Figure 1A in I). The serum Epo concentration increased 3-fold with a 20 mg/kg dose and 6-fold with 60 mg/kg (Figure 1B in I). An oral dose of 100 mg/kg stabilized both HIF-1α and HIF-2α in the kidney and liver (Figure 1A in I) and increased the serum Epo concentration to about 40 times that measured in the vehicle-treated animals (Figure 1C in I). All the subsequent experiments were performed using an oral dose of 100 mg/kg. Figure 13 depicts the cellular and systemic effects of FG-4497.

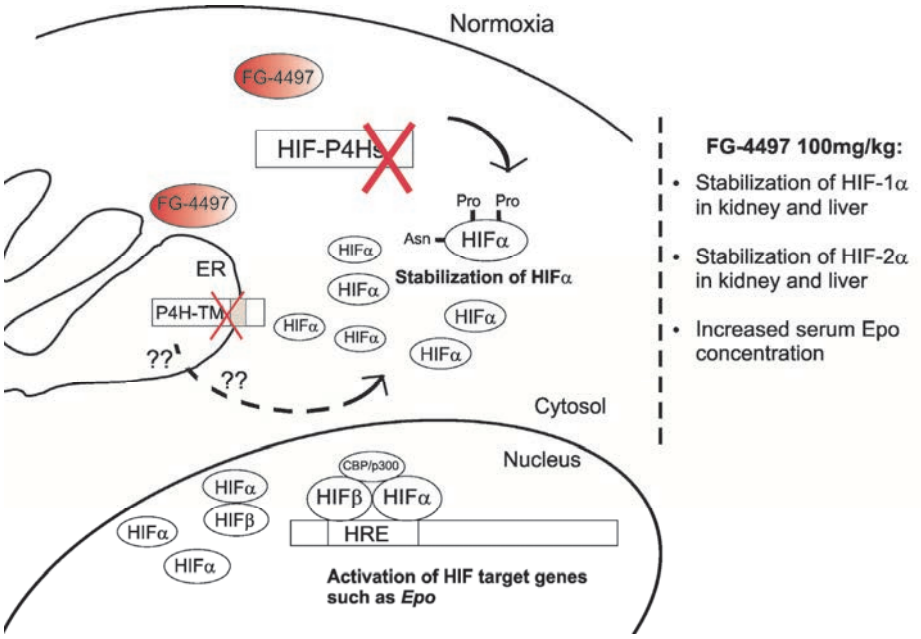

Fig. 13. 2-oxoglutarate analog FG-4497 inhibits both HIF-P4Hs and P4H-TM *in vitro*, but is much less effective P4H-TM inhibitor. Since the catalytic region of P4H-TM is inside the ER lumen the actual molecular mechanism behind the interaction of P4H-TM and HIF-α *in cellulo* is unclear. Repeated administration of FG-4497 for 8 days resulted in stabilization of both HIF-αs in the kidney and liver and in increased serum Epo concentration in mice.

5.1.2 FG-4497-induced erythropoietic effects in P4h-tm null mice

The erythropoietic effects of FG-4497 on genetically modified mice relative to wild-type mice were studied by administering the compound (FG-4497 or vehicle) once and sacrificing the mice 6 h later or three times a week (days 1, 3, and 5) for 3–5 weeks and sacrificing the mice 6 h after the last dose.

The baseline serum Epo concentration was similar in the vehicle-treated *P4h-tm*$^{-/-}$ and wild-type mice. The single-dose experiment resulted in markedly increased serum Epo in both the *P4h-tm*$^{-/-}$ and wild-type mice relative to the vehicle-treated controls, the increase being much larger in the *P4h-tm*$^{-/-}$ mice (Figure 2A in I). No stabilization of Hif-1α or Hif-2α was observed in the kidney or liver of the vehicle-treated *P4h-tm*$^{-/-}$ mice, while FG-4497 stabilized both subunits in both tissues studied. The extent of stabilization of Hif-1α and Hif-2α

in the kidney, but not in the liver, was stronger in *P4h-tm*$^{-/-}$ than in the wild-type mice (Figure 4A in I), and in line with this, no difference in *Epo* mRNA levels in the kidney or liver was seen between the vehicle-treated *P4h-tm*$^{-/-}$ and wild-type mice. FG-4497 treatment induced a rise in kidney *Epo* mRNA level in the *P4h-tm*$^{-/-}$ mice to about 2-fold relative to the wild type at 3 weeks and 4-fold at 5 weeks, but no significant differences between these mice were observed in the liver *Epo* mRNA level (Figure 4A in I). Hemoglobin or hematocrit values were similar in the vehicle-treated *P4h-tm*$^{-/-}$ and wild-type mice, whereas FG-4497 administration for 3 or 5 weeks significantly increased these values in both genotypes in a similar manner. Reticulocyte counts at 3 weeks were significantly higher in the *P4h-tm*$^{-/-}$ mice than in the wild-type mice, however (Figure 5A in I). The *Hepcidin* mRNA level in the FG-4497-treated *P4h-tm*$^{-/-}$ mice was about 40% of that in the FG-4497-treated wild-type mice, whereas no differences were detected between the vehicle-treated animals (Figure 6A in I).

Table 8. FG-4497 induced erythropoietic effects in *P4h-tm*$^{-/-}$ mice after single-dose, 3-weeks or 5-weeks treatment. The table depicts the comparison of the values between the FG-4497-treated *P4h-tm*$^{-/-}$ and similarly treated wild-type mice. The size of the arrow indicates the extent of up- or downregulation of the parameter.

Parameter	Single-dose	3-weeks	5-weeks
Serum Epo	3 x	2.5 x	2.5 x
Hif-1α kidney			↑
Hif-1α liver			No difference
Hif-2α kidney			↑
Hif-2α liver			No difference
Epo mRNA kidney		2 x	4 x
Epo mRNA liver		No difference	No difference
Hepcidin mRNA liver			↓
Hemoglobin		No difference	No difference
Hematocrit		No difference	No difference
Reticulocyte		↑	No difference

5.1.3 FG-4497-induced erythropoietic effects in Hif-p4h-2 hypomorphic and Hif-p4h-3 null mice

The corresponding erythropoietic effects induced by FG-4497 were studied in two other genetically modified mouse lines: *Hif-p4h-2* hypomorphic mice (*Hif-p4h-2*$^{gt/gt}$) and *Hif-p4h-3* null mice. The *Hif-p4h-2*$^{gt/gt}$ mice had been generated earlier

and are known to express lower amounts of wild-type *Hif-p4h-2* mRNA in various tissues (35% of that in wild-type mice in the kidney and 85% in the liver), but they have no increase in serum Epo concentration, kidney Epo mRNA level or blood hemoglobin or hematocrit values (Hyvarinen *et al.* 2010a).

There was no difference in serum Epo concentration between the vehicle-treated *Hif-p4h-2*$^{gt/gt}$, *Hif-p4h-3*$^{-/-}$ and wild-type mice, but a single dose of FG-4497 resulted in a more pronounced increase in serum Epo in the *Hif-p4h-2*$^{gt/gt}$ (Figure 2B in I) and *Hif-p4h-3*$^{-/-}$ mice (Figure 2C in I), the difference between the FG-4497 treated *Hif-p4h-2*$^{gt/gt}$ and wild-type mice being about 12-fold and that between the *Hif-p4h-3*$^{-/-}$ and wild-type mice about 5-fold. The serum Epo concentration at 3 weeks was about 4-fold in the treated *Hif-p4h-2*$^{gt/gt}$ mice relative to the treated wild-type mice (Figure 3B in I), whereas a significant increase to about 5-fold relative to the wild-type mice was seen in the *Hif-p4h-3*$^{-/-}$ mice only at 5 weeks (Figure 3C in I).

A slight baseline stabilization of Hif-1α was detected in the kidney of the *Hif-p4h-2*$^{gt/gt}$ mice, but not in the liver (Figure 4B in I). The 3-week experiment stabilized Hif-1α and Hif-2α in the kidney and liver of both the *Hif-p4h-2*$^{gt/gt}$ and wild-type mice, though to a greater extent in the former, where a slight difference was also seen in the liver (Figure 4B in I). The *Epo* mRNA level in the kidney was induced about 4.5-fold relative to the wild type, there being no significant difference between the genotypes in the liver *Epo* mRNA level (Figure 4B in I).

There was no baseline stabilization of Hif-1α or Hif-2α in the kidney or liver of the *Hif-p4h-3*$^{-/-}$ mice and no difference in the *Epo* mRNA level between the vehicle-treated *Hif-p4h-3*$^{-/-}$ and wild-type mice (Figure 4C in I). Treatment with FG-4497 stabilized both Hif-α subunits in the kidney and liver of both genotypes, the stabilization of Hif-1α being slightly stronger and that of Hif-2α distinctly stronger in the liver of the *Hif-p4h-3*$^{-/-}$ mice than in the wild type, but not in the kidney (Figure 4C in I). The 3, 4 and 5-week experiments increased Epo mRNA levels in the liver of the *Hif-p4h-3*$^{-/-}$ mice about 2.5–5-fold relative to the wild type, while no significant difference was detected in the kidney (Figure 4C in I).

No differences in haemoglobin and haematocrit values were detected between the vehicle-treated *Hif-p4h-2*$^{gt/gt}$ and wild-type (Figure 5B in I) or *Hif-p4h-3*$^{-/-}$ and wild-type mice (Figure 5C in I), but administration of FG-4497 for 3 weeks increased these values in the *Hif-p4h-2*$^{gt/gt}$ mice significantly more than in the wild-type mice. In the *Hif-p4h-3*$^{-/-}$ mice a statistically significant increase relative to the wild type was seen only in the hematocrit value at 5 weeks and in the reticulocyte counts at 4 weeks, but not at 3 or 5 weeks (Figure 5C in I).

FG-4497 administration lowered *Hepcidin* mRNA in the wild-type mice by about 60–65%, and the magnitude of the decrease was even larger in the treated *Hif-p4h-2*$^{gt/gt}$ and *Hif-p4h-3*$^{-/-}$ mice, the values being 30% and 60% of those in the FG-4497 treated wild-type mice, respectively (Figure 6B-C in I). The difference between the *Hif-p4h-3*$^{-/-}$ and wild-type mice was not statistically significant, however (Figure 6C in I).

Table 9. FG-4497 induced erythropoietic effects in *Hif-p4h-2*$^{gt/gt}$ and *Hif-p4h-3*$^{-/-}$ mice after a single-dose treatment or after repeated doses for 3, 4 and 5 weeks. Comparisons between the FG-4497 treated gene-modified and wild-type mice are shown. The size of the arrow indicates the extent of up- or downregulation of the parameter.

Parameter	Single dose		3-weeks		4-weeks	5-weeks
	Hif-p4h-2$^{gt/gt}$	*Hif-p4h-3*$^{-/-}$	*Hif-p4h-2*$^{gt/gt}$	*Hif-p4h-3*$^{-/-}$	*Hif-p4h-3*$^{-/-}$	*Hif-p4h-3*$^{-/-}$
Serum Epo	12 x	5 x	4 x	No difference	No difference	5 x
Hif-1α kidney			↑	No difference		
Hif-1α liver			↑	↑		
Hif-2α kidney			↑	No difference		
Hif-2α liver			↑	↑		
Epo mRNA kidney			4.5 x	No difference	No difference	No difference
Epo mRNA liver			No difference	2.5 x	4-5 x	4-5 x
Hepcidin mRNA liver			↓			No significant difference
Hemoglobin			↑	No difference	No difference	No difference
Hematocrit			↑	No difference	No difference	↑
Reticulocyte			↑	No difference	↑	No difference

5.1.4 Signs of increased erythropoiesis in the *Hif-p4h-2$^{gt/gt}$/P4h-tm$^{-/-}$* mice

In order to further clarify the putative role of P4H-TM in erythropoiesis, a total of 6 double gene-modified *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice were generated. *Hif-p4h-2$^{+/+}$/P4h-tm$^{+/+}$*, *Hif-p4h-2$^{+/+}$/P4h-tm$^{+/-}$*, *Hif-p4h-2$^{+/gt}$/P4h-tm$^{+/+}$* and *Hif-p4h-2$^{+/gt}$/P4h-tm$^{+/-}$* mice, numbering 21 altogether, were used as littermate controls, since the crossings by which the double gene-modified *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice were obtained produced only a few female wild-type mice. A third group consisted of 6 *Hif-p4h$^{+/gt}$/P4h-tm$^{-/-}$* mice. As demonstrated earlier in this work, there was no difference in erythropoiesis between the *Hif-p4h-2$^{gt/gt}$*, *P4h-tm$^{-/-}$* and wild-type mice without FG-4497 treatment, although hemoglobin concentrations were significantly increased in the *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice (mean 159.0 g/L, range 149–180 g/L) and slightly increased in the *Hif-p4h$^{+/gt}$/P4h-tm$^{-/-}$* mice (mean 148 g/L, range 136–161 g/L) relative to the control group (mean 142.9 g/L, range 131–151) (Figure 7A in I). Similar differences between the three groups were also observed in the hematocrit values (Figure 7B in I).

Unexpectedly, the serum Epo concentration was lower in the *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice than in the control group (Figure 7C in I), the lowest concentration being detected in the *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mouse with the highest hemoglobin value. The *Epo* mRNA level was not elevated in the kidneys of the *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice (Figure 7D in I), but that in the liver was very low and there was no increase in the *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice. Although both bone and brain have recently been shown to contribute to systemic Epo and increase hemoglobin values (Rankin *et al.* 2012, Weidemann *et al.* 2009), we did not find *Epo* mRNA to be expressed at detectable levels in the bone of either the control or *Hif-p4h2$^{gt/gt}$/P4h-tm$^{-/-}$* mice, nor were there any differences in *Epo* mRNA levels in the brain between these and the controls.

Table 10. Comparison of parameters that describe the extent of erythropoiesis in *Hif-p4h-2$^{+/gt}$/P4h-tm$^{-/-}$* and *Hif-p4h-2$^{gt/gt}$/P4h-tm$^{-/-}$* mice relative to littermate controls (no FG-4497 administration). Size of the arrow indicates the extent of increase/decrease of the parameter in the mutants compared to control.

Parameter	Hif-p4h-2$^{+/gt}$/P4h-tm$^{-/-}$	Hif-p4h-2$^{gt/gt}$/P4h-tm$^{-/-}$
Hemoglobin	↑	↑
Hematocrit	↑	↑
Serum Epo	No difference	↓
Epo mRNA kidney	No difference	No difference
Epo mRNA liver	No difference	No difference

5.2 Regulation of C-P4H-I and C-P4H-II in chondrocytes by the hypoxia response pathway (II)

5.2.1 Hypoxia induces the expression of P4ha1 and P4ha2 mRNAs

In order to study the effect of HIF-1, HIF-2 and VHL on the expression of C-P4Hs in chondrocytes, we used monolayer cultures of primary chondrocytes isolated from the epiphyseal growth plates of newborn *Vhl*$^{f/f}$, *Hif-1a*$^{f/f}$ and *Hif-2a*$^{f/f}$ mice and exposed them to 1% O_2 (hypoxia) on day 10 or cultured them further in 21% O_2 (normoxia). RT-PCR analysis of the normoxic chondrocytes demonstrated the mRNA expression of *Col2a1* and *Aggrecan* and thus verified the phenotypic identity of the cells (Figure 1 in II). The normoxic chondrocytes were also shown to express detectable mRNA levels of *Glut1* and *Vegfa*, which are two well-characterized HIF target genes, and *P4ha1* and *P4ha2*, encoding the catalytic α(I) and α(II) subunits of C-P4H isoenzymes I and II, respectively (Figure 1 in II). Acute hypoxia resulted in about a 3–3.5-fold increase in *P4ha1* mRNA and a 2.5–5.5 increase in *P4ha2* mRNA in *Hif-1a*$^{f/f}$ and *Hif-2a*$^{f/f}$ chondrocytes infected with the control adenovirus encoding β-galactosidase (Figure 2A and B in II). The hypoxia-induced increase in *Vegfa* and *Glut1* mRNA was about 10–25-fold, while no increase was detected in the case of *Col2a1* mRNA (Figure 2A and B).

To address whether a lack of VHL with subsequent stabilization and activation of HIF-1 and HIF-2 mimics the effects of hypoxia in chondrocytes, primary chondrocytes isolated from newborn epiphyseal growth plates of *Vhl*$^{f/f}$ mice were cultured and transduced with adenoviruses producing either β-galactosidase or Cre recombinase in order to generate control and *Vhl* knockdown chondrocytes, respectively. A lack of *Vhl* upregulated both *P4ha1* and *P4ha2* mRNA levels about 2–3-fold, and a lack of the *Vegfa* and *Glut1* mRNAs about 10 and 3-fold, respectively, thus mimicking the effects of hypoxia (Figure 2C in II). The results indicate that the hypoxia-induced increase in *P4ha1* and *P4ha2* mRNA levels is likely to be dependent on HIF-1α or HIF-2α, or both.

5.2.2 Hypoxia-induced expression of P4ha1 and P4ha2 mRNAs is HIF-1α-dependent

The next step was to study whether the hypoxia inducibility of the *P4ha1* and *P4ha2* mRNAs is dependent on HIF-1α or HIF-2α. For this purpose, primary chondrocytes isolated from growth plates of newborn *Hif-1a*$^{f/f}$ and *Hif-2a*$^{f/f}$ mice were cultured in a monolayer and transduced with adenoviruses producing either β-galactosidase (Bgal) or Cre recombinase. The cells were exposed to hypoxia for 8 h on day 10 or maintained under normoxic conditions. Knockdown of *Hif-1a* completely abolished the hypoxia-induced upregulation of *P4ha1* and *P4ha2* mRNAs (Figure 2A in II), while a lack of *Hif-2a* had no significant effect (Figure 2B in II). The result indicates that the hypoxia-induced increase in *P4ha1* and *P4ha2* mRNAs in newborn mouse growth plate chondrocytes is exclusively HIF-1α-dependent.

In order to confirm the efficacy of HIF-1α and HIF-2α inactivation, the expression of known HIF target genes such as *Vegfa*, *Glut1* and *Epo* was analysed. The hypoxia-induced expression of *Vegfa* and *Glut1* in hypoxic *Hif-1a* knockdown chondrocytes was significantly downregulated relative to controls (Figure 2A in II), and in agreement with this, exposure to hypoxia for 24 h resulted in a 2.6-fold increase in Vegf protein accumulation in the cell culture medium of the *Hif-1a*$^{f/f}$ growth plate chondrocytes transduced with Bgal as compared with normoxic cells, while the increase was only about 1.9-fold in the cells lacking HIF-1α. Inactivation of HIF-2α in the chondrocytes had no significant effect on the hypoxia-induced increase in *Glut1* or *Vegfa* mRNA (Figure 2B in II). By contrast, inactivation of HIF-2α damped the hypoxic induction of *Epo* mRNA (Figure 2B in II). The lack of either HIF-1α or HIF-2α had no effect on *Col2a1* mRNA expression in hypoxia (Figure 2A and B in II).

5.2.3 Hypoxia increases the accumulation of C-P4H-I and C-P4H-II tetramers, which is further reflected in C-P4H activity

The assembly of C-P4H tetramers and C-P4H activity were next analysed in the above growth plate chondrocytes (*Hif-1a*$^{f/f}$, *Hif-2a*$^{f/f}$, *Vhl*$^{f/f}$ transduced with adenoviruses as described above and then exposed to hypoxia or kept in normoxia for the time indicated) to determine whether the hypoxia-related changes in the expression of *P4ha1* and *P4ha2* mRNAs are reflected in similar changes at the protein level.

In order to study the formation of C-P4H tetramers we used novel polyclonal antibodies generated against recombinant peptide substrate binding domains of the human C-P4H α(I) and α(II) subunits (Myllyharju & Kivirikko 1999). The isoenzyme specificity and sensitivity of the C-P4H antibodies were analysed from recombinant insect cell samples and the optimal antibody dilutions were assessed (Figure 4A and B in II). Amounts of C-P4H-I and C-P4H-II in the control growth plate chondrocytes were found to be increased relative to normoxia in all three cell types after 8 or 24 h of exposure to hypoxia (Figure 5A-C in II). Knockdown of HIF-1α completely abolished the hypoxia-induced accumulation of C-P4H-I and C-P4H-II, however, while knockdown of HIF-2α had no effect (Figure 5B in II). Knockdown of VHL resulted in a robust accumulation of C-P4H-I and C-P4H-II even in normoxia, and exposure to hypoxia did not lead to any further increase (Figure 5C in II).

The quantity of active C-P4H tetramers assembled in the *Hif-1a*$^{f/f}$, *Hif-2a*$^{f/f}$, and *Vhl*$^{f/f}$ growth plate chondrocytes was analysed by a method based on the formation of 4-hydroxy [^{14}C]proline in a [^{14}C]proline-labelled substrate consisting of non-hydroxylated procollagen polypeptide chains (Kivirikko and Myllylä 1982). The amount of C-P4H activity had increased approximately 3-fold in the hypoxic control chondrocytes (Figure 6 in II). Inactivation of HIF-1α significantly reduced this increase, while inactivation of HIF-2α had no effect and inactivation of VHL resulted in an increased amount of C-P4H activity even in normoxia (Figure 6 in II).

We then set out to study whether the observed hypoxia-induced increase in C-P4H tetramers is of biological relevance under hypoxic conditions, by assessing the activity of a recombinant human C-P4H-I in 1% O_2. The amount of C-P4H activity generated in hypoxia was about 15% of that generated by the same quantity of the enzyme in normoxia (Figure 7 in II), and a 2–6 fold increase in the amount of C-P4H tetramer increased the C-P4H activity generated in hypoxia to about 20–50% of that in normoxia (Figure 7 in II). Thus the decreased specific activity of C-P4H in hypoxia coud be said to have been partially compensated for by the hypoxia-induced increase in the quantities of the C-P4H tetramers.

Figure 14 depicts the major findings obtained in the article II.

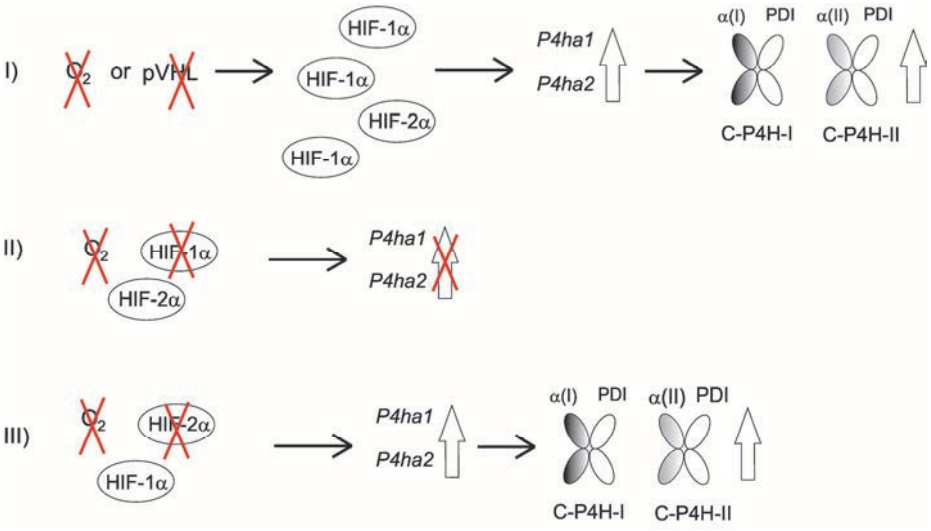

Fig. 14. Summary of hypoxia-dependent regulation of C-P4H-I and C-P4H-II in mouse primary epiphyseal growth plate chondrocytes. I) Hypoxia and/or inactivation of *Vhl* encoding for pVHL result in stabilization of HIF-1α and HIF-2α subunits with subsequent increase in *P4ha1* and *P4ha2* mRNA level and the amount of corresponding C-P4H-I and C-P4H-II enzyme tetramers as well as their activity. II) Knockdown of HIF-1α abolished the hypoxia induced upregulation of *P4ha1* and *P4ha2* mRNAs and accumulation of C-P4H enzyme tetramers, while III) knockdown of HIF-2α had no effect on hypoxia dependent upregulation of C-P4Hs.

5.3 Characterization of mouse lines with complete inactivation of C-P4H-II with or without partial inactivation of C-P4H-I (III)

5.3.1 P4ha1$^{+/-}$;P4ha2$^{-/-}$ mice are smaller than their littermates, while P4ha2$^{-/-}$ mice have no obvious phenotypic alterations

Although homozygous inactivation of the *P4ha1* gene coding for the C-P4H-I α(I) subunit has been reported earlier to result in embryonic lethality between E10.5 and E11.5 due to abnormal assembly of collagen IV and disruption of the BMs, heterozygous *P4ha1*$^{+/-}$ mice have been shown to be viable, fertile and born in the expected Mendelian ratios (Holster *et al.* 2007). In order to study the *in vivo* roles of C-P4H-II, a mouse line with targeted disruption of the *P4ha2* gene coding for the C-P4H-II α(II) subunit was generated. The homozygous *P4ha2*$^{-/-}$ mice were

viable, fertile and born in normal Mendelian ratios. To assess the combined *in vivo* consequences of partial inactivation of C-P4H-I with complete inactivation of C-P4H-II, mice that were heterozygous for the mutant *P4ha1* allele and homozygous for the mutant *P4ha2* allele (*P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice) were generated. Genotyping of the pups obtained from *P4ha1*$^{+/-}$*;P4h2*$^{+/-}$ x *P4ha2*$^{-/-}$ matings indicated that a smaller number of *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ pups were born (12%) than would be expected for normal Mendelian inheritance (25%) (Table 1 in III).

Neither the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ nor the *P4ha2*$^{-/-}$ mice had any overt skeletal phenotype at birth (Figure 3A in III), but the adult *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice appeared to be of a smaller size than their littermates. The growth curve (from 5 to 20 weeks) demonstrated a significantly lower body weight for the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice than for the *P4ha2*$^{-/-}$ mice or the control littermates (Figure 3B in III), the difference persisting throughout their life. The smaller size of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice was characterized by a moderate shortening of their long bones as measured by microcomputed tomography (μCT) (Table 2 in III). Both the femurs and tibia were significantly shorter in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice at 8 weeks of age, whereas no differences were observed between the *P4ha2*$^{-/-}$ and control mice (Table 2 in III). Moreover, the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$, but not the *P4ha2*$^{-/-}$ mice or their control littermates, developed kyphosis that was clearly detectable at the age of 48 weeks (Figure 3C in III).

5.3.2 The growth plate phenotype of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ mice

Because of the observed moderate dwarfism of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice and since C-P4H-II is known to have its highest expression level in chondrocytes (Annunen *et al.* 1997, Annunen *et al.* 1998b), the histology of the limb buds and cartilaginous tibial epiphyseal growth plates of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ mice was analysed and compared with of the situation in their double heterozygous littermates (*P4ha1*$^{+/-}$*;P4ha2*$^{+/-}$). Histological staining demonstrated normal mesenchymal condensations in the limb buds isolated from the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$, *P4ha2*$^{-/-}$ and control *P4ha1*$^{+/-}$*;P4ha2*$^{+/-}$ mice at E13.5 (Figure 4A in III), but during the later stages of development some abnormalities were observed in the tibial epiphyseal cartilage of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice: hypocellular areas in the middle of the proliferative zone and a loss of the columnar organization of the proliferative chondrocytes (Figure 4B in III). The growth plate phenotype was

detectable from the embryonic date E17.5 onwards, being most obvious in the newborn *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice and having resolved by the age of 3 weeks (Figure 4B in III).

No differences in the expression of markers of chondrocyte differentiation or maturation were detected between the genotypes (*P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$, *P4ha2*$^{-/-}$ and *P4ha1*$^{+/-}$*;P4ha2*$^{+/-}$) as studied by qPCR using mRNA isolated from crude growth plate samples or isolated primary chondrocytes as a template, or by *in situ* hybridization of histological growth plate sections (Figure 4C and Supplementary Figure 1 in III). Moreover, there was no significant difference in the proliferation rate of round proliferative chondrocytes in the developing mutant growth plates (*P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$) relative to the controls (*P4ha1*$^{+/-}$*;P4ha2*$^{+/-}$) when analysed by means of a 5-bromo-2'deoxyuridine incorporation assay on histological sections of the proximal epiphysis of the tibia at E18.5 (Figure 5A in III). On the other hand, the proliferation rate of the round proliferative chondrocytes was significantly higher than that of the columnar proliferative chondrocytes in the control specimens, but not in the mutant samples (Figure 5A in III). TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick and labelling) assay revealed apoptotic chondrocytes in the central region of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ newborn growth plate, although the number of TUNEL-positive apoptotic cells was low (Figure 5B in III).

We then set out to study whether uncompensated ER stress with an unfolded protein response (UPR) was triggered in either the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ or *P4ha2*$^{-/-}$ mutant chondrocytes, by investigating the relative levels of expression of the classical UPR markers BiP and CHOP with qPCR in crude growth plate samples isolated from newborn mice. No induction of either marker was detected in the mutant samples as compared with the controls (Supplementary Figure 2 in III). Primary mouse epiphyseal growth plate chondrocytes cultured under hypoxic conditions showed upregulation of CHOP mRNA, which was not significantly exacerbated by the reduced C-P4H activity in chondrocytes (Supplementary Figure 2 in III). Careful electron microscopic analyses of the *P4ha1*$^{+/+}$*;P4ha2*$^{+/-}$ control and *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ proliferative growth plate chondrocytes revealed a well-organized ER in both genotypes and no signs of trapped intracellular procollagen (Supplementary Figure 3 in III).

5.3.3 Quality of growth plate collagen and bone mechanical properties in the mutant mice

To study whether the quality of the collagen in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ growth plate and tibia was impaired, amino acid analyses of the tissues were carried out. The degree of proline hydroxylation, i.e. the ratio 4Hyp/(4Hyp+Pro), was significantly reduced in both the growth plate and tibial tissues of the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ mutants relative to controls (Table 3 in III). Consequently, we expected to observe a reduction in the thermal stability of collagen in these mice, and it was indeed the case that the T_m (midpoint of the thermal transition from a helix to a coil) of the growth plate collagen (mainly type II collagen) was reduced in both the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ samples relative to the controls, the reductions being about 2°C and 1°C, respectively (Figure 6A in III).

A more detailed bone analysis was carried out using μCT scans of the diaphysis and proximal part of the tibia in 12-week-old sex-matched *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ mice and their control littermates, but no differences in cortical bone mineral density were detected between the genotypes, indicating normal mineralization in all cases (Table 4 in III). Cross-sections of the tibial diaphysis showed significant decreases in cross-sectional bone area, medullary area and cortical thickness in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mutant samples, but not in *P4ha2*$^{-/-}$ (Table 4), and a reduction in the mean polar moment of inertia was also detected in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ tibiae but not in their *P4ha2*$^{-/-}$ counterparts, indicating reduced bone strength in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ tibiae (Table 4 in III).

Analysis of the three-dimensional bone microstructure showed a substantial and significant loss of trabecular bone in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice by comparison with their control littermates (Table 4 in III), while a slight but significant reduction in the trabecular bone volume fraction was present in the tibiae of the *P4ha2*$^{-/-}$ mutants (Table 4 in III). Moreover, the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mutants showed significant changes in trabecular number and trabecular separation, whereas no differences in trabecular thickness were detected between the genotypes (Table 4 in III). A moderate but significant reduction in trabecular number was also noted in the *P4ha2*$^{-/-}$ tibia (Table 4 in III).

A three-point bending assay performed on the tibial diaphysis, femoral diaphysis and femoral neck of age and sex-matched animals indicated altered mechanical properties of the bone at all these anatomical sites in the *P4ha1*$^{+/-}$*;P4ha2*$^{-/-}$ mice relative to their control littermates (Table 5 in III), while the

P4ha2$^{-/-}$ mice showed similar changes in the case of the femoral diaphysis but not in the tibial diaphysis or femoral neck (Table 5 in III). Table 11 summarises the phenotypic outcomes of the C-P4H knock-out mice as described in the previous report (Holster *et al.* 2007) and the present study.

Table 11. Summary of the major phenotypic features of C-P4H mutant mice.

Genotype	Phenotype
P4ha1$^{-/-}$	Embryonic lethality between E10.5 and E11.5[1]
	Disruption on BMs due to abnormal assembly of Col IV[1]
P4ha1$^{+/-}$	Viable and fertile[1]
P4ha2$^{-/-}$	Viable and fertile
	Slightly reduced 4Hyp content and reduced thermal stability of collagen in growth plate cartilage
	Moderate impairment in mechanical properties of long bones
P4ha1$^{+/-}$*;P4ha2*$^{-/-}$	Smaller body size with shortening of long bones
	Kyphosis by the age of 48 weeks
	Transient hypocellularity in developing tibial growth plates
	Reduced 4Hyp content and reduced thermal stability of collagen in growth plate cartilage
	Impaired mechanical properties of long bones

[1]Holster *et al.* 2007

6 Discussion

6.1 P4H-TM is involved in the regulation of mammalian Epo synthesis and erythropoiesis

The actions of P4H-TM resemble closely those of the HIF-P4Hs, as it has been shown to hydroxylate HIF-α *in vitro* and *in cellulo*, but its subcellular localization is distinctly different, as it resides in the ER membranes in an orientation in which the catalytic site is inside the lumen while the HIF-P4Hs are found in the cytosol and nucleus (Koivunen *et al.* 2007). It has been suggested that P4H-TM may have additional physiological substrates other than HIF in the ER lumen and that it may play a more important role in the hydroxylation of those other putative compounds (Hyvarinen *et al.* 2010b, Koivunen *et al.* 2007). Zebrafish deficient in *p4h-tm* showed dysfunction of the pronephric kidney, manifested in pericardial oedema, and increased expression levels of several HIF target genes, including the *epo* gene, even though no signs of increased erythropoiesis were detected (Hyvarinen *et al.* 2010b).

It is well established that all three HIF-P4Hs play a role in the regulation of mammalian Epo synthesis and erythropoiesis. Mice with broad-spectrum inactivation of *Hif-p4h-2* showed polycythaemia and large increases in erythropoiesis due to the induction of renal but not hepatic Epo production (Minamishima *et al.* 2008, Minamishima & Kaelin 2010, Takeda *et al.* 2007, Takeda *et al.* 2008). The hypomorphic *Hif-p4h-2* mice (*Hif-p4h-2*$^{gt/gt}$) express varying amounts of wild-type *Hif-p4h-2* mRNA in certain tissues, approximately 35% of that in wild-type mice in the kidney and 85% in the liver (Hyvarinen *et al.* 2010a). No signs of elevated erythropoiesis were detected in these hypomorphic mice, however (Hyvarinen *et al.* 2010a). Individual knockout of either *Hif-p4h-1* or *Hif-p4h-3* did not have any effect on the serum Epo concentration or on blood hemoglobin or hematocrit values (Minamishima & Kaelin 2010, Takeda *et al.* 2008) but the combined inactivation of *Hif-p4h-1* and *Hif-p4h-3* caused moderate polycythemia probably due to induction of the expression of hepatic Epo (Takeda *et al.* 2008). Furthermore, *Hif-p4h-1*$^{-/-}$/*Hif-p4h-2*$^{-/-}$ mice with liver-specific knockout of *Hif-p4h-2* displayed slight increases in serum Epo concentration and erythropoiesis even though hepatic inactivation of *Hif-p4h-2* alone did not have any effect on serum Epo level (Minamishima & Kaelin 2010). Inactivation of all three HIF-P4Hs appears to be required in order to induce dramatic Epo

production in the liver with subsequent massive erythrocytosis (Minamishima & Kaelin 2010). No data have been available for assessing the role of P4H-TM in mammalian erythropoiesis, however.

During embryogenesis liver is the main source of Epo but after birth the kidney assumes this role and hepatic Epo production is largely silenced (Haase 2010). Anemia resulting from renal failure is a major medical problem, and anemia also accompanies many other disease states such as cancer and inflammation (Haase 2010, Muchnik & Kaplan 2011, Myllyharju 2009). The current therapy for stimulating erythropoiesis in such conditions consists of subcutaneous injections of recombinant human Epo, but this also encounters many problems. It is costly and has been associated with increased mortality from cardiovascular complications arising at high hemoglobin levels and with accelerated tumor growth in patients with neoplasias (Haase 2010, Macdougall 2012, Muchnik & Kaplan 2011, Myllyharju 2009). Moreover, approximately 10% of all hemodialysis patients with end-stage renal disease display Epo resistance (Macdougall 2012). Functional iron deficiency accompanying anemia in cases of chronic disease causes resistance to exogenous Epo that cannot be remedied by the administration of intravenous iron (Muchnik & Kaplan 2011). Chemical compounds that stabilize HIF by inhibiting the HIF-P4Hs with respect to their cosubstrate 2-oxoglutarate have been shown recently to provide a potential novel oral therapy for stimulating erythropoiesis in patients with kidney disease (Muchnik & Kaplan 2011, Myllyharju 2009). Of importance in this respect is the fact that HIF stabilization was able to increase erythropoiesis in an inflammation-induced anemia model even though exogenous Epo was ineffective (Muchnik & Kaplan 2011). Moreover, FG-2219, which is a 2-oxoglutarate analogue that inhibits all three HIF-P4Hs, has been shown to increase Epo production in anephric patients as strongly as in healthy subjects, suggesting that it causes effective Epo production in the liver (Muchnik & Kaplan 2011, Myllyharju 2009).

Certain differences in catalytic and inhibitory properties do exist among the three HIF-P4Hs (Hirsila *et al.* 2003, Hirsila *et al.* 2005) and the outcomes of the knockout of individual enzymes are distinctly different (Minamishima *et al.* 2008, Minamishima & Kaelin 2010, Takeda *et al.* 2006, Takeda & Fong 2007, Takeda *et al.* 2008). Furthermore, it has been suggested that the HIF-P4Hs hydroxylate enzyme-specific substrates other than HIF (Cummins *et al.* 2006, Fu *et al.* 2007, Koditz *et al.* 2007). These considerations indicate that it may be possible to

generate pharmaceutical compounds that differentially inhibit individual HIF-P4H isoenzymes in different therapeutic settings (Myllyharju 2009).

In the present study *P4h-tm*$^{-/-}$ mice treated with the 2-oxoglutarate analogue and HIF-P4H inhibitor FG-4497 showed larger increases in serum Epo concentration than did wild-type mice treated in the same way. Moreover, HIF-1 and HIF-2 were stabilized more strongly in the kidneys of the FG-4497-treated *P4h-tm*$^{-/-}$ mice and their *Epo* mRNA level was increased relative to the situation in the treated wild-type controls, whereas no such differences were detected in the livers of either the *P4h-tm*$^{-/-}$ or wild-type mice. On the other hand, the level of liver *Hepcidin* mRNA in the FG-4497-treated *P4h-tm*$^{-/-}$ mice was less than one-half of that in the treated wild-type mice. Administration of FG-4497 to *Hif-p4h-2*$^{gt/gt}$ and *Hif-p4h-3*$^{-/-}$ mice caused similar differences between the treated gene-modified mice and their wild-type controls, but there was also a slight further increase in the HIF-1α and HIF-2α protein levels in the livers of the FG-4497-treated *Hif-p4h-2*$^{gt/gt}$ mice. Furthermore, in the case of the FG-4497-treated *Hif-p4h-3*$^{-/-}$ mice the HIF-1α and HIF-2α stabilization and increased *Epo* mRNA level was seen in the liver rather than in the kidney. In general, the changes detected in the *Hif-p4h-2*$^{gt/gt}$ mice were substantially greater in magnitude than those in the *P4h-tm*$^{-/-}$ mice. In the case of the *Hif-p4h-3*$^{-/-}$ mice the magnitudes of the changes were slightly greater than those seen in the *P4h-tm*$^{-/-}$ mice, but the decrease in the *Hepcidin* mRNA level in the liver was no larger than in the latter.

No increase in the hemoglobin or hematocrit values was found in the FG-4497-treated *P4h-tm*$^{-/-}$ mice relative to treated wild-type mice at 3 or 5 weeks, although the serum Epo concentration and reticulocyte counts showed significant elevation in the former. FG-4497-treated *Hif-p4h-3*$^{-/-}$ mice showed a 5-fold increase in their serum Epo at 5 weeks and a 1.4-fold increase in their reticulocyte counts at 4 weeks by comparison with the treated wild-type controls, but there was no significant further increase in their hemoglobin or hematocrit values at 3 or 4 weeks, although a significant increase in the hematocrit value was detected at 5 weeks. In the case of the *Hif-p4h-2*$^{gt/gt}$ mice, FG-4497 treatment for only 3 weeks caused a 4-fold increase in the hemoglobin and hematocrit values relative to the treated wild type.

The results obtained here when assessing the erythropoietic effects induced in *Hif-p4h-2*$^{gt/gt}$ and *Hif-p4h-3*$^{-/-}$ mice by the HIF-P4H inhibitor FG-4497 agree with previous data obtained by others. Firstly, our findings support the earlier-established primary role of HIF-P4H-2 in the regulation of erythropoiesis with

respect to the other two isoenzymes (Minamishima *et al.* 2008, Minamishima & Kaelin 2010, Takeda *et al.* 2006, Takeda *et al.* 2008). Secondly, it was established that HIF-P4H-2 controls Epo regulation in the kidney but not in the liver (Minamishima *et al.* 2008, Minamishima & Kaelin 2010) and thirdly, *Hif-p4h-1*$^{-/-}$ */Hif-p4h-3*$^{-/-}$ double gene-modified mice had increased HIF-2α protein and *Epo* mRNA levels only in the liver (Takeda *et al.* 2008). Most importantly, the present results show for the first time that P4H-TM also has a role in mammalian erythropoiesis, particularly in regulation of the renal Epo pathway.

Analysis of a *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ double gene-modified mouse line without FG-4497 administration provided data that further supported the suggestion of P4H-TM as a fourth P4H involved in the regulation of erythropoiesis. Neither *Hif-p4h-2*$^{gt/gt}$ nor *P4h-tm* single mutant mice showed any signs of increased erythropoiesis, whereas the double mutant mice had significantly increased hemoglobin and hematocrit values. Surprisingly, the serum Epo concentration was significantly lower in the *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ mice relative to the control group, although a similar situation has been observed earlier in the case of *Hif-p4h-1*$^{-/-}$*/Hif-p4h-3*$^{-/-}$ mice, in which the increased hemoglobin values were accompanied by decreased serum Epo concentration (Takeda *et al.* 2008). It is also possible that we have directly affected the erythroid lineage in these mice and thus they do not display increased serum Epo. Moreover, almost all patients with HIF-P4H-2-associated erythrocytosis have normal or even slightly decreased serum Epo levels (Lee & Percy 2011). The *Epo* mRNA level was not increased in the kidneys of the *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ mice, even though *Epo* mRNA levels were found to be elevated in the kidneys of the *Hif-p4h-2*$^{gt/gt}$ and *P4h-tm*$^{-/-}$ mice following FG-4497 administration. As the increases in hemoglobin and hematocrit values in the double gene-modified mice were small, it is possible that very small increases in the *Epo* mRNA levels could not be detected due to the wide variation in individual values. Furthermore, the increased erythropoiesis in the *Hif-p4h-2*$^{gt/gt}$*/P4htm*$^{-/-}$ mice persisted over a prolonged period of time, while the more dramatic changes seen in the FG-4497-treated mice occurred during a short period of time. Interestingly, it has been demonstrated recently that extrarenal sites such as bone and brain can also produce Epo in sufficient amounts to increase hemoglobin values (Rankin *et al.* 2012, Weidemann *et al.* 2009). In the present study, however, Epo mRNA was not expressed at detectable levels in the bones of either the *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ or

control mice. In the case of the brain no increase in *Epo* mRNA levels was observed in the *Hif-p4h-2*$^{gt/gt}$*/P4htm*$^{-/-}$ mice relative to the controls.

Stabilization of HIF by means of HIF-P4H inhibitors may be of great therapeutic value in the future in a number of pathological states accompanied by hypoxic conditions such as anemia. In the light of previous findings and the present results it seems possible to generate inhibitors that differentially target individual HIF-P4Hs and P4H-TM (Minamishima *et al.* 2008, Minamishima & Kaelin 2010, Myllyharju 2008, Myllyharju 2009, Takeda *et al.* 2007, Takeda *et al.* 2008). As HIF-P4H-2 and P4H-TM are involved in the regulation of Epo production in the kidney but not in the liver, a compound inhibiting several HIF-P4Hs could be more desirable for patients with severe kidney disease (Minamishima *et al.* 2008, Minamishima & Kaelin 2010, Takeda *et al.* 2007, Takeda *et al.* 2008). Based on the present work, a compound inhibiting P4H-TM together with some of the HIF-P4H isoenzymes could produce a more efficient erythropoietic response than that obtained with a HIF-P4H antagonist lacking P4H-TM inhibition. Moreover, this work further demonstrated that P4H-TM inhibition reduces the hepcidin level in a similar manner to inhibition of the HIF-P4Hs. Inhibition of P4H-TM together with the HIF-P4Hs is thus likely to be more beneficial for patients suffering from anemia that is characterized by chronic inflammation and iron deficiency.

6.2 HIF-1 is necessary for the hypoxic induction of C-P4Hs in primary mouse epiphyseal growth plate chondrocytes

Proper accumulation of ECM is crucial for normal organ development, as it provides the tissue with mechanical support and promotes cell differentiation and survival through specific cell-matrix interactions. The ECM of the epiphyseal growth plate, being mostly composed of aggrecan and collagens (especially types II, IX, XI, and X), is quantitatively and functionally a particularly important part of the tissue (Myllyharju & Schipani 2010, Schipani 2010). C-P4Hs are necessary for collagen biosynthesis as they catalyze the formation of 4-hydroxyprolines, which are essential for the thermal stability of all collagen molecules. The vertebrate C-P4Hs are $\alpha_2\beta_2$ tetramers, in which the catalytically active α subunit defines the enzyme functionally. Three isoforms of the α subunit have been identified and shown to form $[\alpha(I)]_2\beta_2$, $[\alpha(II)]_2\beta_2$, and $[\alpha(III)]_2\beta_2$ tetramers with the β subunit (PDI), corresponding to C-P4Hs of types I, II and III, respectively (Myllyharju 2003, Myllyharju 2008).

Low oxygen content upregulates several genes involved in ECM homeostasis including those coding for C-P4Hs, lysyl hydroxylases, lysyl oxidases, plasminogen activator inhibitor-I, and metalloproteinase-1 (Denko *et al.* 2003, Denko *et al.* 2003, Elvidge *et al.* 2006, Hofbauer *et al.* 2003, Kietzmann *et al.* 1999, Koong *et al.* 2000, Wang *et al.* 2005, Wang *et al.* 2005). In human articular chondrocyte cultures transcript levels of *P4HA1* and *P4HA2* genes coding for C-P4H α(I) and α(II) subunits were increased approximately 2-fold and 2.3-fold, respectively (Grimmer *et al.* 2006). Corresponding increases have typically been reported to be between 2 to 8-fold depending on the cell type, but increases as high as 30-fold have been detected in the case of a mouse juxtraglomerular cell line, for example (Elvidge *et al.* 2006, Fahling *et al.* 2006, Grimmer *et al.* 2006, Hofbauer *et al.* 2003, Tajima *et al.* 2001).

Development of long bones occurs through endochondral ossification, in which the cartilaginous growth plate template is first laid down and subsequently replaced by bone. The epiphyseal growth plate is hypoxic for most of its length, and HIF-1 has emerged as a crucial factor not only for survival of the chondrocytes but also for their differentiation and proliferation (Provot & Schipani 2007). Previous *in vivo* experiments with genetically modified mice demonstrated that a lack of HIF-1 in growth plate chondrocytes results in dramatic shortening of the limbs (Schipani *et al.* 2001) the phenotype being characterized by massive cell death at the centre of the developing growth plate due to altered chondrocyte differentiation (Provot & Schipani 2007, Provot *et al.* 2007, Schipani *et al.* 2001). Moreover, the importance of HIF-1α has been highlighted in the limb bud mesenchyme and developing joint (Provot *et al.* 2007). HIF-1α has many downstream effects through which it may support chondrocyte survival and function in a hypoxic environment. It induces angiogenesis, shifts metabolic catabolism to the anaerobic glycolytic pathway, directs autophagy and promotes the deposition of ECM (Myllyharju & Schipani 2010, Provot & Schipani 2007, Schipani 2010).

It is important to note that the main cartilage collagen genes are not direct HIF targets, even though hypoxia results in the accumulation of their protein products (Myllyharju & Schipani 2010). Instead, a hypoxia-responsive element has been identified in the *P4ha1* gene, residing about 120 bp upstream of the transcription start site, and HIF-1 has been shown to bind to this site (Takahashi *et al.* 2000). Consistent with this, no hypoxic induction of either *P4ha1* or *P4ha2* mRNAs was detected in a mouse hepatoma cell line lacking HIF-1 (Hofbauer *et*

al. 2003). Furthermore, chromatin immunoprecipitation (ChIP) coupled to high throughput sequencing applied to a human breast cancer cell line identified HIF-1 binding sites in the *P4HA1* and *P4HA2* genes, while a HIF-2 binding site existed only in the *P4HA1* gene (Schodel *et al.* 2011). In addition, a very recent study using a ChIP assay on mouse chondrocyte lysates demonstrated HIF-1α binding at two active sites on the *P4ha1* promotor, at two sites on the *P4ha2* gene and at three sites on the promoter of the gene encoding the PDI/β subunit of C-P4H (Bentovim *et al.* 2012).

Oxygen is an essential cosubstrate for the C-P4Hs, their K_m for O_2 being approximately 20 μM (Myllyharju 2003, Myllyharju 2008, Myllyharju & Schipani 2010). A hypoxia-induced increase in the amount of C-P4H is likely to be of particular significance in hypoxic tissues that are active in collagen synthesis, such as the avascular epiphyseal growth plate (Myllyharju & Schipani 2010, Schipani 2010). More importantly, regulation of the C-P4Hs could be one key modality through which HIF-1 mediates its survival and differentiation functions in the developing growth plate. It was shown in the present work that the expression of *P4ha1* and *P4ha2* mRNAs was increased approximately 2–6-fold in hypoxic primary mouse growth plate chondrocytes, that a lack of HIF-1α completely abolished this hypoxia-induced upregulation of both *P4ha1* and *P4ha2* mRNAs, and that, by contrast, a lack of HIF-2α had no significant effect on it. These findings indicate that the increase in *P4ha1* and *P4ha2* mRNAs in the growth plate chondrocytes of newborn mice under conditions of hypoxia is exclusively dependent on HIF-1.

It was also studied whether *Col2a1* and three classical HIF target genes, *Vegf*, *Glut1* and *Epo*, show hypoxia inducibility and HIF dependence in these chondrocytes. In line with previous reports, the hypoxia-induced expression of *Vegf* and *Glut1* mRNAs was significantly downregulated in hypoxic HIF-1α knockdown chondrocytes relative to controls (Gordan & Simon 2007, Warnecke *et al.* 2004) whereas the inactivation of HIF-2α in chondrocytes had no significant effect on the hypoxia-induced increase in *Glut1* mRNA, which is known to be an exclusive HIF-1 target, or on *Vegfa* mRNA, which has been reported to be targeted by both HIF-1 and HIF-2 (Gordan & Simon 2007, Warnecke *et al.* 2004). By contrast, knockdown of HIF-2α significantly attenuated the hypoxic induction of *Epo* mRNA, which has also been reported to be expressed in the chondrocytes of developing porcine cartilage and HIF-2 has emerged as the prime isoform regulating the hypoxia-induced expression of Epo (Gruber *et al.* 2007, Rankin *et al.* 2007). In accordance with the well-established

lack of induction of *Col2a1* mRNA by hypoxia (Myllyharju & Schipani 2010) neither the knockdown of HIF-1α nor that of HIF-2α affected this mRNA under conditions of either hypoxia or normoxia.

The present work also demonstrated for the first time that the hypoxia-induced increases in *P4ha1* and *P4ha2* mRNA levels are directly associated with similar increases in the amounts of C-P4H-I and C-P4H-II tetramers. Moreover, the changes in *P4ha1* and *P4ha2* mRNA and corresponding protein levels were reflected in C-P4H activity, which increased approximately 3-fold in the hypoxia-treated control samples. Inactivation of HIF-1α markedly reduced this hypoxia-induced increase in C-P4H activity, while inactivation of HIF-2α had no effect. Inactivation of VHL mimicked the effect of hypoxia.

Interestingly, collagen hydroxylation and its HIF-dependence in hypoxic chondrocytes has been investigated very recently using both *in vitro* and *in vivo* models by Bentovim and co-workers (2012), who demonstrated that *Hif-1a* loss of function results in underhydroxylation of growth plate collagen and protracted collagen secretion (Bentovim *et al.* 2012). Also, hypoxia induced the expression of *P4ha1*, *P4ha2* and the gene coding for the PDI/β subunit of the C-P4H about 3-fold in primary growth plate chondrocytes, an effect that was lost upon *Hif-1a* inactivation (Bentovim *et al.* 2012). Moreover, the *in vivo* expression levels of these subunits were significantly lower in *Hif-1a* deficient murine growth plates, the most dramatic change being detected in the expression level of *P4ha2*, which was reduced by 60% relative to control growth plates (Bentovim *et al.* 2012). These results strongly support our findings and the suggestion that HIF-1α functions as a central regulator of collagen production in chondrocytes under conditions of oxygen deprivation (For a model see Figure 15).

The data obtained here show that HIF-2 has no role in the hypoxic upregulation of the C-P4Hs in newborn mouse growth plate chondrocytes, even though HIF-2α has been reported to have certain roles in cartilage biology in addition to HIF-1α. Hypoxia has been shown to upregulate the key cartilage transcription factor SOX9 in human articular chondrocytes in a HIF-2-dependent manner, leading to enhanced expression of the cartilage matrix genes (Lafont *et al.* 2007, Lafont *et al.* 2008) and HIF-P4H-2 was identified in further experiments as being the dominant HIF-P4H isoform regulating HIF-2α in human chondrocytes (Thoms & Murphy 2010). A lack of HIF-2 in the mouse limb bud mesenchyme has nevertheless been shown to cause only a moderate, transient delay in endochondral bone development, probably due to modest impairment of the

differentiation of hypertrophic growth plate chondrocytes into late hypertrophic cells. Thus HIF-2 is not likely to be crucial for growth plate development. On the other hand, it is necessary for articular cartilage, in which it has been shown to induce a number of catabolic factors and is thus likely to be involved in the development of osteoarthritis.

In conclusion, the data obtained here using primary mouse epiphyseal growth plate chondrocytes with a modified HIF pathway illustrate the significance of this pathway in the regulation of C-P4Hs and thus in the development of cartilage and bone. Moreover, HIF-1 appeared to be the prime factor regulating the hypoxia-induced upregulation of C-P4Hs I and II (Fig. 15).

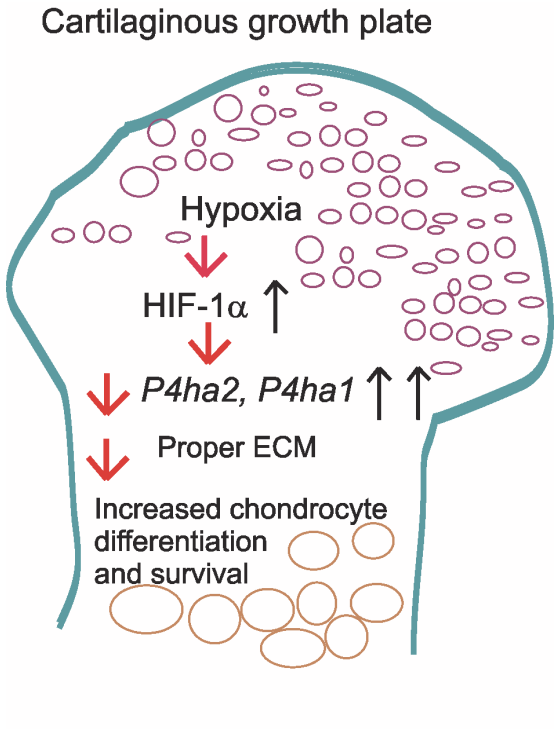

Fig. 15. Model for HIF-1α-dependent regulation of chondrocyte function in cartilaginous growth plate.

6.3 Reduction in C-P4H activity results in mild chondrodysplasia and impaired bone properties in mice

No heritable human diseases associated with mutations in any of the three C-P4H α subunit genes have been identified to date (Myllyharju 2008). As C-P4H was for a long time thought to exist in one type only, with no isoenzymes, and given its fundamental role in collagen biosynthesis, mutations resulting in its inactivation were expected to be lethal. The discovery of the C-P4H isoenzymes II and III, however, aroused interest in the individual roles of the three isoenzymes (Annunen *et al.* 1997, Kukkola *et al.* 2003, Van Den Diepstraten *et al.* 2003). C-P4H-I has emerged as the main isoenzyme in most cell types and tissues, whereas C-P4H-II predominates in chondrocytes, osteoblasts, endothelial cells and cells in epithelial structures (Annunen *et al.* 1998b, Nissi *et al.* 2001) and C-P4H-III is regarded as a minor isoenzyme expressed in various tissues but at much lower levels than C-P4H-I and C-P4H-II (Kukkola *et al.* 2003, Van Den Diepstraten *et al.* 2003).

Increased amounts of C-P4H activity accompanied by excessive collagen formation play a significant role in the pathogenesis of several fibrotic conditions such as liver and pulmonary fibrosis and abnormal wound healing characterized by severe scarring (Myllyharju 2008). In addition, increased expression of C-P4H-II has been reported in osteoarthritic articular chondrocytes relative to healthy chondrocytes (Grimmer *et al.* 2006). Nevertheless, in view of the lack of any specific heritable human disease caused by altered C-P4H function, there is little information available on the accurate individual *in vivo* roles and mutual compensatory capacities of the three vertebrate C-P4H isoenzymes. Mice with inactivation of the *P4ha1* gene coding for the C-P4H-I α(I) subunit die after E10.5 during embryogenesis and show overall developmental delay and rupture of the BMs due to the failure of collagen IV assembly in them (Holster *et al.* 2007). The fibril-forming collagens I and III in the null embryos were still able to assemble into typical cross-striated fibrils, however, although these showed slightly increased diameters (Holster *et al.* 2007). The C-P4H activity level in the null embryos and fibroblasts cultured from them was about 20% of that in the wild type, a figure that may be assumed to represent the contribution of C-P4Hs II and III. This residual C-P4H activity was not sufficient to ensure development of the *P4ha1* null mice after early embryogenesis, however (Holster *et al.* 2007).

A mouse line with targeted inactivation of the α(II) subunit gene (*P4ha2*) was generated in the present study and, given the expression pattern of C-P4H-II, it was hypothesized that the absence of this particular isoenzyme would seriously compromise the development of cartilage and cartilaginous bone. Surprisingly, however, the *P4ha2*$^{-/-}$ mice had no overt skeletal phenotype and appeared to be thoroughly healthy and fertile and to have a normal life span. We therefore set out to study the combined effects of decreased C-P4H-I activity and a lack of C-P4H-II activity by generating mice with heterozygous inactivation of the *P4ha1* gene in combination with homozygous inactivation of *P4ha2* (*P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$). By combining the results obtained with the above mouse lines it was demonstrated that a significant reduction in C-P4H activity leads to mild chondrodysplasia and compromises the structural and mechanical properties of long bones. Also, experimental evidence was provided to indicate that the observed phenotypic alterations were the result of the accumulation of an abnormal collagen matrix in cartilage and bone.

In short, the C-P4H deficiency generated a transient inner cell death phenotype in the developing cartilaginous growth plate. It is important to note that the central part of the early growth plate is hypoxic (Schipani *et al.* 2001) for in line with this, HIF-1α has emerged as a critical factor for the survival, proliferation and differentiation of hypoxic growth plate chondrocytes (Araldi & Schipani 2010, Myllyharju & Schipani 2010, Schipani 2010). The phenotype observed in the *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ growth plate was reminiscent of the phenotypic outcome of a HIF-1α null growth plate, although cell death was admittedly more pronounced in the latter case (Schipani *et al.* 2001).

Since oxygen serves as an essential cosubstrate for the hydroxylation reaction catalyzed by the C-P4Hs (Myllyharju 2003, Myllyharju 2008) it is not suprising that the specific activity of the C-P4Hs decreases under conditions of oxygen deprivation (Aro *et al.* 2012). This is compensated for, however, by a hypoxia-induced HIF-1α-dependent increase in *P4ha1* and *P4ha2* mRNA levels and a concomitant increase in the amount of the corresponding C-P4H tetramers, at least in the case of primary mouse epiphyseal growth plate chondrocytes (Aro *et al.* 2012). Given the hypoxic nature of the developing growth plate chondrocytes and their high activity in collagen synthesis, it is likely that the significance of the HIF-1α-dependent induction of C-P4H expression in these cells is biologically particularly relevant. The data obtained here indicate that the regulation of C-P4H activity could be one of the modalities by which HIF-1α mediates its role as a survival factor in chondrocytes. However, as the growth

plate phenotype, *i.e.* inner cell death, was transient and relatively mild, it is unlikely that the C-P4Hs are the only mediators of HIF-1α survival in growth plate chondrocytes, or even the main ones.

The C-P4Hs control a critical post-translational modification in collagens and are therefore pivotal for the proper folding of collagens in the ER. They also take part in the intrinsic collagen molecule quality control mechanism, as the enzyme associates with non-helical procollagen chains and retains them inside the ER until folding is properly completed (Myllyharju 2003, Myllyharju 2008, Walmsley *et al.* 1999). Excess intracellular accumulation of unfolded/misfolded ECM proteins may result in ER stress, which initiates a further unfolded protein response (UPR) (Hetz 2012). Since this UPR is closely associated with cell death, it has been speculated that reduced amounts of C-P4Hs may trigger uncompensated ER stress, which could in part underlie the hypocellularity observed in the central part of the *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ growth plate. Accordingly, it was demonstrated earlier that *P4ha1* null fibroblasts secrete fibril-forming collagens less efficiently than do wild-type chondrocytes and that *P4ha1*$^{-/-}$ mesenchymal cells display a dilated ER (Holster *et al.* 2007). Most importantly, it has been proposed that UPR may play a key role in the pathogenesis of several genetic chondrodyplasias, including metaphyseal chondrodysplasia of the Schmid type (MCDS) and multiple epiphysial dysplasia (MED), diseases caused by mutations in collagen X and matrilin-3, respectively (Cameron *et al.* 2011, Leighton *et al.* 2007, Rajpar *et al.* 2009). On the other hand, expression of the classical UPR markers Bip and Chop was not increased in either newborn *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ growth plates or chondrocytes isolated from them. Exposure to hypoxia increased the expression of *Chop* in primary mouse epiphyseal chondrocytes *in vitro*, and there was a slight trend for increased *Chop* expression in *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ growth plate chondrocytes relative to *P4ha1*$^{+/+}$;*P4ha2*$^{+/-}$ samples, but this effect was not significant. Furthermore, EM of histological sections of newborn *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ growth plates did not reveal any signs of uncompensated ER stress such as a dilated or fragmented ER or the presence of trapped intracellular procollagen molecules.

Several heritable connective tissue disorders marked by growth retardation and impaired skeletal development and tissue strength are known to result from mutations in genes whose protein products are involved in collagen synthesis (Bateman *et al.* 2009, Myllyharju & Kivirikko 2004). These conditions include EDS VIA, OI and Bruck syndrome (BS). Mutations in the *PLOD1* gene coding

for LH1 cause EDS VIA, which is characterized by kyphoscoliosis, muscular hypotonia, joint laxity, abnormal scarring and an increased risk of fatal arterial ruptures (Bateman *et al.* 2009, Myllyharju & Kivirikko 2004). LH1 null (*Plod1*$^{-/-}$) mice provided a useful disease model for human EDS VIA, as they largely reproduced the phenotype observed in affected humans, but these *Plod1*$^{-/-}$ mice did not display kyphoscoliosis, which in EDS VIA patients is thought to result from muscle hypotonia and joint laxity (Steinmann *et al.* 2002).

Mutations in the human *PLOD2* gene for LH2 have been identified in patients with the extremely rare disease BS, which is manifested in fragile bones, congenital joint contractures, osteoporosis and scoliosis (Bateman *et al.* 2009, Myllyharju & Kivirikko 2004). Bone mineralization is reduced in BS, which is in accordance with the suggested effect of the cross-linking pattern on the mineralization of calcified tissues (Brenner *et al.* 1993). The fragility of BS bones is therefore likely to be a consequence of an altered cross-linking pattern together with a decreased mineral content of the tissue (Brenner *et al.* 1993). The phenotypic outcomes of BS closely resemble those of the more extensively studied disease OI.

OI is a clinically variable and genetically heterogenous disorder mostly caused by mutations in the *COL1A1* and *COL1A2* genes for type I collagen, although mutations in the genes encoding prolyl 3-hydroxylase 1 (P3H1), cartilage-associated protein (CRTAP) or leprecan have been shown to result in a severe form of OI in some rare cases (Baldridge *et al.* 2008, Barnes *et al.* 2006, Morello *et al.* 2006, Willaert *et al.* 2009). Mice with genetic inactivation of *P3h1* had a smaller body size and thinner skull relative to their wild-type littermates, and like *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ mice, they displayed rhizomelia and developed scoliosis, in fact to a, more severe extent than in the latter (Vranka *et al.* 2010). The collagen fibril ultrastructure in various collagen-rich tissues such as bone, tendon and skin was also altered in the *P3h1*-deficient mice (Vranka *et al.* 2010).

The phenotype observed in the *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ mice has certain characteristic features that are common to many genetic diseases affecting connective tissues. Given the expression pattern of C-P4H isoenzymes as described earlier and the obviously smaller body size of the *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ mice, we focused this work on analysing the formation of cartilaginous bone and were able to demonstrate that proper functioning of the C-P4Hs is necessary for the mechanical competence of long bones. It would be important in the future to analyse the biochemical, structural and mechanical properties of other high stress tissues such as the skin and tendons in *P4ha1*$^{+/-}$;*P4ha2*$^{-/-}$ and *P4ha2*$^{-/-}$ mice.

Moreover, the above mouse lines could provide an ideal model for studying the detailed roles of C-P4Hs in wound and fracture healing, which are conditions characterized by markedly increased collagen synthesis.

7 Conclusions and future prospects

Although previous *in vitro* data have demonstrated that, like the HIF-P4Hs, P4H-TM is capable of hydroxylating HIF-α (Koivunen *et al.* 2007, Oehme *et al.* 2002), there has so far been only little data available on the *in vivo* functions of P4H-TM (Hyvarinen *et al.* 2010b). In the first publication associated with this thesis the putative role of P4H-TM in mammalian erythropoiesis was investigated by administering a 2-oxoglutarate analogue, FG-4497, to *P4h-tm* null and wild-type mice. The observed effects were then compared with those seen in FG-4497-treated hypomorphic *Hif-p4h-2* and *Hif-p4h-3* null mice versus their wild-type controls. At the outset, it was demonstrated *in vitro* that FG-4497 actually inhibits both HIF-P4Hs and P4H-TM. Nevertheless, the inhibitor was less effective against P4H-TM than against the three HIF-P4Hs. In FG-4497 treated P4h-tm null mice, larger increases in serum Epo concentration and kidney *Epo* mRNA level were observed than in wild- type mice. HIF-1α and HIF-2α proteins and *Epo* mRNA levels in the kidney, but not in the liver, were more pronounced in the *P4h-tm*$^{-/-}$ mice than in wild-type mice after periods of repeated FG-4497 administration. Moreover, the liver *Hepcidin* mRNA level was lower in the *P4h-tm*$^{-/-}$ mice than in the wild type. Similar differences were observed in the case of *Hif-p4h-2*$^{gt/gt}$ and *Hif-p4h-3*$^{-/-}$ mice relative to wild-type mice. FG-4497 administration elevated hemoglobin and hematocrit values similarly in the *P4h-tm* null and wild-type mice, while the increases were higher in the FG-4497-treated *Hif-p4h-2*$^{gt/gt}$ mice than in the treated wild-type mice. *Hif-p4h-2*$^{gt/gt}$*/P4h-tm*$^{-/-}$ double gene-modified mice had increased hemoglobin and hematocrit values without any FG-4497 administration, even though neither the *Hif-p4h-2*$^{gt/gt}$ nor the *P4h-tm*$^{-/-}$ mice displayed such abnormalities. It may thus be concluded that the data obtained in the first publication indicate for the first time that P4H-TM is involved in regulation of the renal EPO pathway, hepcidin expression, and erythropoiesis.

Since HIF-P4H inhibitors are currently considered promising candidates for the development of drugs to treat anemia, the results obtained here provide valuable new information for pharmaceutical industry, suggesting that the generation of inhibitors that differentially target individual HIF-P4Hs and P4H-TM would appear plausible in the future. With respect to severe anemia, a compound inhibiting P4H-TM together with some of the HIF-P4H isoenzymes could produce a more efficient erythropoietic response than that obtained with a HIF-P4H antagonist lacking P4H-TM inhibition.

HIFs have been shown to play an important role in the normal functioning of chondrocytes in the avascular hypoxic fetal growth plate, which is rich in ECM and its main component collagens (Schipani 2010). C-P4Hs are key enzymes in collagen biosynthesis as they provide the newly synthesized collagen polypeptides with thermal stability. *P4ha1* and *P4ha2* mRNA levels are induced by hypoxia in many cell lines (Myllyharju & Schipani 2010). In the second publication, it was hypothesized that the 4-hydroxylation of proline residues in collagens could be one of the modalities by which HIF regulates the adaptive responses of chondrocytes in hypoxic fetal growth plates. It was demonstrated using primary epiphyseal growth plate chondrocytes isolated from newborn mice with conditionally inactivated genes for HIF-1α, HIF-2α, or the von Hippel-Lindau protein that *P4ha1* and *P4ha2* mRNA levels were increased in chondrocytes exposed to hypoxia in a manner dependent on HIF-1 but not on HIF-2. Moreover, the increases in the C-P4H mRNA levels were associated with both increased amounts of the C-P4H tetramers and augmented C-P4H activity in hypoxia.

As collagen production is essential for normal organ development and function, the presented findings promote our current understanding of bone formation and cartilage homeostasis. This knowledge should be taken into account in future research into pathological conditions in cartilage and bone such as chondrodysplasias, osteoarthritis, impaired fracture healing and osteoporosis.

Lastly, in the third work included in the present thesis mouse lines with complete inactivation of C-P4H-II with or without partial inactivation of C-P4H-I were characterized. Since the expression of C-P4H-II had earlier been reported to predominate in chondrocytes (Nissi *et al.* 2001, Nissi *et al.* 2004), the focus was on analysing the skeletal phenotype of the above mouse lines. The $P4ha2^{-/-}$ mice were viable and fertile and, surprisingly, had no obvious skeletal abnormalities, but the $P4ha1^{+/-};P4ha2^{-/-}$ mice were smaller than their littermates, displayed moderate chondrodysplasia and eventually developed kyphosis. The structural and mechanical properties of their long bones were impaired, probably due to changes in collagen quality.

The results obtained from the third investigation provided new information about the specific *in vivo* functions and mutual compensatory capacities of C-P4H isoenzymes I and II. It is difficult in the light of previous reports and the present study to predict whether human diseases caused by inactivating mutations in the *P4HA1* and *P4HA2* genes will be identified in the future. The $P4ha2^{-/-}$ and

P4ha1$^{+/-}$;*P4ha2*$^{-/-}$ mice produced here could be excellent tools for clarifying the detailed roles of the C-P4Hs in wound and fracture healing which are conditions characterized by markedly increased collagen synthesis. Moreover, when subjected to physical exercise the above mouse lines could provide a good model for studying the role of C-P4H-I and II in degenerative diseases of the musculoskeletal system and connective tissue, for example.

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List of original papers

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

I Laitala A, Aro E, Walkinshaw G, Mäki JM, Rossi M, Heikkilä M, Savolainen ER, Arend M, Kivirikko KI, Koivunen P* & Myllyharju J* (2012) Transmembrane prolyl 4-hydroxylase is a fourth prolyl 4-hydroxylase regulating EPO production and erythropoiesis. Blood 120: 3336–3344.

II Aro E, Khatri R, Gerard-O'Riley R, Mangiavini L, Myllyharju J* & Schipani E* (2012) Hypoxia-inducible factor-1 (HIF-1) but not HIF-2 is essential for hypoxic induction of collagen prolyl 4-hydroxylases in primary newborn mouse epiphyseal growth plate chondrocytes. J Biol Chem 287: 37137–37144.

III Aro E, Salo AM, Khatri R, Finnilä M, Miinalainen I, Sormunen R, Pakkanen O, Holster T, Soininen R, Tuukkanen J, Schipani E* & Myllyharju J* (2012) Mice lacking collagen prolyl 4-hydroxylase isoenzyme II in combination with a reduced amount of isoenzyme I display abnormalities in skeletogenesis. Manuscript.

Original publications are not included in the electrical version of the dissertation.

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