

Structural, Biochemical and Inhibition Studies on Hypoxia-Inducible Factor (HIF) Prolyl Hydroxylases



Kerstin Lippl

Lincoln College, Oxford

Hilary Term 2018

A thesis submitted to the Board of the Faculty of Chemistry at the University of Oxford in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Structural, Biochemical and Inhibition Studies on Hypoxia-Inducible Factor (HIF) Prolyl Hydroxylases

Kerstin Lippl, Lincoln College

Hilary Term 2018

Abstract

In humans and other animals, the chronic hypoxic response is mediated by the hypoxia-inducible transcription factors (HIFs), which regulate the expression of genes that counteract the effects of limiting oxygen. HIF prolyl hydroxylases (PHDs) regulate cellular levels and transcriptional activity of HIFs by catalysing the post-translational hydroxylation of conserved prolyl residues in the HIF α subunits. Based on structural and biophysical studies, it has been proposed that the three human PHDs (PHD1-3) act as oxygen sensors, providing a direct link between cellular oxygen availability and the regulation of the hypoxic response.

Work in this thesis has focused on the *in vitro* characterisation of the human HIF prolyl hydroxylases and the PHD from the simplest existent animal, *Trichoplax adhaerens*. Previous studies on human PHDs suggested that they are not selective for their well established HIF substrates, but accept additional substrates other than HIFs. With PHD inhibitors currently in late-stage clinical trials for treatment of anaemia, investigating the validity of the proposed promiscuous nature of human PHDs is of major medicinal relevance. In systematic and detailed studies, reported non-HIF substrates were probed for reaction with PHDs, using various mass spectrometric and NMR-based approaches. No evidence was found for PHD-catalysed hydroxylation of non-HIF substrates in the studies presented in this thesis, suggesting that PHDs might be more selective for their HIF substrates than previously perceived.

Inhibition studies were carried out on human PHD1-3, to characterise currently available PHD inhibitors, including such as used in clinical trials. In addition, a series of new PHD inhibitors was tested, and a proof of concept was provided that inhibitors capable of discriminating between PHD2 and PHD3 can be developed.

Structural and biochemical studies on human PHD2 variants were performed. As a result, functional relationships between clinically observed mutations in the EGLN1 gene encoding for PHD2 and disease phenotypes, such as cancer and erythrocytosis, were established.

Finally, work was concentrated on the evolution of the HIF pathway in animals and beyond. To investigate if the HIF prolyl hydroxylase in *T. adhaerens* (TaPHD) is capable of sensing hypoxia in a similar way as human PHD2, a comparative biochemical characterisation of TaPHD and human PHD2 was performed. Strong evidence was provided that the special kinetic properties of human PHD2 are conserved in TaPHD, including unusually high *K_m* values for oxygen, low substrate uncoupled 2OG turnover, and structural elements in the active sites of human PHD2 and TaPHD, which enable the unusually slow reaction of human PHD2 with oxygen. Further, evolutionary links between human HIF prolyl hydroxylases and Skp1-mediated oxygen-sensing mechanisms in lower organisms, such as *Dictyostelium discoideum* and *Toxoplasma gondii*, were investigated.

Overall, the work described in this thesis provided new insights into the substrate selectivity of human PHDs, and their potential for therapeutic manipulation. A high degree of conservation between PHDs ranging from humans to *T. adhaerens* was revealed, highlighting the importance of HIF-mediated hypoxia sensing as a prerequisite for the evolution of higher animals.

Acknowledgements

Firstly, I would like to thank my supervisor Professor Chris Schofield for the continuous support that I received from him throughout my DPhil. I am especially grateful for Chris' enthusiasm, for his ideas and encouragement, and for the freedom he gave me to work on my own ideas and projects. I also would like to express my gratitude towards Lincoln College and the Newton-Abraham Board for funding the work in this thesis.

Much of the work presented here was only possible with the help of my collaborators, who supported me by providing materials, by conducting experiments or by training me on new areas of science. In particular, I am indebted to Christoph Loenarz, who trained me when I first arrived in Oxford, and to Martine I. Abboud, Lucía Serrán Aguilera, Hidenori Someya, James Holt-Martyn, Anthony Tumber, Rasheduzzaman Chowdhury, Roman Fischer, Hanna Tarhonskaya, Anna Boleining, and Michael McDonough, for their direct contributions to my work. I am immensely grateful for the rewarding collaborations with Professor Peter Ratcliffe, Ya-Min Tian, Matthew Cockman, and Professor Johanna Myllyharju. I would like to express my warmest thanks to all members of the Mass Spectrometry Facility at the Chemistry Research Laboratory, including James Wickens, and Elisabete Pires. During my time in the Schofield group, I had the pleasure to supervise two students, Patrick Trenkle and William Figg, whom I want to thank for their motivation and eagerness, their hard work and much appreciated feedback.

Most DPhils have their highs and lows, and mine was not an exception. But thanks to the support that I received from people both inside and outside the lab, I had the most enjoyable time. I am extremely thankful to Joanna Bonnici, Leah Taylor Kearney, Martine Abboud, Kristina Aertker, Kate Dunne, Jürgen Brem, and many others for their friendship and for providing me with sweets when my energy levels were particularly low. I am grateful for all my friends who accompanied me through my studies in Munich, Osaka, and Oxford, Kerstin Sigl, Arianna Oddo, Niloofar Karaji, Vanessa Timmermann, Ramona Fritz, and Caro Seefeldt, just to name a few. My deepest thanks to Thomas Hofer for his invaluable encouragement and love, and for always being there for me.

Most importantly, my heartfelt thanks go to my family, my parents, and my sisters, for their endless support and encouragement. This is why I want to dedicate this thesis to them.

Contributions

I would like to express my special thanks to everyone who contributed to the work presented in this thesis. All contributors who carried out or assisted experiments are listed below.

Contributor	Affiliation	Contribution
Martine I. Abboud	Chris Schofield group, University of Oxford	assisted NMR experiments, including displacement experiments, peptide calibrations, and 2OG turnover experiments
Lucía Serrán Aguilera	Chris Schofield group, University of Oxford	contributed to the synthesis of Hyp-containing peptides, performed and assisted VCB fluorescence-polarisation assays
Hidenori Someya	Chris Schofield group, University of Oxford	designed and synthesised dual inhibitors
James Holt-Martyn	Chris Schofield group, University of Oxford	designed and synthesised PHD inhibitors, performed RapidFire-MS inhibition studies on PHD2
Anthony Tumber	Chris Schofield group, University of Oxford	assisted RapidFire-MS experiments
Rasheduzzaman Chowdhury	Chris Schofield group, University of Oxford	performed crystallographic data acquisition and processing on PHD2 variants
Roman Fischer	Benedikt Kessler lab, Target Discovery Institute, University of Oxford	performed LC-MS/MS data acquisition and analysis
Hanna Tarhonskaya	Chris Schofield group, University of Oxford	performed and assisted steady-state kinetic studies on oxygen dependence
Anna Boleininger	Chris Schofield group, University of Oxford	performed crystallisation of <i>Ta</i> PHD
Michael McDonough	Chris Schofield group, University of Oxford	solved all <i>Ta</i> PHD structures
Matthew Cockman	Peter Ratcliffe group, Nuffield Department of Medicine, University of Oxford	assisted cellular studies, including HRE reporter assays and immunoprecipitation experiments
Johanna Myllyharju	Biocenter, University of Oulu, Finland	provided PHD1 and PHD2 full-length enzymes (produced by using a baculovirus expression system)

Table of Contents

Chapter 1 General Introduction	1
1.1 The HIF pathway	1
1.2 The HIF prolyl hydroxylases	7
1.3 HIF prolyl hydroxylases as therapeutic targets	16
1.4 Non-HIF substrates of PHDs	19
1.5 Evolution of the HIF pathway in animals and lower organisms	21
1.6 Objectives of the work described in this thesis	27
1.7 References	28
Chapter 2 Substrate Selectivity of Human HIF Prolyl Hydroxylases	39
2.1 Introduction	39
2.2 Results and Discussion	44
2.2.1 PHD hydroxylation assays	44
2.2.2 Competition of non-HIF substrates with HIF hydroxylation by PHDs	57
2.2.3 Studies on binding of non-HIF substrates to PHD2	61
2.2.4 Influence of non-HIF substrates on 2OG turnover by PHDs	63
2.2.5 VCB binding studies by fluorescence-polarisation	66
2.2.6 Co-expression experiments	73
2.3 Conclusions	77
2.4 References	80
2.5 Appendix	86
Chapter 3 Inhibition Studies on Human HIF Prolyl Hydroxylases	91
3.1 Introduction	91
3.2 Results and Discussion	94
3.2.1 Development of a PHD1-3 inhibition assay	94
3.2.2 Inhibition of PHD1-3 by NOG, FG-2216/IOX3 and IOX2	98
3.2.3 Inhibition of PHD1-3 by clinically-used compounds	100
3.2.4 Inhibition of PHD2 by dual inhibitors	103
3.2.5 Inhibition of PHD2 by clinically-used Fe(II) chelators	107
3.2.6 High-throughput inhibition studies	110
3.2.6.1 Assay development	110
3.2.6.2 Studies on 2OG-oxygenase inhibitors	113
3.2.6.3 Studies on novel PHD inhibitors	115
3.3 Conclusions	120
3.4 References	122
Chapter 4 Studies on Variants of <i>Hs</i>PHD2 and <i>Hs</i>HIF1α CODD	128
4.1 Introduction	128

4.2	Results and Discussion	133
4.2.1	Activity of PHD2 variant enzymes	133
4.2.2	Crystallographic analysis of PHD2 variants	136
4.2.3	Thermal stability of PHD2 variants	143
4.2.4	Activity of PHD2 on HIF1 α CODD variants	145
4.3	Conclusions	147
4.4	References	149
4.5	Appendix	154

Chapter 5 Biochemical Studies on the HIF Prolyl Hydroxylase from *Trichoplax adhaerens*157

5.1	Introduction	157
5.2	Results and Discussion	161
5.2.1	Comparative analyses of <i>Ta</i> PHD crystal structures	161
5.2.1.1	Crystal structures of <i>Ta</i> PHD with and without bound substrate	161
5.2.1.2	Comparison of <i>Ta</i> PHD. <i>Ta</i> ODD and <i>Hs</i> PHD2.CODD/NODD structures	164
5.2.1.3	Comparison with enzyme-substrate structures of other prolyl-4-hydroxylases	167
5.2.2	Comparative biochemical studies on <i>Ta</i> PHD and <i>Hs</i> PHD2	172
5.2.2.1	Production and purification of <i>Ta</i> PHD	172
5.2.2.2	Steady-state kinetic studies on <i>Ta</i> PHD and <i>Hs</i> PHD2	173
5.2.2.3	Substrate binding studies on <i>Ta</i> PHD	176
5.2.2.4	Monitoring the coupled and uncoupled 2OG turnover of <i>Ta</i> PHD and <i>Hs</i> PHD2	177
5.2.2.5	Steady-state oxygen dependence of <i>Ta</i> PHD	178
5.3	Conclusions	180
5.4	References	182
5.5	Appendix	185

Chapter 6 Interaction Studies between *Hs*PHD2 and *Hs*Skp1188

6.1	Introduction	188
6.2	Results and Discussion	192
6.2.1	Competition of <i>Hs</i> Skp1 with HIF α hydroxylation by <i>Hs</i> PHD2 and <i>Hs</i> PHD3	192
6.2.2	HRE luciferase reporter assay	194
6.2.3	Immunoprecipitation experiments	197
6.3	Conclusions	201
6.4	References	203
6.5	Appendix	205

Chapter 7 Materials and Methods206

7.1	Molecular biology	207
7.1.1	Cloning	207
7.1.1.1	Overview of plasmids and primers used in the work	207
7.1.1.2	Ligation independent cloning (LIC)	209

7.1.1.3	Classic cloning	213
7.1.1.4	Site-directed mutagenesis	215
7.1.1.5	Agarose gel electrophoresis	216
7.2	Microbiological techniques	217
7.2.1	Transformation into bacteria	217
7.2.2	Glycerol stocks	217
7.2.3	Starter cultures	217
7.2.4	Expression trials	218
7.2.5	Large-scale protein production	218
7.2.6	Cell lysis	220
7.3	Protein purification	221
7.3.1	Purification strategies	221
7.3.2	Protein chromatography	223
7.3.3	Protease cleavage	225
7.3.4	Buffer exchange.....	225
7.3.5	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	225
7.4	Peptide synthesis	227
7.5	Peptide purification by HPLC	228
7.6	Mass spectrometry	229
7.6.1	MALDI-TOF-MS	229
7.6.2	ESI-LC-MS	231
7.6.3	RapidFire-based enzymatic assays	232
7.6.4	Protein ESI-LC-MS	233
7.6.5	Proteomics	234
7.6.5.1	Sample preparation	234
7.6.5.2	LC-MS/MS analysis	235
7.7	NMR experiments	236
7.7.1	2OG turnover monitoring by ¹ H CPMG NMR experiments	236
7.7.2	¹³ C-HIF1 α CODD and ¹³ C-HIF1 α NODD displacement experiments	236
7.8	Fluorescence-polarisation displacement assays	238
7.9	CD spectroscopy	239
7.10	Crystallography	240
7.10.1	Crystallisation trials	240
7.10.2	Crystal harvesting	240
7.10.3	Data collection and structure solution	240
7.11	Human cell culture	241
7.11.1	Cell passaging.....	241
7.11.2	Transient transfection	241
7.11.3	HRE luciferase assay	242
7.11.4	Immunoprecipitation experiments.....	243

7.12	References	246
Chapter 8	Summary and Conclusions	248

Abbreviations

Abbreviation	Full name
ab	antibody
ACACB	acetyl-CoA carboxylase 2 (ACC2)
ACTB	actin β
ADRB2	adrenoceptor β 2 (β 2AR)
AKT1	AKT serine/threonine kinase 1
ATF4	activating transcription factor 4
ARD	ankyrin repeat domain
bHLH	basic helix-loop-helix motif
BSA	bovine serum albumin
CAD	C-terminal transcriptional activation domain
CBP	cAMP response element binding protein
CD	circular dichroism
CENPN	centromere protein N
CEP192	centrosomal protein 192
c-MET	tyrosine-protein kinase Met
CODD	C-terminal oxygen-dependent degradation domain
CPMG	Carr-Purcell-Meiboom-Gill sequence
<i>Cr</i>	<i>Chlamydomonas reinhardtii</i>
Cul2	cullin box 2
CXCR4	C-X-C chemokine receptor type 4
dCTP	deoxycytidine triphosphate
<i>Dd</i>	<i>Dictyostelium discoideum</i>
DEC1 and DEC2	deleted in esophageal cancer 1 and 2
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMOG	dimethyloxalyl glycine
dNTP	deoxyribonucleotide triphosphate
DSBH	double-stranded β -helix core-fold
DTT	dithiothreitol

Abbreviation	Full name
DYRK1A	dual specificity tyrosine phosphorylation regulated kinase 1A
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
eEF2K	eukaryotic elongation factor 2 kinase
EF-Tu	elongation factor Tu
EGLN	Egl nine homolog
EPAS1	hypoxia-inducible factor 2 α (HIF2 α)
EPO	erythropoietin
EPOR	erythropoietin receptor
ESI-LC-MS	electrospray ionization liquid chromatography mass spectrometry
ETS1	protein C-ets-1
FBS	fetal bovine serum
FIH	factor inhibiting HIF
FKBP10	FK506 binding protein 10
FKBP38	FK506 binding protein 38
fl	full-length
FLNA	filamin A
FOXO3	forkhead box O3
FP	fluorescence-polarisation
FPLC	fast protein liquid chromatography
GST	glutathione-S-transferase
HIF	hypoxia-inducible factor
HK2	hexokinase 2
HPLC	high-performance liquid chromatography
HRE	hypoxia response element
<i>Hs</i>	<i>Homo sapiens</i>
HSP90	heat shock protein 90
HSQC	heteronuclear single-quantum correlation
Hyp	hydroxyproline
IKBKB	inhibitor of nuclear factor kappa B kinase subunit β

Abbreviation	Full name
IP	immunoprecipitation
IPTG	isopropyl β -D-1-thiogalactopyranoside
JmjC	Jumonji-C
k_{cat}	turnover frequency
K_d	dissociation constant
K_m	Michaelis–Menten constant
LC-MS	liquid chromatography mass spectrometry
LIC	ligation independent cloning
MALDI-TOF-MS	matrix assisted laser desorption ionization time of flight mass spectrometry
MAPK6	mitogen-activated protein kinase 6
<i>Mb</i>	<i>Monosiga brevicollis</i>
MBP	maltose-binding protein
MTHFD1	methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyl-tetrahydrofolate synthetase 1
MYH10	myosin heavy chain 10
MYND	myeloid, Nervy, and DEAF-1 motif
NDRG3	NDRG family member 3
Nip3	nineteen kD interacting protein-3
NMR	nuclear magnetic resonance
NODD	N-terminal oxygen-dependent degradation domain
NOG	N-oxalylglycine
ODD	oxygen-dependent degradation domain
PAC	PAS-associated C-terminal domain
PAS	Per-ARNT-Sim domain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PDE4D	phosphodiesterase 4D
PH	prolyl hydroxylase
PHD	prolyl hydroxylase domain containing protein
PKM	pyruvate kinase, muscle

Abbreviation	Full name
PKM2	pyruvate kinase, muscle isoform 2
POLR2A	RNA polymerase II subunit A (Rpb1)
<i>Pp</i>	<i>Pseudomonas putida</i>
PPHD	<i>Pseudomonas (putida)</i> prolyl hydroxylase domain containing protein
PPP2R2A	protein phosphatase 2 regulatory subunit Ba
P4H	prolyl-4-hydroxylase
p35srj	protein 35-kD serine-glycine rich junction
Rbx1	RING box protein 1
rt	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
Skp1	S-phase kinase-associated protein 1
SOCS1	suppression of cytokine signaling 1
SPRY2	sprouty RTK signaling antagonist 2
t	truncated
<i>Ta</i>	<i>Trichoplax adhaerens</i>
TCEP	tris(2-carboxyethyl)phosphine
TELO2	telomere maintenance 2
TEMED	tetramethylethylenediamine
<i>Tg</i>	<i>Toxoplasma gondii</i>
THRA	thyroid hormone receptor α
T _m	melting temperature
TP53	tumour protein p53
Tris	tris(hydroxymethyl)aminomethane
TRPA1	transient receptor potential cation channel subfamily A member 1
VHL	von Hippel-Lindau protein
VCB complex	pVHL, elongins B and C
wt	wild-type
2OG	2-oxoglutarate
8UY	1,2,4-triazolo-(1,5- α)pyridine

Chapter 1

General Introduction

1.1 The HIF pathway

The development of mechanisms to utilise atmospheric oxygen for bioenergetic processes (e.g. oxidative phosphorylation) is proposed to have been a key event in the evolution of higher organisms, such as metazoans (1). Since then, oxygen has become a major factor in the survival of all aerobic organisms ranging from mycobacteria to humans, and higher animals have evolved complex respiratory and circulatory systems for maintaining the supply of oxygen to their tissues and cells. Limitations in oxygen availability cause major cellular stress and are associated with the development of pathologic conditions, such as cancer and cardiovascular diseases (2-3). Aerobic organisms have developed various mechanisms to overcome hypoxic conditions in their cells and organs, i.e. it has been known since the late 19th century that animals react to low oxygen levels at high altitudes by an increased production of red blood cells (4). However, the molecular mechanisms behind this observation were unclear until the discovery of the hypoxia-inducible transcription factors (HIFs) in the 1990s. HIFs up-regulate the production of red blood cells as a response to chronic hypoxia *via* the glycoprotein hormone erythropoietin (EPO) (5-6).

The HIFs are heterodimeric transcription factors that consist of a α and a β subunit (HIF α and HIF β , respectively). Under hypoxic conditions, HIF α forms a heterodimer with HIF β in the nucleus and the transcription factor complex binds to hypoxia response elements (HREs) within the DNA. The HIFs recognise a characteristic consensus sequence (5'-RCGTG-3') in the HREs and recruit the transcriptional coactivator proteins p300 and cAMP response element binding protein (CBP) (7-9). The human HIF system (and in other animals) does not only activate the gene expression of erythropoietin, but regulates hundreds of different genes in a context and cell-type dependent manner (**Table 1.1**, (10-11)). The large set of HIF-regulated genes include components of metabolic pathways, signaling pathways for cell growth and apoptosis, and angiogenesis (11-12). Thus, the HIF system induces the adaptation of organisms to hypoxia at both cellular and physiological levels.

Table 1.1 Examples of genes regulated by the human HIF system in a hypoxia-dependent manner (adopted from (11)).

Pathway	Gene Product
Cell migration	C-X-C chemokine receptor type 4 (CXCR4) Tyrosin-protein kinase Met (c-Met)
Hormonal regulation	Erythropoietin Leptin
Energy metabolism	Glucose transporter-1 Hexokinase-2 6-Phosphofructo-1-kinase L 6-Phosphofructo-2-kinase Glyceraldehyde-3-phosphate dehydrogenase Aldolase A Lactate dehydrogenase A Carbonic anhydrase-9
Angiogenic signaling	Vascular endothelial growth factor A Vascular-endothelial-growth-factor receptor-1 Endothelial-gland-derived vascular endothelial growth factor Plasminogen-activator inhibitor-1
Growth and apoptosis	Insulin-like growth-factor-binding protein-1 Nineteen kD interacting protein-3 (Nip3) Endoglin Wilm's tumour suppressor Calcitonin-receptor-like receptor
Transcriptional regulation	Deleted in esophageal cancer 1 and 2 (DEC1 and DEC2) Protein C-ets-1 (ETS1) protein 35-kD serine-glycine rich junction (p35srj)
Virus related	Retrotransposon VL30
Transport	Transferrin Transferrin receptor Ceruloplasmin Multidrug-resistance P-glycoprotein
Matrix and barrier functions	Procollagen prolyl hydroxylase- α 1 Intestinal trefoil factor
Vasomotor regulation	Adrenomedullin Tyrosine hydroxylase Haem oxygenase

The HIF transcription factors can be considered as ‘master regulators’ of the chronic hypoxic response. However, the HIFs cannot induce a response to hypoxia on a timescale shorter than the time needed for protein biosynthesis (acute hypoxia). Therefore, under acute hypoxia, it is likely that other processes apart from the HIF pathway are involved in the maintenance of oxygen homeostasis.

The level of activity of the HIF transcription factors is regulated in an oxygen dependent manner, by the HIF prolyl hydroxylases (PHDs, **Figure 1.1**). The PHDs belong to the family of 2-oxoglutarate (2OG) dependent oxygenases, members of which use 2OG as a co-factor to catalyse a variety of reactions on substrates ranging from proteins to DNA/RNA, metabolites and other compounds (13). The PHDs act as cellular oxygen sensors by hydroxylating two conserved prolyl residues in the *N*- and *C*-terminal oxygen-dependent degradation domains of HIF α (NODD and CODD, respectively) under normoxic conditions (11, 14). HIF α prolyl hydroxylation strongly promotes its binding to the von-Hippel Lindau protein (pVHL), a targeting component of an E3 ubiquitin ligase complex. Subsequent ubiquitination of HIF α signals for degradation of the protein *via* the ubiquitin proteasome system (15-17).

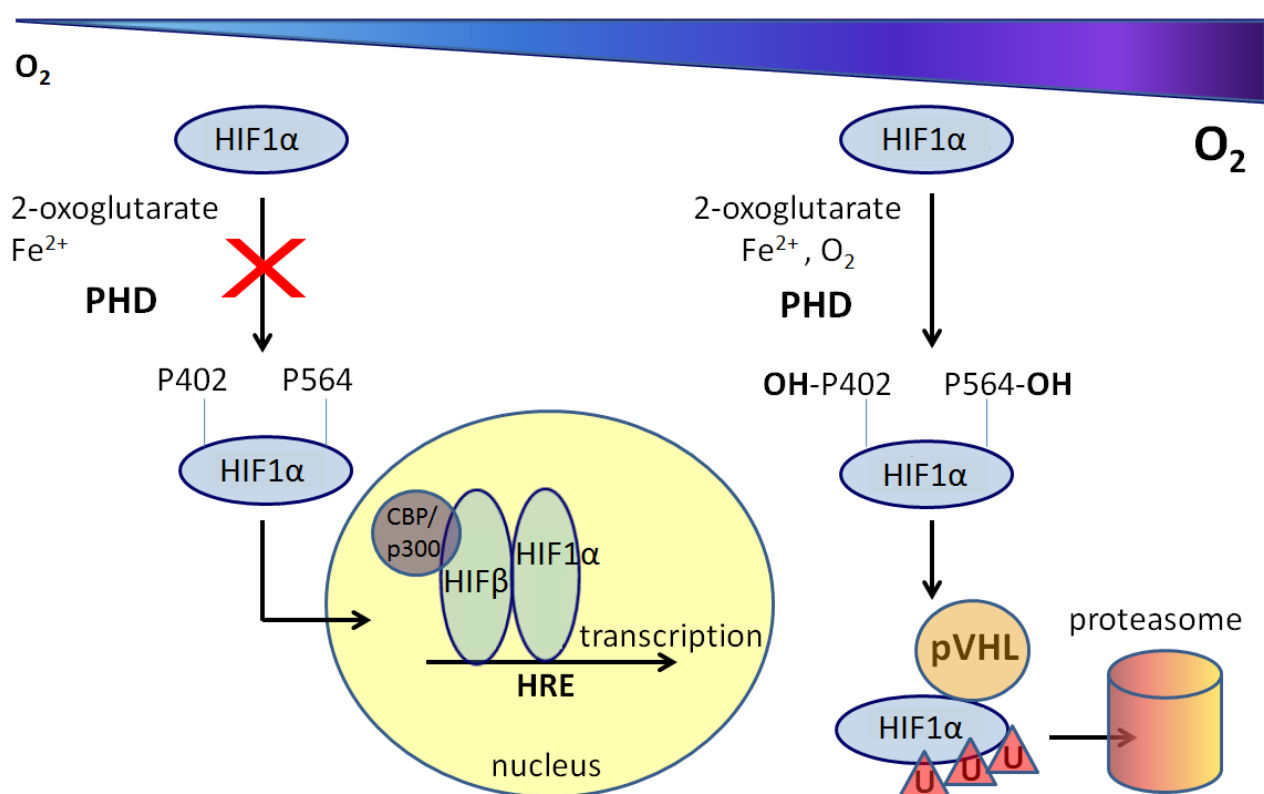


Figure 1.1: Overview of the HIF system. The hypoxia-inducible transcription factors (HIFs) are regulated by prolyl hydroxylase domain (PHD) catalysed hydroxylation of prolyl residues in the ODD(s) of HIF α under normoxia. Recognition of the hydroxylated prolyl residues, followed by ubiquitylation by the von-Hippel Lindau protein (pVHL) E3 ubiquitin ligase labels HIF α for proteasomal degradation.

In addition, HIF transcriptional activity is regulated by a second 2OG oxygenase, factor inhibiting HIF (FIH), that catalyses asparaginyl-hydroxylation in a C-terminal transcriptional activation domain (CAD) of HIF α (18-19); this modification inhibits the interaction between HIF and transcriptional coactivators (CBP/p300) (19). FIH is active at lower oxygen concentrations than the PHDs and is likely less important from a hypoxia sensing perspective (20-21).

In humans, there are three isoforms of HIF α (HIF1 α -HIF3 α , **Figure 1.2**), but only one HIF β which is continuously expressed independently of cellular oxygen levels. HIF1 α and HIF2 α each have two ODDs (Codd and Nodd, respectively), which manifest differential sensitivities to hypoxia (20, 22). Extensive studies conducted on HIF1 α and HIF2 α have identified specific sets of (partly overlapping) target genes for HIF1 α and HIF2 α (23-25). For instance, the production of erythropoietin is predominantly regulated by HIF2 α , whereas HIF1 α appears to be more important for regulation of gene expression of pyruvate dehydrogenase and other glycolysis-related proteins (26-27). HIF1 α and HIF2 α are both broadly expressed in human cancers, however, there is emerging evidence suggesting different roles for HIF1 α and HIF2 α in tumour growth and progression. The opposing effects of HIF1 α /HIF2 α activity are mediated through regulation of different target genes, as well as interactions with tumour suppressors, such as p53 (28).

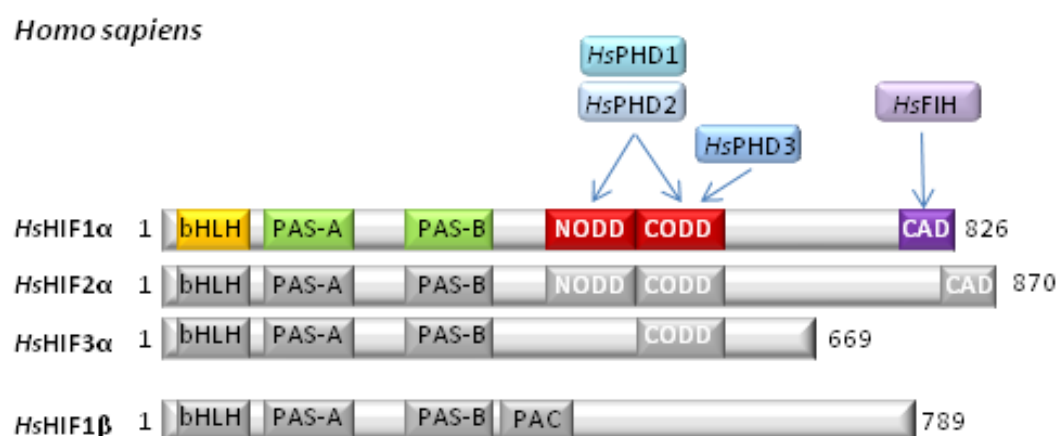


Figure 1.2: Domain structures of human HIF α and HIF β . There are three HIF α -isoforms and one HIF β present in the human HIF system. By contrast to HIF1 α and HIF2 α , that contain both N-terminal and C-terminal oxygen-dependent degradation domains (NODD and CODD, respectively), there is only one ODD in HIF3 α . Arrows indicate assigned prolyl- and asparaginyl-hydroxylation sites. Domain acronyms: basic helix-loop-helix (bHLH) motif, Per-ARNT-Sim (PAS) domain, C-terminal transactivation domain (CAD), PAS-associated C-terminal (PAC) domain.

In contrast to the well-characterised HIF1 α and HIF2 α , the biological role of HIF3 α , which contains a single C-terminal ODD, is less understood. HIF3 α is usually regarded as a negative regulator of HIF1 α and HIF2 α , and a differential role for HIF3 α splice variants in the regulation of the hypoxic response has been proposed (29-31).

Under normoxia, HIF α is targeted for proteasomal degradation by the pVHL E3 ubiquitin ligase complex, that consists of pVHL, elongins B and C (VCB complex), Cullin box 2 (Cul2), and RING box protein 1 (Rbx1) (**Figure 1.3**, (32)). HIF α interacts with pVHL/VCB, that catalyses the ubiquitination of HIF α , leading to the degradation of the protein via hydrolysis in the proteasome (15, 33). Prolyl hydroxylation on either NODD, CODD, or both ODDs causes a substantial increase in the binding affinity of HIF α to pVHL (22, 34-35). The oxygen-dependent binding mechanism of ODDs to the VCB complex has been investigated in structural studies (34, 36). The crystal structure of the VCB-HIF1 α CODD complex (**Figure 1.4**, (36)) shows the hydroxylated prolyl residue (Hyp564) positioned in a hydrophobic cleft in the β -domain of pVHL. The hydroxyl group interacts with the pVHL-residues Ser111 and His115 by forming several hydrogen bonds to these residues, thus increasing the binding affinity of the HIF1 α CODD (relative to the unhydroxylated ODD) to the VCB complex (34, 36).

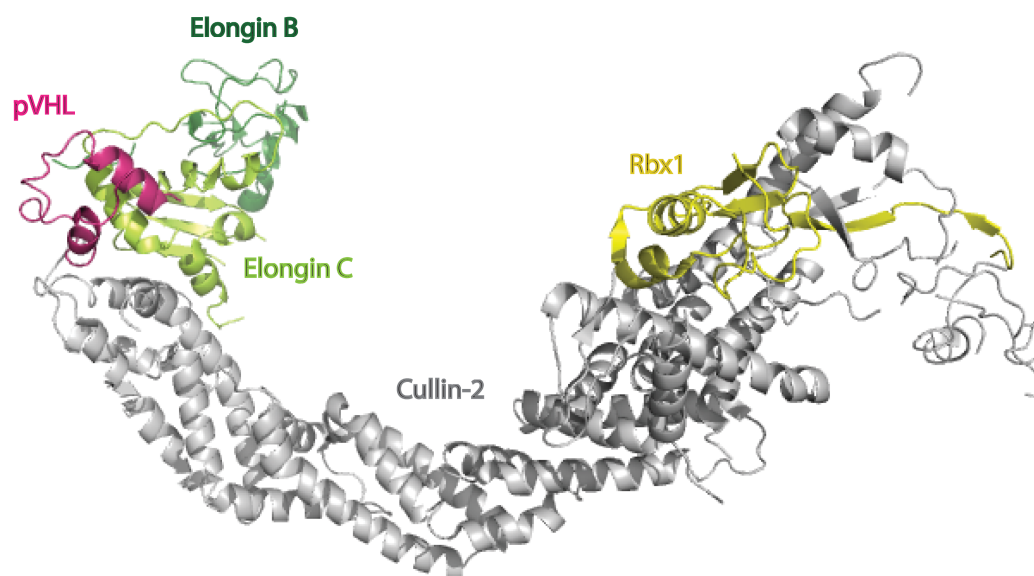


Figure 1.3: View from a crystal structure of the pVHL E3 ubiquitin ligase complex (PDB: 5N4W, (32)). Protein and domain acronyms: von-Hippel-Lindau protein (pVHL), RING box protein 1 (Rbx1), Cullin box 2 (Cullin-2). The VCB-complex (pVHL, Elongin B and C) is responsible for targeting and ubiquitination of HIF α . Cullin-2 and Rbx1 have mainly scaffolding roles.

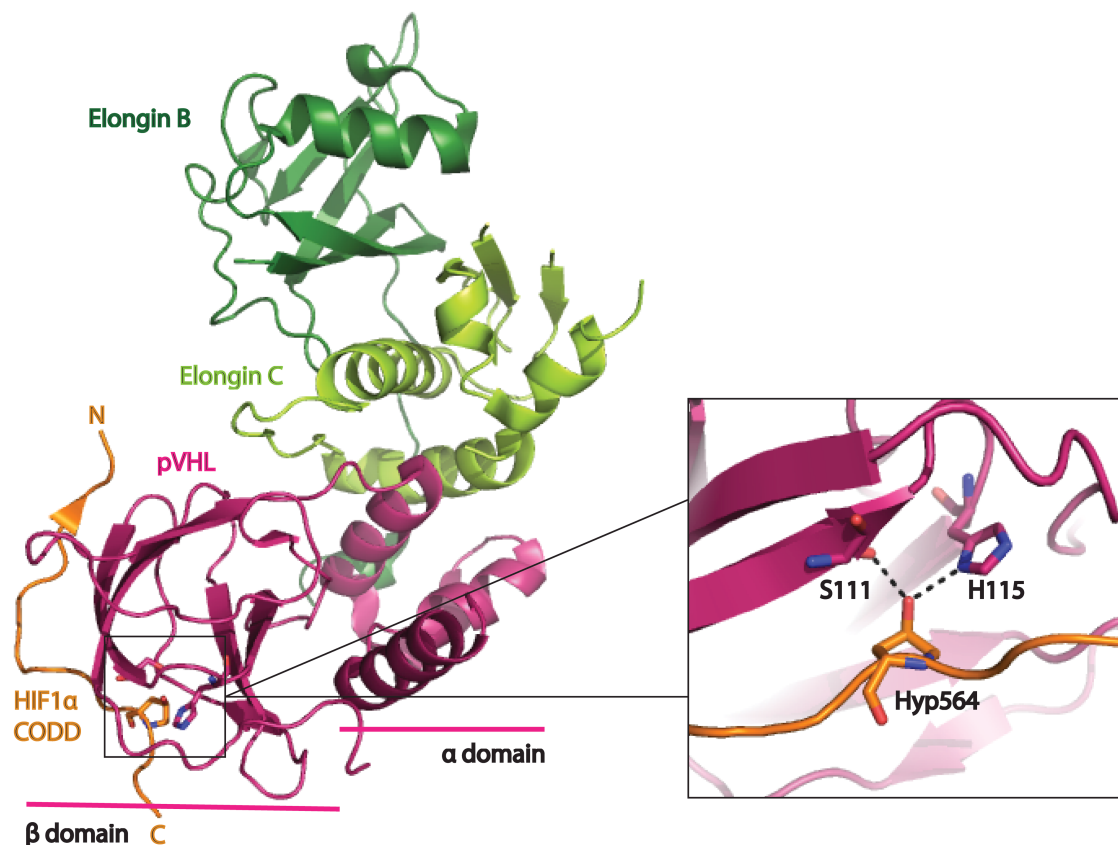
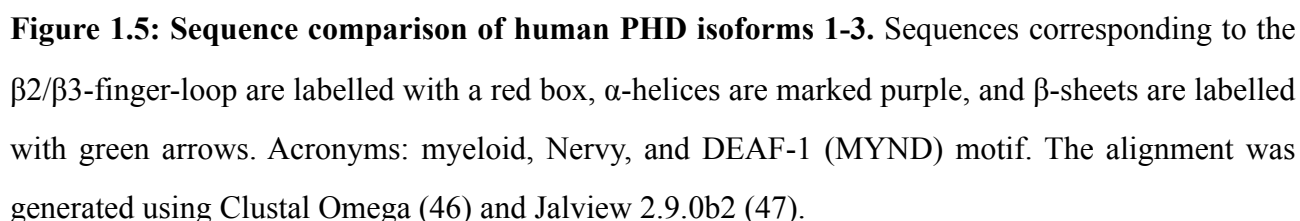


Figure 1.4: Views from a crystal structure of the VCB complex bound to a hydroxylated HIF1 α CODD peptide (PDB:1LM8, (36)). Overall structure of the VCB.HIF1 α CODD complex. Protein and domain acronyms: von-Hippel-Lindau protein (pVHL), VCB-complex (pVHL, Elongin B and C). A close-up at the HIF α binding site in pVHL reveals the mechanism of Hyp-induced binding of HIF1 α CODD to pVHL, as Hyp564 forms hydrogen bonds with Ser111_{pVHL} and His115_{pVHL}.

1.2 The HIF prolyl hydroxylases

In humans, the HIF transcription factors are regulated in an oxygen-dependent manner by three different prolyl hydroxylases (PHD1, 2, and 3, respectively) (16, 37-38). The three PHD isoforms have different substrate selectivities, with PHD1 and 2 accepting both CODD and NODD in HIF α , but PHD3 having a strong preference for CODD over NODD (39-40). Extensive studies in cells and animals have shown that PHD2 is the most abundant of the three PHD isoforms, as its mRNA was found to be ubiquitously expressed, unlike PHD1, that is predominantly expressed in the testes, and PHD3 that is mostly expressed in the heart (41-42). Moreover, animal studies suggest that PHD2 is the most important of the PHDs, as PHD2 knockout mice, unlike PHD1/3 knockouts, are not viable beyond embryogenesis (43). Cellular studies on distribution of the PHD isoforms found that PHD1 is predominantly located in the nucleus, whereas PHD2 was observed mostly in the cytoplasm, and PHD3 was present in both compartments (44).

Sequence alignment of PHD1-3 shows a high degree of homology in their 2OG-oxygenase domain, yet the three PHD isoforms differ significantly at their *N*-terminus (**Figure 1.5**). PHD2 contains a MYND-type zinc-finger domain at its *N*-terminus, which is not present in any of the other PHDs. The *N*-terminus of PHD1 is predicted to be disordered, and only a very short *N*-terminal domain is present in PHD3. To date, the function of the *N*-terminal domains of the PHDs is not well understood. In case of the MYND domain in PHD2, an interaction with heat shock protein 90 (HSP90) co-chaperones p23 and FKBP38 has been proposed (45).



In order to catalyse prolyl hydroxylation, the PHDs utilise 2OG as a co-substrate, which is converted to succinate during the catalytic cycle (48). The active site of the three PHD isoforms is predicted to be very similar. Structural studies on PHD2 provide insight into the mechanism of 2OG-dependent prolyl hydroxylation by the enzyme (49-50). In the active site of PHD2, Mn(II) (substituting for the natural co-factor Fe(II)) is octahedrally coordinated by water molecules and a triad of residues, consisting of one aspartate and two histidine residues (HXD/E...H motif), which

is highly conserved among 2OG-dependent oxygenases (**Figure 1.6**, (48-49)). Upon binding of the 2OG analogue *N*-oxalylglycine (NOG) and the substrate HIF1 α CODD, the 6-coordinate state of Mn(II) is retained by the conserved triad of residues, and by a remaining metal-bound water molecule, that is stabilised via hydrogen bonds to an aspartate residue (D315_{PHD2}, (50)). NOG is coordinated to the metal ion in a bidentate manner (**Figure 1.6**), and interacts by its C5 carboxylate with R383_{PHD2}. The 1-carboxylate of NOG is positioned at the coordination site closest to the hydroxylation target, P564_{HIF1 α CODD}, making the remaining metal coordination site (which is occupied by a water molecule in **Figure 1.6**) less accessible for oxygen binding to the metal ion. It was found in kinetic studies that the active site Fe(II) in PHD2 reacts slowly with oxygen, as compared to other 2OG oxygenases such as FIH (51-52). Structural features in the active site of PHD2, including the positioning of NOG/2OG in metal-coordination, along with the strongly-bound water molecule at the metal ion, that has to be displaced in order for oxygen to bind, are proposed to play a role in the observed slow reaction of PHD2 with oxygen (50); these properties of PHD2 are considered as crucial for the oxygen sensing capability of the enzyme.

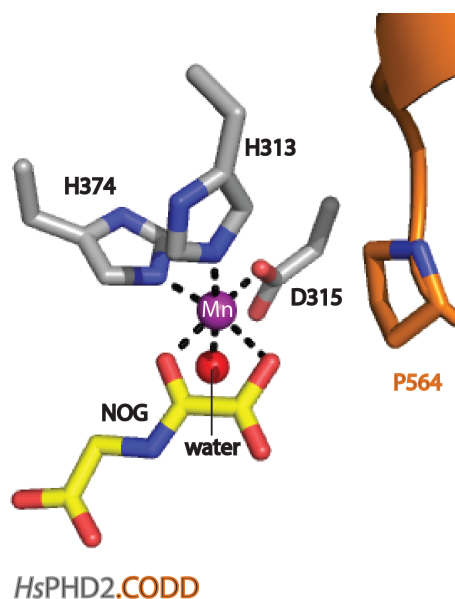


Figure 1.6: View of the active site from the crystal structure of PHD2 with its substrate bound. Active site arrangement in the *Homo sapiens* PHD2:Mn(II):NOG:HIF1 α CODD complex (referred to as *Hs*PHD2.CODD, PDB:3HQR, (50)). The metal ion (manganese substituting for iron, purple sphere) is octahedrally coordinated by a triad of residues (H313_{PHD2}, D315_{PHD2}, and H374_{PHD2}), *N*-oxalylglycine (NOG, substituting for 2OG), and a water molecule (red sphere). The stable metal-water coordination is proposed to enable the oxygen sensing ability in PHD2 (50).

2OG-dependent oxygenases employ a consensus mechanism, that was first proposed in 1982 (53). Spectroscopic studies on the taurine 2OG dioxygenase (tauD) lead to the direct detection of reaction intermediates, providing further insight into the mechanism of 2OG oxygenases (54-61). **Figure 1.7** shows a (simplified) outline of the conserved mechanism, with the most important features including:

- (A) Upon binding of 2OG to the enzyme, a ternary complex is formed (enzyme:Fe(II):2OG), in which 2OG is coordinated to the metal ion in a bidentate manner.
- (B) Binding of the substrate leads to the generation of an open coordination site on Fe(II) (62).
- (C) The last metal-coordinated water molecule is displaced upon binding of oxygen to Fe(II).
- (D) - (E) Oxidative decarboxylation of 2OG yields a reactive Fe(IV)=O intermediate.
- (F) The ferryl intermediate promotes the hydroxylation of the substrate via a radical mechanism.
- (G) Succinate is released.

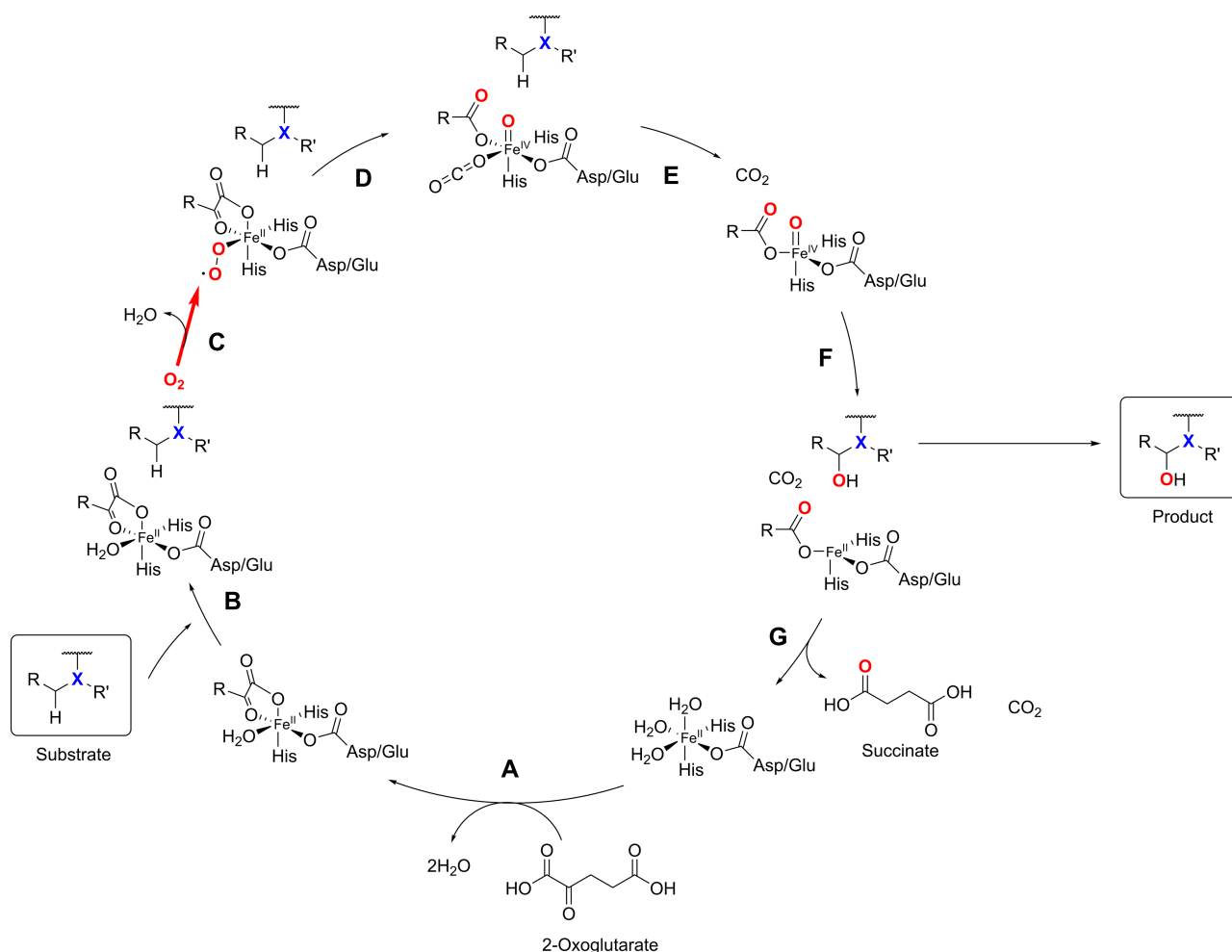


Figure 1.7: Outline of the 2OG oxygenase mechanism (adopted from Richard Hopkinson, University of Leicester, UK). Within the active site, Fe(II) is initially bound in a 6-coordinate manner by a conserved HX(D/E)...H motif and three water molecules, two of which get displaced upon binding of 2-oxoglutarate (2OG, **A**). Subsequent binding of the substrate (**B**) and oxygen (**C**) leads to the displacement of the third water molecule. Note that binding of oxygen to the active site Fe(II) in *Hs*PHD2 has been proposed to be the rate limiting step of catalysis (51). Oxidative decarboxylation of 2OG generates CO₂ and succinate, and leads to the formation of a reactive Fe(IV)=O intermediate, which promotes hydroxylation of the substrate (**D-G**).

Biochemical studies on the PHDs revealed unique kinetic properties of the enzyme (compared to other 2OG oxygenases), that are central to their oxygen sensing roles. The PHDs have been reported to have a low affinity to oxygen as judged by *K_m* values, which are substantially above physiological oxygen levels (*K_m*(PHD2, O₂) > 400 μM, physiological oxygen concentrations ~100 μM, (39, 63-65), allowing the enzyme to react to small changes in cellular oxygen levels. Moreover, studies *in vivo* have shown a graded dependence of PHD-catalysed HIF-hydroxylation on oxygen concentrations (20); by contrast, the second HIF hydroxylase, FIH, was found to be less sensitive to changes in oxygen levels. In addition, there is evidence that the human PHDs form a stable PHD:Fe(II):2OG complex, and are less sensitive to changes in iron or 2OG availability (66). Together with structural features in the active site that allow for slow binding of oxygen to Fe(II) (as discussed above), these findings give insights into how PHDs achieve their 'oxygen sensing' capability.

Crystal structures of PHD2 in complex with Mn(II) (substituting for Fe(II)) and inert 2OG analogues reveal that the enzyme contains the conserved double-stranded β-helix core-fold (DSBH, or “jellyroll”-motif), which is present in all characterised 2OG dependent oxygenases (**Figure 1.8**, (49, 67)). The DSBH is composed of a major (βI, βVIII, βIII, βVI) and a minor (βII, βVII, βIV, βV) β-sheet, and the metal- and 2OG-binding sites are sandwiched between the two β-sheets. The major β-sheet core is stabilised by three α-helices (α1-3). Notably, the active site appears deeply embedded within the β-sheet core, when compared with other 2OG oxygenases, such as FIH (see **Figure 1.10 A and B**). The buried nature of the active site in PHD2 may rationalise the observed formation of a relatively stable PHD2:Fe(II):2OG complex (66).

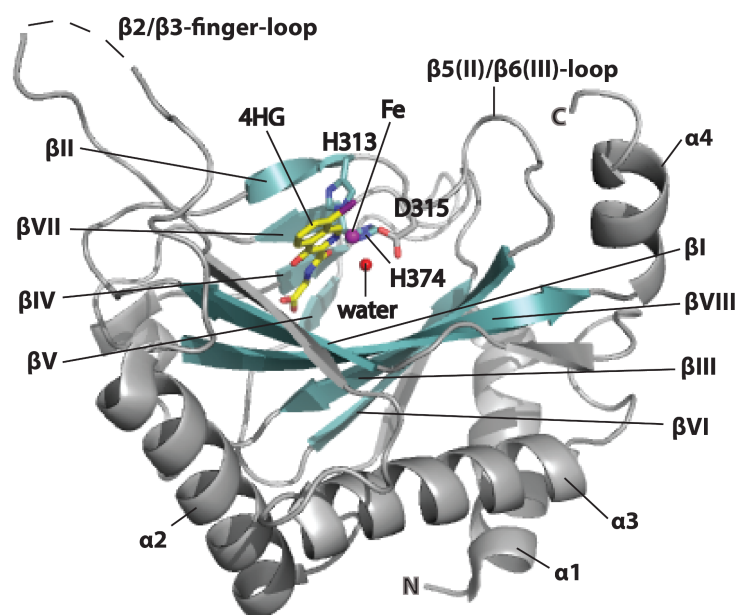


Figure 1.8: Views from the crystal structure of PHD2 in complex with Fe(II) and N-[(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]glycine (4HG, substituting for 2OG) (PDB:2G1M, (49)). Secondary structural elements in PHD2 comprise four α -helices and ten β -strands of which eight form the double-stranded β -helix core fold (DSBH, cyan, Roman numerals).

Structures of PHD2 in complex with its peptidic substrates HIF1 α CODD and HIF1 α NODD give insights into the substrate-binding mode of PHD2. The main substrate binding elements are comprised of the active site containing groove, the β 2/ β 3-finger-loop, and the C-terminal α 4-helix (**Figure 1.9**, (50, 68)). In both the PHD2.CODD and PHD2.NODD enzyme-substrate complex structures, the β 2- β 3 residues (observed to be partially disordered in the substrate-unbound structure), folds to form a loop that encloses the substrate at the active site (50, 68). Various studies suggest that the β 2/ β 3-finger-loop is not only important for substrate recognition, but it also plays a significant role in determining NODD/CODD selectivity in PHDs (50, 68, 69).

In addition to the β 2/ β 3-finger-loop, the C-terminal α 4-helix in PHD2 was found to be crucial for substrate selectivity. In the PHD2.CODD structure, a salt-bridge between an arginine residue in the α 4-helix and an aspartate residue in HIF1 α CODD (R396_{PHD2}/D571_{HIF1 α} CODD) is observed (50). However, this salt-bridge is not present in the PHD2.NODD complex structure, where the HIF1 α NODD-peptide interacts with the C-terminal region of PHD2 via hydrophobic contacts (68).

At the active site of PHD2, the target proline residues P564_{CODD} and P406_{NODD} adopt a C-4 *endo*-conformation, with the 4*R* C-H bond that is cleaved during hydroxylation, positioned close to the metal centre (**Figure 1.9**). Upon hydroxylation by PHD2, the conformation of the pyrrolidine ring in the target proline residues is proposed to switch from C-4 *endo* to C-4 *exo* (70), and structural studies have shown that the C-4 hydroxylated proline adopts the C-4 *exo* conformation when bound to pVHL (**Figure 1.4**, (34-36, 70)).

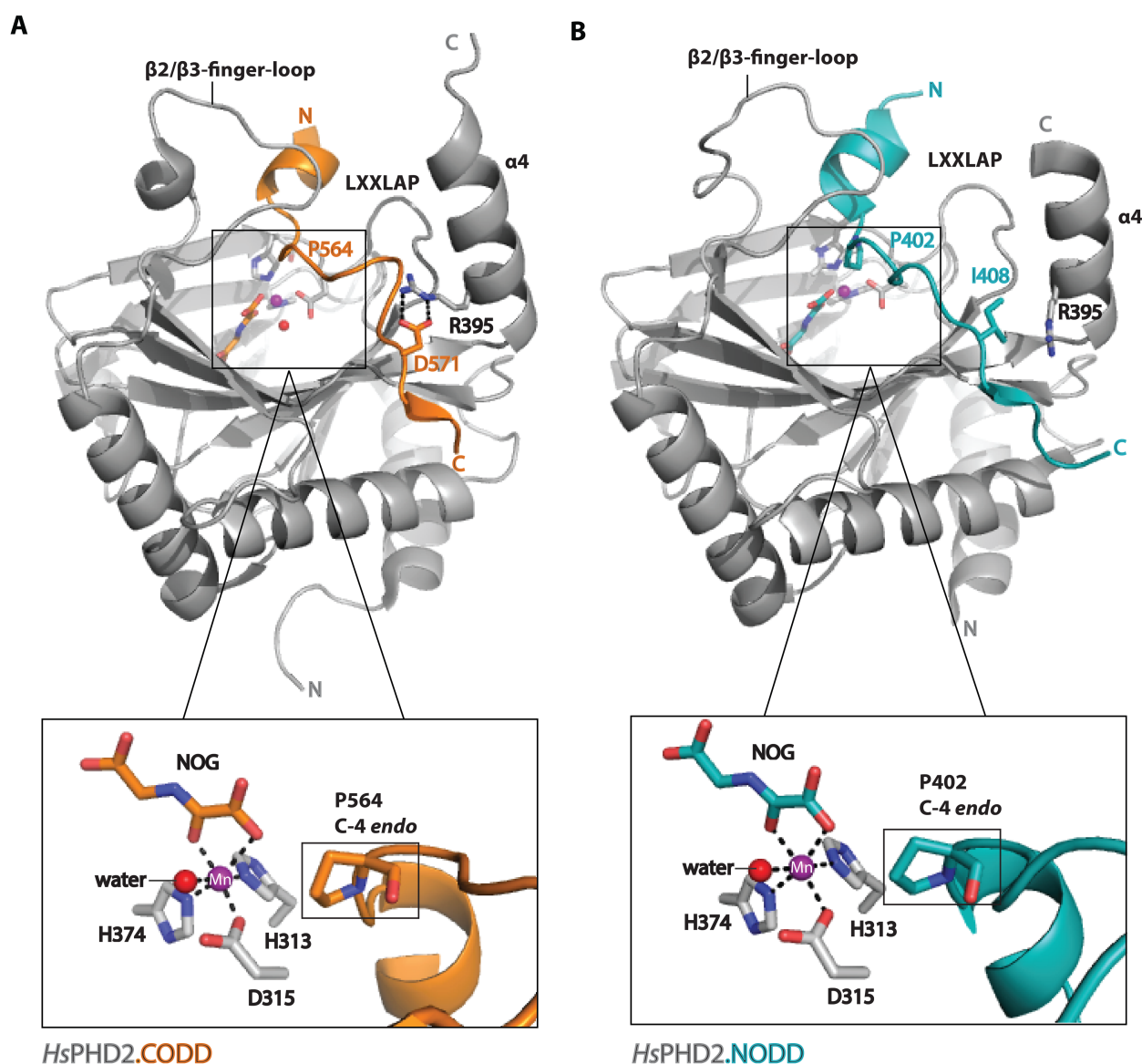


Figure 1.9: Comparison of ODD binding modes in PHD2.CODD and PHD2.NODD substrate structures. (A) PHD2.CODD (PDB:3HQY, (50)), and **(B)** PHD2.NODD (PDB:5L9V, (68)). Major differences in the substrate binding modes are found in the flexible β2/β3-finger-loop and at the C-terminal binding interface. The active site close-ups show that the target proline residues P564_{CODD} and P405_{NODD} adopt a C-4 *endo*-conformation.

To date, no structural information is available for PHD3; however, recently the structure of PHD1 in complex with Fe(II) and the inhibitor 1,2,4-triazolo-(1,5- α)pyridine (8UY) has been solved (**Figure 1.10 A**, (71)). The overall structure of PHD1 contains the conserved double-stranded β -helix core-fold (DSBH, or “jellyroll”-motif), and the major β -sheet core is stabilised by three α -helices. As with PHD2, the β 2/ β 3-finger-loop is partially disordered in the PHD1 structure without bound substrate. Notably, the region C-terminal of helix α 4, which has not been observed in any of the PHD2 structures to date, is visible in the PHD1 structure, where it forms an additional short α -helix. The position of the C-terminus in PHD1 overlaps with that of HIF1 α CODD in the PHD2.CODD substrate-complex structure (**Figure 1.10 A**), suggesting that the region C-terminal of helix α 4 needs to be displaced upon substrate binding to PHD1. Considering the position of the C-terminus in PHD1, it is likely that this region forms contacts to HIF α in the substrate-bound state, and thus plays a role in substrate recognition by PHD1 (the same is possibly the case for PHD2/PHD3). In the active site of PHD1, a metal-coordinated water molecule is present, positioned at the same coordination site as previously observed for PHD2, although more distant to the metal centre than is the case for PHD2 (distance (Fe-O₂, PHD1) = 2.7 Å, distance (Mn-O₂, PHD2) = 2.2 Å); note that in FIH this position is occupied by 2OG/NOG (**Figure 1.10 B**, (72)). By contrast to the bidentate binding mode observed with most 2OG analogues, the inhibitor 8UY in the PHD1 structure coordinates in a monodentate manner to the metal ion. Overall, the arrangement in the active site of PHD1 suggests that major structural features necessary for its oxygen sensing role are present in the enzyme.

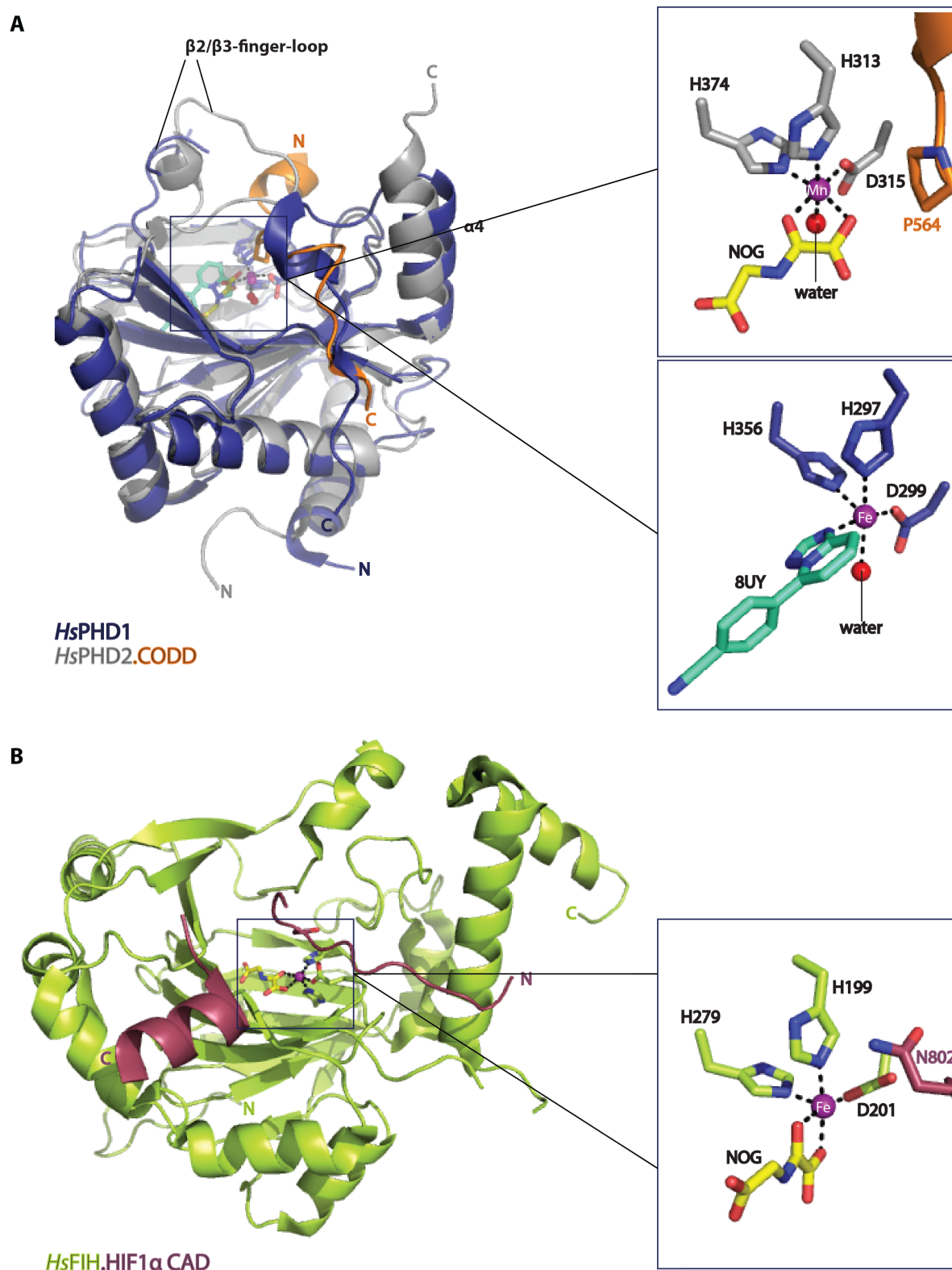


Figure 1.10: Comparison of the overall architecture of PHD1, PHD2, and FIH. (A) Superimposition of structural views of PHD2 in complex with Mn(II), NOG, and HIF1 α CODD (PDB:3HQR, (50)) with the structure of PHD1 in complex with Fe(II) and 1,2,4-triazolo-(1,5- α)pyridine (8UY) (PDB:5V1B, (71)). **(B)** Structure of FIH in complex with Fe(II), NOG and HIF1 α CAD (PDB:1H2K, (72)).

1.3 HIF prolyl hydroxylases as therapeutic targets

Hypoxia plays a major role in various human diseases, i.e. in the development of cancers and in cardiovascular diseases, such as ischemia, stroke, and heart attacks (73). The modulation of the HIF pathway, by either up- or down-regulating HIF activity, is therefore interesting from a medicinal perspective. Activation of the HIF transcriptional response promotes EPO production, which can be exploited for the treatment of anaemia (73). Indeed, small molecule inhibitors of the HIF prolyl hydroxylases for treatment of anaemia in chronic kidney disease are currently in the very late stage clinical trials (74).

In general, the HIF pathway can be modulated at different levels, either by targeting HIFs, pVHL, or the HIF hydroxylases FIH and PHDs. To date, most pharmaceutical approaches focused on inhibition of the latter, and a variety of PHD inhibitors have been developed both by companies and academic groups. The mode of action of most PHD inhibitors involves competition with the co-substrate 2OG (75), and various crystal structures of PHD2 in complex with 2OG analogue inhibitors give insight into the binding mode of the inhibitors to the enzyme (**Figure 1.11**, (50, 76-77)). Most of the PHD inhibitors studied so far bind to the active site of PHD2 by chelating Fe(II) in a bidentate manner, thus blocking 2OG binding to the metal centre. However, many of the 2OG analogues used for inhibition of PHDs also target other 2OG-dependent oxygenases, raising the issue for the design of more selective PHD inhibitors. It has been shown that the mode of 2OG 'side chain' binding to the respective enzyme is characteristic for each 2OG oxygenase subfamily (78), which can be exploited for the design of more selective 2OG analogue-inhibitors.

In contrast to the general use of 2OG as a co-factor, the human 2OG-dependent oxygenases catalyse reactions on a broad range of substrates (13, 48). As a consequence, differences in the substrate binding modes of the enzymes can be exploited for the design of more selective inhibitors (78-79). There is emerging evidence suggesting that different types of PHD inhibitors not only compete with 2OG, but also interfere with binding of CODD and/or NODD to PHDs. Combined crystallographic and NMR-based studies revealed that the PHD inhibitor FG-2216/IOX3 (Fibrogen) prevents binding of HIF1 α CODD to the enzyme (**Figure 1.11 B**, (68, 80)). Biophysical studies on the clinical PHD inhibitors suggest that they bind to the active site metal of the PHDs in a bidentate manner and form electrostatic interactions in the 2OG C-5 carboxyl binding pocket, thus efficiently competing with 2OG-binding (77). However, significant differences have been observed in the extents to which the inhibitors block the substrate-binding pocket in the PHDs and interfere with CODD/

NODD binding to the enzymes. In contrast to the clinical PHD inhibitor Molidustat (Bayer), that binds to PHD2 without causing any substantial effect on substrate binding, other inhibitors in clinical trials, including FG-4592 (Fibrogen), Vadadustat (Akebia Therapeutics, **Figure 1.11 C**), and GSK1278863 (GSK) were found to interfere significantly with substrate binding to PHD2, with FG-4592 exerting the strongest effect on CODD-binding (77).

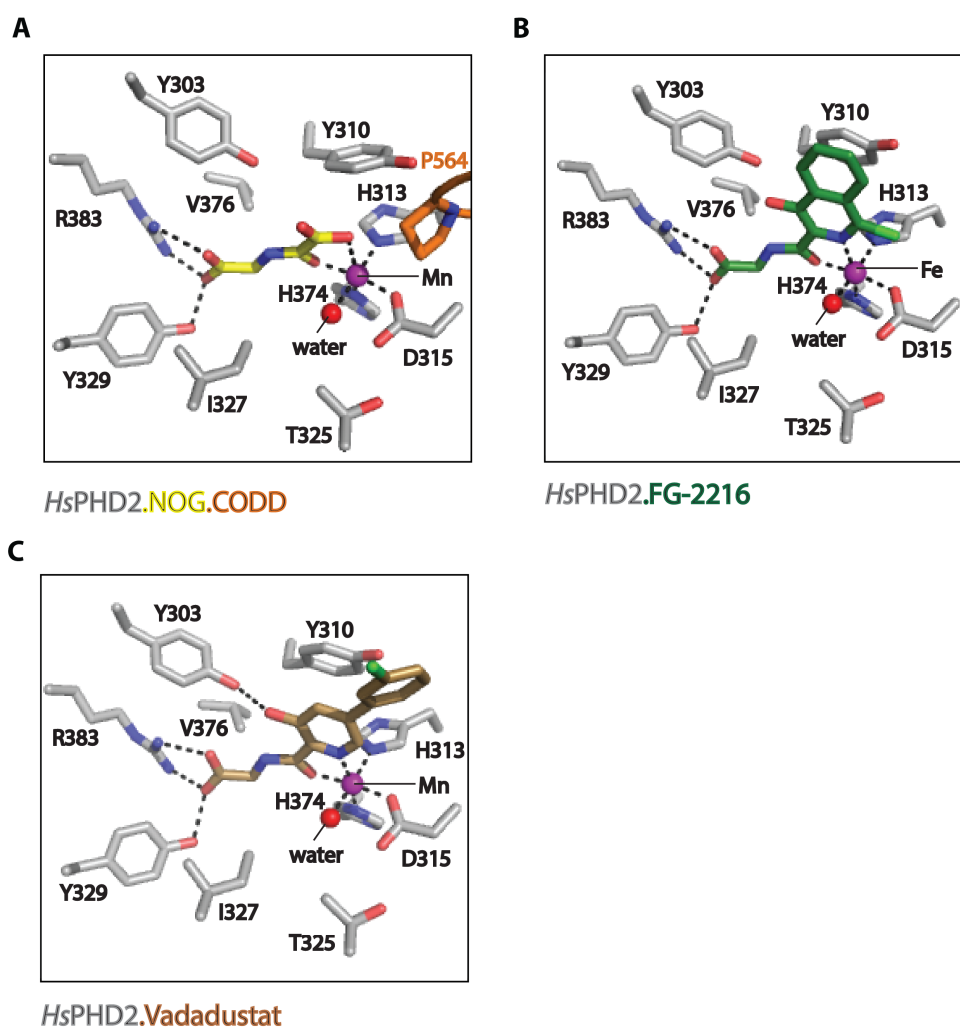


Figure 1.11: Crystallographically observed binding modes of 2OG analogues and clinical PHD inhibitors with PHD2. Views from crystal structures of PHD2 in complex with **(A)** NOG (PDB: 3HQR, (50)), **(B)** FG-2216/IOX3 (PDB:2HBT, (76)), and **(C)** Vadadustat (PDB:5OX6, (77)) reveal differences in the binding modes of the inhibitors (B-C: structures are superimposed with the PHD2.CODD enzyme-substrate complex structure (PDB:3HQR)). In contrast to NOG, that mainly acts by competing with 2OG binding to PHD2, FG-2216/IOX3 and the clinically-used inhibitor Vadadustat have a dual mode of action, both as 2OG competitors and by interfering with substrate-binding to PHD2.

Considering the emerging evidence that implies disparate roles of the three PHD isoforms in the hypoxic response, including different substrate selectivities (39-40) and differential expression of the three isoforms (41-42), the development of isoform-selective PHD inhibitors might be of great pharmaceutical interest. By use of inhibitors that selective inhibit PHD1/3 over PHD2, an effective differentiation of clinical effects may be achieved, e.g. by regulating HIF2 α versus HIF1 α (81). In this regard, the differences in the *N*-terminal regions of the PHDs are noticeable, as they might provide a route to isoform-selective inhibition of the PHDs. To date, the role of the *N*-terminal regions in the PHDs is not well understood and no structural information is available, making it difficult to design inhibitors that target the *N*-terminus of PHDs. However, the structurally observed differences in NODD/CODD binding modes to PHD2 (50, 68) could be exploited for the design of inhibitors selective for PHD3.

1.4 Non-HIF substrates of PHDs

Over the last years, there have been multiple reports that describe additional substrates of the HIF prolyl hydroxylases, other than their *bona fide* HIF substrates. With the PHDs being current targets for the therapeutic manipulation of the oxygen sensing machinery and PHD inhibitors in the late stage Phase 3 clinical trials, the validation of these reports has gained major medicinal relevance. Among the reported non-HIF substrates of PHDs are key elements of the transcription machinery, including the large subunit of RNA Polymerase II (82-83), and several transcription factors including forkhead box O3 (FOXO3a, (84)), and activating transcription factor 4 (ATF4, (85)). Other reported PHD substrates are components of signaling pathways such as the β 2-adrenergic receptor (ADRB2, (86)), erythropoietin receptor (EPOR, (87)), and thyroid hormone receptor, α (THRA, (88)). Multiple reports suggest that PHDs accept various kinases as their substrates, amongst them AKT serine/threonine kinase 1 (AKT1, (89)), dual specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A, (90)), *eukaryotic* elongation factor 2 kinase (*e*EF2K, (91-92)), and pyruvate kinase, muscle 2 (PKM2, (93)). Given the high number of non-HIF PHD substrates reported in the literature (up to date, ~ 30 reports), most of them being involved in fundamental biological pathways, the possibility arises that clinical inhibition of PHDs could lead to a disruption of these pathways, thus causing side-effects in the treated patients.

Most of the apparent non-HIF substrates have been identified in cell-based studies, by analysis of O₂-dependent changes in the cell proteome, or by co-immunoprecipitation of the substrates with the PHDs or pVHL. Further evidence for an interaction between the identified proteins and PHDs were usually gained by use of, e.g., knockdowns of PHDs, inhibition of the enzymatic activity by the 2OG oxygenase inhibitor dimethyloxalyl glycine (DMOG), or exposure of cells to hypoxia. However, some studies are lacking direct evidence for PHD-catalysed hydroxylation of the proposed substrate. In other cases, evidence for the hydroxylation of the non-HIF PHD substrates is provided by mass spectrometry, yet identification of prolyl hydroxylation by MS presents a technical challenge. Owing to widespread isobaric methionine oxidations (causing the same mass shift as hydroxylation in the MS spectra), the risk for “false positives” is high (94). In this context, the question arises of how selective PHDs are for their established HIF substrates.

Notably, the second HIF hydroxylase, factor inhibiting HIF (FIH) accepts a broad range of non-HIF substrates (95). Multiple FIH-dependent hydroxylation sites within consensus sequences corresponding to ankyrin repeat domains (ARD) have been identified in non-HIF substrates, including members of the I κ B and Notch protein families (95). ARD-containing substrates have been shown to bind to the wide substrate binding site of FIH in a similar manner as the C-terminal activation domain (CAD) of HIF1 α that contains the target asparagyl residue (see **Figure 1.10 B**, (96)) Unlike PHDs, FIH catalyses the hydroxylation of various amino acids, including His, Leu, and Ser residues (95-97). The highly promiscuous FIH and the PHDs belong to structurally distinct 2OG oxygenase subfamilies, with FIH being part of the Jumonji-C (JmjC) 2OG oxygenase subfamily IX (67, 72), and the PHDs belonging to the PH (prolyl hydroxylase) subfamily VIII (67) (**Figure 1.12**).

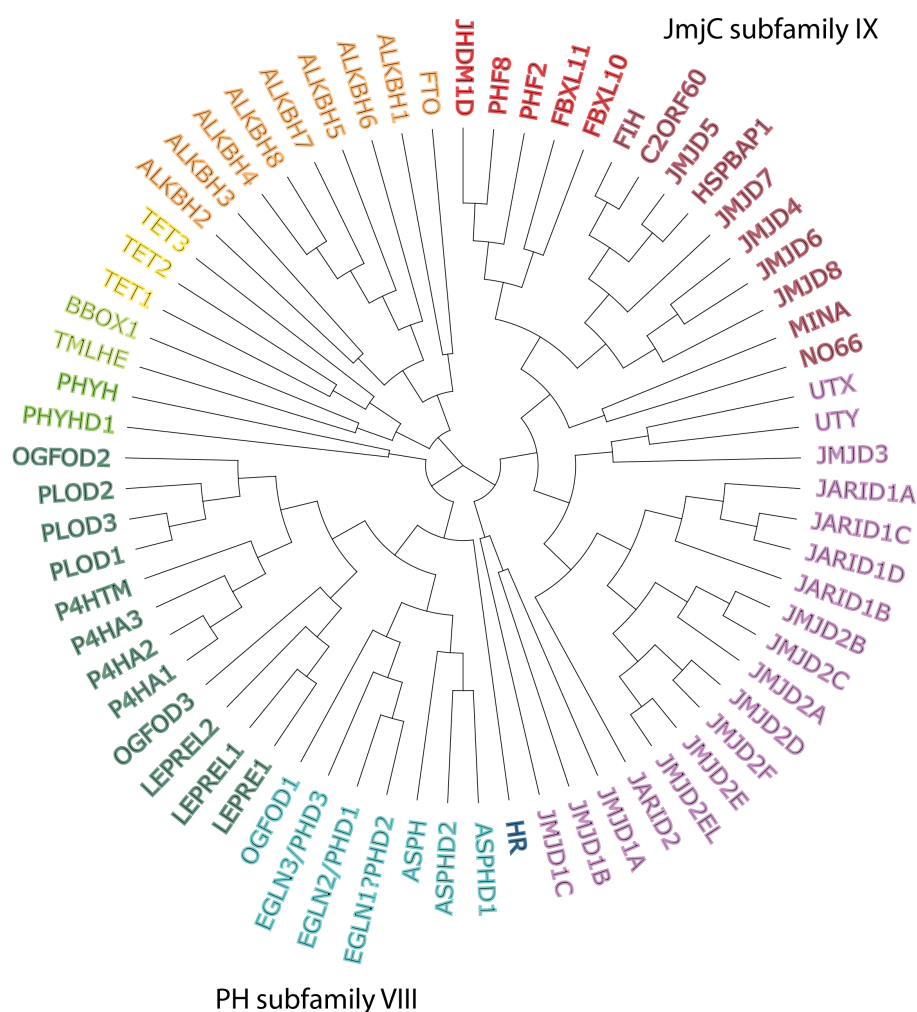


Figure 1.12: 2OG dependent oxygenases family tree. 2OG oxygenases were clustered into distinct subfamilies by bioinformatic analysis of their catalytic domains (Figure adopted from Christoph Loenarz, Universität Freiburg, Germany).

1.5 Evolution of the HIF pathway in animals and lower organisms

During the history of earth, starting 4500 million years ago with the formation of the planet, the composition of the terrestrial atmosphere has varied greatly, and changing oxygen levels in the atmosphere had a significant impact on the development of life on earth (**Figure 1.13**, 98-102). The first primitive form of life developed from a single source within the first 500 million years after formation of the planet, marking the starting point for the evolution of single-celled prokaryotes (102-103). With the development of cyanobacteria 3000 million years ago, the first (known) photosynthetic organisms evolved, thus generating molecular oxygen (102, 104). The photosynthetically-produced oxygen initially reacted with inorganic minerals, such as iron, and it took until the reserves of readily-oxidised material on the surface of earth were depleted, before oxygen accumulated in the lower terrestrial atmosphere (102).

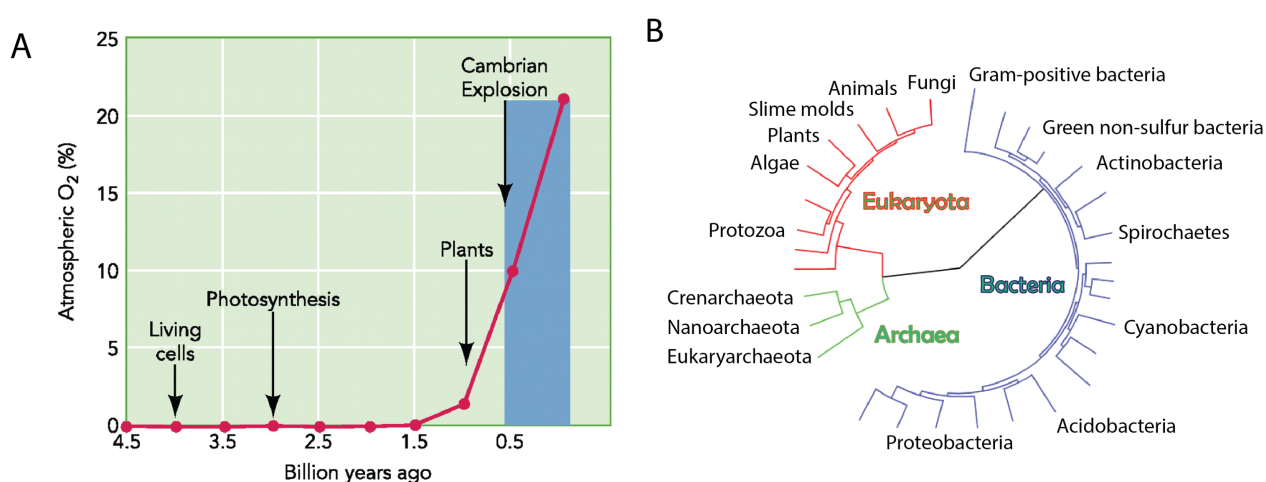


Figure 1.13: Evolution of life. (A) Changes in atmospheric oxygen levels and their impact on evolution of complex life forms (figure adapted from (102)). **(B)** Phylogenetic tree (figure modified from (105)) showing the three domains of life, archaea, bacteria, and eukaryota.

Initially, the reactive properties of oxygen proved lethal for most of the anaerobic prokaryotes inhabiting the planet. However, it fueled the evolution of more complex, eukaryotic organisms, which were formed by symbiosis between different kinds of bacterial cells (102). Eukaryotes were shielded from the harmful effects of molecular oxygen by compartmentalisation; moreover, they evolved mechanism to utilise oxygen for the efficient production of ATP (102, 106). Between 550

and 600 million years ago, the oxygen levels in the atmosphere reached 15-20 %, leading to the 'Cambrian explosion', i. e. the onset of a rapid evolution and diversification of multicellular, differentiated life forms, such as metazoans (99, 102).

With the evolution of aerobic lifeforms, that rely on a steady oxygen supply for their energy metabolism, the development of hypoxia sensing mechanisms likely became a major factor of evolutionary selection (102). Hypoxia sensing mechanisms have been identified in various organisms, including *Escherichia coli*, where the balance between anaerobic and aerobic metabolism is regulated by the fumarate and nitrate reduction (FNR) pathway (107). In the single-celled eukaryote *Saccharomyces cerevisiae*, components of aerobic metabolism are regulated by the Hap1 pathway (108), and in the slime mold *Dictyostelium discoideum*, the PHD-ortholog prolyl-4-hydroxylase (*DdP4H* or *DdPhyA*) was identified, that catalyses the hydroxylation of the S-phase kinase-associated protein 1 (Skp1), playing a role in the oxygen-dependent development of *D. discoideum* (109).

However, the available evidence is that only humans and other animals use the HIF pathway for hypoxia sensing, as bioinformatic studies suggest that HIF is not present in organisms lower than animals (though homologues of the PHDs are present in earlier organisms (110-112)). Despite the apparent conservation of the HIF pathway in all animals, the HIF system is much less complex in non-bilateria (animals without bilateral symmetry such as 'head' and 'tail') and non-vertebrates, than in the higher evolved vertebrates and mammals (**Figure 1.14**, 112). Most of the organisms that developed before the gene duplication event took place only possess one copy of a HIF α gene and one PHD as determined by bioinformatic analysis, whereas multiple HIF α and PHD isoforms are present in vertebrates and mammals (112).

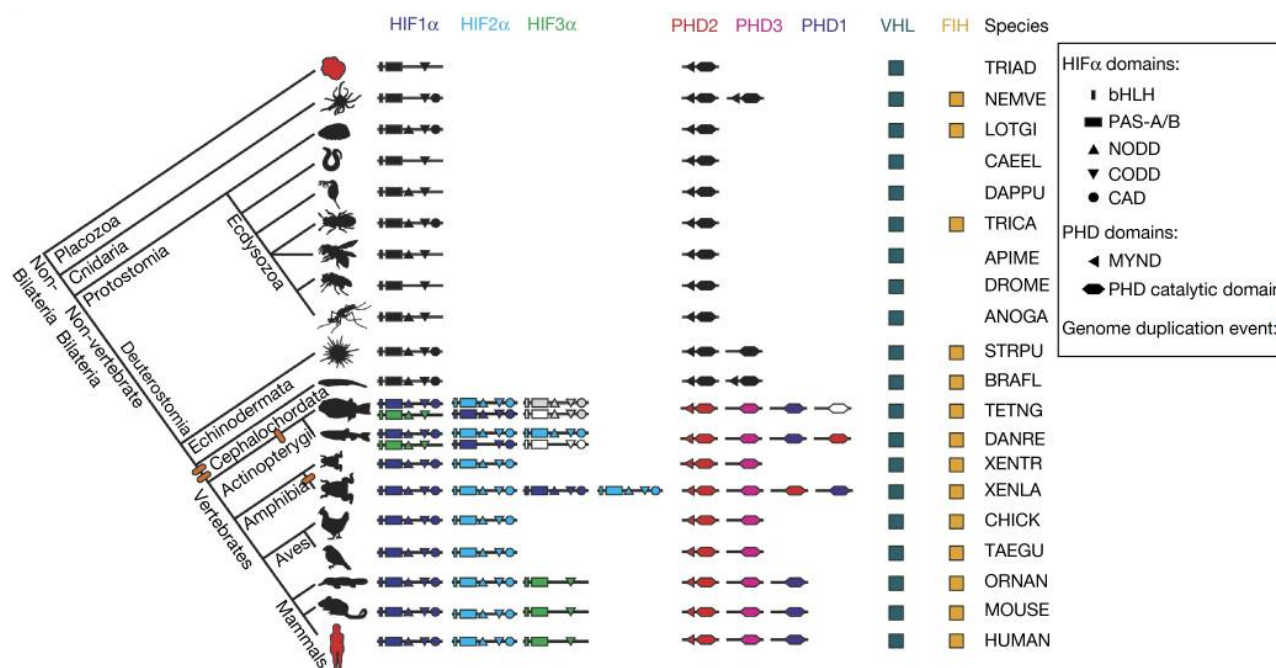


Figure 1.14: Bioinformatic analysis of the HIF pathway (Figure adapted from (112)). The available evidence is that the HIF pathway is apparently conserved in animals, but is unique to this kingdom. While most non-bilateria and non-vertebrates only have one copy of HIF α and one PHD, there are multiple isoforms of HIF α and PHDs present in higher animals, such as vertebrates and mammals, that developed after the gene duplication event took place. The simplest animal, the plactozoan *Trichoplax adhaerens* is marked red.

The simplest existent animal, the placozoan *Trichoplax adhaerens*, is a flat, multicellular organism of about 1 mm diameter. It consists of five different cell-types, and lacks any symmetry or internal structures, such as organs (113). The natural habitat of *T. adhaerens* is found in seawater, where the animal feeds on small algae. (113) A functional HIF-system in *T. adhaerens*, composed of one HIF α isoform with one single ODD and one single PHD isoform (*TaPHD*), has been demonstrated (**Figure 1.15**, (112)). Whereas the single ODD in *T. adhaerens* more closely resembles the sequences of CODDs than the one of NODDs, the sequence of *TaPHD* suggest that it is closely related to the human PHD2 (112). The study of components of the HIF pathway in *T. adhaerens* is of great interest from a basic science perspective, as it might provide insights into the evolution of oxygen sensing in animals.

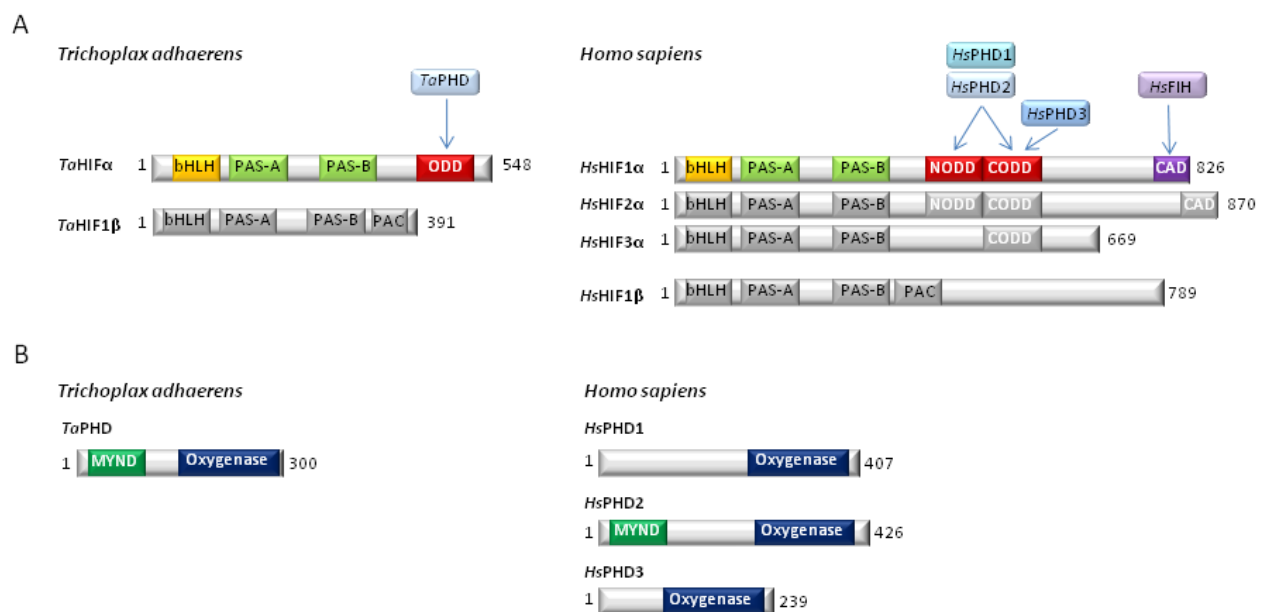


Figure 1.15: Comparison of components of the HIF system in *T. adhaerens* and humans. (A) Domain structures of HIF α and HIF β in *T. adhaerens* and humans. Arrows indicate prolyl- and asparaginyl-hydroxylation sites. Domain acronyms: oxygen-dependent degradation (ODD) domain, basic helix-loop-helix (bHLH) motif, Per-ARNT-Sim (PAS) domain, C-terminal transactivation domain (CAD), PAS-associated C-terminal (PAC) domain. **(B)** Domain structures of HIF prolyl hydroxylases (PHDs) in *T. adhaerens* and humans. Domain acronyms: 2OG-dioxygenase domain (oxygenase), myeloid, Nervy, and DEAF-1 (MYND)-type zinc finger domain (MYND).

By contrast to HIFs, that (apparently) only exist in animals, PHD-like enzymes have been found in organisms ranging from early eukaryotes, such as the slime mold *Dicystelium discoideum*, to prokaryotes such as *Pseudomonas* (**Figure 1.16**).

Organism	<i>P. aeruginosa</i>	<i>D.discoideum</i>	<i>M. brevicollis</i>	<i>T. adhaerens</i>	<i>H. sapiens</i>
Prolyl-4-hydroxylase	PPHD	DdP4H	MbP4H	TaPHD	PHD1,2,3
Substrate	EF-Tu	DdSkp1	?	TaHIFα	HIFα
		Amoebozoa	Choanoflagellata	Animals	
	Eubacteria	Archaea	Eukaryota		

Figure 1.16: Overview of PHDs and PHD-related enzymes in different domains and kingdoms of life.

In various *Pseudomonas* strains, a PHD-related enzyme, the *Pseudomonas* prolyl hydroxylase domain containing protein (PPHD), catalyses the hydroxylation of elongation factor Tu (EF-Tu, (110)). A crystal structure of the PPHD from *Pseudomonas putida*, complexed with its protein substrate EF-Tu, reveals striking similarities in the active site and in the substrate binding modes between PPHD and human PHD2 (*HsPHD2*, (110)). The biological function of the hydroxylation in EF-Tu remains to be elucidated; however a *Pseudomonas aeruginosa* PPHD knockout mutant has been associated with increased production of pyocyanin, the virulence factor that plays a role in lung infections caused by *P. aeruginosa* (110).

In the unicellular eukaryote *D. discoideum*, a role of the PHD-related *DdPhyA* (*DdP4H*) in prolyl-4-hydroxylation of S-phase kinase associated protein 1 (Skp1) has been reported (111). The hydroxyprolyl residue in Skp1 subsequently is modified by addition of five sugars, a reaction that is catalysed by three different glycosyltransferases (114-116). It has been demonstrated that the hydroxylation and glycosylation of Skp1 is essential for development of *D. discoideum*, which is ultra-sensitive to oxygen levels (**Figure 1.17**, 117-119).

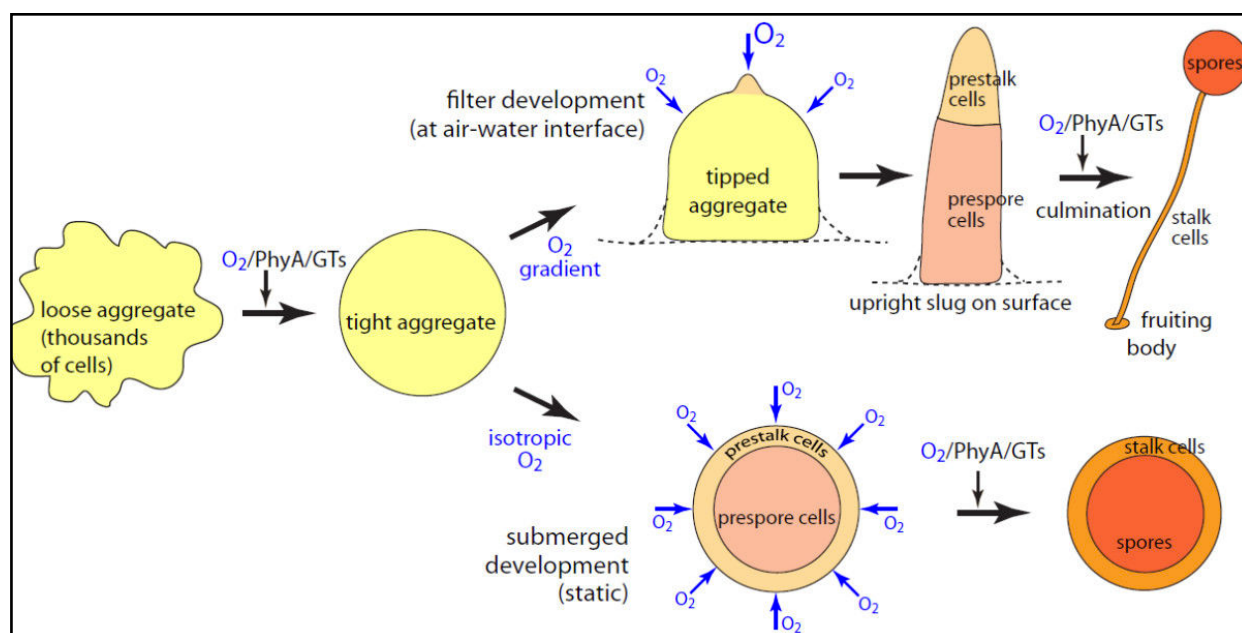


Figure 1.17: Model of the O_2 -dependent, Skp1-mediated development of the slime mold *Dictyostelium discoideum* (figure adopted from (118)). Acronyms: Prolyl-4-hydroxylase from *D. discoideum* (PhyA), glycosyltransferases (GTs).

Under starvation, the normally solitary slime mold forms a multicellular aggregate, that migrates through the soil until it reaches the surface, where it culminates into a fruiting body (119). Culmination is triggered by high oxygen levels, and genetic manipulation of Skp1 and DdP4H suggests that the oxygen requirement for culmination is mediated by the Skp1-modification pathway (118). The Skp1 pathway also exists in the human parasite *Toxoplasma gondii*, where hydroxylation (and subsequent glycosylation) of Skp1 by the PHD-related enzyme TgPhyA has been associated with optimal parasite growth (120).

The choanoflagellate *Monosiga brevicollis* is widely considered as one of the closest relatives to metazoans (121). The study of this organism therefore presents a great chance to gain insights into the evolution of metazoan life. Bioinformatic analyses of the *M. brevicollis* genome identified the sequence of a potential prolyl-4-hydroxylase (*MbP4H*) that is related to the sequences of TaPHD and other animal PHDs (122). Although there is no HIF present in the *M. brevicollis* genome, it was shown that recombinantly produced *MbP4H* catalyses the hydroxylation of a peptide containing the ODD of *Trichoplax adhaerens* HIF α (122). To date, the natural substrate of *MbP4H* has not been identified, yet it might provide another link in the evolution of oxygen sensing in animals by the HIF-system.

1.6 Objectives of the work described in this thesis

Extensive studies on the HIF system provided a detailed understanding on the mechanisms that underlie oxygen sensing in humans and other animals, and established PHDs as major targets for the therapeutic manipulation of the HIF pathway. However, emerging evidence suggests that PHDs accept substrates other than their well-established HIF-substrates, implying an increased risk of side-effects for patients treated with PHD inhibitors. Moreover, it is desirable to improve the selectivity profile of current-generation PHD inhibitors against different PHD isoforms and other 2OG oxygenases. Therefore, two objectives of the work described in this thesis were:

1. Investigations on the substrate selectivity of human PHDs, including validation of reported non-HIF substrates and substrate binding studies on PHDs and pVHL (**Chapter 2**).
2. Inhibition studies on the human PHD isoform 1-3, aiming to establish screening methods that allow the detection of isoform-selective inhibitors (**Chapter 3**).

Mutations in the Egl nine homolog 1 (EGLN1) gene encoding for PHD2 have been associated with erythrocytosis (123). However, the exact role of different PHD2 variants in the development of disease is poorly understood. Therefore, the third aim of this thesis was:

3. To conduct structural and biophysical studies on clinically observed variants of human PHD2. The goal of this study was to investigate to what extent mutations in the EGLN1 gene affect the structure, activity, or substrate binding mode of *Hs*PHD2 (**Chapter 4**).

The development of hypoxia sensing mechanisms likely played a major role in the evolution of aerobic lifeforms (102). Two objectives of the work described in this thesis on this topic were:

4. To generate an understanding of the evolution of HIF prolyl hydroxylases in animals ranging from humans to *Trichoplax adhaerens*. Comparative kinetic and mechanistic studies on *Hs*PHD2 and *Ta*PHD were performed (**Chapter 5**).
5. Exploring biochemical evolutionary links between human HIF prolyl hydroxylases and the Skp1-mediated oxygen sensing mechanisms in *Dicyostelium discoideum* and *Toxoplasma gondii*. Interaction studies between *Hs*PHD2 and *Hs*Skp1 were carried out (**Chapter 6**).

1.7 References

- (1) Conway Morris, S. (2006). Darwin's dilemma: the realities of the Cambrian "explosion." *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1470), 1069–1083.
- (2) Michiels, C. (2004). Physiological and Pathological Responses to Hypoxia. *The American Journal of Pathology*, 164(6), 1875–1882.
- (3) Semenza, G. L. (2001). Hypoxia-Inducible Factor 1: Control of Oxygen Homeostasis in Health and Disease. *Pediatric Research*, 49(5), 614–617.
- (4) Bert, P. (1882). Sur la richesse en hémoglobine du sang des animaux vivant sur les haux lieux. *Comptes Rendus de l'Académie des Sciences Paris*, 94, 805–807.
- (5) Semenza, G. L., Nejfelt, M. K., Chi, S. M., & Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proceedings of the National Academy of Sciences of the United States of America*, 88(13), 5680–5684.
- (6) Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology*, 12(12), 5447–5454.
- (7) Semenza, G. L., Jiang, B.-H., Leung, S. W., Passantino, R., Concordet, J.-P., Maire, P., & Giallongo, A. (1996). Hypoxia Response Elements in the Aldolase A, Enolase 1, and Lactate Dehydrogenase A Gene Promoters Contain Essential Binding Sites for Hypoxia-inducible Factor 1. *Journal of Biological Chemistry*, 271(51), 32529–32537.
- (8) Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., ... Livingston, D. M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*, 93(23), 12969–12973.
- (9) Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., & Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1 α . *The EMBO Journal*, 17(22), 6573–6586.
- (10) Maxwell, P. H., Pugh, C. W., & Ratcliffe, P. J. (1993). Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, 90(6), 2423–2427.
- (11) Schofield, C. J., & Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. *Nature Reviews Molecular Cell Biology*, 5(5), 343–354.
- (12) Semenza, G. L. (2007). Hypoxia-Inducible Factor 1 (HIF-1) Pathway. *Science Signaling*, 2007(407), pp. cm8.

- (13) Hausinger, R. P. (2015). CHAPTER 1 Biochemical Diversity of 2-Oxoglutarate-Dependent Oxygenases. In 2-Oxoglutarate-Dependent Oxygenases (pp. 1–58). The Royal Society of Chemistry.
- (14) Chowdhury, R., Hardy, A., & Schofield, C. J. (2008). The human oxygen sensing machinery and its manipulation. *Chemical Society Reviews*, 37(7), 1308–1319.
- (15) Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., ... Kaelin Jr., W. G. (2001). HIF α Targeted for VHL-Mediated Destruction by Proline Hydroxylation: Implications for O₂ Sensing. *Science*, 292(5516), 464–468.
- (16) Jaakkola, P., Mole, D. R., Tian, Y.-M., Wilson, M. I., Gielbert, J., Gaskell, S. J., ... Ratcliffe, P. J. (2001). Targeting of HIF- α to the von Hippel-Lindau Ubiquitylation Complex by O₂-Regulated Prolyl Hydroxylation. *Science*, 292(5516), 468–472.
- (17) Maxwell, P. H., Wiesener, M. S., Chang, G.-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., ... Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, 399(6733), 271–275.
- (18) Hewitson, K. S., McNeill, L. A., Riordan, M. V, Tian, Y.-M., Bullock, A. N., Welford, R. W., ... Schofield, C. J. (2002). Hypoxia-inducible Factor (HIF) Asparagine Hydroxylase Is Identical to Factor Inhibiting HIF (FIH) and Is Related to the Cupin Structural Family. *Journal of Biological Chemistry*, 277(29), 26351–26355.
- (19) Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., & Whitelaw, M. L. (2002). Asparagine Hydroxylation of the HIF Transactivation Domain: A Hypoxic Switch. *Science*, 295(5556), 858–861.
- (20) Tian, Y.-M., Yeoh, K. K., Lee, M. K., Eriksson, T., Kessler, B. M., Kramer, H. B., ... Ratcliffe, P. J. (2011). Differential Sensitivity of Hypoxia Inducible Factor Hydroxylation Sites to Hypoxia and Hydroxylase Inhibitors. *The Journal of Biological Chemistry*, 286(15), 13041–13051.
- (21) Koivunen, P., Hirsilä, M., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2004). Catalytic Properties of the Asparaginyl Hydroxylase (FIH) in the Oxygen Sensing Pathway Are Distinct from Those of Its Prolyl 4-Hydroxylases. *Journal of Biological Chemistry*, 279(11), 9899–9904.
- (22) Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., & Ratcliffe, P. J. (2001). Independent function of two destruction domains in hypoxia-inducible factor- α chains activated by prolyl hydroxylation. *The EMBO Journal*, 20(18), 5197–5206.
- (23) Ratcliffe, P. J. (2007). HIF-1 and HIF-2: working alone or together in hypoxia? *Journal of Clinical Investigation*, 117(4), 862–865.
- (24) Biswas, S., Troy, H., Leek, R., Chung, Y.-L., Li, J., Raval, R. R., ... Harris, A. L. (2010). Effects of HIF-1 α and HIF2 α on Growth and Metabolism of Clear-Cell Renal Cell Carcinoma 786-0 Xenografts. *Journal of Oncology*, 2010, 757908.

- (25) Loboda, A., Jozkowicz, A., & Dulak, J. (2012). HIF-1 versus HIF-2 — Is one more important than the other? *Vascular Pharmacology*, 56(5), 245–251.
- (26) Kim, J., Tchernyshyov, I., Semenza, G. L., & Dang, C. V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*, 3(3), 177–185.
- (27) Kapitsinou, P. P., Liu, Q., Unger, T. L., Rha, J., Davidoff, O., Keith, B., ... Haase, V. H. (2010). Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. *Blood*, 116(16), 3039–3048.
- (28) Keith, B., Johnson, R. S., & Simon, M. C. (2011). HIF1 α and HIF2 α : sibling rivalry in hypoxic tumor growth and progression. *Nature Reviews. Cancer*, 12(1), 9–22.
- (29) Heikkilä, M., Pasanen, A., Kivirikko, K. I., & Myllyharju, J. (2011). Roles of the human hypoxia-inducible factor (HIF)-3 α variants in the hypoxia response. *Cellular and Molecular Life Sciences*, 68(23), 3885–3901.
- (30) Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., ... Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature*, 414(6863), 550–554.
- (31) Yamashita, T., Ohneda, O., Nagano, M., Iemitsu, M., Makino, Y., Tanaka, H., ... Yamamoto, M. (2008). Abnormal Heart Development and Lung Remodeling in Mice Lacking the Hypoxia-Inducible Factor-Related Basic Helix-Loop-Helix PAS Protein NEPAS. *Molecular and Cellular Biology*, 28(4), 1285–1297.
- (32) Cardote, T. A. F., Gadd, M. S., & Ciulli, A. (2017). Crystal Structure of the Cul2-Rbx1-EloBC-VHL Ubiquitin Ligase Complex. *Structure*, 25(6), 901–911.
- (33) Srinivas, V., Zhang, L.-P., Zhu, X.-H., & Caro, J. (1999). Characterization of an Oxygen/Redox-Dependent Degradation Domain of Hypoxia-Inducible Factor α (HIF- α) Proteins. *Biochemical and Biophysical Research Communications*, 260(2), 557–561.
- (34) Hon, W.-C., Wilson, M. I., Harlos, K., Claridge, T. D. W., Schofield, C. J., Pugh, C. W., ... Jones, E. Y. (2002). Structural basis for the recognition of hydroxyproline in HIF-1[α] by pVHL. *Nature*, 417(6892), 975–978.
- (35) Illingworth, C. J. R., Loenarz, C., Schofield, C. J., & Domene, C. (2010). Chemical Basis for the Selectivity of the von Hippel Lindau Tumor Suppressor pVHL for Prolyl-Hydroxylated HIF-1 α . *Biochemistry*, 49(32), 6936–6944.
- (36) Min, J.-H., Yang, H., Ivan, M., Gertler, F., Kaelin, W. G., & Pavletich, N. P. (2002). Structure of an HIF-1 α -pVHL Complex: Hydroxyproline Recognition in Signaling. *Science*, 296(5574), 1886–1889.

- (37) Epstein, A. C. R., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., ... Ratcliffe, P. J. (2017). *C. elegans* EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation. *Cell*, 107(1), 43–54.
- (38) Bruick, R. K., & McKnight, S. L. (2001). A Conserved Family of Prolyl-4-Hydroxylases That Modify HIF. *Science*, 294(5545), 1337–1340.
- (39) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (40) Huang, J., Zhao, Q., Mooney, S. M., & Lee, F. S. (2002). Sequence Determinants in Hypoxia-inducible Factor-1 α for Hydroxylation by the Prolyl Hydroxylases PHD1, PHD2, and PHD3. *Journal of Biological Chemistry*, 277(42), 39792–39800.
- (41) Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R., & Taubman, M. B. (2002). Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. *Biochemistry and Cell Biology*, 80(4), 421–426.
- (42) Willam, C., Maxwell, P. H., Nichols, L., Lygate, C., Tian, Y. M., Bernhardt, W., ... Pugh, C. W. (2017). HIF prolyl hydroxylases in the rat; organ distribution and changes in expression following hypoxia and coronary artery ligation. *Journal of Molecular and Cellular Cardiology*, 41(1), 68–77.
- (43) Takeda, K., Cowan, A., & Fong, G.-H. (2007). Essential Role for Prolyl Hydroxylase Domain Protein 2 in Oxygen Homeostasis of the Adult Vascular System. *Circulation*, 116(7), 774–781.
- (44) Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., ... Fandrey, J. (2003). Intracellular localisation of human HIF-1 α hydroxylases: implications for oxygen sensing. *Journal of Cell Science*, 116(7), 1319–1326.
- (45) Song, D., Li, L.-S., Heaton-Johnson, K. J., Arsenault, P. R., Master, S. R., & Lee, F. S. (2013). Prolyl Hydroxylase Domain Protein 2 (PHD2) Binds a Pro-Xaa-Leu-Glu Motif, Linking It to the Heat Shock Protein 90 Pathway. *The Journal of Biological Chemistry*, 288(14), 9662–9674.
- (46) Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7, 539.
- (47) Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25(9), 1189–1191.
- (48) Hausinger, R. P. (2004). Fe(II)/ α -Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 39(1), 21–68.

- (49) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (50) Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., ... Schofield, C. J. (2009). Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*, 17(7), 981–989.
- (51) Flashman, E., Hoffart, L. M., Hamed, R. B., Bollinger, J. M., Krebs, C., & Schofield, C. J. (2010). Evidence for the Slow Reaction of Hypoxia-Inducible Factor Prolyl Hydroxylase 2 with Oxygen. *The FEBS Journal*, 277(19), 4089–4099.
- (52) Tarhonskaya, H., Hardy, A. P., Howe, E. A., Loik, N. D., Kramer, H. B., McCullagh, J. S. O., ... Flashman, E. (2015). Kinetic Investigations of the Role of Factor Inhibiting Hypoxia-inducible Factor (FIH) as an Oxygen Sensor. *The Journal of Biological Chemistry*, 290(32), 19726–19742.
- (53) Hanauske-Abel, H. M., & Günzler, V. (1982). A stereochemical concept for the catalytic mechanism of prolylhydroxylase: Applicability to classification and design of inhibitors. *Journal of Theoretical Biology*, 94(2), 421–455.
- (54) Price, J. C., Barr, E. W., Hoffart, L. M., Krebs, C., & Bollinger, J. M. (2005). Kinetic Dissection of the Catalytic Mechanism of Taurine:α-Ketoglutarate Dioxygenase (TauD) from *Escherichia coli*. *Biochemistry*, 44(22), 8138–8147.
- (55) Bollinger, J. M., Price, J. C., Hoffart, L. M., Barr, E. W., & Krebs, C. (2005). Mechanism of Taurine: α-Ketoglutarate Dioxygenase (TauD) from *Escherichia coli*. *European Journal of Inorganic Chemistry*, 2005(21), 4245–4254.
- (56) Price, J. C., Barr, E. W., Glass, T. E., Krebs, C., & Bollinger, J. M. (2003). Evidence for Hydrogen Abstraction from C1 of Taurine by the High-Spin Fe(IV) Intermediate Detected during Oxygen Activation by Taurine:α-Ketoglutarate Dioxygenase (TauD). *Journal of the American Chemical Society*, 125(43), 13008–13009.
- (57) Proshlyakov, D. A., Henshaw, T. F., Monterosso, G. R., Ryle, M. J., & Hausinger, R. P. (2004). Direct Detection of Oxygen Intermediates in the Non-Heme Fe Enzyme Taurine/α-Ketoglutarate Dioxygenase. *Journal of the American Chemical Society*, 126(4), 1022–1023.
- (58) Riggs-Gelasco, P. J., Price, J. C., Guyer, R. B., Brehm, J. H., Barr, E. W., Bollinger J. Martin, & Krebs, C. (2004). EXAFS Spectroscopic Evidence for an FeO Unit in the Fe(IV) Intermediate Observed during Oxygen Activation by Taurine:α-Ketoglutarate Dioxygenase. *Journal of the American Chemical Society*, 126(26), 8108–8109.

- (59) Grzyska, P. K., Appelman, E. H., Hausinger, R. P., & Proshlyakov, D. A. (2010). Insight into the mechanism of an iron dioxygenase by resolution of steps following the Fe(IV)=O species. *Proceedings of the National Academy of Sciences of the United States of America*, 107(9), 3982–3987.
- (60) Price, J. C., Barr, E. W., Tirupati, B., Bollinger J. Martin, & Krebs, C. (2003). The First Direct Characterization of a High-Valent Iron Intermediate in the Reaction of an α -Ketoglutarate-Dependent Dioxygenase: A High-Spin Fe(IV) Complex in Taurine/ α -Ketoglutarate Dioxygenase (TauD) from *Escherichia coli*. *Biochemistry*, 42(24), 7497–7508.
- (61) Krebs, C., Price, J. C., Baldwin, J., Saleh, L., Green, M. T., & Bollinger, J. M. (2005). Rapid Freeze-Quench Fe Mössbauer Spectroscopy: Monitoring Changes of an Iron-Containing Active Site during a Biochemical Reaction. *Inorganic Chemistry*, 44(4), 742–757.
- (62) Pavel, E. G., Zhou, J., Busby, R. W., Gunsior, M., Townsend, C. A., & Solomon, E. I. (1998). Circular Dichroism and Magnetic Circular Dichroism Spectroscopic Studies of the Non-Heme Ferrous Active Site in Clavaminic Synthase and Its Interaction with α -Ketoglutarate Cosubstrate. *Journal of the American Chemical Society*, 120(4), 743–753.
- (63) Ehrismann, D., Flashman, E., Genn, D. N., Mathioudakis, N., Hewitson, K. S., Ratcliffe, P. J., & Schofield, C. J. (2007). Studies on the activity of the hypoxia-inducible-factor hydroxylases using an oxygen consumption assay. *Biochemical Journal*, 401(1), 227–234.
- (64) Tarhonskaya, H., Chowdhury, R., Leung, I. K. H., Loik, N. D., McCullagh, J. S. O., Claridge, T. D. W., ... Flashman, E. (2014). Investigating the contribution of the active site environment to the slow reaction of hypoxia-inducible factor prolyl hydroxylase domain 2 with oxygen. *Biochemical Journal*, 463(3), 363–372.
- (65) Dao, J. H., Kurzeja, R. J. M., Morachis, J. M., Veith, H., Lewis, J., Yu, V., ... Tagari, P. (2009). Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology. *Analytical Biochemistry*, 384(2), 213–223.
- (66) McNeill, L. A., Flashman, E., Buck, M. R. G., Hewitson, K. S., Clifton, I. J., Jeschke, G., ... Schofield, C. J. (2005). Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. *Molecular BioSystems*, 1(4), 321–324.
- (67) Aik, W. S., Chowdhury, R., Clifton, I. J., Hopkinson, R. J., Leissing, T., McDonough, M. A., ... Walport, L. J. (2015). CHAPTER 2 Introduction to Structural Studies on 2-Oxoglutarate-Dependent Oxygenases and Related Enzymes. In *2-Oxoglutarate-Dependent Oxygenases* (pp. 59–94). The Royal Society of Chemistry.

- (68) Chowdhury, R., Leung, I. K. H., Tian, Y.-M., Abboud, M. I., Ge, W., Domene, C., ... Schofield, C. J. (2016). Structural basis for oxygen degradation domain selectivity of the HIF prolyl hydroxylases. *Nature Communications*, 7, 12673.
- (69) Flashman, E., Bagg, E. A. L., Chowdhury, R., Mecinović, J., Loenarz, C., McDonough, M. A., ... Schofield, C. J. (2008). Kinetic Rationale for Selectivity toward N- and C-terminal Oxygen-dependent Degradation Domain Substrates Mediated by a Loop Region of Hypoxia-Inducible Factor Prolyl Hydroxylases. *Journal of Biological Chemistry*, 283(7), 3808–3815.
- (70) Loenarz, C., Mecinović, J., Chowdhury, R., McNeill, L. A., Flashman, E., & Schofield, C. J. (2009). Evidence for a Stereoelectronic Effect in Human Oxygen Sensing. *Angewandte Chemie International Edition*, 48(10), 1784–1787.
- (71) Ahmed, S., Ayscough, A., Barker, G. R., Canning, H. E., Davenport, R., Downham, R., ... Hendrick, A. G. (2017). 1,2,4-Triazolo-[1,5-a]pyridine HIF Prolylhydroxylase Domain-1 (PHD-1) Inhibitors With a Novel Monodentate Binding Interaction. *Journal of Medicinal Chemistry*, 60(13), 5663–5672.
- (72) Elkins, J. M., Hewitson, K. S., McNeill, L. A., Seibel, J. F., Schlemminger, I., Pugh, C. W., ... Schofield, C. J. (2003). Structure of Factor-inhibiting Hypoxia-inducible Factor (HIF) Reveals Mechanism of Oxidative Modification of HIF-1 α . *Journal of Biological Chemistry*, 278(3), 1802–1806.
- (73) Hewitson, K. S., & Schofield, C. J. (2004). The HIF pathway as a therapeutic target. *Drug Discovery Today*, 9(16), 704–711.
- (74) Chan, M. C., Holt-Martyn, J. P., Schofield, C. J., & Ratcliffe, P. J. (2016). Pharmacological targeting of the HIF hydroxylases – A new field in medicine development. *Molecular Aspects of Medicine*, 47(Supplement C), 54–75.
- (75) Rose, N. R., McDonough, M. A., King, O. N. F., Kawamura, A., & Schofield, C. J. (2011). Inhibition of 2-oxoglutarate dependent oxygenases. *Chem. Soc. Rev.*, 40(8), 4364–4397.
- (76) Evdokimov, A.G., Walter, R.L., Mekel, M., Pokross, M.E., Kawamoto, R., Boyer, A. (2006). Crystal structure of HIF prolyl hydroxylase in complex with a biologically active inhibitor. Retrieved from: <http://www.rcsb.org/pdb/explore.do?structureId=2HBT>.
- (77) Yeh, T.-L., Leissing, T., Abboud, M. I., Thinnies, C. C., Atasoylu, O., Holt-Martyn, J. P., ... Schofield, C. J. (2017). Molecular and cellular mechanisms of HIF prolyl hydroxylase inhibitors in clinical trials. *Chemical Science*, 8, 7651–7668.
- (78) Aik, W., McDonough, M. A., Thalhammer, A., Chowdhury, R., & Schofield, C. J. (2012). Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases. *Current Opinion in Structural Biology*, 22(6), 691–700.

- (79) McDonough, M. A., Loenarz, C., Chowdhury, R., Clifton, I. J., & Schofield, C. J. (2010). Structural studies on human 2-oxoglutarate dependent oxygenases. *Current Opinion in Structural Biology*, 20(6), 659–672.
- (80) Leung, I. K. H., Flashman, E., Yeoh, K. K., Schofield, C. J., & Claridge, T. D. W. (2010). Using NMR Solvent Water Relaxation to Investigate Metalloenzyme–Ligand Binding Interactions. *Journal of Medicinal Chemistry*, 53(2), 867–875.
- (81) Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., ... Gleadle, J. M. (2004). Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 279(37), 38458–38465.
- (82) Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., ... Czyzyk-Krzeska, M. F. (2003). von Hippel–Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. *Proceedings of the National Academy of Sciences*, 100(5), 2706–2711.
- (83) Mikhaylova, O., Ignacak, M. L., Barankiewicz, T. J., Harbaugh, S. V., Yi, Y., Maxwell, P. H., ... Czyzyk-Krzeska, M. F. (2008). The von Hippel-Lindau Tumor Suppressor Protein and Egl-9-Type Proline Hydroxylases Regulate the Large Subunit of RNA Polymerase II in Response to Oxidative Stress. *Molecular and Cellular Biology*, 28(8), 2701–2717.
- (84) Zheng, X., Zhai, B., Koivunen, P., Shin, S. J., Lu, G., Liu, J., ... Zhang, Q. (2014). Prolyl hydroxylation by EglN2 destabilizes FOXO3a by blocking its interaction with the USP9x deubiquitinase. *Genes & Development*, 28(13), 1429–1444.
- (85) Köditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., ... Katschinski, D. M. (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. *Blood*, 110(10), 3610–3617.
- (86) Xie, L., Xiao, K., Whalen, E. J., Forrester, M. T., Freeman, R. S., Fong, G., ... Stamler, J. S. (2009). Oxygen-Regulated $\beta(2)$ -Adrenergic Receptor Hydroxylation by EGLN3 and Ubiquitylation by pVHL. *Science Signaling*, 2(78), pp. ra33.
- (87) Heir, P., Srikumar, T., Bikopoulos, G., Bunda, S., Poon, B. P., Lee, J. E., ... Ohh, M. (2016). Oxygen-dependent Regulation of Erythropoietin Receptor Turnover and Signaling. *The Journal of Biological Chemistry*, 291(14), 7357–7372.
- (88) Xie, L., Pi, X., Townley-Tilson, W. H. D., Li, N., Wehrens, X. H. T., Entman, M. L., ... Patterson, C. (2015). PHD2/3-dependent hydroxylation tunes cardiac response to β -adrenergic stress via phospholamban. *The Journal of Clinical Investigation*, 125(7), 2759–2771.

- (89) Guo, J., Chakraborty, A. A., Liu, P., Gan, W., Zheng, X., Inuzuka, H., ... Wei, W. (2016). pVHL suppresses kinase activity of Akt in a proline-hydroxylation-dependent manner. *Science*, 353(6302), 929–932.
- (90) Lee, S. B., Frattini, V., Bansal, M., Castano, A. M., Sherman, D., Hutchinson, K., ... Lasorella, A. (2016). An ID2-dependent mechanism for VHL inactivation in cancer. *Nature*, 529(7585), 172–177.
- (91) Romero-Ruiz, A., Bautista, L., Navarro, V., Heras-Garvín, A., March-Díaz, R., Castellano, A., ... Pascual, A. (2012). Prolyl Hydroxylase-dependent Modulation of Eukaryotic Elongation Factor 2 Activity and Protein Translation under Acute Hypoxia. *The Journal of Biological Chemistry*, 287(12), 9651–9658.
- (92) Moore, C. E. J., Mikolajek, H., Regufe da Mota, S., Wang, X., Kenney, J. W., Werner, J. M., & Proud, C. G. (2015). Elongation Factor 2 Kinase Is Regulated by Proline Hydroxylation and Protects Cells during Hypoxia. *Molecular and Cellular Biology*, 35(10), 1788–1804.
- (93) Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., ... Semenza, G. L. (2011). Pyruvate Kinase M2 is a PHD3-stimulated Coactivator for Hypoxia-Inducible Factor 1. *Cell*, 145(5), 732–744.
- (94) Arsenault, P. R., Heaton-Johnson, K. J., Li, L., Song, D., Ferreira, V. S., Patel, N., ... Lee, F. S. (2015). Identification of Prolyl Hydroxylation Modifications in Mammalian Cell Proteins. *Proteomics*, 15(7), 1259–1267.
- (95) Cockman, M. E., Webb, J. D., Kramer, H. B., Kessler, B. M., & Ratcliffe, P. J. (2009). Proteomics-based Identification of Novel Factor Inhibiting Hypoxia-inducible Factor (FIH) Substrates Indicates Widespread Asparaginyl Hydroxylation of Ankyrin Repeat Domain-containing Proteins. *Molecular & Cellular Proteomics : MCP*, 8(3), 535–546.
- (96) Yang, M., Chowdhury, R., Ge, W., Hamed, R. B., McDonough, M. A., Claridge, T. D. W., ... Schofield, C. J. (2011). Factor-inhibiting hypoxia-inducible factor (FIH) catalyses the post-translational hydroxylation of histidiny residues within ankyrin repeat domains. *The FEBS Journal*, 278(7), 1086–1097.
- (97) Yang, M., Hardy, A. P., Chowdhury, R., Loik, N. D., Scotti, J. S., McCullagh, J. S. O., Claridge, T. D. W., McDonough, M. A., Ge, W. and Schofield, C. J. (2013), Substrate Selectivity Analyses of Factor Inhibiting Hypoxia-Inducible Factor. *Angewandte Chemie International Edition*, 52(6), 1700–1704.
- (98) Beerling, D. J., & Berner, R. A. (2000). Impact of a Permo-Carboniferous high O(2) event on the terrestrial carbon cycle. *Proceedings of the National Academy of Sciences of the United States of America*, 97(23), 12428–12432.
- (99) Berner, R. A., VandenBrooks, J. M., & Ward, P. D. (2007). Oxygen and Evolution. *Science*, 316(5824), 557–558.

- (100) Falkowski, P. G., Katz, M. E., Milligan, A. J., Fennel, K., Cramer, B. S., Aubry, M. P., ... Zapol, W. M. (2005). The Rise of Oxygen over the Past 205 Million Years and the Evolution of Large Placental Mammals. *Science*, 309(5744), 2202-2204.
- (101) Ward, P., Labandeira, C., Laurin, M., & Berner, R. A. (2006). Confirmation of Romer's Gap as a low oxygen interval constraining the timing of initial arthropod and vertebrate terrestrialization. *Proceedings of the National Academy of Sciences of the United States of America*, 103(45), 16818-16822.
- (102) Taylor, C. T., & McElwain, J. C. (2010). Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans. *Physiology*, 25(5), 272-279.
- (103) Hedges, S. B., Marin, J., Suleski, M., Paymer, M., & Kumar, S. (2015). Tree of Life Reveals Clock-Like Speciation and Diversification. *Molecular Biology and Evolution*, 32(4), 835-845.
- (104) Semenza, G. L. (2007). Life with Oxygen. *Science*, 318(5847), 62-64.
- (105) Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Snel, B., & Bork, P. (2006). Toward Automatic Reconstruction of a Highly Resolved Tree of Life. *Science*, 311(5765), 1283-1287.
- (106) Rich, P. R. (2003). The molecular machinery of Keilin's respiratory chain. *Biochemical Society Transactions*, 31(6), 1095-1105.
- (107) Kiley, P. J., & Beinert, H. (2003). The role of Fe-S proteins in sensing and regulation in bacteria. *Current Opinion in Microbiology*, 6(2), 181-185.
- (108) Hon, T., Dodd, A., Dirmeier, R., Gorman, N., Sinclair, P. R., Zhang, L., & Poyton, R. O. (2003). A Mechanism of Oxygen Sensing in Yeast: Multiple oxygen-responsive steps in the heme biosynthetic pathway affect Hap1 activity. *Journal of Biological Chemistry*, 278(50), 50771-50780.
- (109) Xu, Y., Wang, Z. A., Green, R. S., & West, C. M. (2012). Role of the Skp1 prolyl-hydroxylation/glycosylation pathway in oxygen dependent submerged development of *Dictyostelium*. *BMC Developmental Biology*, 12, 31.
- (110) Scotti, J. S., Leung, I. K. H., Ge, W., Bentley, M. A., Paps, J., Kramer, H. B., ... Schofield, C. J. (2014). Human oxygen sensing may have origins in prokaryotic elongation factor Tu prolyl-hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America*, 111(37), 13331-13336.
- (111) West, C. M., Wang, Z. A., & van der Wel, H. (2010). A cytoplasmic prolyl hydroxylation and glycosylation pathway modifies Skp1 and regulates O(2)-dependent development in *Dictyostelium*. *Biochimica et Biophysica Acta*, 1800(2), 160-171.
- (112) Loenarz, C., Coleman, M. L., Boleining, A., Schierwater, B., Holland, P. W. H., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Reports*, 12(1), 63-70.

- (113) Miller, D. J., & Ball, E. E. (2017). Animal Evolution: The Enigmatic Phylum Placozoa Revisited. *Current Biology*, 15(1), R26–R28.
- (114) van der Wel, H., Morris, H. R., Panico, M., Paxton, T., Dell, A., Kaplan, L., & West, C. M. (2002). Molecular Cloning and Expression of a UDP-N-acetylglucosamine (GlcNAc):Hydroxyproline Polypeptide GlcNAc-transferase That Modifies Skp1 in the Cytoplasm of Dictyostelium. *Journal of Biological Chemistry*, 277(48), 46328–46337.
- (115) Zhang, D., van der Wel, H., Johnson, J. M., & West, C. M. (2012). Skp1 Prolyl 4-Hydroxylase of Dictyostelium Mediates Glycosylation-independent and -dependent Responses to O(2) without Affecting Skp1 Stability. *The Journal of Biological Chemistry*, 287(3), 2006–2016.
- (116) Wang, Z. A., van der Wel, H., Vohra, Y., Buskas, T., Boons, G.-J., & West, C. M. (2009). Role of a Cytoplasmic Dual-function Glycosyltransferase in O(2) Regulation of Development in Dictyostelium. *The Journal of Biological Chemistry*, 284(42), 28896–28904.
- (117) Wang, Z. A., Singh, D., Wel, H. van der, & West, C. M. (2011). Prolyl hydroxylation- and glycosylation-dependent functions of Skp1 in O(2)-regulated development of Dictyostelium. *Developmental Biology*, 349(2), 283–295.
- (118) Xu, Y., Wang, Z. A., Green, R. S., & West, C. M. (2012). Role of the Skp1 prolyl-hydroxylation/ glycosylation pathway in oxygen dependent submerged development of Dictyostelium. *BMC Developmental Biology*, 12, 31.
- (119) West, C. M., Wang, Z. A., & van der Wel, H. (2010). A cytoplasmic prolyl hydroxylation and glycosylation pathway modifies Skp1 and regulates O(2)-dependent development in Dictyostelium. *Biochimica et Biophysica Acta*, 1800(2), 160–171.
- (120) Xu, Y., Brown, K. M., Wang, Z. A., van der Wel, H., Teygong, C., Zhang, D., ... West, C. M. (2012). The Skp1 Protein from Toxoplasma Is Modified by a Cytoplasmic Prolyl 4-Hydroxylase Associated with Oxygen Sensing in the Social Amoeba Dictyostelium. *The Journal of Biological Chemistry*, 287(30), 25098–25110.
- (121) Hoffmeyer, T. T., & Burkhardt, P. (2016). Choanoflagellate models — *Monosiga brevicollis* and *Salpingoeca rosetta*. *Current Opinion in Genetics & Development*, 39, 42–47.
- (122) Boleininger, A. (2012). Evolution of PHDs as oxygen sensors: mechanistic and structural studies of the PHD of *Trichoplax adhaerens*, the simplest animal, and mechanistic studies of a PHD-like enzyme of the protist *Monosiga brevicollis*. DPhil. University of Oxford.
- (123) Gardie, B., Percy, M. J., Hoogewijs, D., Chowdhury, R., Bento, C., Arsenault, P. R., ... Lee, F. S. (2014). The role of PHD2 mutations in the pathogenesis of erythrocytosis. *Hypoxia*, 2, 71–90.

Chapter 2

Substrate Selectivity of Human HIF Prolyl Hydroxylases

2.1 Introduction

The overall objective of the work described in this Chapter was to investigate the validity of the reported non-HIF substrates of the PHDs. To date, there have been approximately 30 reports of non-HIF PHD substrates (listed in **Table 2.1**), published in peer reviewed journals. In most cases, the non-HIF substrates were initially identified *in vivo* by use of PHD or pVHL interaction screens, such as GST-pull-down assays (1), yeast 2-hybrid assays (1), or by immunoprecipitation (IP) followed by either Western Blotting using pan-hydroxyproline antibodies or by MS analysis of trypsin digested peptides (2-3). In other approaches, the cellular proteome was analysed for changes in protein expression levels under hypoxic and normoxic conditions, or by treating cells with the cell-penetrating 2OG oxygenase inhibitor DMOG (4-6).

The risk of false positives presents a major caveat of these approaches, since unspecific, or non-biologically relevant, interactions can be assigned based on IP screens and the specificity of pan-hydroxyproline-residue antibodies has not been investigated in detail. The use of the broad-spectrum 2OG oxygenase inhibitor DMOG can be misleading, as the observed effects might not be due to PHD inhibition, but inhibition of other members of the 2OG oxygenase family, like FIH, histone demethylases, or isocitrate dehydrogenase (7-10). Another difficulty manifests in the analysis of mass spectrometric data, e.g. in distinguishing prolyl hydroxylations from other common amino acid oxidations, such as frequently occurring non-enzymatic methionine, tyrosine, or tryptophan oxidations (11).

Some of the PHD substrate candidates were initially identified by searching the human genome for proteins that contain a conserved LXXLAP motif (12-13), which is present in all vertebrate HIF1 α and HIF2 α isoforms (14). However, it has been shown that this sequence is not an essential property for all PHD substrates, e.g. the human PHD2 accepts the YXXLAP sequence present in the HIF α from *Trichoplax adhaerens* (15).

In some studies, the PHD-dependence of the observed hydroxylation was further validated *in vitro*, mostly by incubating the full-length protein or peptidic substrates with the respective PHD isoforms, followed by MS analysis or detection by pan-hydroxyproline antibodies. Alternatively, a radioactive [$1\text{-}^{14}\text{C}$] 2OG decarboxylation assay was applied, which measures the turnover of 2OG in the presence and absence of the potential substrate. However, decarboxylation of 2OG precedes the hydroxylation event in the catalytic cycle of 2OG oxygenases, and is not always (completely) coupled to hydroxylation (see *General Introduction*, **Chapter 1.2**). Thus, an observed stimulation of 2OG turnover can be falsely attributed to enzyme-catalysed substrate hydroxylation.

Given the potential issues with the methods used for the assignment of the hydroxylation sites in non-HIF PHD substrates, detailed future work is required to definitely assign non-HIF PHD substrates.

Table 2.1 Reported PHD target substrates. Abbreviations: mass spectrometry (MS), tandem mass spectrometry (MS/MS), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), electrospray (ES), immunoprecipitation (IP), antibody (ab), hydroxyproline (Hyp).

Gene Symbol	Protein	Hydroxylation site	PHD isoform	<i>in vitro</i> validation of hydroxylation	Ref.
HIF1A	hypoxia-inducible factor 1 α (HIF1 α)	P402 (NODD) P564 (Codd)	1, 2, 3	PHD-catalysed hydroxylation is well established by	(35-43)
EPAS1	hypoxia-inducible factor 2 α (HIF2 α)	P405 (NODD) P531 (Codd)	1, 2, 3	- radioactive [$1\text{-}^{14}\text{C}$] 2OG decarboxylation	
HIF3A	hypoxia-inducible factor 3 α (HIF3 α)	P492 (Codd)	1, 2, 3	- mass spectrometry-based evidence of PHD-catalysed hydroxylation (including amino acid analysis and MS/MS) - kinetic studies - crystallographic structures of PHD2-HIF1 α complexes	
ACACB	acetyl-CoA carboxylase 2 (ACC2)	P450	3	radioactive [$1\text{-}^{14}\text{C}$] 2OG decarboxylation	(2)
ACTB	actin β	P307, P322	3	incubation of ACTB protein with recombinant PHD3, detection with pan-Hyp-ab	(6)
ADRB2	adrenoceptor β 2 (β 2AR)	P382, P395	3	incubation of ADRB2 protein with PHDs in cell lysates, followed by MALDI-MS	(16)
AKT1	AKT serine/threonine kinase 1 (Akt1)	P125, P313	2	incubation of AKT1 peptides with recombinant PHD2, detection with Hyp-ab	(17)

Gene Symbol	Protein	Hydroxylation site	PHD isoform	<i>in vitro</i> validation of hydroxylation	Ref.
ATF4	activating transcription factor 4	P156, P162, P164, P167, P174	3	—	(1)
CENPN	centromere protein N, isoform 3	P311	2	incubation of CENPN peptides with recombinant PHD2, followed by MS/MS	(12)
CEP192	centrosomal protein 192	P1717	1	incubation of CEP192 peptides with recombinant PHD1, followed by ES-MS	(18)
DYRK1A	dual specificity tyrosine phosphorylation regulated kinase 1A	not specified	1	—	(3)
eEF2K	<i>eukaryotic</i> elongation factor 2 kinase	P98	2	—	(4, 19)
EPOR	erythropoietin receptor	P419, P426	3	incubation of EPOR protein with purified PHD3, followed by MS analysis	(20)
FKBP10	FK506 binding protein 10	P36	not specified	—	(21)
FLNA	filamin A	P2309, 2316	2	incubation of FLNA peptides with PHDs in cell lysates, detection of interaction with PHD2 by IP	(22)
FOXO3	forkhead box O3 (FOXO3A)	P426, P437	1	radioactive [1- ¹⁴ C] 2OG decarboxylation	(23)
HK2	hexokinase 2	P326	not specified	—	(21)
IKBKB	inhibitor of nuclear factor kappa B kinase subunit β	P191	1	—	(13)
MAPK6	mitogen-activated protein kinase 6	P25	3	incubation of MAPK6 protein with PHDs in cell lysates, followed by MS analysis	(5)
MTHFD1	methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1	P806	not specified	—	(21)

Gene Symbol	Protein	Hydroxylation site	PHD isoform	<i>in vitro</i> validation of hydroxylation	Ref.
MYH10	myosin heavy chain 10	P733	not specified	—	(21)
NDRG3	NDRG family member 3	P294	2	—	(24)
PDE4D	phosphodiesterase 4D	P29, P382, P419	2	—	(25)
PKM	pyruvate kinase, muscle	P477	not specified	—	(21)
PKM2	pyruvate kinase, muscle 2	P403, P408	3	incubation of PKM2 protein with PHDs in cell lysates, followed by MS analysis	(26)
POLR2A	RNA polymerase II subunit A (Rpb1)	P1465	1, (2)	—	(27-28)
SPRY2	sprouty RTK signaling antagonist 2	P18, P144, P160	1, 2, 3	—	(29)
TELO2	telomere maintenance 2	P374, P419, P422	3	radioactive [1- ¹⁴ C] 2OG decarboxylation	(30)
THRA	thyroid hormone receptor, α (TR- α)	P160, P162	2, 3	incubation of THRA protein with recombinant PHD2 and PHD3, followed by MS analysis	(31)
TRPA1	transient receptor potential cation channel subfamily A member 1	P394	2	incubation of TRPA1 peptides with PHD2, followed by MS analysis	(32)
PPP2R2A	protein phosphatase 2 regulatory subunit B α	P319	2	radioactive [1- ¹⁴ C] 2OG decarboxylation	(33)
TP53	tumour protein p53	P359	1	—	(34)

To investigate the validity of the reported PHD substrates, detailed and systematic studies were carried out.

1. Due to the high number of reports, all potential substrates were first tested at the peptide level in an ESI-LC-MS-based hydroxylation screen against all three PHD isoforms.
2. The non-HIF peptide substrates were further probed for interaction/binding to PHD2 and inhibitory effects of non-HIF peptide substrates on PHDs were investigated.
3. Binding of the non-HIF substrates to the VCB complex was examined by fluorescence-polarisation.
4. A selection of the potential PHD substrates was examined on the protein level, by co-expressing GST-tagged proteins with the respective PHD isoform in *E. coli*, followed by LC-MS/MS analysis of the purified proteins.
5. In parallel, extensive hydroxylation studies using full-length non-HIF substrates produced by *in vitro* transcription and translation (IVTT) were conducted by Ya-Min Tian, Matthew Cockman, and Peter Ratcliffe (Nuffield Department of Medicine, University of Oxford, UK and Francis Crick Institute, UK).

2.2 Results and Discussion

2.2.1 PHD hydroxylation assays

Owing to the high number of reported non-HIF PHD substrates, it was decided, in an initial approach, to screen synthetic peptides containing the reported PHD catalysed hydroxylation sites for reaction with PHDs. In most studies of non-HIF PHD substrates, it was reported that only one of the three human PHD isoforms interacts with the potential substrate, and catalyses the hydroxylation of prolyl residues in the non-HIF substrate (**Table 2.1**). Despite this apparent PHD isoform selectivity, it was decided to conduct an extensive study and test each reported hydroxylation site for reaction with each of the three recombinantly produced human PHD isoforms (PHD1-3).

Previous work on PHD2 has focused on the characterisation of a *N*-terminally truncated construct that contains the catalytic domain, but lacks the *N*-terminal MYND domain (tPHD2, residues 181-426, (43)). The truncated construct tPHD2 can be produced efficiently in *E. coli*, and accepts 19-20 mer HIF α peptides containing the respective hydroxylation sites as substrates (43). However, the function of the cysteine-rich MYND-type zinc finger domain in PHD2 is not fully understood (44). A role for the MYND domain in the interaction with heat shock protein 90 (HSP90) co-chaperones p23 and FKBP38 has been proposed (44), yet it cannot be excluded that the *N*-terminus in PHD2 or *N*-terminal domains in the other two PHD isoforms plays a role in substrate recognition. For this reason, the potential PHD substrates were screened against full-length PHD enzymes. There have been efforts in the past to produce recombinant PHD1 and PHD2 full-length enzymes in *E. coli*, yet with limited success, due to the low solubility of the full-length proteins and low activity (in case of PHD1). Work by Johanna Myllyharju (University of Oulu, Finland) has shown that highly active full-length PHD1 and PHD2 can be obtained using baculovirus expression systems. Consequently, the peptide screen was conducted using PHD1 and PHD2 enzymes that were produced in baculovirus (kindly provided by Johanna Myllyharju).

Active full-length PHD3 has been previously produced by Rasheduzzaman Chowdhury (University of Oxford, UK; unpublished data), yet the construct was mutated at two cysteine residues at the surface of PHD3, in order to increase the solubility of the enzyme. It cannot be excluded that the mutations in PHD3 could affect the substrate selectivity of the enzyme, therefore a strategy for production and purification of unmutated (wild-type) full-length PHD3 was developed.

Production of wild-type full-length PHD3

The production of full-length PHD3 (PHD3^{fl}) in a stable, active form for use in the peptide screen, first constituted a major challenge. In order to achieve an optimal protein yield, the sequence of EGLN3 (encoding for PHD3) was codon-optimised for expression in *E. coli* (GenScript) and subsequently cloned into various vectors containing different protein tags (*Materials and Methods*, **Chapter 7.1.1**). It was first tried to express EGLN3 using a pNIC28 vector with a *N*-terminal His₆-tag, however, the yield of produced and soluble recombinant PHD3^{fl} was low (*Materials and Methods*, **Chapter 7.2**, see **Table 2.2** for expression conditions). The solubility of the protein could be increased by cloning EGLN3 in a pNH-TrxT vector and producing PHD3^{fl} with a thioredoxin-tag (with a TEV-protease susceptible linker) that turned out to stabilise the enzyme.

Table 2.2 EGLN3 expression trials. The optimised expression condition is labelled in red.

Vector	Tag	Expression conditions	Result
pNIC28	<i>N</i> -terminal His ₆ -tag	18 °C, 0.5 mM IPTG, 4 h	low expression levels, low solubility
		18 °C, 0.5 mM IPTG, 0 _N	
		18 °C, 0.05 mM IPTG, 4 h	
		18 °C, 0.05 mM IPTG, 0 _N	
		28 °C, 0.5 mM IPTG, 4 h	
		28 °C, 0.5 mM IPTG, 0 _N	
		28 °C, 0.05 mM IPTG, 4 h	
		28 °C, 0.05 mM IPTG, 0 _N	
		37 °C, 0.5 mM IPTG, 4 h	
		37 °C, 0.5 mM IPTG, 0 _N	
		37 °C, 0.05 mM IPTG, 4 h	
		37 °C, 0.05 mM IPTG, 0 _N	
pNH-TrxT	<i>N</i> -terminal	18 °C, 0.5 mM IPTG, 4 h	medium expression levels, medium-high solubility
		18 °C, 0.5 mM IPTG, 0 _N	
		18 °C, 0.05 mM IPTG, 4 h	
		18 °C, 0.05 mM IPTG, 0_N	
		28 °C, 0.5 mM IPTG, 4 h	
		28 °C, 0.5 mM IPTG, 0 _N	
	His ₆ -thioredoxin tag	28 °C, 0.05 mM IPTG, 4 h	medium expression levels, high solubility
		28 °C, 0.05 mM IPTG, 0 _N	
		28 °C, 0.05 mM IPTG, 0 _N	
		37 °C, 0.5 mM IPTG, 4 h	
		37 °C, 0.5 mM IPTG, 0 _N	
		37 °C, 0.05 mM IPTG, 4 h	
		37 °C, 0.05 mM IPTG, 0 _N	

Optimal production conditions for His₆-thioredoxin tagged PHD3^{fl} were found at 18 °C, 0_N, and 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). At higher temperatures and IPTG concentrations, the PHD3^{fl} levels were increased, yet the proportion of soluble PHD3^{fl} was reduced.

The yield of soluble PHD3^{fl} was further increased by letting the *E. coli* culture grow to OD₆₀₀ 1.2 instead of OD₆₀₀ 0.6-0.8, before protein production was induced by adding IPTG.

PHD3^{fl} was purified by affinity chromatography via a HisTrap™ column (GE Healthcare) as described in *Materials and Methods*, **Chapter 7.3**. The protein was analysed by SDS-PAGE and the molecular weight of the purified protein was confirmed by ESI-LC-MS (**Figure 2.1**, *Materials and Methods*, **Chapter 7.6.2**). The initial yield of purified PHD3^{fl} from a 8L large-scale culture was 6 mg. In order to increase the protein yield, the buffer conditions for lysis and affinity purification were optimised. A substantial increase in protein yield (> 20 mg per 8 L culture) was achieved by adding 5 mM dithiothreitol (DTT) to the lysis buffer, and the protein was further stabilised by adding 5 % glycerol to every buffer used in lysis and purification. It is likely that DTT helps to keep the surface cysteine residues in a reduced state, thus increasing the solubility of the enzyme. Effort were made to increase the purity of the PHD3^{fl} by applying a second purification step (size-exclusion chromatography (SEC) using a Superdex 75 300 mL column (Amershan Pharmacia Biosciences), or anion exchange chromatography using a Mono Q column (GE Healthcare)), yet the enzymatic activity substantially dropped after the second purification step (in case of SEC, the PHD3^{fl} likely precipitated on the column).

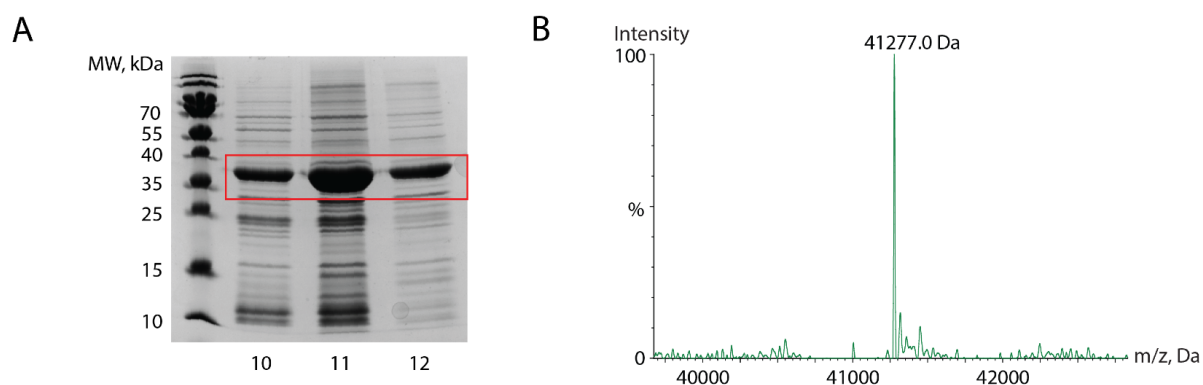


Figure 2.1: PHD3^{fl} purification. (A) SDS-PAGE gel and (B) deconvoluted protein LC-MS spectrum. Affinity purification of PHD3^{fl} (41.3 kDa) yielded the enzyme in ~80 % purity. The identity of the purified protein was verified by ESI-LC-MS (expected mass: 41275.2 Da).

Efforts to cleave the thioredoxin tag by incubating PHD3^{fl} with the TEV-protease led to inactive protein (*Materials and Methods*, **Chapter 7.3**); it is likely that the tag is needed to keep the enzyme in a soluble and reduced state. PHD3^{fl} was found to be sensitive to freeze-thaw cycles, and the activity of the enzyme slowly decreased after 2-3 weeks (as shown by MS analysis) when stored at -80 °C.

To assess the activity of PHD3^{fl}, hydroxylation of 19-20mer HIF α peptides that contain the C- and N-terminal oxygen dependent degradation domains (CODD/NODD) was studied under standard conditions previously optimised for tPHD2 (see *Materials and Methods*, **Chapter 7.6.2**), using ESI-LC-MS based assays. Hydroxylation of the peptides leads to a +16 mass shift in the MS spectra relative to the non-hydroxylated peptide controls (**Figure 2.2**). PHD3^{fl} was found to be highly active on peptides that contain the CODD sequences of HIF1 α -HIF3 α , yet no reaction with HIF1 α /HIF2 α NODD was observed above the detection limits of the assay. This finding is in accordance with previous studies on the enzyme that suggest a high degree of selectivity for CODD over NODD (37-38).

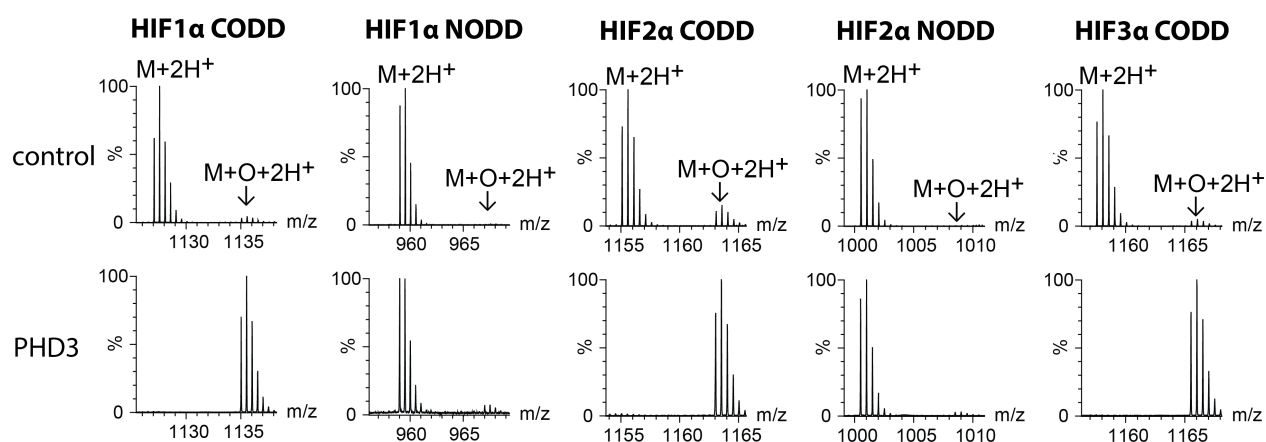


Figure 2.2: Activity of PHD3^{fl} on 19-20mer HIF α peptides. PHD3^{fl}-catalysed hydroxylation of 19-20mer HIF α peptides was assessed by use of ESI-LC-MS. Assay conditions: 10 μ M PHD3^{fl} enzyme, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 300 μ M 2-oxoglutarate disodium salt, 300 μ M sodium L-ascorbate, and 50 μ M 19-20mer peptides in 50 mM Tris, pH 7.5. Reactions were incubated at 37 °C for 4 h and quenched with formic acid (1 % v/v). Samples were analysed by ESI-LC-MS, and hydroxylation levels were determined using MassLynx™ V4.1.

Design of synthetic peptides

In order to screen all reported PHD-dependent hydroxylation sites, synthetic peptides with C-terminal amides were either prepared as described in *Materials and Methods*, **Chapter 7.4** and **7.5**, or commercially purchased (for complete list of all screened peptides, refer to *Appendix*, **Chapter 2.5**, **Table 2.7** and **Table 2.8**). The human PHD isoforms efficiently catalyse the hydroxylation of 19-20mer HIF α CODD/NODD peptides (**Table 2.3**, (43)). The sequences contain the conserved LXXLAP motif (bold letters, target proline is underlined), 2-4 residues N-terminal to the LXXLAP motif, and 10-11 residues C-terminal to the target proline.

Table 2.3 HIF α peptide sequences.

Peptide, hydroxylation site	Sequence	Length
HIF1 α CODD P564	DLD LEMLAP <u>Y</u> IPMDDDFQL	19mer
HIF1 α NODD P402	DAL TLLAP <u>A</u> AGDTIISLDF	19mer
HIF2 α CODD P531	ELD LETLP <u>A</u> YIPMDGEDFQL	20mer
HIF2 α NODD P405	EEL AQLAP <u>T</u> PGDAIISLDF	19mer
HIF3 α CODD P492	DALD LEMLAP <u>Y</u> ISMDDDFQL	20mer

The non-HIF substrate peptides were designed based on the information gained from the HIF α CODD/NODD peptides (**Table 2.3**, (43)), with at least 9 N-terminal and 11 C-terminal residues relative to the target proline residue. The 21-25mer peptides were synthesised as C-terminal amides for consistency with previous studies. In some reports, the non-HIF substrates were tested for reaction with PHDs at the peptide level, in which case peptides with the exact same sequences as reported were included into the study presented in this Chapter. In addition to the published PHD-dependent prolyl hydroxylation sites, isoforms of reported substrates (e.g. acetyl-CoA carboxylase isoform 1 (ACC1), centromere protein N isoform 1 (CENPN i1), inhibitor of nuclear factor kappa B kinase subunit α (IKBKA) and proteins that are functionally related to the reported substrates (e.g. *eukaryotic* elongation factor 1 and 2 (eEF1, eEF2) related to *eukaryotic* elongation factor 2 kinase (eEF2K)) were tested for reaction with PHDs (**Table 2.1**). Previous efforts in this group and Peter Ratcliffe's group (The Francis Crick Institute, UK) to identify new substrates of PHDs by the use of pull-down assays, has led to the identification of BLES03 as a potential PHD substrate (Wei-Shen Aik, Ming Yang, University of Oxford, UK, data unpublished). Therefore, a peptide containing this potential hydroxylation site was also included into the study.

PHD hydroxylation assay conditions and results

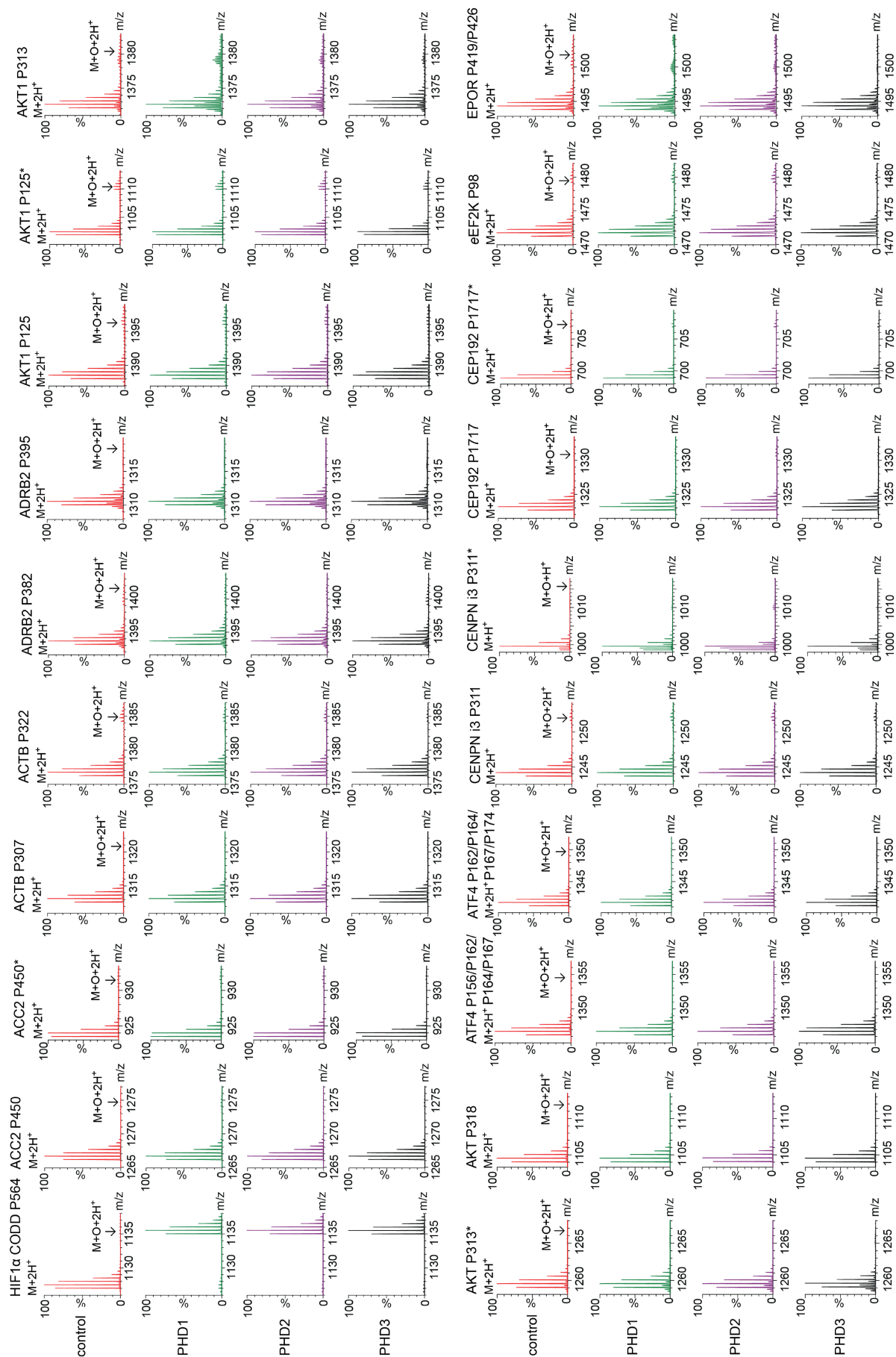
Peptide hydroxylation assays were carried out using the assay conditions described in *Materials and Methods*, **Chapter 7.6.2**. Peptides, enzymes, and co-factors (Fe(II), ascorbate, and 2OG) were incubated for 4 h at 37 °C, followed by electrospray-ionization liquid chromatography-mass spectrometry (ESI-LC-MS). The results confirmed the high activity of all three PHD isoforms on their well-established HIF1 α CODD P564 substrate. The assay conditions were optimised (**Table 2.4**) to maximise the enzymatic activity on the putative peptidic substrates and to minimise non-enzymatic background oxidation (discussed in more detail below). PHD1 and 2 were incubated at an enzyme:substrate ratio of 1:25, and PHD3 was incubated at a ratio of 1:10, to reach complete hydroxylation of the control HIF1 α CODD peptide.

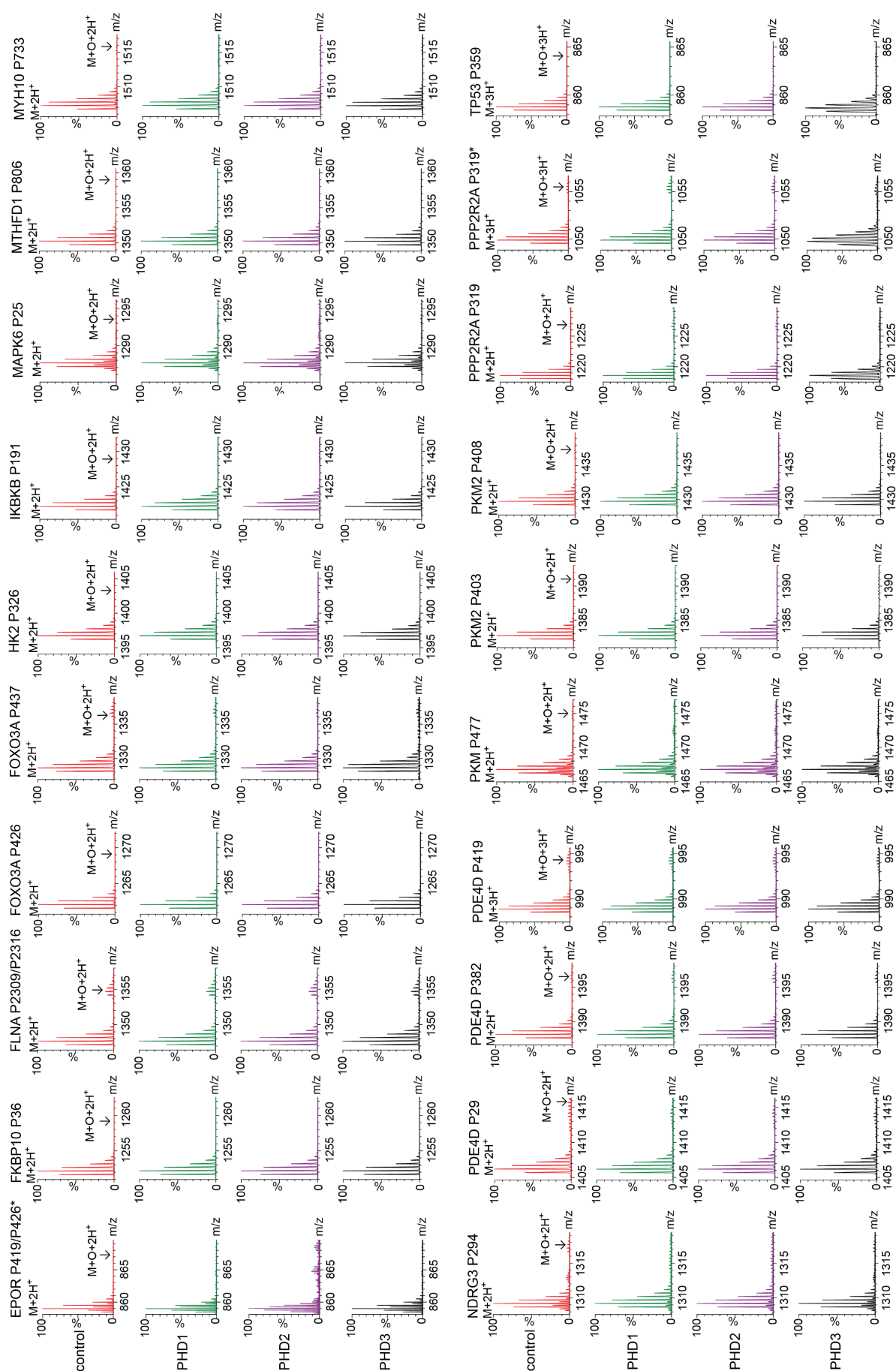
Table 2.4 Optimisation of the PHD hydroxylation assays. Abbreviations: mass spectrometry (MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), electrospray-ionization liquid chromatography-mass spectrometry (ESI-LC-MS).

Optimisation step	Conditions	Results
incubation time	O_N 4 h (final condition)	- hydroxylation of HIF1α CODD P564 by PHD1-3 complete after 4 h (and after O_N incubation) - less non-enzymatic oxidation in enzyme-free control samples after 4 h, compared to O_N incubation
temperature	25 °C 37 °C (final condition)	- no differences observed
enzyme:peptide ratio	1:10 1:25	- PHD1/2-catalysed hydroxylation of HIF1α CODD complete using 1:10 and 1:25 ratios - PHD3-catalysed hydroxylation of HIF1α CODD only complete using an enzyme:peptide ratio of 1:10
sodium L-ascorbate concentration	4 mM 300 μ M (final condition)	- reduced +16 peak in enzyme-free control samples, indicating less non-enzymatic oxidation takes place when ascorbate concentration is decreased
buffer composition	5 % glycerol 0 % glycerol (final condition)	- no differences observed
MS analysis	MALDI-TOF-MS ESI-LC-MS (final condition)	- better signal to noise ratio in ESI-LC-MS spectra - improved sensitivity for small changes in the intensity of +16 peaks using ESI-LC-MS

The results of the PHD hydroxylation screen are presented in **Figure 2.3**. The data were analysed for +16 Da shifts relative to the original peptide peak, indicating the incorporation of an oxygen atom. Despite extensive attempts, no hydroxylation of any of the tested 58 non-HIF peptides was detected. In many cases, a + 16 peak (< 10 % of total peptide signal) was observed (ACTB P322, AKT1 P125, AKT1 P125*, CENPN i3 P311, eEF2K P98, EPOR P419/P426, FLNA P2309/P2316, FOXO3A P437, NDRG3 P294, PDE4D P29, PDE4D P382, PDE4D P419, PPP2R2A P319*, SPRY2 P160, THRA P160/P162, IKBKA P190). However, these peaks were also found in the respective control samples without enzyme, suggesting that the +16 shift is due to a non-enzymatic oxidation on e.g. methionine during sample incubation. Under initial, un-optimised assay conditions, the signal of the non-enzymatic oxidation in some cases was up to 20-30 % of the total peptide signal. The extent of the + 16 shift was decreased substantially by lowering the amount of ascorbate in the assay samples from 4 mM to 300 μ M, indicating that high ascorbate concentrations promote the non-enzymatic oxidation of amino acids. For two peptides, BLES03 P135 and eEF2 P446, the level of non-enzymatic oxidation could not be decreased below 25 % of the total peptide signal, implying that these two peptide sequences are easily oxidised under the conditions used in this assay; notably, the sequence of eEF2 P446 contains two methionine residues, and the sequence of BLES03 P135 contains one methionine residue (see *Appendix, Chapter 2.5, Table 2.8*).

Overall, the results presented here suggest that the HIF prolyl hydroxylases are potentially highly selective for their well established HIF substrates. Thus, the results of the study differ from the current view in the literature, where a promiscuous nature of the PHDs is proposed.





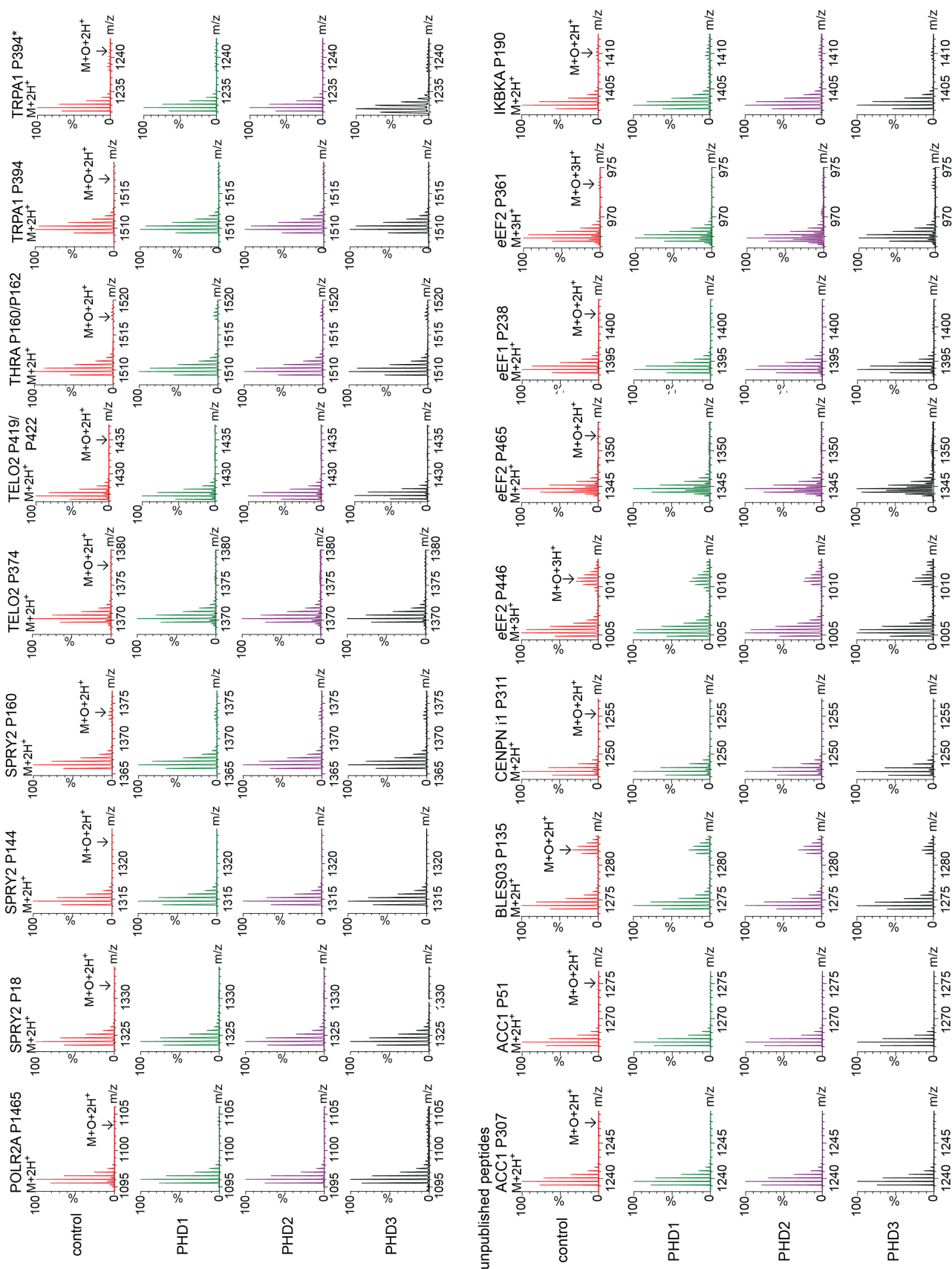


Figure 2.3: PHD hydroxylation assays. PHD1-3 full-length enzymes were incubated 4 h at 37 °C with peptides of reported substrates under the following conditions: 2 µM PHD1/2 or 10 µM PHD3, 50 µM (NH₄)₂Fe(II)(SO₄)₂, 300 µM 2-oxoglutarate disodium salt, 300 µM sodium L-ascorbate and 50 µM substrate peptides, in 50 mM Tris, pH 7.5. (In case of PHD3, 100 µM of the substrate peptides were used). The samples were analysed by electrospray-ionization liquid chromatography–mass spectrometry (ESI-LC-MS), and the data was scrutinised for +16 peaks (+‘O’), potentially indicating hydroxylation of the peptide.

Assay validation

As a consequence of the negative results obtained in the PHD hydroxylation assays, it was decided to investigate the detection limits of hydroxylated peptides under the used conditions in the assay. For that purpose, 4-hydroxyproline (Hyp) was synthetically introduced into a selection of PHD substrate peptides (see *Materials and Methods*, **Chapter 7.4**). Samples containing the Hyp peptides were then analysed by ESI-LC-MS, along with samples of non-hydroxylated peptides. The retention times of the peptides in the ESI-LC-MS chromatograms were compared; yet no substantial shift in the retention time of Hyp peptides, relative to the non-hydroxylated peptides, was observed under the tested conditions (**Figure 2.4**). The difference in retention time between the HIF1α CODD P564 and the HIF1α CODD Hyp564 peptides only amounted to 0.01 min (0.6 sec) at a total run time of 10 min, and the observed maximal shift in retention time was 0.48 min (28.8 sec, NDRG3 P294/ NDRG3 Hyp294). As expected, the introduction of two Hyp residues into a peptide resulted in a larger shift in retention time than single hydroxylations of the same peptide (see FLNA, FOXO3A, **Figure 2.4**). Overall, the results suggest that the introduction of one or multiple Hyp residues into a peptide affects the retention time of the peptide, most likely due to the increased polarity of the Hyp peptides, yet the effect on the retention times was not substantial under the used conditions in the PHD substrate screen.

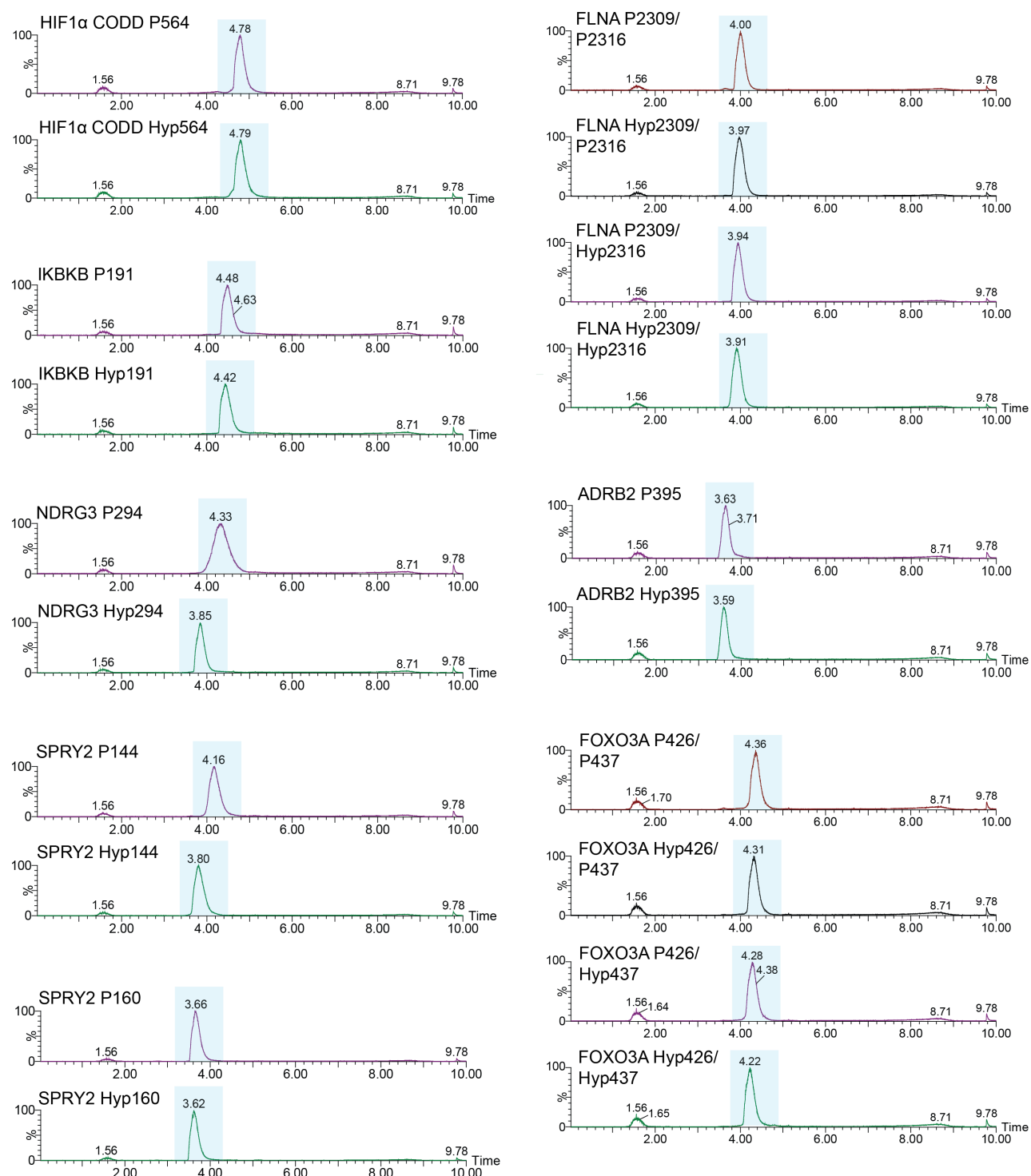


Figure 2.4: Retention times of hydroxylated and non-hydroxylated peptides by ESI-LC-MS. Shifts in the retention times between hydroxylated and the respective non-hydroxylated peptides were determined from ESI-LC-MS chromatograms; total run time: 10 min, maximum shift detected: 0.48 min (28.8 sec, NDRG3 P294/NDRG3 Hyp294).

In another approach, differences in detection efficiency of Hyp peptides and non-hydroxylated peptides were investigated. Samples containing equal molarity of a Hyp peptide and the respective non-hydroxylated peptide were analysed by ESI-LC-MS, and the intensity of the peptide peaks in the ESI-LC-MS spectra were compared. The ratio of the peak intensities of the hydroxylated peptide relative to the non-hydroxylated peptide were determined (**Table 2.5**, see *Appendix*, **Figure 2.13** for ESI-LC-MS spectra), with values ranging from 0.63 (Intensity (IKBKB Hyp191) : Intensity (IKBKB P191)) to 1.76 (Intensity (FOXO3A P426/P437) : Intensity (FOXO3A Hyp426/P437)).

Table 2.5 Relative detection efficiency of hydroxylated and non-hydroxylated peptides by ESI-LC-MS. Differences in the purities of individual peptides were taken into account for calculating the ratios below.

Non-hydroxylated peptide	Hydroxylated peptide	Ratio Intensity (hydroxylated peptide) : Intensity (non-hydroxylate peptide)
HIF1α CODD P564	HIF1α CODD Hyp564	0.71
IKBKB P191	IKBKB Hyp191	0.63
SPRY2 P144	SPRY2 Hyp144	0.65
SPRY2 P160	SPRY2 Hyp160	0.64
FLNA P2309/P2316	FLNA Hyp2309/P2316	1.34
FLNA P2309/P2316	FLNA P2309/Hyp2316	1.34
FLNA P2309/P2316	FLNA Hyp2309/Hyp2316	0.69
FOXO3A P426/P437	FOXO3A Hyp426/P437	1.76
FOXO3A P426/P437	FOXO3A P426/Hyp437	0.88
FOXO3A P426/P437	FOXO3A Hyp426/Hyp437	0.71

Overall, these results suggest that introducing Hyp residues into the tested peptide sequences does not cause any substantial effect on the detection efficiency of the peptides relative to the unmodified peptides. Taken together, the results confirm that the chosen conditions in the study presented here are suitable for detection of PHD-dependent hydroxylations in putative substrate peptides.

2.2.2 Competition of non-HIF substrates with HIF hydroxylation by PHDs

To investigate if the putative PHD substrate peptides interact with PHDs, and influence the rate of HIF1 α Codd/Nodd hydroxylation, competition screens with tPHD2 and PHD3^{fl} were performed. The enzymes were incubated with co-factors, HIF1 α Codd or HIF1 α Nodd (50 μ M), and putative substrate peptides (500 μ M) under the conditions described in *Materials and Methods*, **Chapter 7.6.2**; the reactions were quenched at a time point within the linear region of enzyme activity. Hydroxylation levels in the presence and absence of putative non-HIF substrates were analysed by ESI-LC-MS (**Figure 2.5**).

The HIF1 α Codd/Nodd : putative substrate peptide ratio of 1 : 10 was chosen, because it was assumed that some of the non-HIF substrate peptides might be weak competitors of HIF1 α Codd/Nodd. Likewise, initial competition screens with tPHD2 were conducted using the weaker PHD2 substrate HIF1 α Nodd instead of HIF1 α Codd.

A substantial inhibitory effect on tPHD2 was observed for peptide sequences from the *eukaryotic* elongation factor 2 (*eEF2* P465), the RNA polymerase II subunit (POLR2A P1465), and in control samples containing HIF1 α Codd and *Ta*HIF1 α ODD (**Figure 2.5 A**). *eEF2* P465 and POLR2A P1465 peptides reduced the hydroxylation of HIF1 α Nodd to < 20 % (**Figure 2.5 A**). For comparison, HIF1 α Nodd hydroxylation levels were reduced to ~ 10 % in samples containing HIF1 α Codd, and ~ 15 % in the presence of *Ta*HIF1 α ODD (**Figure 2.5 A**).

Notably, none of the tested peptides markedly affected tPHD2-catalysed HIF1 α Codd hydroxylation levels (**Figure 2.5 B**). No stimulatory effect on HIF1 α hydroxylation by any of the putative substrate peptides was observed.

By studying PHD3^{fl} catalysed HIF1 α Codd hydroxylation, it was found that POLR2A P1465 did not have any effect on HIF1 α Codd hydroxylation levels, unlike *eEF2* P465, that reduced hydroxylation levels to < 30 % (**Figure 2.5 C**).

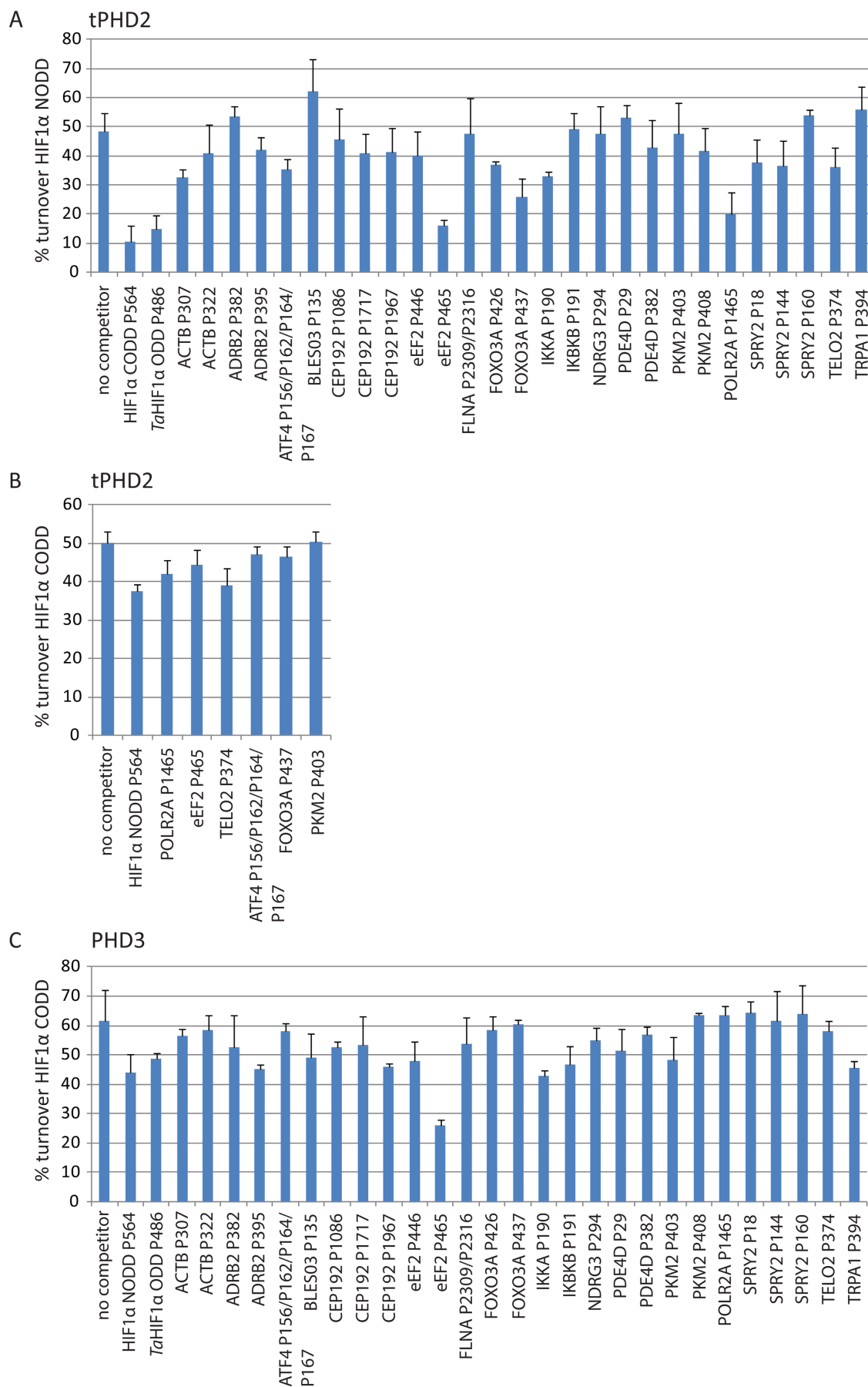


Figure 2.5: Competition assays. Peptides of reported substrates were screened for their ability to alter HIF-hydroxylation by PHDs. **(A)** HIF1 α NODD hydroxylation by tPHD2, **(B)** HIF1 α CODD hydroxylation by tPHD2, **(C)** HIF1 α CODD hydroxylation by PHD3^{fl}. Conditions: 2 μ M PHD2/3, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 300 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate, 50 μ M HIF1 α CODD/NODD, and 500 μ M peptides of reported substrates in 50 mM Tris, pH 7.5. The samples were quenched with 1% FA (v/v) at a time point in the linear region of enzyme activity, and analysed by electrospray-ionization liquid chromatography–mass spectrometry (ESI-LC-MS). Hydroxylation levels were quantified using MassLynx™ V4.1, and non-enzymatic background oxidation was subtracted. Errors are depicted as standard deviations (n=3).

As a consequence, further studies were conducted on the *e*EF2 P465 and POLR2A P1465 peptides, in order to investigate if the competitive effect of *e*EF2 P465 and POLR2A P1465 was dependent on the peptide concentration in the assay (**Figure 2.6**). The obtained results suggest that *e*EF2 P465 and POLR2A P1465 inhibit tPHD2-catalysed HIF1 α NODD hydroxylation in a concentration-dependent manner, as HIF1 α NODD hydroxylation levels are subsequently decreased by increasing the concentrations of *e*EF2 P465 and POLR2A P1465. Notably, the inhibitory effect of *e*EF2 P465 and POLR2A P1465 on tPHD2-catalysed HIF1 α NODD hydroxylation is already observable at a 1 : 1 ratio of HIF1 α NODD : competitive peptide (50 μ M *e*EF2 P465/ POLR2A P1465, **Figure 2.6**, second row). At a 1 : 1 ratio, HIF1 α NODD hydroxylation levels are decreased by ~ 45 % in the presence of POLR2A P1465, and by ~ 25 % in the presence of *e*EF2 P465.

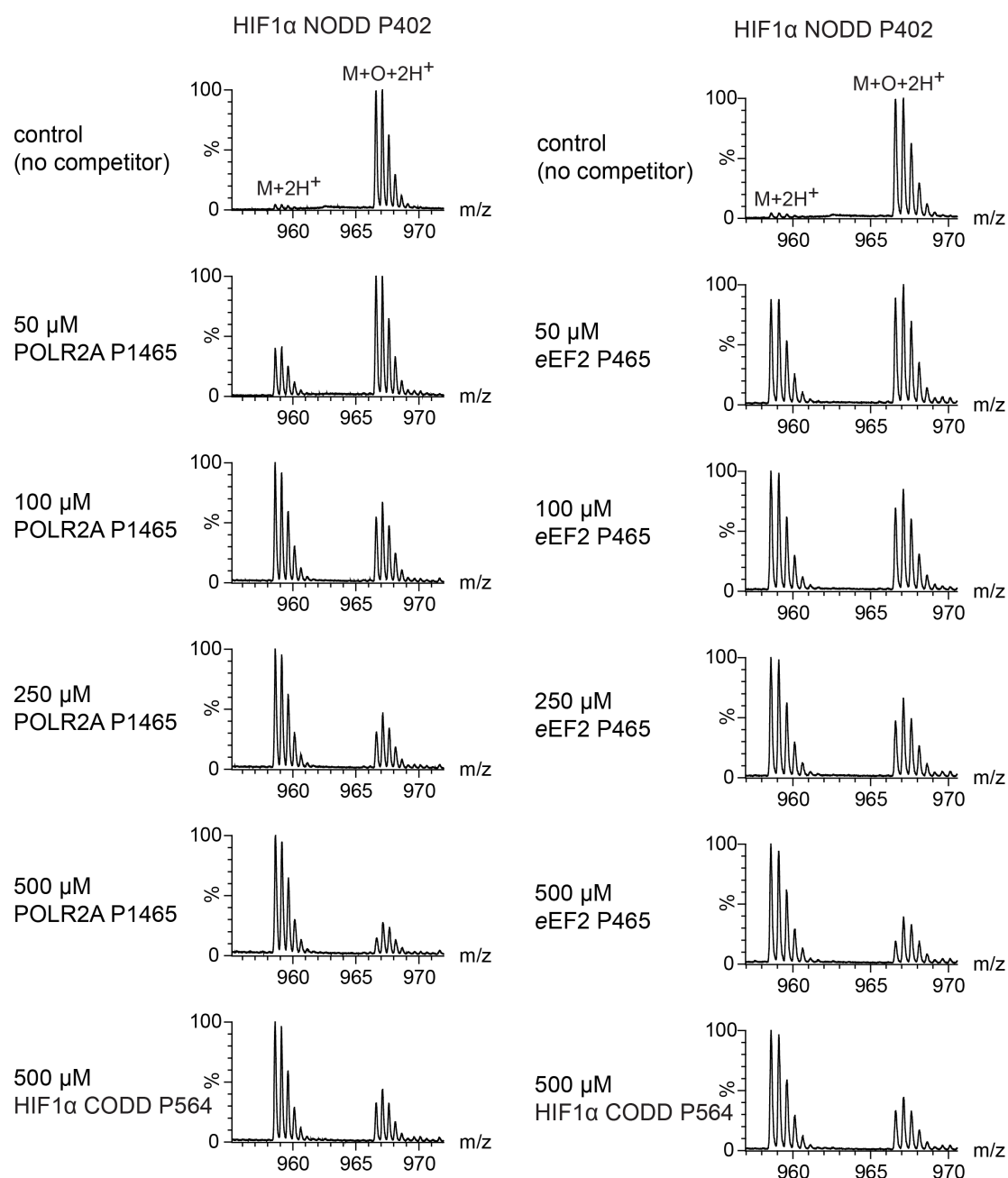


Figure 2.6: Concentration dependent effect of *e*EF2 P465 and POLR2A P1465 peptides on HIF1 α NODD hydroxylation by tPHD2. Conditions: 2 μ M tPHD2, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 300 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate, 50 μ M HIF1 α NODD, and varying concentrations of *e*EF2 P465 or POLR2A P1465, in 50 mM Tris, pH 7.5. The samples were quenched with 1% FA (v/v) after near completion of the reaction, and analysed by electrospray-ionization liquid chromatography–mass spectrometry (ESI-LC-MS). Hydroxylation levels were analysed using MassLynx™ V4.1.

2.2.3 Studies on binding of non-HIF substrates to PHD2

The binding modes of the two inhibitory peptides *e*EF2 P465 and POLR2A P1465 were investigated by NMR-displacement experiments (for assay conditions see *Materials and Methods*, **Chapter 7.7.2**, (45)). Reporter HIF1 α CODD and NODD peptides were used that were ^{13}C labelled on all carbons in their proline residues, to produce a characteristic signal of the peptides in the NMR spectra (**Figure 2.7**, peptide signal labelled with ^{13}C -CODD and ^{13}C -NODD, respectively).

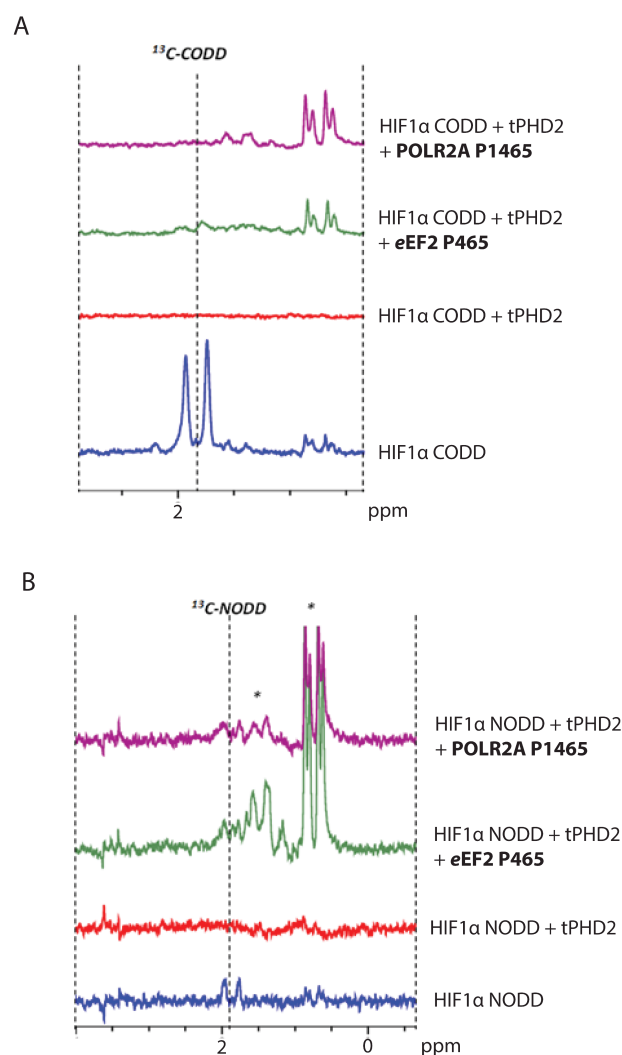


Figure 2.7: Qualitative single concentration screening by ^{13}C -CODD and ^{13}C -NODD displacement as monitored by 1D CLIP HSQC NMR. (A) ^{13}C -CODD displacement, (B) ^{13}C -NODD displacement. The assay mixtures contained 50 μM apo-tPHD2, 400 μM Zn(II), 50 μM 2OG, 50 μM ^{13}C -CODD or ^{13}C -NODD, 800 μM peptidic substrate and 50 mM Tris- D_{11} , pH 7.5 in 10 % D_2O and 90 % H_2O . * Denotes intrinsic peptide signals.

Upon addition of apo-tPHD2 into the assay mix, a stable complex was formed with the [^{13}C]-labelled HIF1 α Codd or Nodd peptides, and Zn(II) (substituting for Fe(II)). As a consequence, the reporter signal disappeared. By adding *e*EF2 P465 or POLR2A P1465 to the assay mix, the HIF1 α Nodd reporter signal was regained (**Figure 2.6 B**), suggesting that these peptides directly bind to tPHD2 and displace HIF1 α Nodd from the enzyme. By contrast, no displacement of HIF1 α Codd by *e*EF2 P465 or POLR2A P1465 was observed within the detection limits (**Figure 2.6 A**).

The obtained results are in accordance with the observation of an inhibitory effect of the *e*EF2 P465 and POLR2A P1465 peptides on HIF1 α Nodd hydroxylation by tPHD2 (**Chapter 2.2.2**). Previous studies have found that the HIF1 α Codd and HIF1 α Nodd peptides differ in their binding affinity to PHD2, with HIF1 α Codd being the better binder as judged by K_d comparison (K_d (HIF1 α Nodd) = 1.06 ($\pm$ 0.39) s $^{-1}$, K_d (HIF1 α Codd) = 0.14 ($\pm$ 0.04) s $^{-1}$, (38)), and HIF1 α Codd was shown to be more efficiently hydroxylated by PHD2 than HIF1 α Nodd (38). It is possible that the observed selective displacement of Nodd is due to the differences in binding strengths and turnover rates between HIF1 α Codd and HIF1 α Nodd. Recently, structural studies on the PHD2.Codd and PHD2.Nodd enzyme-substrate complexes revealed differences in the binding modes of the substrate peptides, e.g. in the C-terminal helix α 4 and the β 2 β 3-loop of PHD2 (39-40). As a consequence, the selective displacement of HIF1 α Nodd by *e*EF2 P465 and POLR2A P1465 might be due to differences in the binding modes of HIF1 α Codd/Nodd to PHD2.

2.2.4 Influence of non-HIF substrates on 2OG turnover by PHDs

2OG dependent oxygenases catalyse the uncoupled turnover of 2OG to succinate and CO₂ in the absence of their prime substrate to varying extents (42). In the case of PHD2, it has been shown that 2OG decarboxylation is strongly coupled to CDD or NODD hydroxylation, with only a small degree of substrate-uncoupled turnover observed (41). In previous studies, the strong coupling between substrate hydroxylation and 2OG turnover has been used to identify new substrates of the PHDs, by use of a radioactive [1-¹⁴C] 2OG decarboxylation assay (2, 23, 30, 33). To investigate if the reported non-HIF substrates can stimulate the 2OG turnover by the PHDs, succinate formation and 2OG depletion were monitored by ¹H-NMR in the presence and absence of the putative substrates (see *Materials and Methods*, **Chapter 7.7.1**, (46-47)). A selection of the non-HIF substrate peptides was chosen, including the hydroxylation sites in acetyl-CoA carboxylase 2 (ACACB/ ACC2 P450), and protein phosphatase 2 regulatory subunit B α (PPP2R2A P319); both of them have been reported to stimulate 2OG turnover in the radioactive [1-¹⁴C] 2OG decarboxylation assay (2, 33).

The results in **Figure 2.8** demonstrate that none of the tested putative substrate peptides stimulate 2OG turnover by tPHD2 (for supplementary spectra see *Appendix*, **Figure 2.14**). Consistent with previous studies (41), only a small degree of succinate formation was observed in the absence of HIF1 α CDD/NODD. Upon addition of HIF1 α CDD/NODD peptides, the rate of succinate formation (and 2OG depletion) increased substantially. However, this effect was not observed with any of the studied non-HIF substrates, thus the results obtained here differ from previous studies (2, 33).

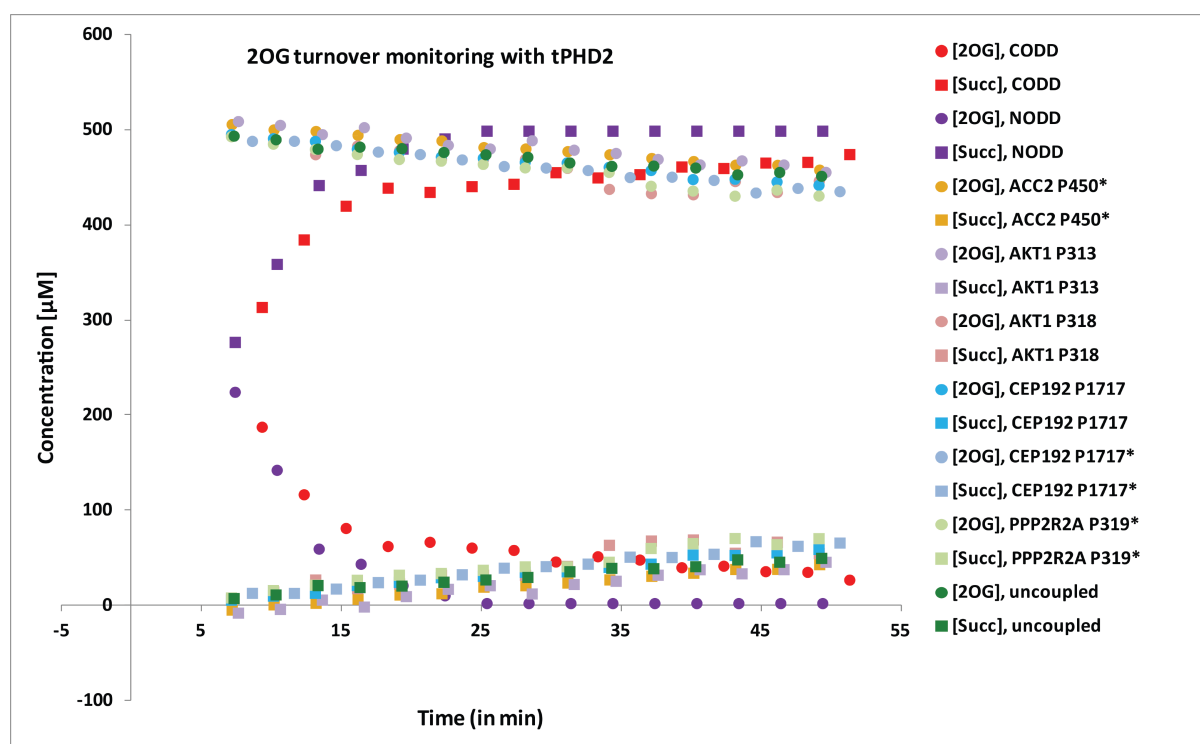


Figure 2.8: Monitoring the coupled and uncoupled turnover of 2OG by tPHD2 in the presence and absence of HIF and non-HIF substrates. 2OG turnover was monitored by ^1H CPMG NMR, conditions: 20 μM PHD2, 125 μM Fe(II), 1 mM sodium L-ascorbate, 500 μM peptides where necessary, 400 μM 2OG, in 10 % D_2O and 90 % H_2O , 50 mM Tris- D_{11} , pH 7.5; [Succ]=Succinate.

By contrast to the extensive studies of 2OG turnover by PHD2, the extent to which substrate-uncoupled 2OG turnover is catalysed by PHD1 and PHD3 is less well understood. Therefore, a similar study was conducted with PHD3 (due to limited availability of PHD1 and high enzyme consumption by the 2OG turnover assay, the study was not performed with PHD1).

The results suggest that 2OG decarboxylation by PHD3, like PHD2, is strongly coupled to CODD hydroxylation (**Figure 2.9**, supplementary spectra in *Appendix*, **Figure 2.14**). The degree of both uncoupled and coupled turnover was decreased as compared to tPHD2, likely due to lower activity of the recombinant PHD3. Notably, upon addition of HIF1 α NODD, an increase in 2OG turnover was observed (although the effect is less when compared to HIF1 α CODD). Studies on PHD3 activity to date suggest that the enzyme is highly selective for CODD over NODD, and hydroxylation of a NODD sequence by PHD3 has not been observed *in vitro* or *in vivo* (36-37, 48).

The results presented here suggest that, even though PHD3 does not accept HIF1 α NODD as a substrate, the NODD peptide interacts with PHD3, thus stimulating the turnover of 2OG. As is the case with PHD2, no 2OG turnover by PHD3 was stimulated upon addition of the non-HIF substrate peptides.

Overall, these results support the proposal that PHDs are highly selective for their *bona fide* HIF1 α substrates, implying that they do not accept any of the other tested, less well established, substrates.

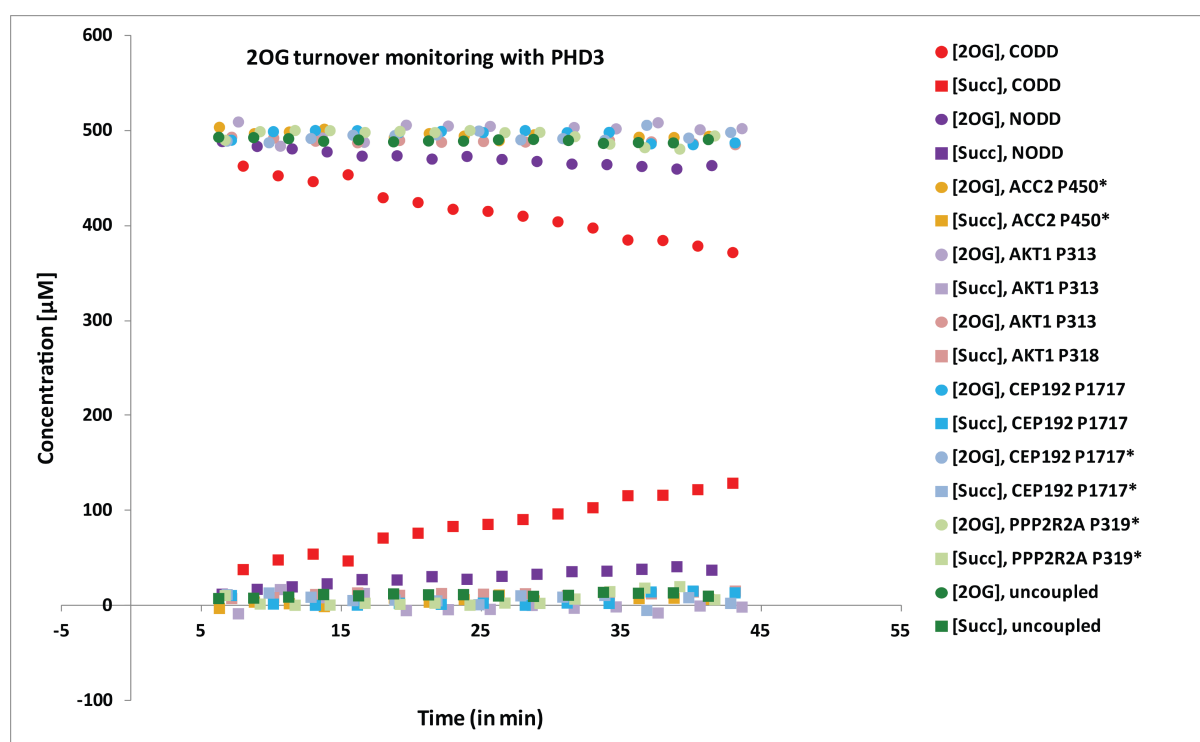


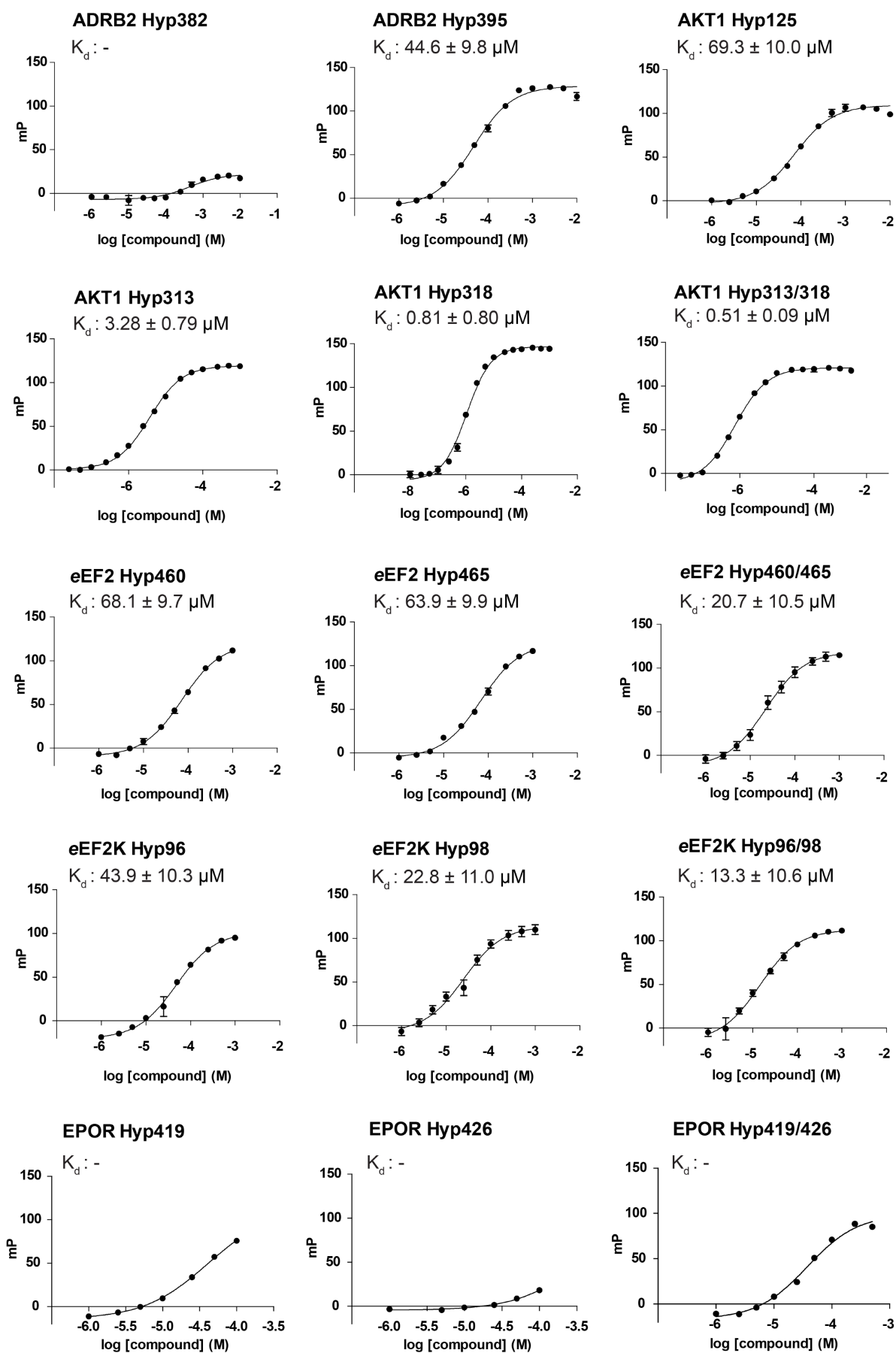
Figure 2.9: Monitoring the coupled and uncoupled turnover of 2OG by PHD3 in the presence and absence of HIF and non-HIF substrates. 2OG turnover was monitored by ^1H CPMG NMR, conditions: 20 μM PHD2, 125 μM Fe(II), 1 mM sodium L-ascorbate, 500 μM peptides where necessary, 400 μM 2OG, in 10 % D_2O and 90 % H_2O , 50 mM Tris- D_{11} , pH 7.5; [Succ]=Succinate.

2.2.5 VCB binding studies by fluorescence-polarisation

The results presented in this Chapter suggest that, at least in isolated form, the PHDs are more selective for their HIF α substrates than previously assumed, contrasting the reports in the literature of multiple PHDs substrates other than HIFs. In some of these reports, pVHL/VCB interaction studies were carried out showing that putative PHD substrates bind to the pVHL or the VCB complex (ADRB2 (16), AKT1 (17), EPOR (20), FLNA (22), NDRG3 (24), POLR2A (27-28), SPRY2 (29)); in some cases, prolyl hydroxylation-dependent binding to pVHL/VCB was reported (ADRB2 (16), AKT1 (17), EPOR (20), POLR2A (27-28)).

In order to validate these reports, putative PHD substrates were tested for binding to the VCB complex by use of a fluorescence-polarization (FP) assay. To assess if the binding to VCB is dependent on the presence of Hyp in the peptide sequence, single and double hydroxylated peptides, as well as un-modified peptides were synthesised (*Materials and Methods*, **Chapter 7.4**) and tested in the FP assay. The VCB protein used in the FP assay was prepared by Lucía Serrán Aguilera (University of Oxford, UK), following the procedure in (49).

The ability of the non-HIF substrates to bind to VCB was measured by competition with a fluorescence-labelled HIF-derived peptide (FAM-DEALAHypYIPD, for assay conditions see *Materials and Methods*, **Chapter 7.8**, (50)). FAM-DEALAHypYIPD was incubated with VCB and increasing amounts of a non-HIF substrate peptide. The degree of displacement of FAM-DEALAHypYIPD from the VCB complex by the competitive binder (non-HIF substrate peptide) was determined by measuring the difference in fluorescence-polarisation (mP) between samples containing VCB and FAM-DEALAHypYIPD (maximum polarisation, minimum displacement), and samples containing the competitive binder. The data were normalised to a sample containing FAM-DEALAHypYIPD without VCB (minimum polarisation, maximum displacement). IC₅₀ values were calculated using GraphPad Prism, and K_d values for the competitive binder-VCB complex were calculated from the IC₅₀ values as described in (50).



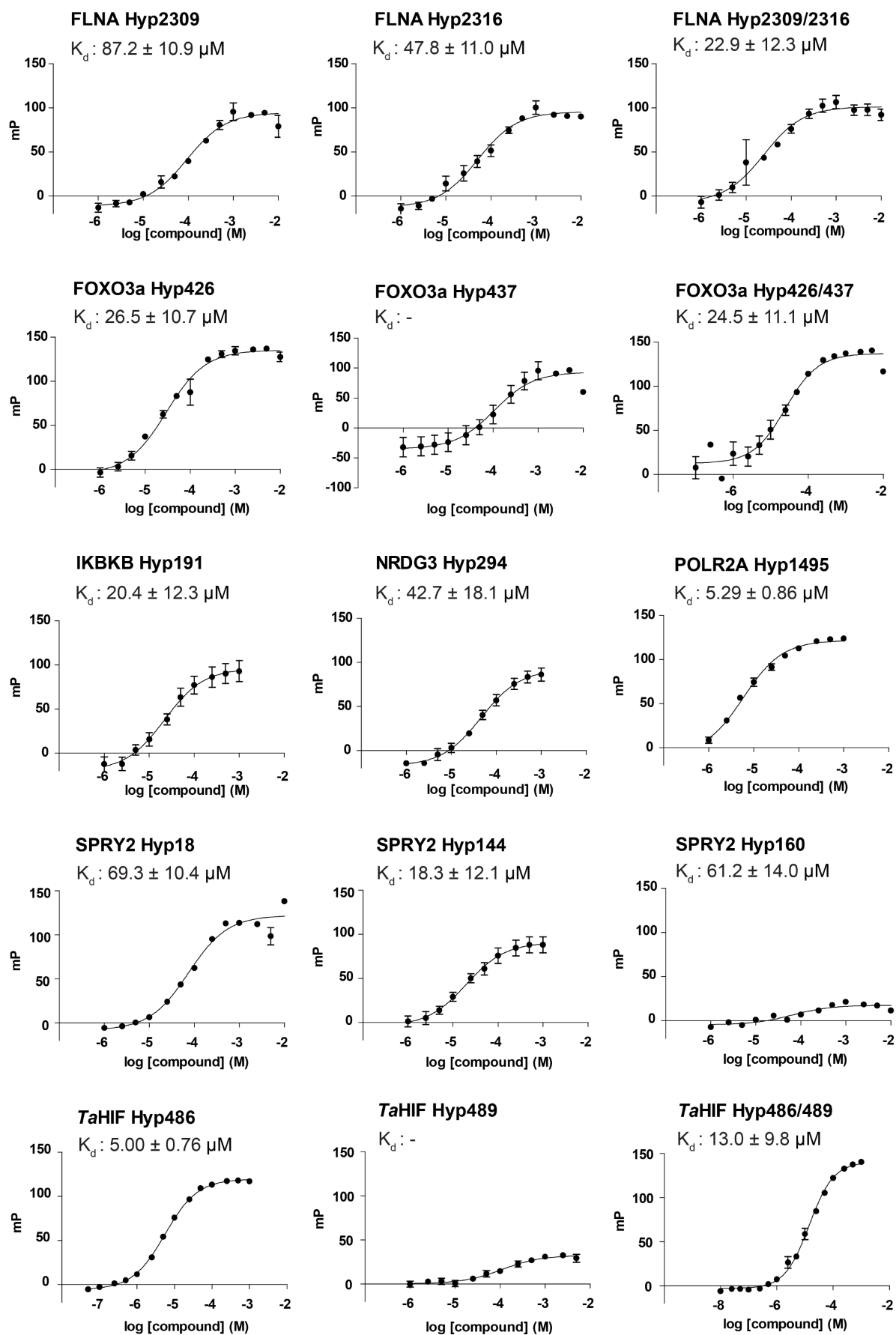


Figure 2.10: Binding of Hyp-containing non-HIF substrate peptides to the VCB complex. K_d values were determined by measuring the degree of displacement of a 6-fluorescein amidite-labelled HIF-derived peptide (FAM-DEALAHypYIPD) from VCB by a competitive binder (non-HIF substrate peptide). The plotted values represent the difference in fluorescence-polarisation (mP) between samples containing FAM-DEALAHypYIPD and VCB and samples additionally containing increasing concentrations of a competitive binder; conditions: 0.1 - 100 μ M competitive binder (non-HIF peptide), 0.5 μ M VCB, 139 nM FAM-DEALAHypYIPD. Error bars represent standard error of the mean (SEM).

Table 2.6 Relative binding strengths of hydroxylated peptides to VCB as determined by fluorescence-polarisation; labelling: LXXLAP and LAP motifs (red), $K_d < 10 \mu$ M (orange), sequence identity to HIF1 α CODD > 30 % (yellow). K_d values labelled with * were determined by Lucía Serrán Aguilera (University of Oxford, UK, unpublished data).

Peptide	Sequence	K_d (μ M)	Sequence identity to HIF1 α CODD
HIF1 α CODD Hyp564	DLDLEMLA Hyp YIPMDDDFQL	0.05 $\pm$ 0.01 *	100 %
HIF2 α CODD Hyp531	ELDLET LAHyp YIPMDGEDFQL	0.14 $\pm$ 0.03 *	84.2 %
HIF3 α CODD Hyp492	ALDLEMLA Hyp YISMDDDFQL	0.14 $\pm$ 0.04 *	89.5 %
HIF1 α NODD Hyp402	DALTLLA Hyp AAGDTIISLDF	1.32 $\pm$ 0.16 *	20 %
HIF2 α NODD Hyp405	EELAQLA Hyp TPGDAIISLDF	0.18 $\pm$ 0.02 *	33.3 %
ADRB2 Hyp382	QEKENKLLCEDL Hyp GTEDFVGHQGT	-	21.1 %
ADRB2 Hyp395	GTEDFVGHQGT Hyp SDNIDSQGRNCS	44.6 $\pm$ 9.8	28.6 %
AKT1 Hyp125	QEEEEMDFRSGS Hyp SDNSGAEEMEVS	69.3 $\pm$ 10.0	10.5 %
AKT1 Hyp313	KDGATMKTFCGT Hyp EY LAP EVLEDND	3.28 $\pm$ 0.79	37.5 %
AKT1 Hyp318	KDGATMKTFCGT PEYLAHyp EVLEDND	0.81 $\pm$ 0.80	37.5 %
AKT1 Hyp313/318	KDGATMKTFCGT HypEYLAHyp EVLEDND	0.51 $\pm$ 0.09	37.5 %
eEF2 Hyp460	GRYVE Hyp IEDVPCGNIVGLVGVDQFL	68.1 $\pm$ 9.7	16.7 %
eEF2 Hyp465	GRYVE PIEDVHyp CGNIVGLVGVDQFL	63.9 $\pm$ 9.9	16.7 %
eEF2 Hyp460/465	GRYVE Hyp IEDV Hyp CGNIVGLVGVDQFL	20.7 $\pm$ 10.5	16.7 %
eEF2K Hyp96	KHAIQKAKH MHyp DPWAEFHLEDIATE	43.9 $\pm$ 10.3	26.7 %
eEF2K Hyp98	KHAIQKAKH MPDHyp WAEFHLEDIATE	22.8 $\pm$ 11.0	26.7 %
eEF2K Hyp96/98	KHAIQKAKH MHypDHyp WAEFHLEDIATE	13.3 $\pm$ 10.6	26.7 %

Peptide	Sequence	K _d (μM)	Sequence identity to HIF1α CODD
EPOR Hyp419	QLLRPWTLC Hyp ELPPTP Hyp HLKYLYLV	-	13.3 %
EPOR Hyp426	QLLRPWTLC PE LPPT Hyp HLKYLYLV	-	13.3 %
EPOR Hyp419/426	QLLRPWTLC Hyp ELPPT Hyp HLKYLYLV	-	13.3 %
FLNA Hyp2309	VKFNEEH I Hyp DSPFVV P VASPSGDAR	87.2 ± 10.9	16.7 %
FLNA Hyp2316	VKFNEEH I P DSPFVV Hyp VASPSGDAR	47.8 ± 11.0	16.7 %
FLNA Hyp2309/2316	VKFNEEH I Hyp DSPFVV Hyp VASPSGDAR	22.9 ± 12.3	16.7 %
FOXO3A Hyp426	SGLGS Hyp TSSFNSTVFG P SSLNSL	26.5 ± 10.7	13.3 %
FOXO3A Hyp437	SGLGS P TSSFNSTVFG Hyp SSLNSL	-	13.3 %
FOXO3A Hyp426/437	SGLGS Hyp TSSFNSTVFG Hyp SSLNSL	24.5 ± 11.1	13.3 %
IKBKB Hyp191	TSFVGT L QY LA Hyp ELLEQQKYTVTD	20.4 ± 12.3	21.1 %
NDRG3 Hyp294	TTLLKMADCGGL Hyp QVVQPGKLTEAF	42.7 ± 18.1	25 %
POLR2A Hyp1495	ENIM L GQ LA Hyp AGTGCFDLLLLD	5.29 ± 0.86	29.4 %
SPRY Hyp18	QSGNGSQPLLQT Hyp RDGGRQRGEPDP	69.3 ± 10.4	22.2 %
SPRY Hyp144	EQRLLGSSFS S Hyp VADGIIRVQPKS	18.3 ± 12.1	17.7 %
SPRY Hyp160	GIIRVQPKSEL K Hyp GELKPLSKEDLG	61.2 ± 14.0	25 %
TaHIF Hyp486	PINEKEDYDD LA Hyp FVPPPSFDNRLY	5.00 ± 0.76	31.6 %
TaHIF Hyp489	PINEKEDYDD LA P FV Hyp PPPSFDNRLY	-	31.6 %
TaHIF Hyp486/489	PINEKEDYDD LA Hyp FV Hyp PPPSFDNRLY	13.0 ± 9.8	31.6 %

The results of the VCB-binding assays are shown in **Table 2.6** and **Figure 2.10**. It was found that most of the tested putative PHD substrates bind to VCB, although with varying strengths (K_d values ranging from 0.51 ± 0.09 to 87.2 ± 10.9 μM). No substantial binding to VCB was observed for the single- and double hydroxylated peptides that contain the reported hydroxylation site in the erythropoietin receptor (EPOR). Most importantly, it was found that binding of the peptides to VCB was dependent on the hydroxylation of at least one prolyl residue in the sequence, as none of the un-modified peptides was able to displace the labelled HIF1α peptide from VCB (data not shown).

The strongest affinity to VCB (lowest K_d value) was obtained for peptides from AKT serine/threonine kinase 1 (AKT1). Two PHD2-dependent prolyl hydroxylation sites in AKT1 have been reported in the literature, i. e. residues P125 and P313, both of which are proposed to stimulate binding to VCB in their hydroxylated forms (17). However, in the study presented in this Chapter, no PHD-catalysed hydroxylation of AKT1 peptides was observed (**Chapter 2.2.1, Figure 2.3**). Similarly, no PHD2-catalysed hydroxylation was found in assays using full-length AKT1 produced by IVTT (Ya-Min Tian, Matthew Cockman, and Peter Ratcliffe, Nuffield Department of Medicine, University of Oxford, UK and Francis Crick Institute, UK, unpublished data).

However, in agreement with the literature, it was found in this study that AKT1 Hyp313 efficiently binds to VCB ($K_d = 3.28 \pm 0.79 \mu\text{M}$), yet the peptide containing the other reported hydroxylation site, AKT1 Hyp125, bound substantially less well to VCB ($K_d = 69.3 \pm 10.0 \mu\text{M}$). The sequence of AKT1 contains a LAP motif adjacent to the reported hydroxylation site P313, and hydroxylation of the prolyl residue within the LAP motif (AKT1 Hyp318), stimulated the binding to VCB even more than was the case for the reported site (K_d (AKT1 Hyp318) = $0.81 \pm 0.80 \mu\text{M}$ vs. K_d (AKT1 Hyp313) = $3.28 \pm 0.79 \mu\text{M}$). Binding to VCB was further increased by introducing two adjacent hydroxylations, both in the reported site and in the LAP motif (AKT1 Hyp313/318, $K_d = 0.51 \pm 0.09 \mu\text{M}$). The binding strengths of the AKT1 Hyp318 and AKT1 Hyp313/318 peptides are comparable to the binding strength of the natural VCB substrate HIF1 α NODD Hyp402 ($K_d = 1.32 \pm 0.16 \mu\text{M}$), yet are lower than for HIF α CODD (K_d values ranging from 0.05 ± 0.01 - $0.14 \pm 0.04 \mu\text{M}$). As a consequence, the obtained K_d values for AKT1 are in the biologically relevant range, however further studies are necessary to investigate a potential physiological role of AKT1 binding to the VCB complex.

Overall, the following trends regarding the binding strength of hydroxylated peptides to VCB were observed (**Table 2.6**):

1. Double hydroxylation improves the binding affinity to VCB in most cases, but not all (in case of the HIF sequence from *Trichoplax adhaerens* (TaHIF P486), hydroxylation of the unnatural site P486 led to a substantial decrease in binding).
2. The best VCB binders all contain a LAP motif (i.e. sequences exhibiting a leucine and an alanine residue positioned *N*-terminal to the target proline residue), suggesting that this motif is a crucial criterion for VCB affinity. However, the presence of a LAP motif in the peptide sequence is not a sufficient criterion for efficient VCB binding (e.g. K_d (IKBKB Hyp191) = $21 \pm 2 \mu\text{M}$).
3. The best VCB binders have a sequence similarity > 30 % to HIF1 α CODD.

Taken together, the results obtained in the VCB-binding study suggest that VCB has the potential to be highly promiscuous and likely accepts additional substrates other than HIFs. The data presented in this Chapter is also relevant from a medicinal chemistry point of view, since the identified criteria for VCB binding can be exploited for the design of new (e.g. cyclic peptide) inhibitors of VCB.

2.2.6 Co-expression experiments

So far, the work presented in this Chapter suggests that the PHDs do not accept any of the reported non-HIF substrates. However, it cannot be excluded that the PHDs require longer substrate sequences than those used in the peptide screens (although 19-20mer HIF α peptides are efficiently hydroxylated by the PHDs). To follow up the work on peptidic substrate screens, a selection of the reported non-HIF substrates were tested for hydroxylation at the protein level.

The initial set of putative substrates included full-length pyruvate kinase isoform 2 (PKM2), full-length actin β (ACTB), adrenoceptor β 2, residues 330-413 (ADRB2), and HIF1 α , residues 530-698 as a control. The codon-optimised genes of the putative substrates were cloned into a GST-vector, and co-expressed in *E. coli* with the respective PHD isoform that was found responsible for the hydroxylation in the literature (tPHD2 or PHD3^{fl}, *Materials and Methods*, **Chapter 7.1.1.3**, and **7.2.5**). After optimisation of the co-expression conditions, the putative substrates were purified over a GST-affinity column, the proteins were treated with trypsin, and samples of the digested proteins were subjected to analysis for prolyl hydroxylation by LC-MS/MS (*Materials and Methods*, **Chapter 7.2**, **7.3**, and **7.6.5**). Data acquisition and analysis were performed by Roman Fischer, (Benedikt Kessler Lab, Target Discovery Institute, University of Oxford). The data were analysed by conducting automated standard searches for post-translational modifications using PEAKS studio.

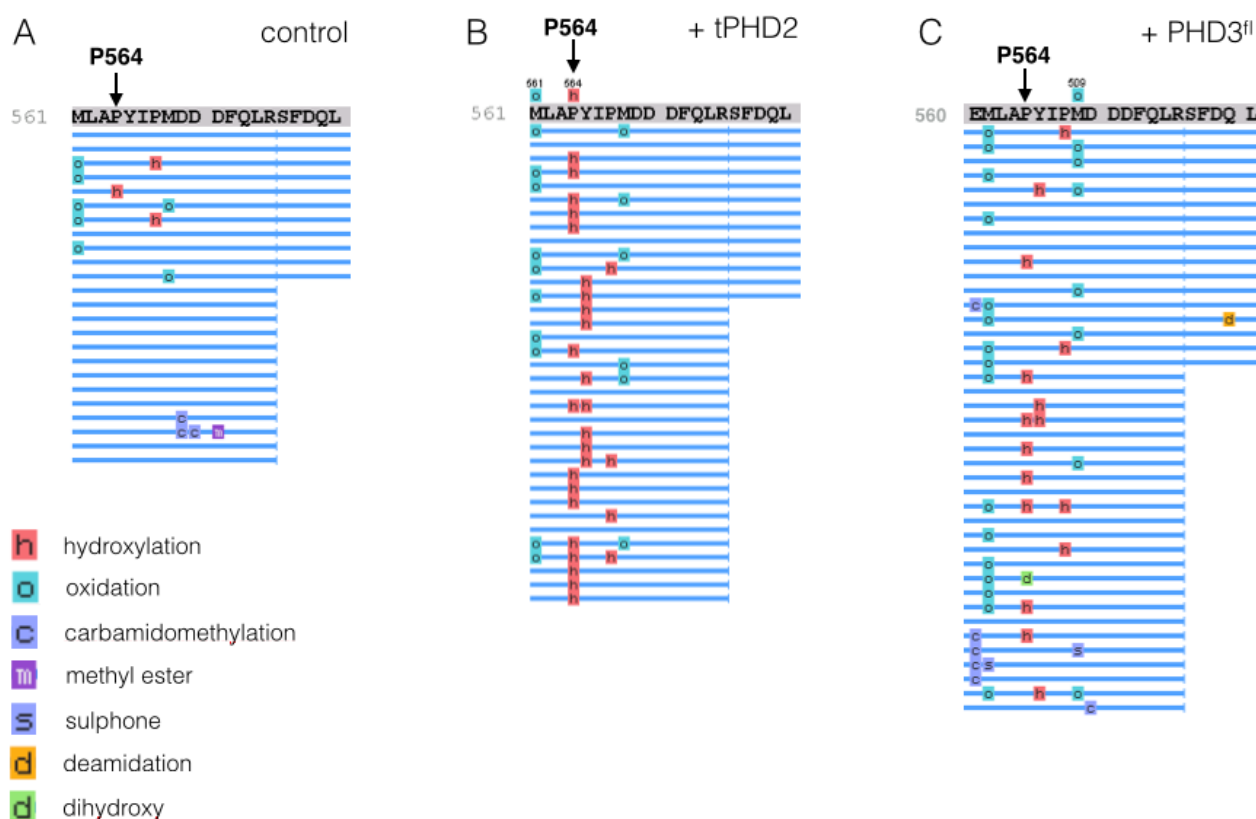
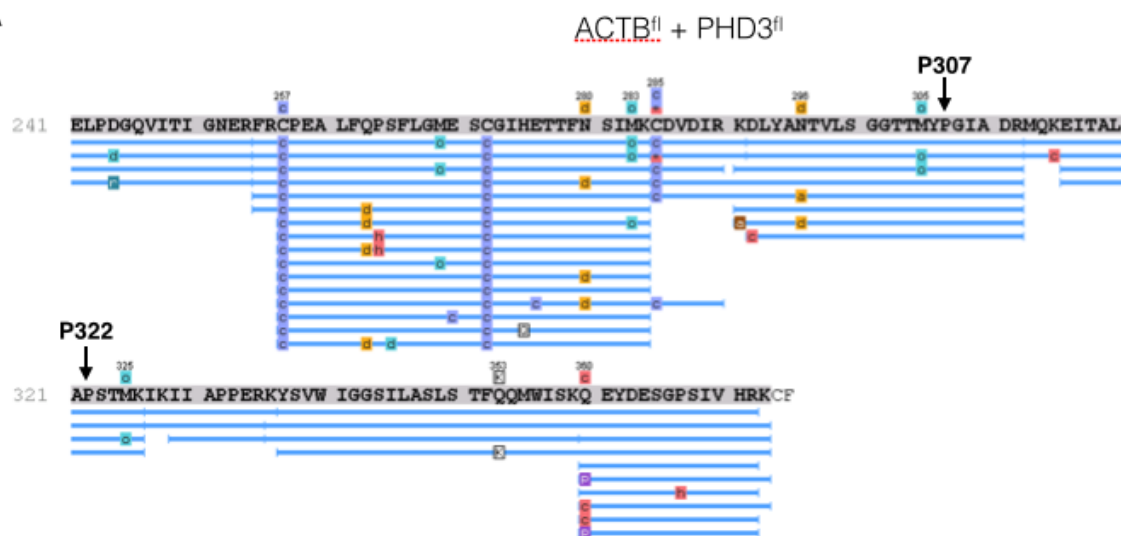


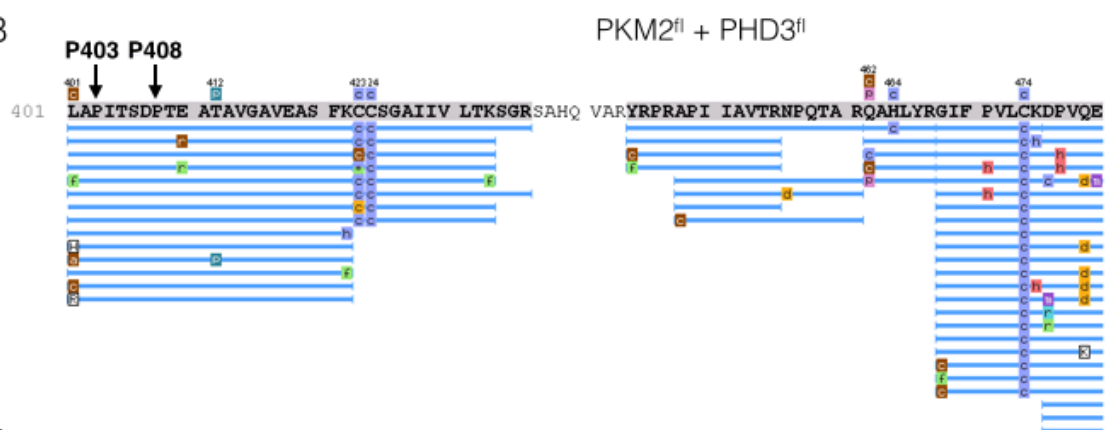
Figure 2.11: LC-MS/MS data from co-expressions of HIF1 $\alpha^{530-698}$ with tPHD2 and PHD3^{fl}. GST-tagged HIF1 $\alpha^{530-698}$ was co-expressed in *E. coli* with PHDs. (A) control (without PHD), (B) tPHD2, and (C) PHD3^{fl}. HIF1 $\alpha^{530-698}$ was subsequently purified, and tryptic digests of the protein were analysed for PTMs by LC-MS/MS. Hydroxylation at the prolyl residue P564 in the tryptic peptides was detected (increased abundance in samples from co-expressions with PHD3^{fl} and tPHD2).

Initial data confirmed the activity of tPHD2 and PHD3^{fl} on HIF1 $\alpha^{530-698}$ in the co-expressions, since multiple peptides from the tryptic digest containing hydroxylation of the prolyl residue P564 were detected (**Figure 2.11**). In some tryptic peptides, the hydroxylation was (automatically) assigned to the adjacent residue Y565, or to P567. It is likely that these hydroxylation sites were wrongly assigned as a result of the automated search for PTMs, highlighting the risk of detection of false positives in LC-MS/MS data analysis.

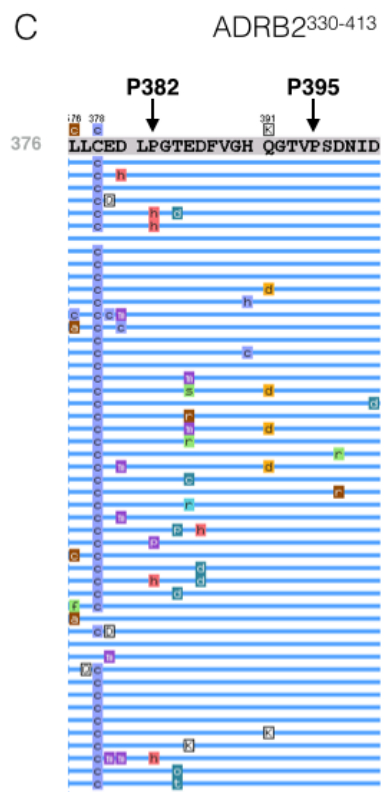
A



B



C



D



Figure 2.12: LC-MS/MS data from co-expressions of putative substrates with PHD3^{fl}. (A) ACTB^{fl}, (B) PKM2^{fl}, (C) ADRB2⁵³⁰⁻⁶⁹⁸ (no-enzyme control), and (D) ADRB2⁵³⁰⁻⁶⁹⁸ (co-expressed with PHD3^{fl}). GST-tagged proteins were co-expressed with PHD3^{fl} in *E. coli*, purified, and tryptic digests of the protein were analysed for PTMs by LC-MS/MS. Reported prolyl hydroxylation sites are labelled with black arrows.

The initial data on the co-expressions of PKM2^{fl} and ACTB^{fl} with PHD3^{fl} (**Figure 2.12 A and B**) suggests that the two reported substrates are not hydroxylated at the prolyl residues assigned in the literature (PKM2^{fl} P403, P408, and ACTB^{fl} P307, P322, (6, 26)) However, there is weak evidence for prolyl hydroxylations at other sites in the proteins (e.g. PKM2^{fl} P471 and P477, ACTB^{fl} P366); this can be due to wrong assignment of the PTM, yet it cannot be excluded that other sites than the ones reported in the literature are hydroxylated by PHD3^{fl}.

By contrast, in co-expressions of ADRB2⁵³⁰⁻⁶⁹⁸ with PHD3^{fl} (**Figure 2.12 D**), tryptic peptides containing hydroxylations at the reported prolyl residue P382 (16), but not at the second reported site, P395 (16), were detected. However, the same result was obtained when ADRB2⁵³⁰⁻⁶⁹⁸ was expressed alone (**Figure 2.12 C**), suggesting that the observed modification of P382 is independent of the enzymatic activity of PHD3^{fl}.

In order to test all reported PHD substrates at the protein level, the study presented in this Chapter was followed up in collaboration with Peter Ratcliffe, Ya-Min Tian, and Matthew Cockman (Nuffield Department of Medicine, University of Oxford). The collaborators conducted an extensive study using IVTT-produced protein substrates, that were incubated with the respective reported PHD isoform. Data analysis by LC-MS/MS confirmed that none of the reported non-HIF substrates were modified in the IVTT-assays (unpublished data).

2.3 Conclusions

The HIF prolyl hydroxylases (PHDs) play a crucial role in the adaptation of organisms to hypoxia. The enzymes function as 'oxygen sensors', by catalysing the hydroxylation of HIF α in an oxygen-regulated manner. However, to date there have been ~ 30 reports suggesting that the PHDs are highly promiscuous enzymes, that accept multiple substrates other than HIFs. With PHD inhibitors for the treatment of anaemia in late stage clinical trials, the question on how these additional substrates potentially influence the HIF pathway is gaining major medicinal relevance (51). The apparent PHD substrates have been mostly characterised in cells, and crucially, many studies lack hard evidence for PHD-dependent hydroxylation of the proposed substrate (see **Table 2.1**). Moreover, characterisation of substrate hydroxylation *in vitro* by use of radioactive [1- ^{14}C] 2OG decarboxylation can be misleading, as the decarboxylation of 2OG precedes substrate hydroxylation in the catalytic cycle (see *General Introduction*, **Chapter 1.2**). In addition, the detection of prolyl hydroxylations by mass spectrometry entails some difficulties, due to isobaric oxidations of other amino acids in the substrate protein (e.g. widespread non-enzymatic methionine oxidations). Given the high relevance for basic science and medicine, the aim of the work in this Chapter was to validate the PHD-dependent hydroxylation of reported non-HIF substrates.

An extensive *in vitro* study was carried out for the validation of alternative (non-HIF) PHD substrates. The reported hydroxylation sites were examined in four different types of assays, including a peptide-based hydroxylation screen, measurement of the 2OG turnover by PHDs in the presence of reported substrates, co-expression of the apparent substrates with PHDs, and screening of IVTT-produced full-length proteins for reaction with PHDs (the latter was performed by collaborators (Peter Ratcliffe group)). All the results of these studies suggest that none of the reported hydroxylation sites in non-HIF substrates are accepted by any of the three PHD isoforms. As a consequence, the study presented here strongly contrasts with previous reports and challenges the current view in the field. The study also points out the risk of detection of false positives, i. e. wrong annotations of the +16 mass shift, when searches for post-translational modifications are performed using standard search parameters in PEAKS or MASCOT. For correct annotation of the +16 modification it is crucial to perform MS/MS and amino acid analysis, which should become a gold standard for the detection of prolyl hydroxylations (and other amino acid modifications).

Given the results from the *in vitro* study, it cannot be excluded that PHDs catalyse the hydroxylation of the reported non-HIF substrates *in vivo*. For instance, hydroxylation of the reported substrates could be mediated in the cell by an interaction partner that facilitates the formation of an (active) PHD-non-HIF substrate complex, or via PTMs on PHDs that are necessary for hydroxylation of the non-HIF substrate. Yet, to date there is no evidence of a PHD interactor or a PTM that enhances binding or hydroxylation of HIFs in cells. In addition, the rate of reproducibility of the reported substrates is low, as there are hardly any follow-up studies on any of the non-HIF substrates, although many reports have been published more than 10 years ago.

By contrast to the PHDs, the factor inhibiting HIF (FIH) asparagyl hydroxylase has been previously found to be highly promiscuous, as it hydroxylates various amino acids in a broad range of substrates (52-54). The PHDs and FIH belong to two structurally different 2OG-oxygenase subfamilies, the first belonging to the PH (prolyl hydroxylase) subfamily VIII (55) and the latter being part of the Jumonji-C (JmjC) 2OG oxygenase subfamily IX (55). Notably, FIH has a wide active site cleft that can probably accommodate different substrates more easily, unlike the active site of PHD2, which is highly buried (see *General Introduction*, **Chapter 1.2**, (35, 39, 56)).

The results from competition experiments suggest that most of the peptides containing reported prolyl hydroxylation sites did not influence the hydroxylation kinetics of PHD2 or PHD3 to a substantial degree. However, two peptides belonging to the *eukaryotic* elongation factor 2 (*eEF2*) and the large subunit of RNA Polymerase II (POLR2A), were identified which substantially inhibited HIF1 α NODD hydroxylation by PHD2 (as well as HIF1 α CODD hydroxylation by PHD3 in case of *eEF2*). NMR-displacement experiments showed that the two peptides directly bind to PHD2 by displacing HIF1 α NODD from the enzyme. Notably, both of the identified PHD2 interactors are part of the transcription-translation machinery. The finding that the transcription factor *eEF2* competes with HIF1 α hydroxylation *in vitro* is of potential interest from an evolutionary perspective, as it emphasises evolutionary links between human PHDs and the prolyl hydroxylase from *Pseudomonas* PPHD, which accepts the transcription factor EF-Tu as its natural substrate (57). However, *eEF2* is not directly related to the *Pseudomonas* EF-Tu, but is a functional ortholog of *Pseudomonas* EF-G.

Future work could aim at exploring the potential biological relevance of the interaction between PHDs and *eEF2/POLR2A* *in vivo*. This could include immunoprecipitation (IP) experiments from human cells followed by western blotting and antibody detection of the interacting proteins. A potential influence of over-expression or knockdown of *eEF2/POLR2A* on HIF activity could be investigated by use of HRE luciferase assays (although any manipulation of *eEF2/POLR2A* levels in cells will likely change the expression level of the cell proteome).

VCB binding studies indicate that the von-Hippel-Lindau protein (pVHL) may be highly promiscuous as it accepts a broad range of the reported PHD substrates; this is (partly) in agreement with previous studies on non-HIF PHD substrates in the literature, where an interaction between apparent PHD substrates and pVHL was observed ((16-17, 20, 22, 24, 27-29)); however no PHD-catalysed hydroxylation of these reported substrates was found in the studies described in this Chapter. Notably, binding of some of the peptides to pVHL was dependent on prolyl hydroxylation, as no un-modified peptides were accepted. Provided that the observed interactions between at the non-HIF peptides and pVHL also exist *in vivo*, the question arises on how the peptides can be hydroxylated in cells. It is possible that an additional (potentially uncharacterised) prolyl hydroxylase, other than PHDs, accepts the non-HIF substrates in cells and catalyses the hydroxylation of prolyl residues in the proteins, which then can be recognised by pVHL. Another possible explanation is that hydroxylations on prolyl residues are introduced by non-enzymatic oxidation. Synthetic studies on small-molecule iron catalysts have shown that these compounds are capable of facilitating the selective C–H oxidative modification of amino acids and peptides, including the oxidation of proline to 5-hydroxyproline (58). Future work could aim at investigating to what degree prolyl residues can be oxidised under cellular conditions, including studies on the stereochemistry of the oxidation products.

The results from the VCB binding studies also provide insights into the substrate preferences of pVHL, such as the presence of a LAP motif in the sequence of the peptide, and a high sequence similarity to HIF1 α CDD. The identified substrate preferences can be exploited for medicinal manipulation of the HIF-pathway, i.e. for the design of cyclic peptide inhibitors of pVHL. Highly selective cyclic peptide inhibitors of the histone demethylases KDM4A-C have been reported (59), suggesting that the development of cyclic peptide inhibitors might constitute a promising approach for the selective targeting of other proteins, such as pVHL.

2.4 References

- (1) Köditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., ... Katschinski, D. M. (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. *Blood*, 110(10), 3610 LP-3617.
- (2) German, N. J., Yoon, H., Yusuf, R. Z., Murphy, J. P., Finley, L. W. S., Laurent, G., ... Haigis, M. C. (2017). PHD3 Loss in Cancer Enables Metabolic Reliance on Fatty Acid Oxidation via Deactivation of ACC2. *Molecular Cell*, 63(6), 1006–1020.
- (3) Lee, S. B., Frattini, V., Bansal, M., Castano, A. M., Sherman, D., Hutchinson, K., ... Lasorella, A. (2016). An ID2-dependent mechanism for VHL inactivation in cancer. *Nature*, 529(7585), 172–177.
- (4) Romero-Ruiz, A., Bautista, L., Navarro, V., Heras-Garvín, A., March-Díaz, R., Castellano, A., ... Pascual, A. (2012). Prolyl Hydroxylase-dependent Modulation of Eukaryotic Elongation Factor 2 Activity and Protein Translation under Acute Hypoxia. *The Journal of Biological Chemistry*, 287(12), 9651–9658.
- (5) Rodriguez, J., Pilkington, R., Garcia Munoz, A., Nguyen, L. K., Rauch, N., Kennedy, S., ... von Kriegsheim, A. (2016). Substrate-Trapped Interactors of PHD3 and FIH Cluster in Distinct Signaling Pathways. *Cell Reports*, 14(11), 2745–2760.
- (6) Luo, W., Lin, B., Wang, Y., Zhong, J., O’Meally, R., Cole, R. N., ... Semenza, G. L. (2014). PHD3-mediated prolyl hydroxylation of nonmuscle actin impairs polymerization and cell motility. *Molecular Biology of the Cell*, 25(18), 2788–2796.
- (7) Thinnes, C. C., England, K. S., Kawamura, A., Chowdhury, R., Schofield, C. J., & Hopkinson, R. J. (2014). Targeting histone lysine demethylases — Progress, challenges, and the future. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1839(12), 1416–1432.
- (8) Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., & Whitelaw, M. L. (2002). Asparagine Hydroxylation of the HIF Transactivation Domain: A Hypoxic Switch. *Science*, 295(5556), 858-861.
- (9) Hamada, S., Kim, T.-D., Suzuki, T., Itoh, Y., Tsumoto, H., Nakagawa, H., ... Miyata, N. (2009). Synthesis and activity of N-oxalylglycine and its derivatives as Jumonji C-domain-containing histone lysine demethylase inhibitors. *Bioorganic & Medicinal Chemistry Letters*, 19(10), 2852–2855.
- (10) Zhdanov, A. V., Okkelman, I. A., Collins, F. W. J., Melgar, S., & Papkovsky, D. B. (2015). A novel effect of DMOG on cell metabolism: direct inhibition of mitochondrial function precedes HIF target gene expression. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1847(10), 1254–1266.
- (11) Dunlop, R. A., Brunk, U. T., & Rodgers, K. J. (2009). Oxidized proteins: Mechanisms of removal and consequences of accumulation. *IUBMB Life*, 61(5), 522–527.

- (12) Moser, S. C., Bensaddek, D., Ortmann, B., Rocha, S., Lamond, A. I., & Swedlow, J. R. (2015). The Prolylhydroxylase PHD2 Modulates Centromere Function through the Hydroxylation of CENP-N. *bioRxiv*. Retrieved from <http://biorxiv.org/content/early/2015/11/02/030452.abstract>.
- (13) Cummins, E. P., Berra, E., Comerford, K. M., Ginouves, A., Fitzgerald, K. T., Seeballuck, F., ... Taylor, C. T. (2006). Prolyl hydroxylase-1 negatively regulates I κ B kinase- β , giving insight into hypoxia-induced NF κ B activity. *Proceedings of the National Academy of Sciences of the United States of America*, 103(48), 18154–18159.
- (14) Epstein, A. C. R., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., ... Ratcliffe, P. J. (2017). *C. elegans* EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation. *Cell*, 107(1), 43–54.
- (15) Loenarz, C., Coleman, M. L., Boleininger, A., Schierwater, B., Holland, P. W. H., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Reports*, 12(1), 63–70.
- (16) Xie, L., Xiao, K., Whalen, E. J., Forrester, M. T., Freeman, R. S., Fong, G., ... Stamler, J. S. (2009). Oxygen-Regulated $\beta(2)$ -Adrenergic Receptor Hydroxylation by EGLN3 and Ubiquitylation by pVHL. *Science Signaling*, 2(78), ra33-ra33.
- (17) Guo, J., Chakraborty, A. A., Liu, P., Gan, W., Zheng, X., Inuzuka, H., ... Wei, W. (2016). pVHL suppresses kinase activity of Akt in a proline-hydroxylation-dependent manner. *Science*, 353(6302), 929–932.
- (18) Moser, S. C., Bensaddek, D., Ortmann, B., Maure, J.-F., Mudie, S., Blow, J. J., ... Rocha, S. (2013). PHD1 Links Cell-Cycle Progression to Oxygen Sensing through Hydroxylation of the Centrosomal Protein Cep192. *Developmental Cell*, 26(4), 381–392.
- (19) Moore, C. E. J., Mikolajek, H., Regufe da Mota, S., Wang, X., Kenney, J. W., Werner, J. M., & Proud, C. G. (2015). Elongation Factor 2 Kinase Is Regulated by Proline Hydroxylation and Protects Cells during Hypoxia. *Molecular and Cellular Biology*, 35(10), 1788–1804.
- (20) Heir, P., Srikumar, T., Bikopoulos, G., Bunda, S., Poon, B. P., Lee, J. E., ... Ohh, M. (2016). Oxygen-dependent Regulation of Erythropoietin Receptor Turnover and Signaling. *The Journal of Biological Chemistry*, 291(14), 7357–7372.
- (21) Arsenault, P. R., Heaton-Johnson, K. J., Li, L., Song, D., Ferreira, V. S., Patel, N., ... Lee, F. S. (2015). Identification of Prolyl Hydroxylation Modifications in Mammalian Cell Proteins. *Proteomics*, 15(7), 1259–1267.
- (22) Segura, I., Lange, C., Knevels, E., Moskalyuk, A., Pulizzi, R., Eelen, G., ... Carmeliet, P. (2016). The Oxygen Sensor PHD2 Controls Dendritic Spines and Synapses via Modification of Filamin A. *Cell Reports*, 14(11), 2653–2667.

- (23) Zheng, X., Zhai, B., Koivunen, P., Shin, S. J., Lu, G., Liu, J., ... Zhang, Q. (2014). Prolyl hydroxylation by EglN2 destabilizes FOXO3a by blocking its interaction with the USP9x deubiquitinase. *Genes & Development*, 28(13), 1429–1444.
- (24) Lee, D. C., Sohn, H. A., Park, Z.-Y., Oh, S., Kang, Y. K., Lee, K., ... Yeom, Y. I. (2017). A Lactate-Induced Response to Hypoxia. *Cell*, 161(3), 595–609.
- (25) Huo, Z., Ye, J.-C., Chen, J., Lin, X., Zhou, Z.-N., Xu, X.-R., ... Li, J. (2012). Prolyl hydroxylase domain protein 2 regulates the intracellular cyclic AMP level in cardiomyocytes through its interaction with phosphodiesterase 4D. *Biochemical and Biophysical Research Communications*, 427(1), 73–79.
- (26) Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., ... Semenza, G. L. (2011). Pyruvate Kinase M2 is a PHD3-stimulated Coactivator for Hypoxia-Inducible Factor 1. *Cell*, 145(5), 732–744.
- (27) Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., ... Czyzyk-Krzeska, M. F. (2003). von Hippel–Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. *Proceedings of the National Academy of Sciences*, 100(5), 2706–2711.
- (28) Mikhaylova, O., Ignacak, M. L., Barankiewicz, T. J., Harbaugh, S. V., Yi, Y., Maxwell, P. H., ... Czyzyk-Krzeska, M. F. (2008). The von Hippel-Lindau Tumor Suppressor Protein and Egl-9-Type Proline Hydroxylases Regulate the Large Subunit of RNA Polymerase II in Response to Oxidative Stress. *Molecular and Cellular Biology*, 28(8), 2701–2717.
- (29) Anderson, K., Nordquist, K. A., Gao, X., Hicks, K. C., Zhai, B., Gygi, S. P., & Patel, T. B. (2011). Regulation of Cellular Levels of Sprouty2 Protein by Prolyl Hydroxylase Domain and von Hippel-Lindau Proteins. *The Journal of Biological Chemistry*, 286(49), 42027–42036.
- (30) Xie, L., Pi, X., Mishra, A., Fong, G., Peng, J., & Patterson, C. (2012). PHD3-dependent hydroxylation of HCLK2 promotes the DNA damage response. *The Journal of Clinical Investigation*, 122(8), 2827–2836.
- (31) Xie, L., Pi, X., Townley-Tilson, W. H. D., Li, N., Wehrens, X. H. T., Entman, M. L., ... Patterson, C. (2015). PHD2/3-dependent hydroxylation tunes cardiac response to β -adrenergic stress via phospholamban. *The Journal of Clinical Investigation*, 125(7), 2759–2771.
- (32) Takahashi, N., Kuwaki, T., Kiyonaka, S., Numata, T., Kozai, D., Mizuno, Y., ... Mori, Y. (2011). TRPA1 underlies a sensing mechanism for O₂. *Nature Chemical Biology*, 7(10), 701–711.
- (33) Di Conza, G., Trusso Cafarello, S., Zheng, X., Zhang, Q., & Mazzone, M. (2017). PHD2 Targeting Overcomes Breast Cancer Cell Death upon Glucose Starvation in a PP2A/B55 α -Mediated Manner. *Cell Reports*, 18(12), 2836–2844.

- (34) Deschoemaeker, S., Di Conza, G., Lilla, S., Martín-Pérez, R., Mennerich, D., Boon, L., ... Mazzone, M. (2015). PHD1 regulates p53-mediated colorectal cancer chemoresistance. *EMBO Molecular Medicine*, 7(10), 1350–1365.
- (35) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (36) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (37) Huang, J., Zhao, Q., Mooney, S. M., & Lee, F. S. (2002). Sequence Determinants in Hypoxia-inducible Factor-1 α for Hydroxylation by the Prolyl Hydroxylases PHD1, PHD2, and PHD3. *Journal of Biological Chemistry*, 277(42), 39792–39800.
- (38) Flashman, E., Bagg, E. A. L., Chowdhury, R., Mecinović, J., Loenarz, C., McDonough, M. A., ... Schofield, C. J. (2008). Kinetic Rationale for Selectivity toward N- and C-terminal Oxygen-dependent Degradation Domain Substrates Mediated by a Loop Region of Hypoxia-Inducible Factor Prolyl Hydroxylases. *Journal of Biological Chemistry*, 283(7), 3808–3815.
- (39) Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., ... Schofield, C. J. (2017). Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*, 17(7), 981–989.
- (40) Chowdhury, R., Leung, I. K. H., Tian, Y.-M., Abboud, M. I., Ge, W., Domene, C., ... Schofield, C. J. (2016). Structural basis for oxygen degradation domain selectivity of the HIF prolyl hydroxylases. *Nature Communications*, 7, 12673.
- (41) Flashman, E., Hoffart, L. M., Hamed, R. B., Bollinger, J. M., Krebs, C., & Schofield, C. J. (2010). Evidence for the Slow Reaction of Hypoxia-Inducible Factor Prolyl Hydroxylase 2 with Oxygen. *The FEBS Journal*, 277(19), 4089–4099.
- (42) Hausinger, R. P. (2004). Fe(II)/ α -Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 39(1), 21–68.
- (43) Hewitson, K. S., Schofield, C. J., & Ratcliffe, P. J. (2007). Hypoxia-Inducible Factor Prolyl-Hydroxylase: Purification and Assays of PHD2. *Methods in Enzymology*, 435, 25–42.
- (44) Song, D., Li, L.-S., Heaton-Johnson, K. J., Arsenault, P. R., Master, S. R., & Lee, F. S. (2013). Prolyl Hydroxylase Domain Protein 2 (PHD2) Binds a Pro-Xaa-Leu-Glu Motif, Linking It to the Heat Shock Protein 90 Pathway. *The Journal of Biological Chemistry*, 288(14), 9662–9674.

- (45) Yeh, T.-L., Leissing, T., Abboud, M. I., Thinnies, C. C., Atasoylu, O., Holt-Martyn, J. P., ... Schofield, C. J. (2017). Molecular and cellular mechanisms of HIF prolyl hydroxylase inhibitors in clinical trials. *Chemical Science*, 8, 7651-7668.
- (46) Carr, H. Y., & Purcell, E. M. (1954). Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments. *Physical Review*, 94(3), 630-638.
- (47) Aguilar, J. A., Nilsson, M., Bodenhausen, G., & Morris, G. A. (2012). Spin echo NMR spectra without J modulation. *Chemical Communications*, 48(6), 811-813.
- (48) Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., ... Gleadle, J. M. (2004). Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 279(37), 38458-38465.
- (49) Buckley, D. L., Van Molle, I., Gareiss, P. C., Tae, H. S., Michel, J., Noblin, D. J., ... Crews, C. M. (2012). Targeting the von Hippel-Lindau E3 ubiquitin ligase using small molecules to disrupt the VHL/HIF-1 α interaction. *Journal of the American Chemical Society*, 134(10), 4465-4468.
- (50) Van Molle, I., Thomann, A., Buckley, D. L., So, E. C., Lang, S., Crews, C. M., & Ciulli, A. (2012). Dissecting Fragment-Based Lead Discovery at the von Hippel-Lindau Protein:Hypoxia Inducible Factor 1 α Protein-Protein Interface. *Chemistry & Biology*, 19(10), 1300-1312.
- (51) Chan, M. C., Holt-Martyn, J. P., Schofield, C. J., & Ratcliffe, P. J. (2016). Pharmacological targeting of the HIF hydroxylases – A new field in medicine development. *Molecular Aspects of Medicine*, 47(Supplement C), 54-75.
- (52) Cockman, M. E., Webb, J. D., Kramer, H. B., Kessler, B. M., & Ratcliffe, P. J. (2009). Proteomics-based Identification of Novel Factor Inhibiting Hypoxia-inducible Factor (FIH) Substrates Indicates Widespread Asparaginyl Hydroxylation of Ankyrin Repeat Domain-containing Proteins. *Molecular & Cellular Proteomics : MCP*, 8(3), 535-546.
- (53) Yang, M., Chowdhury, R., Ge, W., Hamed, R. B., McDonough, M. A., Claridge, T. D. W., ... Schofield, C. J. (2011). Factor-inhibiting hypoxia-inducible factor (FIH) catalyses the post-translational hydroxylation of histidiny residues within ankyrin repeat domains. *The FEBS Journal*, 278(7), 1086-1097.
- (54) Yang, M., Hardy, A. P., Chowdhury, R., Loik, N. D., Scotti, J. S., McCullagh, J. S. O., Claridge, T. D. W., McDonough, M. A., Ge, W. and Schofield, C. J. (2013), Substrate Selectivity Analyses of Factor Inhibiting Hypoxia-Inducible Factor. *Angewandte Chemie International Edition*, 52: 1700-1704.
- (55) Aik, W. S., Chowdhury, R., Clifton, I. J., Hopkinson, R. J., Leissing, T., McDonough, M. A., ... Walport, L. J. (2015). CHAPTER 2 Introduction to Structural Studies on 2-Oxoglutarate-Dependent Oxygenases and Related Enzymes. In *2-Oxoglutarate-Dependent Oxygenases* (pp. 59-94). The Royal Society of Chemistry.

- (56) Elkins, J. M., Hewitson, K. S., McNeill, L. A., Seibel, J. F., Schlemminger, I., Pugh, C. W., ... Schofield, C. J. (2003). Structure of Factor-inhibiting Hypoxia-inducible Factor (HIF) Reveals Mechanism of Oxidative Modification of HIF-1 α . *Journal of Biological Chemistry*, 278(3), 1802–1806.
- (57) Scotti, J. S., Leung, I. K. H., Ge, W., Bentley, M. A., Paps, J., Kramer, H. B., ... Schofield, C. J. (2014). Human oxygen sensing may have origins in prokaryotic elongation factor Tu prolyl-hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America*, 111(37), 13331–13336.
- (58) Osberger, T. J., Rogness, D. C., Kohrt, J. T., Stepan, A. F., & White, M. C. (2016). Oxidative diversification of amino acids and peptides by small-molecule iron catalysis. *Nature*, 537, 214.
- (59) Kawamura, A., Münzel, M., Kojima, T., Yapp, C., Bhushan, B., Goto, Y., ... Schofield, C. J. (2017). Highly selective inhibition of histone demethylases by de novo macrocyclic peptides. *Nature Communications*, 8, 14773.

2.5 Appendix

Table 2.7 Sequences of PHD substrate peptides. Peptide sequences labelled with * correspond to peptides reported in the literature. Proposed hydroxylation sites are bold, underlined.

Peptides with putative hydroxylation site (published)	Sequence	MW g/mol
HIF1 α CODD-P564	DLDLEMLAP <u>Y</u> IPMDDDFQL	2254.6
ACC2-P450	DEGLEAAERIGF <u>P</u> LMIKASEGGGGK	2532.9
ACC2-P450*	ERIGF <u>P</u> LMIKASEGGGGK	1847.2
ACTB-P307	ANTVLSSGGTTMY <u>P</u> GIADRMQKEITA	2625.0
ACTB-P322	A DRMQKEITALA <u>P</u> STMKIKIAPPE	2752.3
ADRB2-P382	QEKENKLLCEDL <u>P</u> GTEDFVGHQGTV	2786.1
ADRB2-P395	GTEDFVGHQGT <u>P</u> SDNIDSQGRNCS	2619.7
AKT1-P125	QEEEEMDFRSGS <u>P</u> SDNSGAEEMEVS	2776.8
AKT1-P125*	EEEMDFRSGS <u>P</u> SDNSGAEEM	2204.2
AKT1-P313	KDGATMKTFCGT <u>P</u> EYLAPEVLEDND	2745.0
AKT1-P313*	KTFCGT <u>P</u> EYLAPEVLEDNDYGR	2517.8
AKT1-P318	GT <u>P</u> EYLA <u>P</u> EVLEDNDYGRAV	2208.4
ATF4-P156/P162/P164/P167	LPESLTKPDQVAP <u>P</u> FTFLQ <u>P</u> L <u>P</u> LS <u>P</u> G	2691.4
ATF4-P162/P164/P167/P174	TFLQ <u>P</u> L <u>P</u> LS <u>P</u> GVLSST <u>P</u> DHSFSLEL	2683.1
CENPN-P311, isoform 3	SPHLLEALKSLA <u>P</u> AALVCRIQKLLC	2688.3
CENPN-P311, isoform 3*	SLA <u>P</u> AALVCR	1000.2
CEP192-P1717	DVKWHLSSLA <u>P</u> PYVKGVDES GDVF	2645.0
CEP192-P1717*	WHLSSLA <u>P</u> PYVK	1397.6
eEF2K-P98	KHAIQKAKHMPD <u>P</u> WAEFHLEDIATE	2941.4
EPOR-P419/P426	QLLRPWTLC <u>P</u> ELPPT <u>P</u> PHLKYLVLV	2988.6
EPOR-P419/P426*	LC <u>P</u> ELPPT <u>P</u> PHLKYL	1718.1
FKBP10-P36	VQAVGRGLGRAS <u>P</u> AGGPLEDVVIER	2503.8
FLNA-P2309/2316	VKFNEEH <u>I</u> PDSPFVV <u>P</u> VASPSGDAR	2693.3
FOXO3a-P426	FPYTTKGSGLS <u>P</u> TSSFNSTVFG <u>P</u> S	2522.8
FOXO3a-P437	S <u>P</u> TSSFNSTVFG <u>P</u> SSLNSLRQSPMQ	2655.9
HK2-P326	AKEELLFGGKLS <u>P</u> ELLNTGRFETK	2793.1
IKBKB-P191	TSFVGTLQYLA <u>P</u> ELLEQQKYTVTV	2842.5

Peptides with putative hydroxylation site (published)	Sequence	MW g/mol
MAPK6-P25	GFDLGSR YM DLK P LGCGGNGLVFSA	2574.9
MTHFD1-P806	ALAQAVQRAAQA P SSFQLLYDLKLP	2700.1
MYH10-P733	FQEFRQRYEILT P NAIPKGFMDGKQ	3014.4
NDRG3-P294	TTLLKMADCGGL P QVVQPGKLTEAF	2617.0
PDE4D-P29 HEK293	GSDSAGGATLKAP K HLWRHEQHHQY	2810.4
PDE4D-P382 HEK293	LMHSSSLTNSSI P RFVVKTEQEDVL	2775.2
PDE4D-P419 HEK293	HVFRIAELSGNR P LTVMHTIFQER	2965.5
PKM-P477	LYRGIFPVLC KD P VQEAWAEDVDLR	2933.3
PKM2-P403	HLQLFEELRRLA P ITSD P TEATAVG	2764.2
PKM2-P408	EELRRLA P ITSD P TEATAVGAVEASFK	2858.2
POLR2A-P1465	ENIMLGQLA P AGTGCFDLLLLD	2191.5
SPRY2-P18	QSGNG SQPLLQT P RDGGRQRGEPDP	2646.8
SPRY2 -P144	EQRLLGSSFSSG P VADGIIRVQPKS	2628.0
SPRY2-P160	GIIRVQPKSELK P GELKPLSKEDLG	2730.6
TELO2 (HCLK2)-P374	KAVLICLAQLGE P ELRDSRDELLAS	2739.2
TELO2 (HCLK2)-P419/422	VAEVVSARIH P EG P PLKFQYEE DEL	2853.2
THRA-P160/P162	EMIRSLQQR P EP P EEWDLIHATE	3019.3
TRPA1-P394	TVQQPYGLKNLR P EFMQMQQIKELV	3018.6
TRPA1-P394*	PYGLKNLR P EFMQMQQIKEL	2463.9
PPP2R2A-P319*	WDLNMENR P VETYQVHEYL	2436.7
PPP2R2A-P319	VKIWDLNMENR P VETYQVHEYLRSK	3148.6
TP53-P359	ALELKDAQAGKE P GGSSRAHSSHLKS	2574.8

Table 2.8 Sequences of PHD substrate peptides (extended study). Proposed hydroxylation sites are bold, underlined.

Peptides with putative hydroxylation site (unpublished)	Sequence	MW g/mol
ACC1-P307	DDGLQAAEEVGY <u>P</u> VMIKASEGGGK	2478.7
ACC1-P51 (NODD-like)	LDLLEEKEGSL <u>S</u> PASVGSDTLSDLG	2532.7
BLES03-P135	VLSGKWLMLH <u>L</u> APGFKLDHAWAGI	2546.4
CENPN-P311, isoform 1	SPHLLLEALKSL <u>A</u> PAGIADAPLSPLL	2494.9
øEF2-P446	YTPGKKEDLYLK <u>P</u> IQRTILMMGRYV	3012.6
øEF2-P465	GRYVEPIEDV <u>P</u> CGNIVGLVGVDQFL	2687.4
øEF1-P238	TTLLEALDCIL <u>P</u> TRPTDKPLRLPL	2787.3
øEF2-P361	ALLQMITIHL <u>P</u> SVTAQKYRCELLY	2902.5
IKBKA-P190	TSFVGTLQYL <u>A</u> PELFENKPYTATVD	2803.4

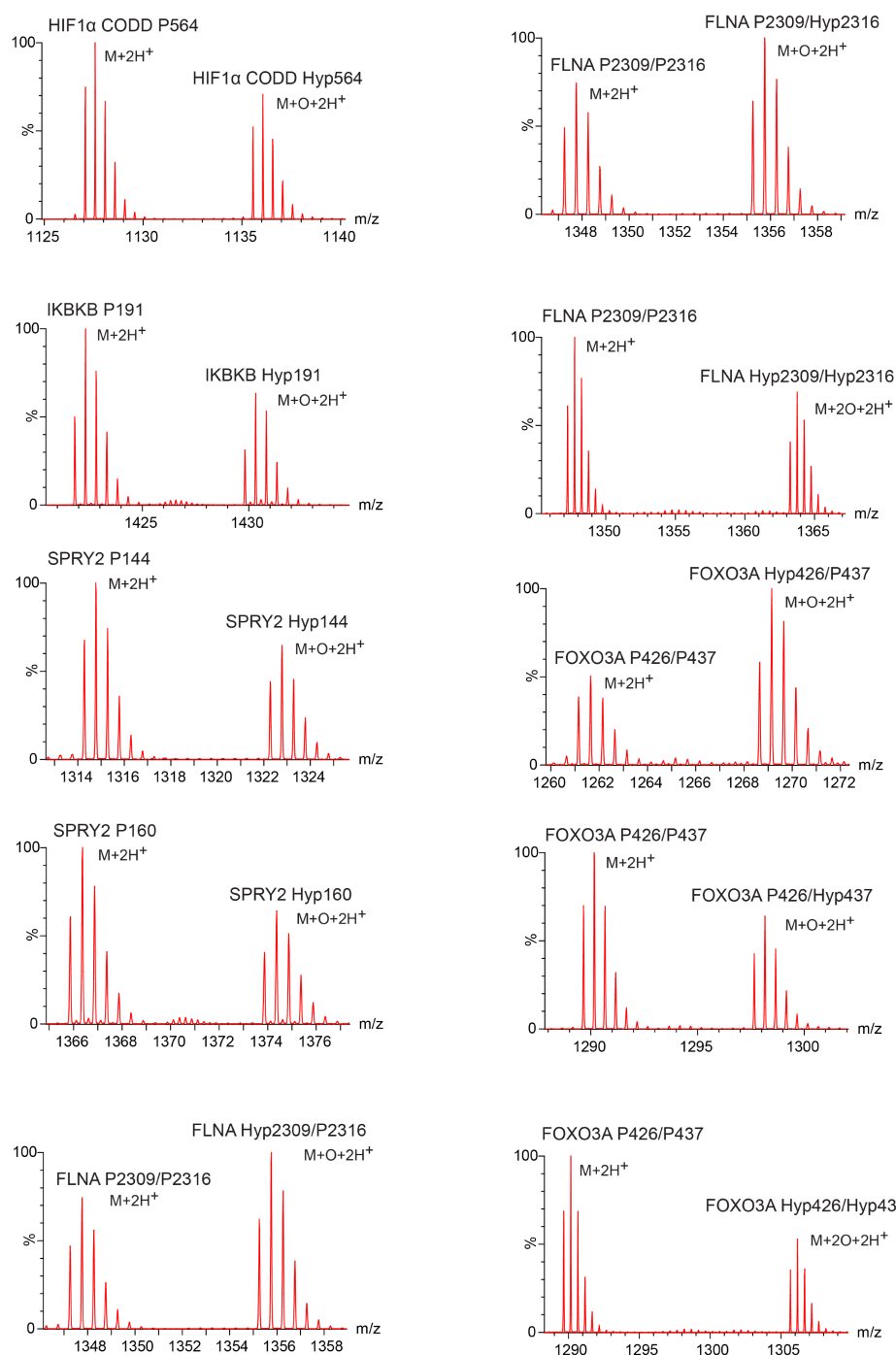


Figure 2.13: ESI-LC-MS spectra corresponding to Table 2.5 (Relative detection efficiency of hydroxylated and non-hydroxylated peptides by ESI-LC-MS). Samples containing equal molarity of a Hyp peptide and the respective non-hydroxylated peptide were analysed using ESI-LC-MS.

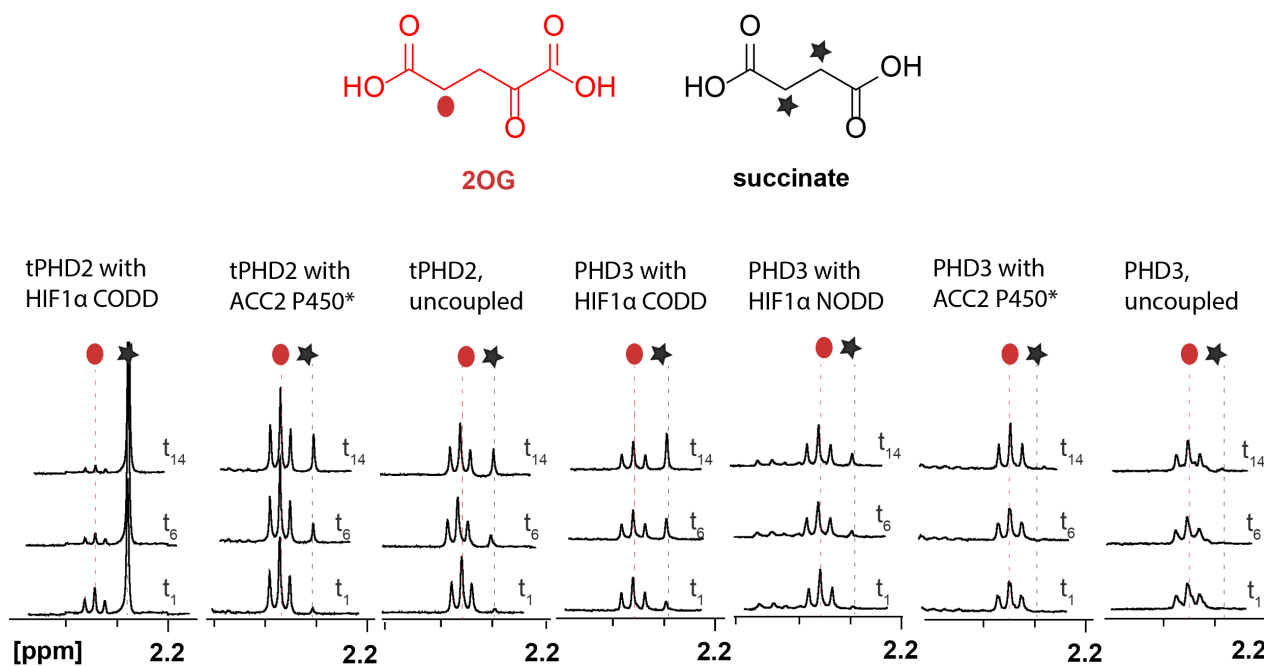


Figure 2.14: Monitoring the coupled and uncoupled turnover of 2OG by tPHD2 and PHD3 in the presence and absence of HIF and non-HIF substrates - supplementary spectra. For assay conditions, see legend of **Figure 2.8** and **Figure 2.9**.

Chapter 3

Inhibition Studies on Human HIF Prolyl Hydroxylases

3.1 Introduction

The HIF prolyl hydroxylases are pharmacological targets for treatment of anaemia in renal disease (chronic kidney disease, (1)). In anaemia associated with renal disease, erythropoietin (EPO) production is reduced due to changes in the renal interstitium (2). By inhibiting HIF prolyl hydroxylases, the native erythropoietin production in the diseased kidneys or in the liver can be augmented (1). The ability of HIF prolyl hydroxylase inhibitors to promote erythropoietin production and erythropoiesis in mice, rats, non-human primates, and humans has been established in various studies, reporting subsequent increases in haematocrit and haemoglobin levels (1, 3-8).

To date, anaemia associated with renal disease is mainly treated by injecting recombinant human erythropoietin (rhEPO) into the patient (1). By contrast, HIF prolyl hydroxylases inhibitors offer the advantage of an oral route of delivery (1). Treatment of patients with higher doses of rhEPO, to achieve high target haemoglobin levels, has been associated with an increased incidence of cardiovascular events (9-11), and vascular toxicity caused by very high levels of plasma erythropoietin has been observed (12). Several studies in animals and humans suggest that target haemoglobin levels can be achieved with more modest rises in plasma EPO levels by use of HIF prolyl hydroxylase inhibitors (3-8), hence potentially decreasing the risk of adverse effects in patients. Clinical and experimental observation suggest that the systematic activation of the HIF pathway by use of PHD inhibitors stimulates not only the production of EPO, but may have additional beneficial effects on erythropoiesis (1-2). HIF target genes are involved in iron absorption, transport and metabolism, potentially enhancing iron availability for erythropoiesis (2, 13), and direct actions of the HIF pathway on the bone marrow have been suggested (1, 14). However, the clinical use of PHD inhibitors also comprises risks. Above all, it is not certain if, by use of PHD inhibitors, sufficient selectivity on erythropoietin can be achieved to avoid the general effects of HIF activation (1).

Several PHD inhibitors from different pharmaceutical companies have entered clinical trials for the treatment of anaemia (**Table 3.1**, (1)) The first compound in clinical trials was FG-2216/IOX3 (Fibrogen), yet the agent caused adverse events and was replaced by the structurally related compound FG-4592 (15-16). Studies on PHD inhibitors in animals and humans confirmed the ability of these agents to promote erythropoietin production and erythropoiesis (3-8, 17-19).

Table 3.1 PHD inhibitors in clinical trials (1, 20).

Company	Compound	Clinical Trials (Anaemia)
Fibrogen	FG-2216/IOX3	Phase I (ended)
	FG-4592	Phase III
GSK	GSK1278863	Phase III
Bayer	Molidustat	Phase II
Akebia	Vadadustat (AKB-6548)	Phase III
	Fluoro-vadadustat (AKB-6899)	Phase I
	AKB-4924 (Fe-chelator)	Phase I

Selective inhibition of one or two of the three PHD isoforms might offer a way to differentially modulate the HIF pathway, yet to date it is not known for most of the clinically-used PHD inhibitors if they act equally on all three PHD isoforms. A recent study on Fluoro-vadadustat (AKB-6899, Akebia), suggests that compound inhibits PHD3 preferentially, and the apparent isoform selectivity of Fluoro-vadadustat has been associated with specific up-regulation of HIF2 α (21). Therefore, one aim of the work presented in this Chapter was to investigate a potential isoform selectivity of clinically-used inhibitors.

PHD inhibitors are widely used in academia for studies on the HIF pathway. A recent study investigated the cellular transcriptional response to hypoxia by high-throughput RNA sequencing (RNA-seq), and compared it to the effects of PHD/FIH inhibition on the transcriptome (22). The study included the broad spectrum 2OG oxygenase inhibitor NOG, a selective FIH inhibitor (NOFD), and the PHD inhibitors FG-2216/IOX3 and IOX2, both of which are (partly) selective for PHDs over other 2OG oxygenases. As a result of the study, the broad-spectrum 2OG oxygenase inhibitor NOG was found to be the best mimic of the overall transcriptional response to hypoxia.

Additionally, a subset of genes was defined that was not fully responsive to PHD inhibition alone, suggesting that there are substantial differences in the role of prolyl and asparaginyl hydroxylation in regulating hypoxia-responsive genes (22). As a contribution to the study, it was tested if the used inhibitors are selective for any of the three PHD isoforms, the results of which are presented in this Chapter.

The aims of the work described in this Chapter were:

1. To investigate to which extent currently available inhibitors (including such used in clinical trials) exhibit PHD isoform selectivity, using an inhibition assay with PHD1-3.
2. To assess the potency of dual inhibitors that contain a PHD inhibitor domain fused to either a pVHL inhibitor or a bromodomain inhibitor. Hence, a contribution was made to the development of inhibitors that can target PHDs and pVHL/bromo domain containing enzymes simultaneously.
3. To investigate the effect of clinically-used iron chelators on PHD activity.
4. To develop a RapidFire high-throughput assay that allows to test novel PHD inhibitors for isoform selectivity.

3.2 Results and Discussion

3.2.1 Development of a PHD1-3 inhibition assay

A variety of methods have been developed to date for testing inhibitors of 2OG dependent oxygenases (23). Some of these assays monitor enzyme activity indirectly by measuring 2OG turnover ((24), with the risk of detecting false positives due to uncoupled 2OG turnover), others employ mass spectrometry or antibody-based methods (i.e. AlphaScreen, (25-28)). For developing a PHD isoform inhibition assay, mass spectrometry methods were chosen, as they directly monitor the hydroxylation of a HIF α substrate peptide, and do not suffer from the drawbacks of indirect methods, such as the monitoring of 2OG turnover (23, 24).

In order to establish a PHD isoform inhibition assay, recombinant enzymes (PHD1^{fl}, tPHD2, PHD3^{fl}) were produced in *E. coli* and purified by protein chromatography as described in *Materials and Methods*, **Chapter 7.2** and **7.3** (see **Chapter 2** for production of PHD3^{fl}). The *N*-terminally truncated tPHD2 was produced following an established protocol (29). For production of full-length PHD1, the enzyme was fused with maltose-binding protein (MBP) to increase protein solubility. PHD1^{fl} was produced in *E. coli*, and subsequently purified via amylose affinity chromatography and size-exclusion chromatography as reported (22, 30). To assess the activity of the produced PHD isoforms, hydroxylation of HIF1 α CODD 19mer was determined under standard conditions using a MALDI-TOF-MS-based assay (*Materials and Methods*, **Chapter 7.6.1**). A time-course of HIF1 α CODD hydroxylation was measured by analysing the ratio of hydroxylated to non-hydroxylated peptide at different time points ranging from 2 - 60 min (**Figure 3.1**). All enzymes were found to be highly active on the 19mer HIF1 α CODD peptide, yet the isoforms differed in their reaction rates, with tPHD2 showing the highest rate of HIF1 α CODD hydroxylation, and PHD1^{fl} reacting the slowest with the substrate peptide.

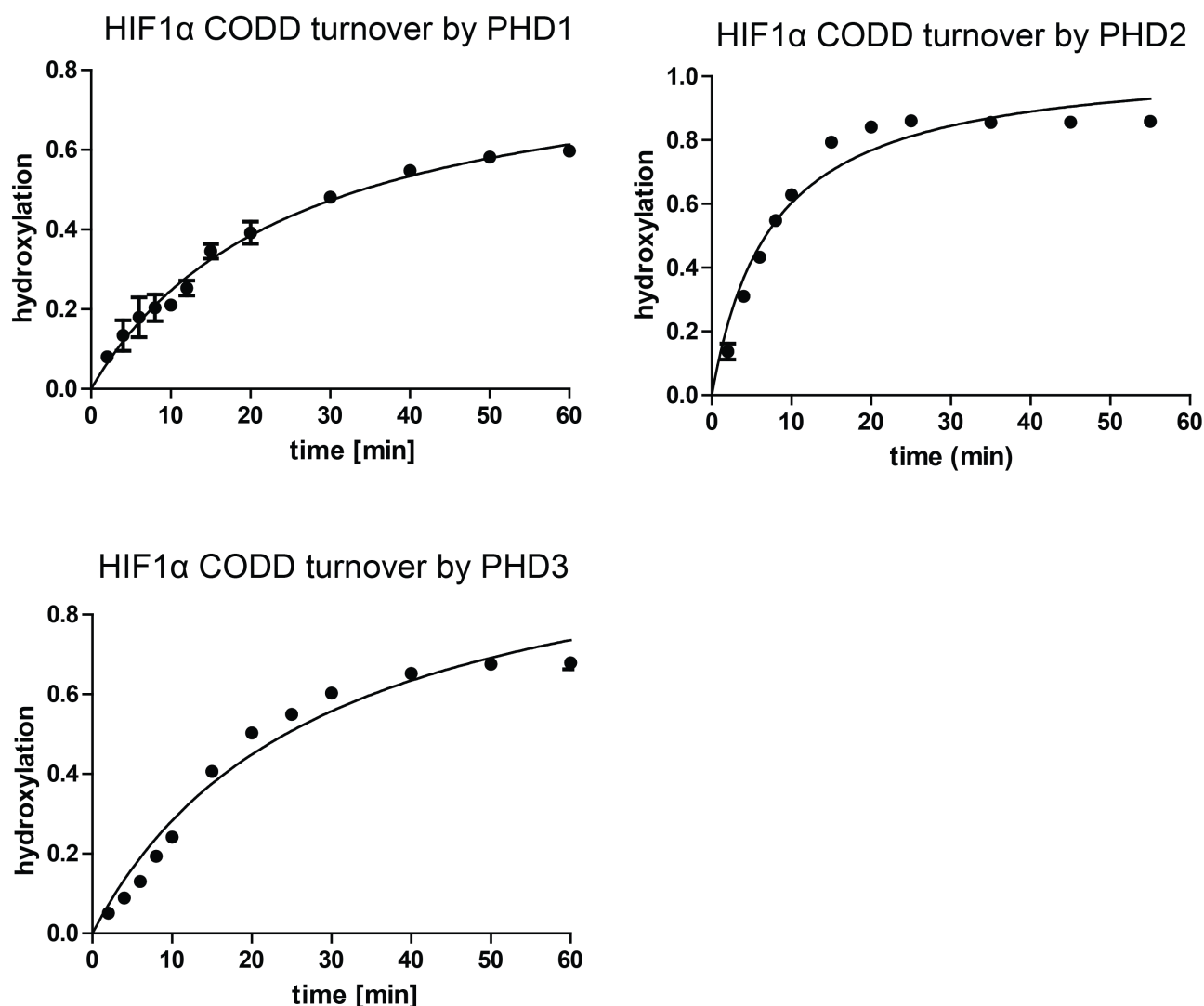


Figure 3.1: Time-course assays of PHD-catalysed hydroxylation of HIF1α CODD. (A) PHD1^{fl}, (B) tPHD2, (C) PHD3^{fl}. Assay conditions: 1 μM PHD enzyme, 50 μM (NH₄)₂Fe(II)(SO₄)₂, 10 μM 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μM 19mer CODD-peptide (sequence: DLDLEMLAPYIPMDDDFQL) in 50 mM Tris, pH 7.5. Reactions were incubated at 37 °C and quenched with 1 % (v/v) formic acid at different time points. Hydroxylation levels were quantified using MassLynx™ V4.0, and non-enzymatic background oxidation was subtracted. The curves were fitted using GraphPad Prism (errors are indicated as standard deviations, n=3).

The most commonly used method for quantifying the potency of an inhibitor is by measuring its half maximal inhibitory concentration (IC₅₀), i.e. the inhibitor concentration that is required to reduce enzyme activity by 50 %. In order to determine IC₅₀ values, the enzyme activity at varying inhibitor concentrations is measured, and a dose-response curve is established.

The following kinetic factors must be taken in account when choosing the assay conditions for IC₅₀ determination.

- The effect of an inhibitor on enzyme activity is determined at a time point within the linear range of enzyme activity, in order to maximise the sensitivity of the assay.
- To minimise the influence of co-factor availability on the assay result, Fe(II) and ascorbate are provided at saturated concentrations ($\gg K_m$).
- PHD inhibitors usually compete with 2OG and/or substrate binding. Therefore, substrate and 2OG concentrations are chosen at concentrations $\leq K_m$. (At higher concentrations, the substrate/2OG may outcompete inhibitor binding to the enzyme.)
- The highest measurable potency (lowest IC₅₀ value) equals half of the enzyme concentration, assuming 100% enzyme activity. Therefore, it is aimed to minimise the used enzyme concentration to maximise assay sensitivity.
- IC₅₀ values are dependent on the used enzyme concentration, hence inhibitor assays with PHD1-3 were carried out at the same enzyme concentration.

PHD2 has been the subject of extensive kinetic studies, and K_m values for its co-factor 2OG, and HIF α substrates have been determined (**Table 3.2**, (31)). Comparative studies on PHD1-3 suggest that K_m values of substrates and co-factors for all three enzymes lie in a similar range (Rasheduzzaman Chowdhury, University of Oxford, UK, unpublished data). As a consequence, the conditions in **Table 3.3** were chosen for determining IC₅₀ values of PHD1-3 inhibition in a MALDI-TOF-MS-based assay.

Table 3.2 Steady-state kinetic parameters of PHD2.

Co-factor/substrate	K_m (μ M)
2OG	13 ± 2 ⁽³¹⁾
HIF1 α CODD	9 ± 3 ⁽³¹⁾

Table 3.3 Conditions for PHD1-3 inhibition assays.

* minimal concentration within the detection limit by MALDI-TOF-MS was chosen.

Reagent	Final concentration
PHD1-3	1 μ M
HIF1 α CDD	10 μ M *
inhibitor	0.03 μ M, 0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 100 μ M
(NH ₄) ₂ Fe(II)(SO ₄) ₂	50 μ M
2-oxoglutarate disodium salt	10 μ M *
sodium L-ascorbate	4 mM
	in 50 mM Tris, pH 7.5

3.2.2 Inhibition of PHD1-3 by NOG, FG-2216/IOX3 and IOX2

To compare the potency of the 2OG analogue inhibitors NOG, IOX2, and FG-2216/IOX3 on PHD1-3, IC_{50} values were determined. The structure of the broad-spectrum 2OG oxygenase inhibitor NOG most closely resembles that of 2OG, with the ethyl at position C-3 being substituted by an amine group, whereas the more selective PHD inhibitors IOX2 and FG-2216/IOX3 contain additional aromatic residues, that protrude into the substrate binding pockets of the enzymes (**Figure 3.2**, (22)).

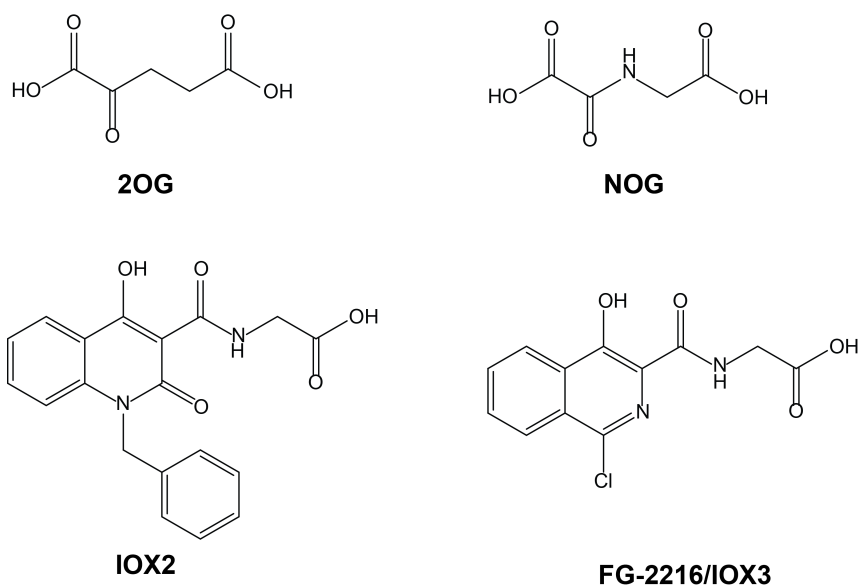


Figure 3.2: Structures of 2OG and the 2OG analogues NOG, IOX2, and FG-2216/IOX3 (22).

The results (shown in **Figure 3.3**) suggest that the tested compounds potently inhibit all three PHD isoforms, with IC_{50} values lying within the one digit micromolar to high nanomolar range. Some of the obtained values for PHD1 and PHD3 are below the detection limit of the assay ($IC_{50} = 0.5 \mu M$), indicating that the actual concentration of active enzyme is lower than the total concentration of PHD1/PHD3 used in the assay. Notably, in some cases, the inhibition was incomplete as up to 20 % of the enzymatic activity was retained. Possibly this is due to precipitation of the inhibitor at higher concentrations. All in all, given that no substantial PHD isoform selectivity of the tested compounds was found, it can be hypothesised that the three tested inhibitors employ a similar binding mode to PHD1, 2, and 3.

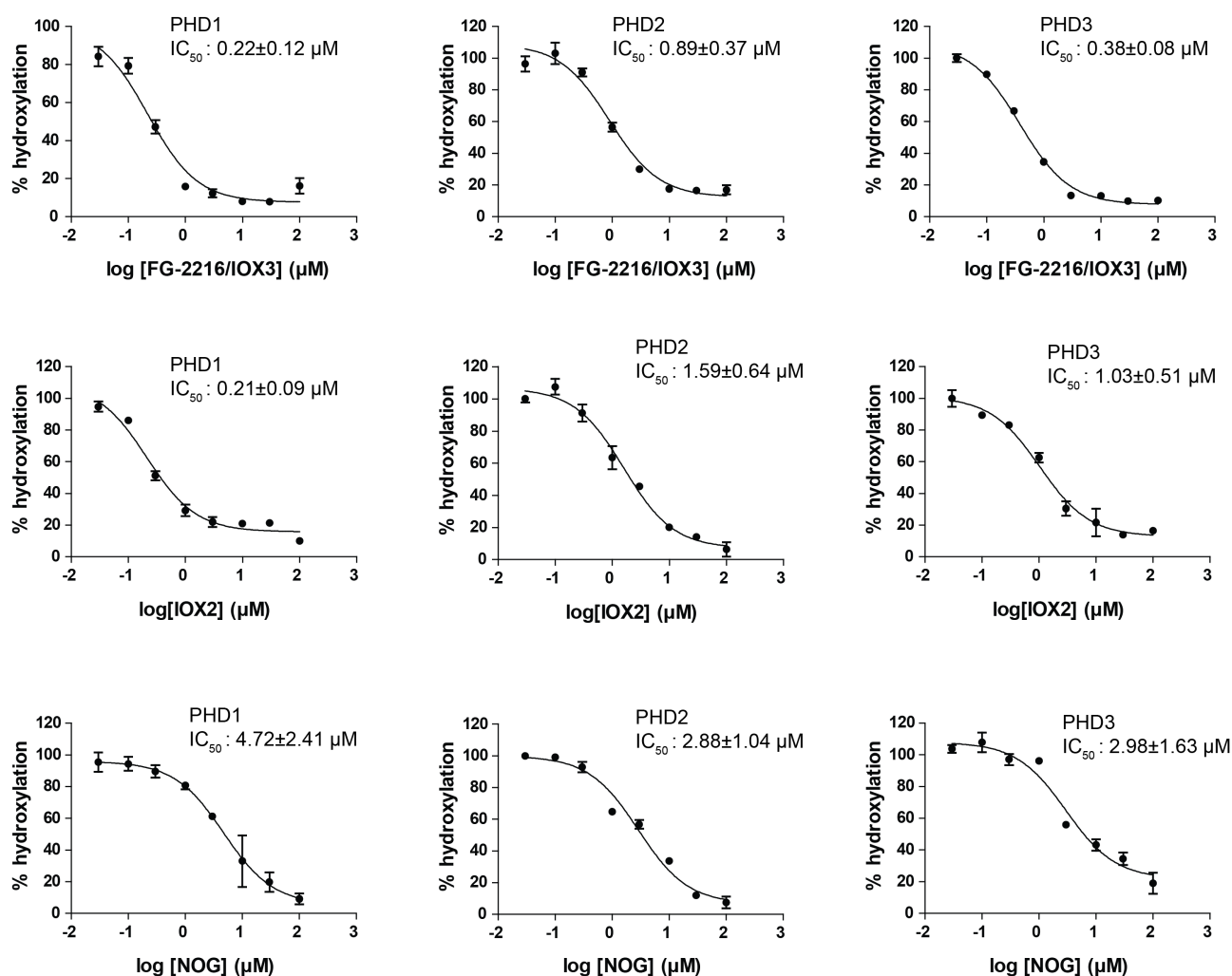


Figure 3.3: IC₅₀ values for inhibition of PHD1-3 by NOG, IOX2, and FG-2216/IOX3. Dose responses were assessed by incubation of 1 μM PHD1-3 with increasing concentrations of inhibitor (0.03 μM, 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM) in the presence of 50 μM (NH₄)₂Fe(II)(SO₄)₂, 10 μM 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μM 19mer CODD-peptide in 50 mM Tris, pH 7.5 at 37 °C. Samples were measured by MALDI-TOF-MS and hydroxylation levels were quantified using MassLynx™ V4.0, non-enzymatic background oxidation was subtracted, and IC₅₀ values were determined with GraphPad Prism.

3.2.3 Inhibition of PHD1-3 by clinically-used compounds

Of the compounds used in clinical studies for treatment of anaemia in chronic kidney disease (**Figure 3.4**), four share the same backbone as 2OG (FG-4592, GSK1278863, Vadadustat, and Fluoro-vadadustat), whereas the clinical inhibitor Molidustat and the structurally related IOX4 (a potent and selective PHD inhibitor that was added to the study for reasons of comparison) have a tricyclic core that (likely) coordinates the metal ion in PHD *via* the nitrogens of their pyrazolone rings (32).

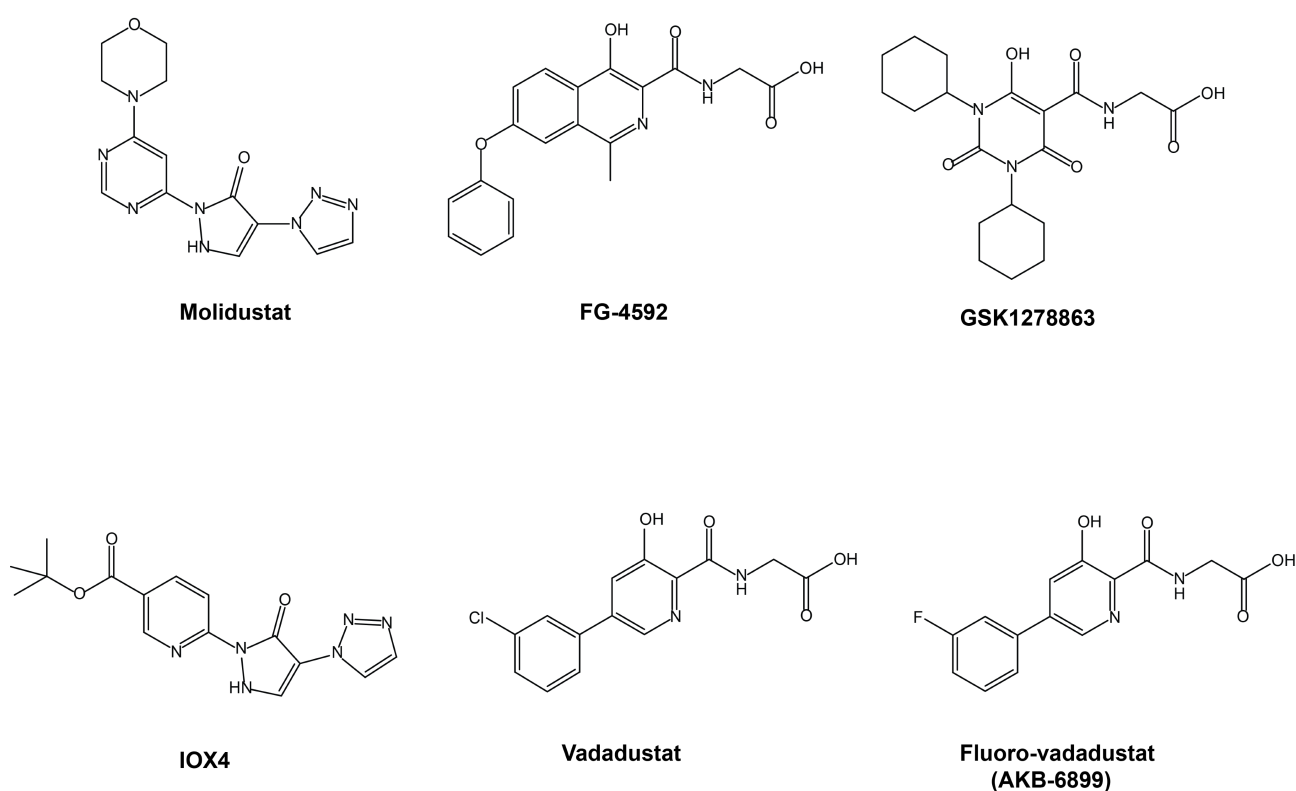


Figure 3.4: Structures of clinically-used PHD inhibitors and IOX4 (32-33).

The inhibition assays on the clinical compounds against PHD1-3 show that all inhibitors potently inhibit the three PHD isoforms with IC_{50} values around the single-digit micromolar or high sub-micromolar range at the used PHD1-3 concentration of 1 μ M (**Figure 3.5**).

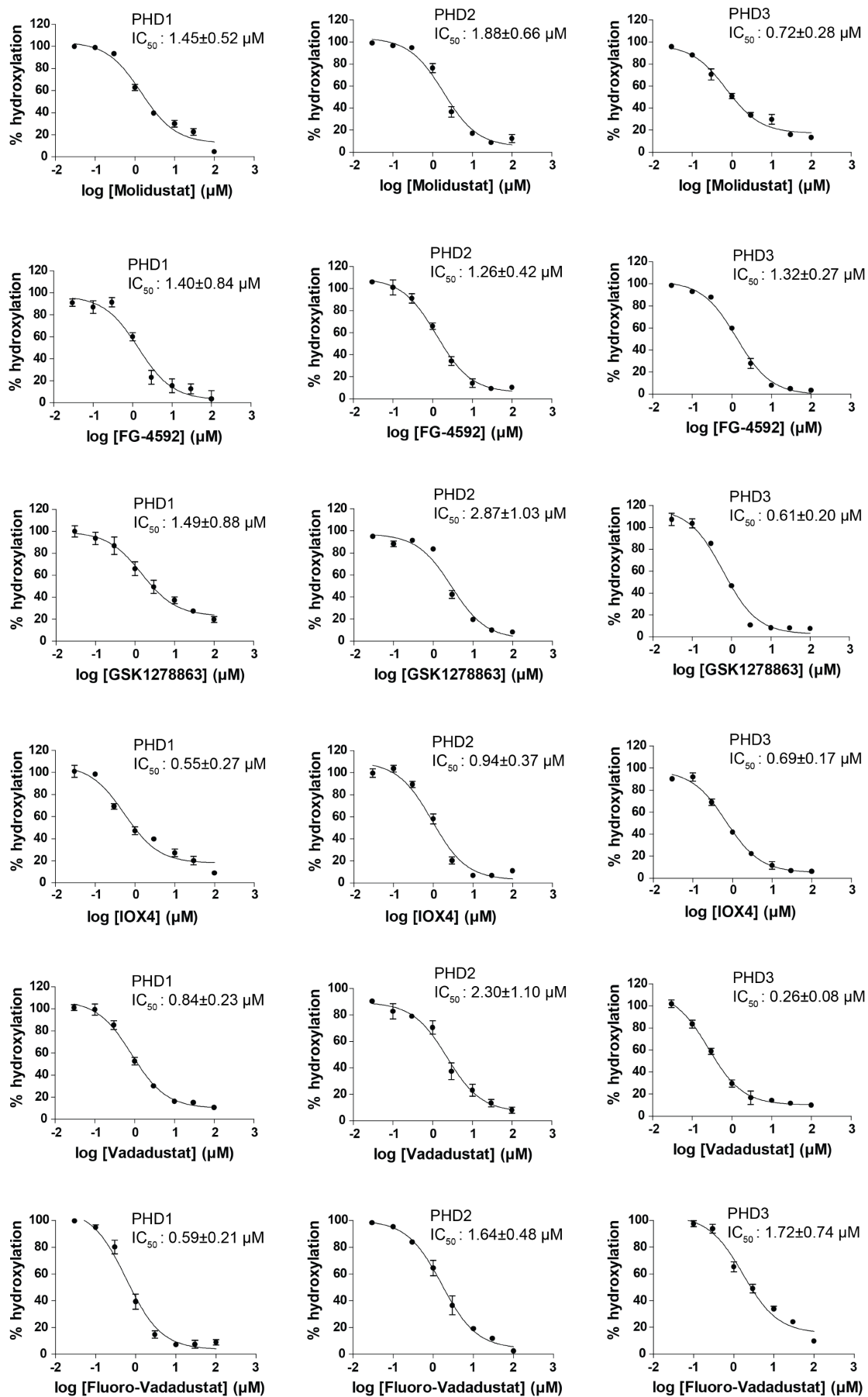


Figure 3.5: IC₅₀ values for inhibition of PHD1-3 by clinically-used compounds (IOX4 was added for reasons of comparison). Assay conditions: 1 μ M PHD1-3, inhibitor (0.03 μ M, 0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 100 μ M), 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 10 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μ M 19mer CODD-peptide in 50 mM Tris, pH 7.5. Samples were measured by MALDI-TOF-MS and hydroxylation levels were quantified using MassLynx™ V4.0, non-enzymatic background oxidation was subtracted, and IC₅₀ values were determined with GraphPad Prism.

Notably, no substantial selectivity of Fluoro-vadadustat for PHD3 over the other PHDs was found within the detection limits of the assay, contrasting with previous studies in macrophages, where stabilisation of HIF2 α , but not of HIF1 α , was observed upon treatment of the macrophages with Fluoro-vadadustat (21). In the study, it was concluded that the up-regulation of HIF2 α vs. HIF1 α was due to selective inhibition of PHD3 by Fluoro-vadadustat (21). However, it is possible that the observed up-regulation of HIF2 α vs. HIF1 α is cell-type specific or not related to selective inhibition of PHD3.

Overall, the results suggest that currently used PHD inhibitors are equally potent on all three PHD isoforms. The mode of action of the clinically-used PHD inhibitors is by competing with 2OG binding to PHDs, however FG-4592, Vadadustat, and GSK1278863 additionally protrude into the substrate-binding pocket of PHD2 and substantially interfere with substrate binding to PHD2 (see *General Introduction*, **Chapter 1.3**, (32)). The results imply that, in order to design isoform-selective inhibitors, new binding modes, apart from 2OG competition, need to be explored, e.g. by developing PHD inhibitors that exploit differences in the substrate binding modes between PHD isoforms.

3.2.4 Inhibition of PHD2 by dual inhibitors

Besides extensive studies on PHD inhibition, there have been alternative approaches to modulating the HIF pathway, i.e. inhibitors against pVHL have been developed that block HIF α binding to the E3 ubiquitin ligase complex. In order to simultaneously inhibit PHDs and pVHL in the cell, dual inhibitors were designed by Hidenori Someya (University of Oxford, UK), that target both PHDs and pVHL in one molecule (unpublished data). These inhibitors are the product of fusing the potent PHD inhibitor HS1.46 (**Figure 3.6**, orange box) via a linker region to a pVHL inhibitor that mimics a hydroxyproline peptide backbone (VH032, (34), **Figure 3.6**, blue box). PHD-pVHL inhibitors were designed with different linker lengths to optimise the distance between the two inhibitor domains and to maximise the cell permeability of the compounds. Cell permeability was further increased by use of prodrugs, that are protected at the terminal carboxyl group (**Figure 3.6**, HS3.60-HS3.62, Hidenori Someya, Tzu-Lan Yeh, University of Oxford, UK, unpublished data). The protecting groups reduce the ionic potential of the carboxyl group, thus facilitating cell entrance through the hydrophobic membrane. Inside the cell, the protecting groups are hydrolysed by ester cleaving enzymes, resulting in the free active compounds.

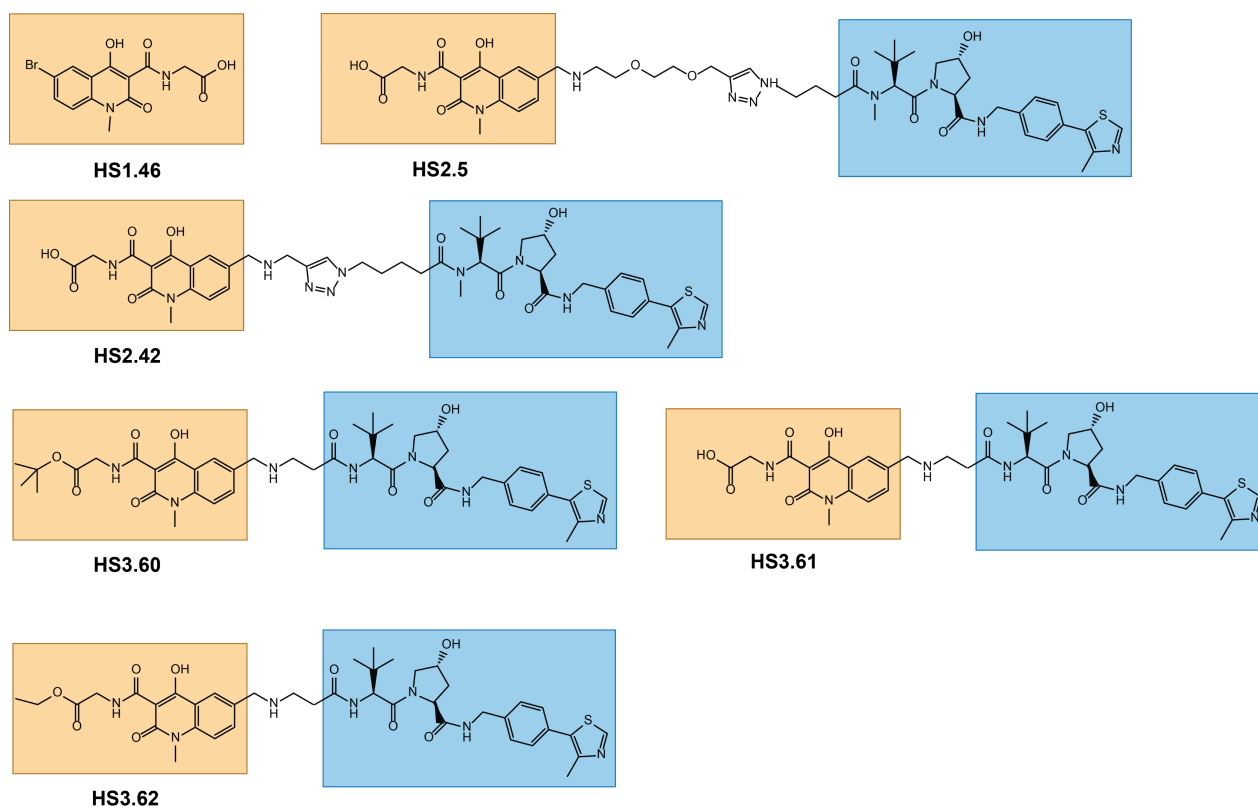


Figure 3.6: Structures of PHD-pVHL dual inhibitors (Hidenori Someya, University of Oxford, UK). Colour scheme: PHD inhibitor domain (orange), pVHL inhibitor domain (blue).

Emerging evidence suggests links between the HIF pathway and the epigenetic regulation of gene transcription (35-36). The transcriptional activity and stability of HIF1 α has been shown to be tightly regulated by the lysine acetylation-deacetylation system (36-37). Multiple members of the lysine acetylase and deacetylase families interact with HIF1 α (38-41), and various acetylation sites within HIF1 α have been discovered (39, 41-42). However, the biological consequence of HIF1 α acetylation/deacetylation is not fully understood, and differential outcomes on HIF1 α transcriptional activity and stability, depending on the acetylation site, have been proposed (37, 39-40, 43).

The acetylation of lysines is an important post-translational modification of DNA-packing histones, that plays a central role in regulating chromatin structure and gene transcription (44). Acetyl-lysine residues are recognised by conserved structural modules, the bromodomains (BRDs), which are present in chromatin-associated proteins and histone acetyltransferases (44).

It was of interest to investigate if and how HIF1 α is regulated by bromodomain-containing proteins. For this purpose, dual inhibitors were developed by Hidenori Someya (University of Oxford, UK, unpublished data, **Figure 3.7**), by fusing the PHD inhibitor HS1.46 to the bromodomain inhibitor JQ1 (45).

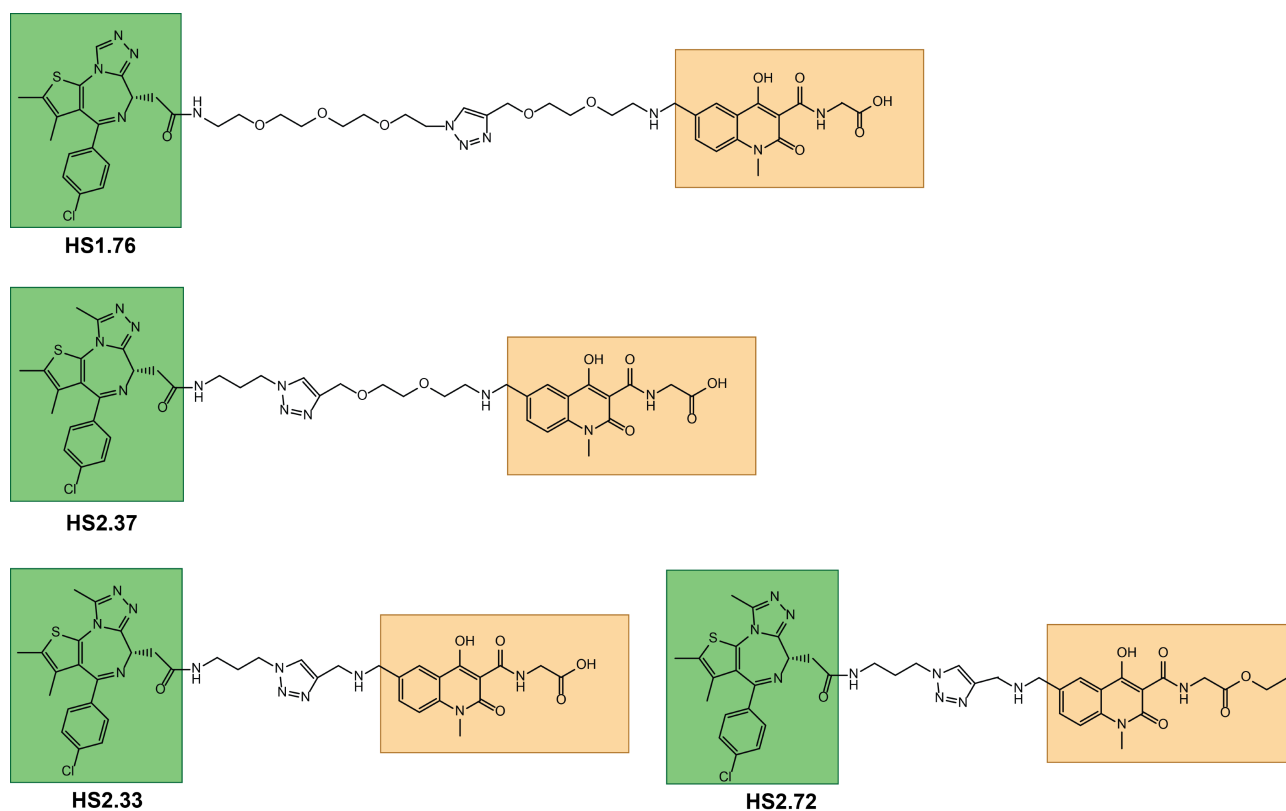


Figure 3.7: Structures of PHD-BRD dual inhibitors (Hidenori Someya, University of Oxford, UK). Colour scheme: PHD inhibitor domain (orange), BRD inhibitor domain (green).

These dual inhibitors were used to study the effect of simultaneously inhibiting HIF α prolyl hydroxylation, and bromodomain-containing proteins. Cell permeability of the PHD-BRD dual inhibitors was optimised as discussed above.

To support the development of PHD-pVHL and PHD-BRD dual inhibitors, the potency of the compounds against PHD2 was determined *in vitro*. The results in **Figure 3.8** show that PHD2 is efficiently inhibited by most of the tested compounds. However, the capability of the fused compounds to inhibit PHD2 is decreased when compared to the free PHD inhibitor ($IC_{50} = 0.63 \pm 0.21 \mu M$), with IC_{50} values of dual PHD-pVHL and PHD-BRD inhibitors ranging from 4-16 μM . As expected, the prodrugs with protected terminal carboxyl groups (HS3.60, HS3.62, and HS2.72) do not inhibit PHD2 in the tested concentration range. This finding is in accordance with the previously observed role of the free terminal carboxyl group (C-5) of 2OG and 2OG analogues, that forms contacts with PHD residues R383 and Y329 in the 2OG binding pocket of the enzyme (*General Introduction*, **Chapter 1.3**, **Figure 1.11**).

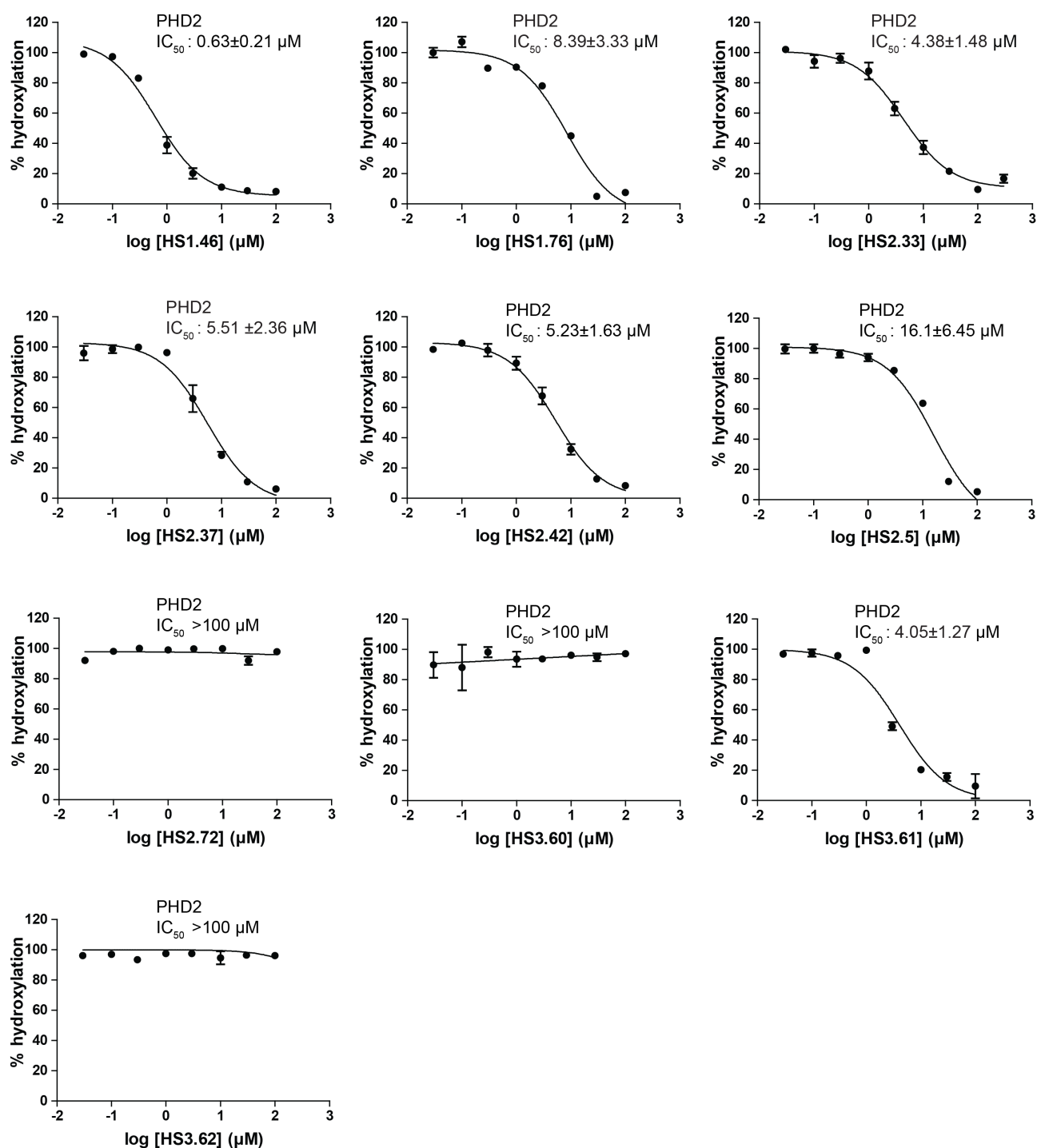


Figure 3.8: IC₅₀ values for inhibition of PHD2 by dual inhibitors. Assay conditions: 1 μM PHD2, inhibitor (0.03 μM, 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM), 50 μM (NH₄)₂Fe(II)(SO₄)₂, 10 μM 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μM 19mer CODD-peptide in 50 mM Tris, pH 7.5. Samples were measured by MALDI-TOF-MS and hydroxylation levels were quantified using MassLynx™ V4.0, non-enzymatic background oxidation was subtracted, and IC₅₀ values were determined with GraphPad Prism.

3.2.5 Inhibition of PHD2 by clinically-used Fe(II) chelators

The iron chelators Deferasirox and Deferoxamine are used for treatment of chronic iron overload, usually in patients receiving multiple blood transfusions (46-48). In addition, Deferoxamine efficiently binds aluminium ions and therefore is used for treatment of aluminium toxicity (49). The structures of both compounds are shown in **Figure 3.9**.

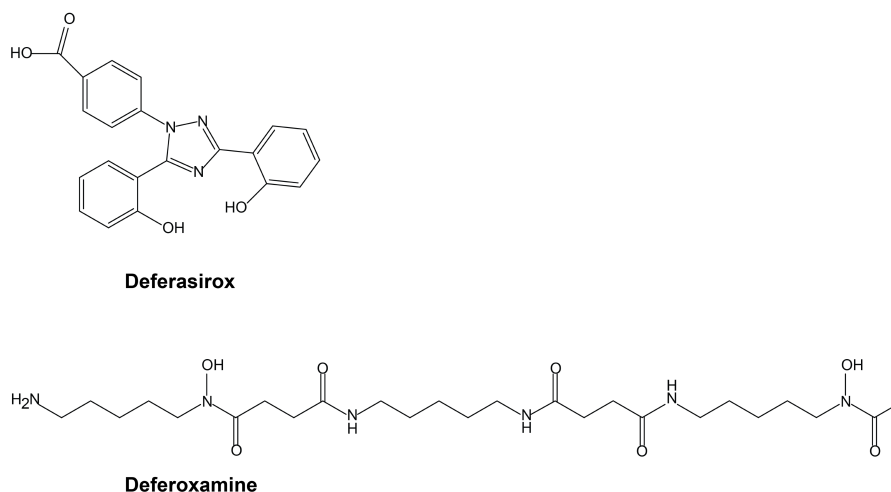


Figure 3.9: Structures of the iron chelators Deferasirox and Deferoxamine (46-49).

In vitro studies on Deferoxamine showed that this compound inhibits PHD2 ($IC_{50} = 7.64 \pm 1.35 \mu M$, (41)) and FIH ($IC_{50} = 8.41 \pm 0.82 \mu M$, (41)). In Hep3B cells treated with Deferoxamine, the inhibitor enhanced HIF1 α accumulation under normoxia (50). Due to the low cell-permeability of Deferoxamine, high concentrations of the inhibitor (at least 150 μM) were necessary to observe HIF1 α accumulation (50). Deferoxamine mediated HIF1 α accumulation was prevented by treating Hep3B cells with Fe(II/III), suggesting that the mode of action of Deferoxamine is via chelating Fe(II/III) ions, thus inhibiting PHDs that use Fe(II) as a co-factor (50).

Following up on this study, it was investigated if and how the two iron chelators Deferasirox and Deferoxamine influence the activity of PHDs. For this purpose, IC_{50} values of Deferasirox and Deferoxamine were determined (**Figure 3.10 A**). It was found that both compounds potently inhibit PHD2 with IC_{50} values in the low micromolar range (Deferoxamine: $IC_{50} = 2.38 \pm 0.99 \mu M$, Deferasirox: $IC_{50} = 2.44 \pm 0.68 \mu M$, concentration of Fe(II) in the assay: 1 μM).

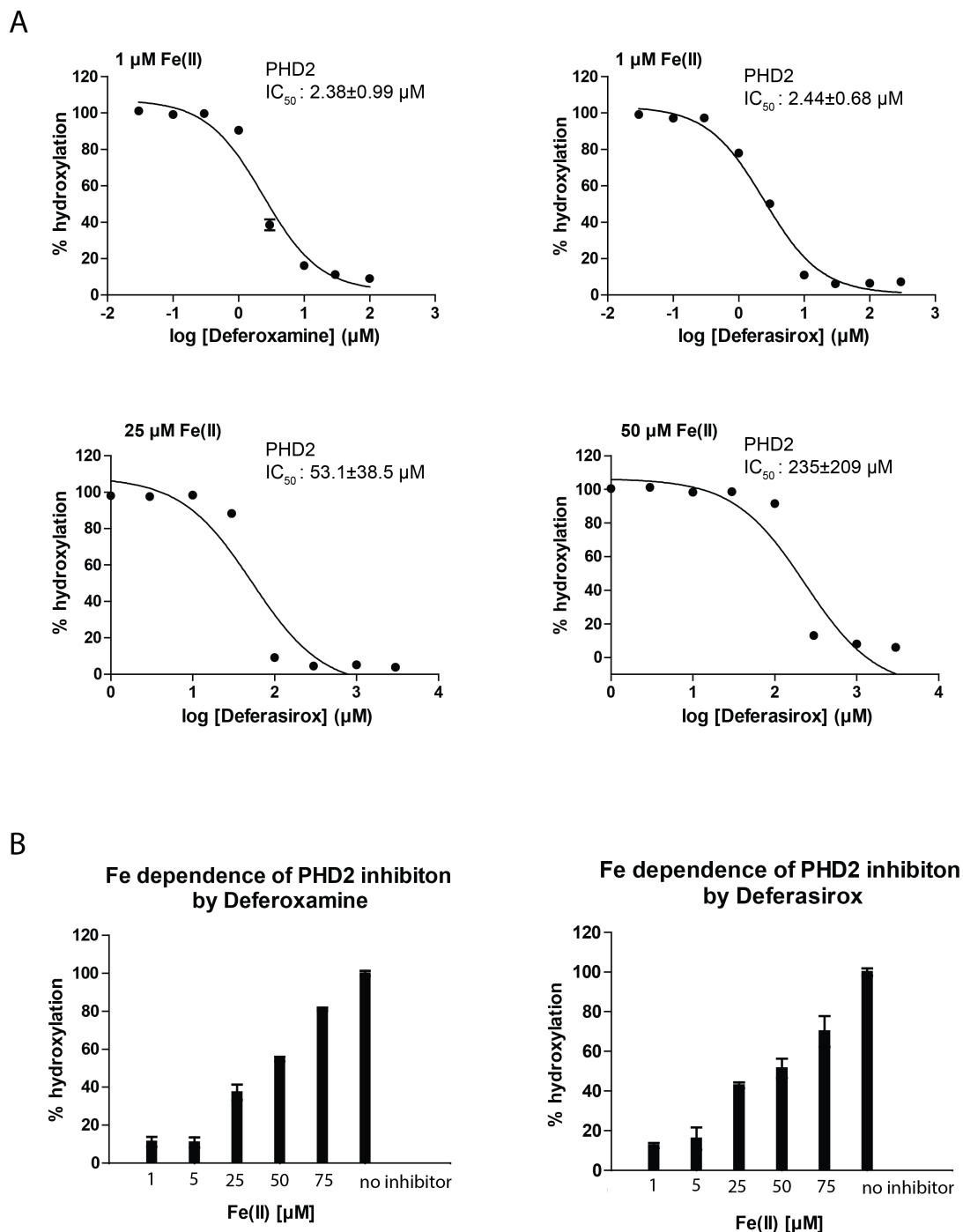


Figure 3.10: Inhibition of PHD2 by the iron chelators Deferoxamine and Deferasirox. (A) IC_{50} values. Assay conditions: 1 μM PHD2, inhibitor (0.03 μM , 0.1 μM , 0.3 μM , 1 μM , 3 μM , 10 μM , 30 μM , 100 μM), 10 μM 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μM 19mer CODD-peptide in 50 mM Tris, pH 7.5. Samples were measured by MALDI-TOF-MS and hydroxylation levels were quantified using MassLynx™ V4.0, non-enzymatic background oxidation was subtracted, and IC_{50} values were determined with GraphPad Prism. **(B)** Inhibition potency at different Fe(II) concentrations. Assay conditions as above, 100 μM inhibitor used.

NMR studies revealed that Deferasirox, unlike Deferoxamine, binds to PHD2 (Martine I. Abboud, University of Oxford, UK, unpublished data). Notably, Deferasirox has a tricyclic core that contains a pyrazolone ring, a feature shared with the potent PHD inhibitors IOX4 and Molidustat (see **Figure 3.4**). Further studies on Deferasirox showed that the potency of the inhibitor is strongly decreased at higher Fe(II) concentrations (IC_{50} at 25 μ M Fe(II) = 53.1 ± 38.5 μ M, IC_{50} at 50 μ M Fe(II) = 235 ± 209 μ M). Notably, the typical (physiological) plasma iron concentration is approximately 18 μ M (51), suggesting that Deferasirox possibly inhibits PHD2 under physiological conditions, yet (likely far) less potently as clinically-used PHD inhibitors. In order to compare the potency of Deferasirox and Deferoxamine at different iron concentrations, endpoint assays using 100 μ M inhibitor and 1-75 μ M Fe(II), were performed (**Figure 3.10 B**). It was found that the inhibitor potency of both compounds was similarly decreased by stepwise increases in iron concentrations, suggesting that the two compounds inhibit PHD2 by competing for iron binding with the enzyme.

3.2.6 High-throughput inhibition studies

3.2.6.1 Assay development

To establish an assay platform for PHD selective high-throughput inhibition screening, the RapidFire RF360 Agilent6530 QTOF system (Agilent) was used. Initial inhibition assays were set up with tPHD2 and PHD3^{fl}, using the conditions described in *Materials and Methods*, **Chapter 7.6.3**, at enzyme concentrations of 150 nM in the presence of HIF1 α CODD (5 μ M in case of PHD2, and 2 μ M in case of PHD3).

However, the initially measured IC₅₀ values were below the theoretical threshold of 75 nM (= 1/2 enzyme concentration), suggesting that the actual concentration of catalytically active PHD2/3 in the assay was lower than the (calculated) total enzyme concentration. In order to generate comparative inhibitor IC₅₀ values between different PHD isoforms, it is crucial to work with identical concentrations of catalytically active enzyme. Therefore, it was necessary to determine the fraction of catalytically active PHD2/3 in total enzyme batches.

For that purpose, active site titrations of PHD2 and PHD3 were performed as described in *Materials and Methods*, **Chapter 7.6.3**, (52). The titrations involved measuring initial rates of enzyme activity (HIF1 α CODD turnover) in the presence of increasing concentrations of a potent PHD inhibitor. The used PHD inhibitors (JHM174 in case of PHD2 and JHM532 in case of PHD3, **Figure 3.11**) were selected based on preliminary results from inhibitor screens that suggest high potency of the inhibitors towards the respective PHD isoform. Morrison plots of the data were created by plotting the measured enzyme activity vs. inhibitor concentration, and active site concentrations were determined using the Morrison equation in GraphPad Prism (52).

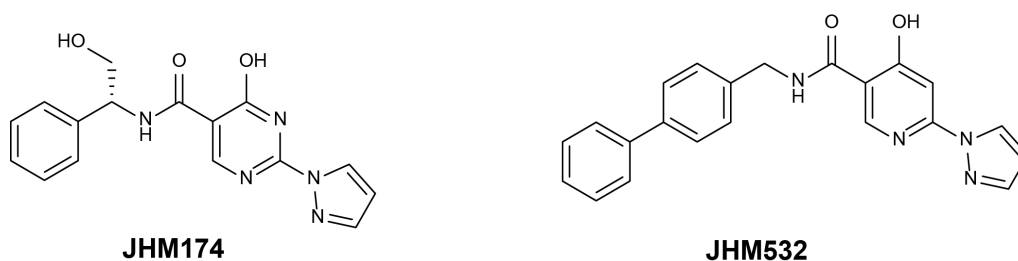


Figure 3.11: Structures of PHD inhibitors JHM174 and JHM532 used for determining active site concentrations of PHD2 and PHD3.

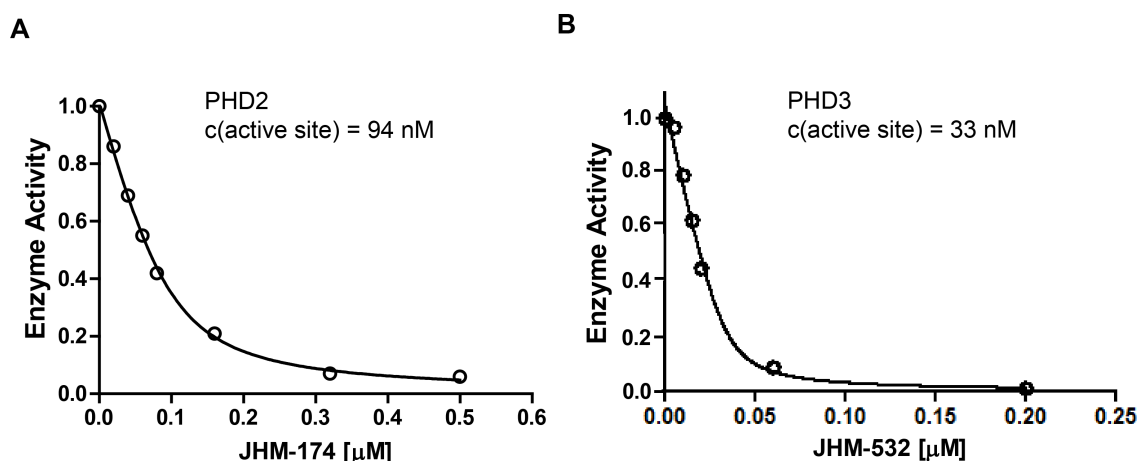


Figure 3.12: Determination of active site concentrations in batches of PHD2 and PHD3.

Morrison plot of (A) PHD2, and (B) PHD3. Conditions: 150 nM total enzyme (tPHD2/PHD3ⁿ), 5 μ M HIF1 α CODD (in case of tPHD2), 2 μ M HIF1 α CODD (in case of PHD3ⁿ), 0-500 nM inhibitor, 10 μ M (NH₄)₂Fe(II)(SO₄)₂, 10 μ M sodium L-ascorbate, 10 μ M 2-oxoglutarate disodium salt, 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM TCEP (in case of PHD3ⁿ). Inhibitors at varying concentrations (at least 6 different concentrations in a suitable range) were first incubated with PHD enzyme for 25 min, to allow for complete binding to the enzyme. The reaction then was started by addition of a substrate-co-factor mix, incubated at 25 °C, and hydroxylation levels were determined at various time points in the linear region. Initial rates of hydroxylation were determined using RapidFire Integrator, and the active site concentration was calculated from the Morrison Plot, using GraphPad Prism.

The results in **Figure 3.12** show that at a total PHD2 concentration of 150 nM, the concentration of catalytically active PHD2 was 94 nM, corresponding to 63 % active enzyme. In case of PHD3, 33 nM in 150 nM of total enzyme were catalytically active (22 % active enzyme). The reasons for the observed levels of active PHDs below 100 % are unclear, but could be due to inactivated enzyme or aggregation. The active site titration was repeated with various batches of PHD3, yet no substantial differences in enzyme activity between the batches were observed. All subsequent experiments were conducted using 100 nM active PHD2 or PHD3 enzyme, with the final assay conditions listed in **Table 3.4**, (32).

Table 3.4 Conditions for comparative PHD2-3 high-throughput inhibition screens (32).

Reagent	Final concentration
PHD2-3	100 nM active enzyme
HIF1 α CDD inhibitor	5 μ M (assays with PHD2), 2 μ M (assays with PHD3) varying concentration ranges
(NH ₄) ₂ Fe(II)(SO ₄) ₂	10 μ M
2-oxoglutarate disodium salt	10 μ M
sodium L-ascorbate	4 mM
Tris(2-carboxyethyl)phosphin (TCEP)	2 mM (only in assays with PHD3)
	in 50 mM Tris, pH 7.5, 50 mM NaCl

3.2.6.2 Studies on 2OG-oxygenase inhibitors

A range of 2OG-oxygenase inhibitors (and related compounds) were tested against PHD2 and PHD3 using the RapidFire-based high-throughput screen. The chosen inhibitors are listed in **Table 3.5** and structures are displayed in **Figure 3.13** (unless shown before in this Chapter).

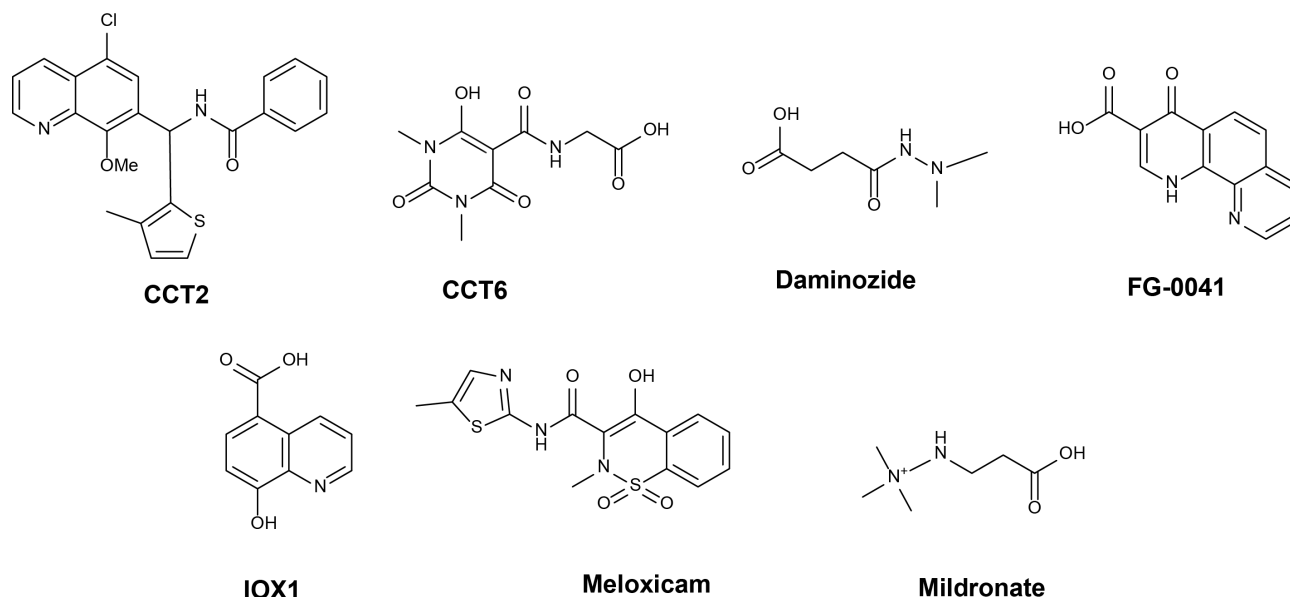


Figure 3.13: Structures of inhibitors tested against PHD2 and PHD3 (15, 32, 53-58).

The results of the inhibition screens are shown in **Table 3.5**. No inhibition of PHD2/3 was observed in the presence of Mildronate, Meloxicam, CCT2, CCT6, and Daminozide. In case of CCT2, the obtained results are in agreement with previous studies in HeLa cells, where no effect of the compound on HIF1 α and HIF2 α levels was observed (57). However, the inhibitor CCT6 (structurally related to the clinically-used GSK1278863), was active against PHD2 in previous studies and a PHD2.Mn.CCT6 complex crystal structure is available (PDB:5OX5, (32)). The inactivity of CCT6 against PHD2/3 in the study presented in this Chapter is likely due to either degradation of the compound during storage, or low solubility of the compound under the used assay conditions. In agreement with results from MALDI-TOF-MS-based assays (**Chapter 3.2.2** and **3.2.3**), the clinically-used PHD inhibitors FG-4592, FG-2216/IOX3, Vadadustat, and GSK1278863 potently inhibited PHD2 and PHD3 with IC₅₀ values in the nanomolar range. In case of FG-4592, a 5-fold increased potency against PHD2 (IC₅₀ (PHD2, FG-4592) = 0.16 μ M), when compared to PHD3 (IC₅₀ (PHD3, FG-4592) = 0.82 μ M), was measured.

The PHD inhibitors IOX2 and IOX4, the 2OG oxygenase inhibitor IOX1, and the prolyl-4-hydroxylase inhibitor FG-0041 were active on PHD2 and PHD3, with IC_{50} values in the nanomolar to low micromolar range. The measured IC_{50} values suggest a 5-fold higher potency of IOX1 against PHD3 (IC_{50} (PHD3, IOX1) = 4.90 μ M), when compared to PHD2 (IC_{50} (PHD2, IOX1) = 24.2 μ M). Apart from FG-4592 and IOX1, no substantial differences in potency against PHD2 vs. PHD3 were observed.

Table 3.5 IC_{50} values determined for inhibition of PHD2 and PHD3.

Compound ID/ name	Inhibition profiles	Structure	IC_{50} for PHD2 (μ M)	IC_{50} for PHD3 (μ M)
IOX1	broad-spectrum 2OG oxygenase inhibitor (53)	Figure 3.13	24.2	4.90
IOX2	PHD inhibitor (22)	Figure 3.2	0.20	0.17
IOX4	PHD inhibitor (33)	Figure 3.4	0.25	0.28
FG-4592	clinically-used PHD inhibitor (32)	Figure 3.4	0.16	0.82
FG-0041	orally available P4H inhibitor (54)	Figure 3.13	10.7	11.7
Mildronate	inhibitor of carnitine biosynthesis (55)	Figure 3.13	>100	>100
Meloxicam	cyclooxygenase (COX) inhibitor (56)	Figure 3.13	>100	>100
FG-2216/IOX3	clinically-used PHD inhibitor (study stopped, 15-16)	Figure 3.2	0.26	0.15
Vadadustat	clinically-used PHD inhibitor (32)	Figure 3.4	0.28	0.31
GSK1278863	clinically-used PHD inhibitor (32)	Figure 3.4	0.24	0.41
CCT2	compound initially designed as KDM inhibitor (57)	Figure 3.13	>100	>100
CCT6	GSK1278863 related inhibitor (32)	Figure 3.13	>100	>100
Daminozide	KDM inhibitor (58)	Figure 3.13	>100	>100

3.2.6.3 Studies on novel PHD inhibitors

A series of PHD inhibitors was designed and synthesised by James Holt-Martyn (University of Oxford, UK, unpublished data). The structures of the PHD inhibitors are shown in **Figure 3.14** and **3.15**. RapidFire-based inhibition studies on PHD2 (conducted by James Holt-Martyn and Anthony Tumber (University of Oxford, UK) using identical assay conditions as described in **Chapter 3.2.6.1**, data in **Table 3.6**) revealed that, while the majority of the compounds potentially inhibited PHD2, some of the putative PHD inhibitors did not reduce activity of the enzyme, but instead increased the rate of PHD2-catalysed HIF1 α CODD turnover. The observed effect of increased HIF1 α CODD hydroxylation by PHD2 in the presence of the compounds JHM377, JHM675, and JHM682 (61 %, 65 %, and 41 % increase of PHD2-catalysed HIF1 α CODD hydroxylation over the measured timeframe, respectively) was further investigated in cellular studies, where increased HIF α degradation was observed when Hep3B cells were treated with the compounds (Tzu-Lan Yeh, James Holt-Martyn, University of Oxford, UK, unpublished data). Therefore, it was of interest to examine if compounds JHM377, JHM675, and JHM682 exhibit a similar effect on PHD3. Additionally, a selection of potent PHD2 inhibitors was tested against PHD3 using the RapidFire-based high-throughput screen (**Chapter 3.2.6.1** and *Materials and Methods*, **Chapter 7.6.3**).

The results of the study (**Table 3.6**) revealed that all tested PHD2 inhibitors potentially inhibited PHD3. The majority of the inhibitors thereby did not discriminate between PHD2 and PHD3. However, four inhibitors (JHM773, JHM885, JHM997, and JHM1037) exhibited a 5-fold to 37-fold difference in potency against PHD2 vs. PHD3. Further studies on these compounds using alternative assay methods such as AlphaScreen are necessary to validate the observed PHD2/3 isoform selectivity.

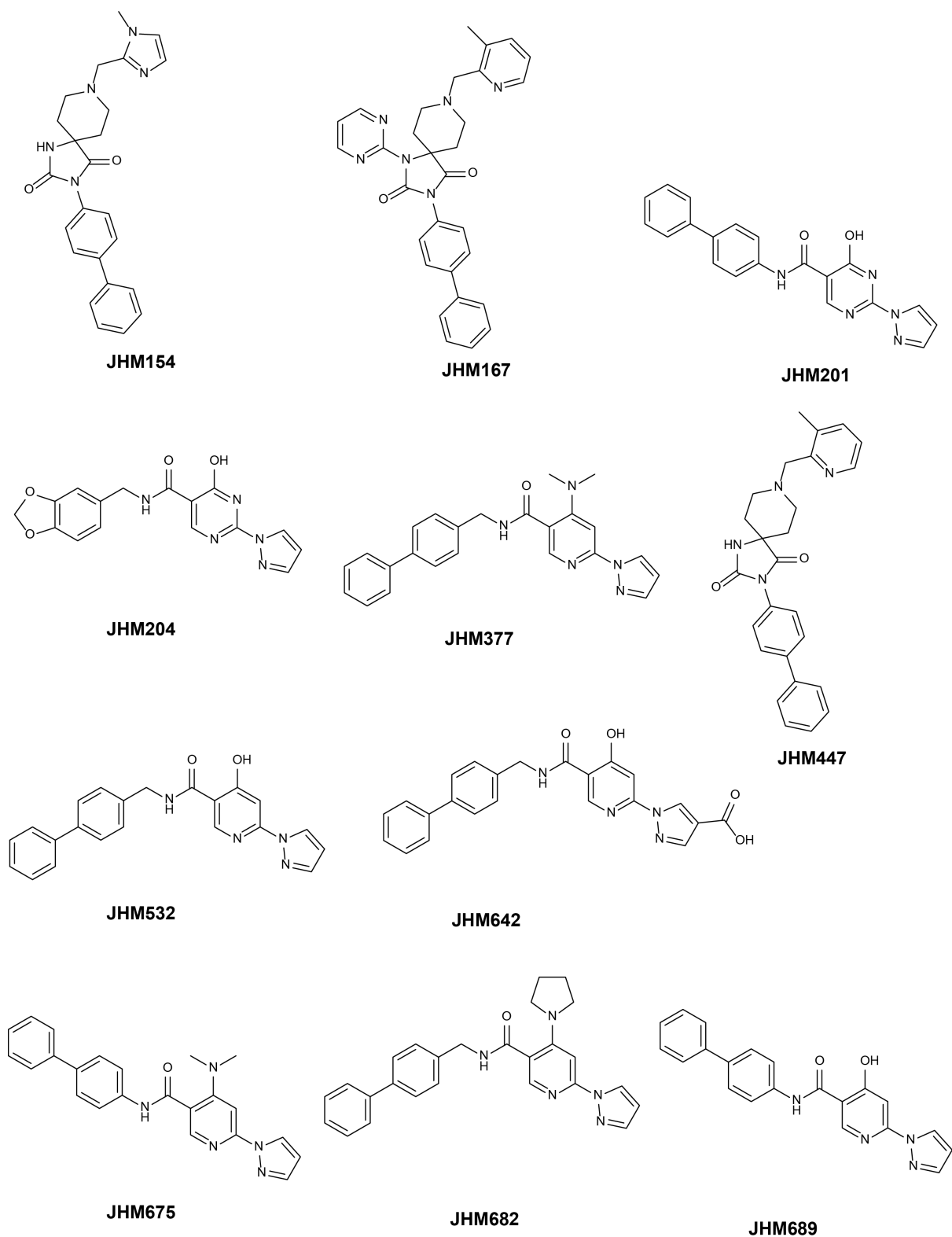


Figure 3.14: Structures of PHD inhibitors (designed and synthesised by James Holt-Martyn, University of Oxford, UK).

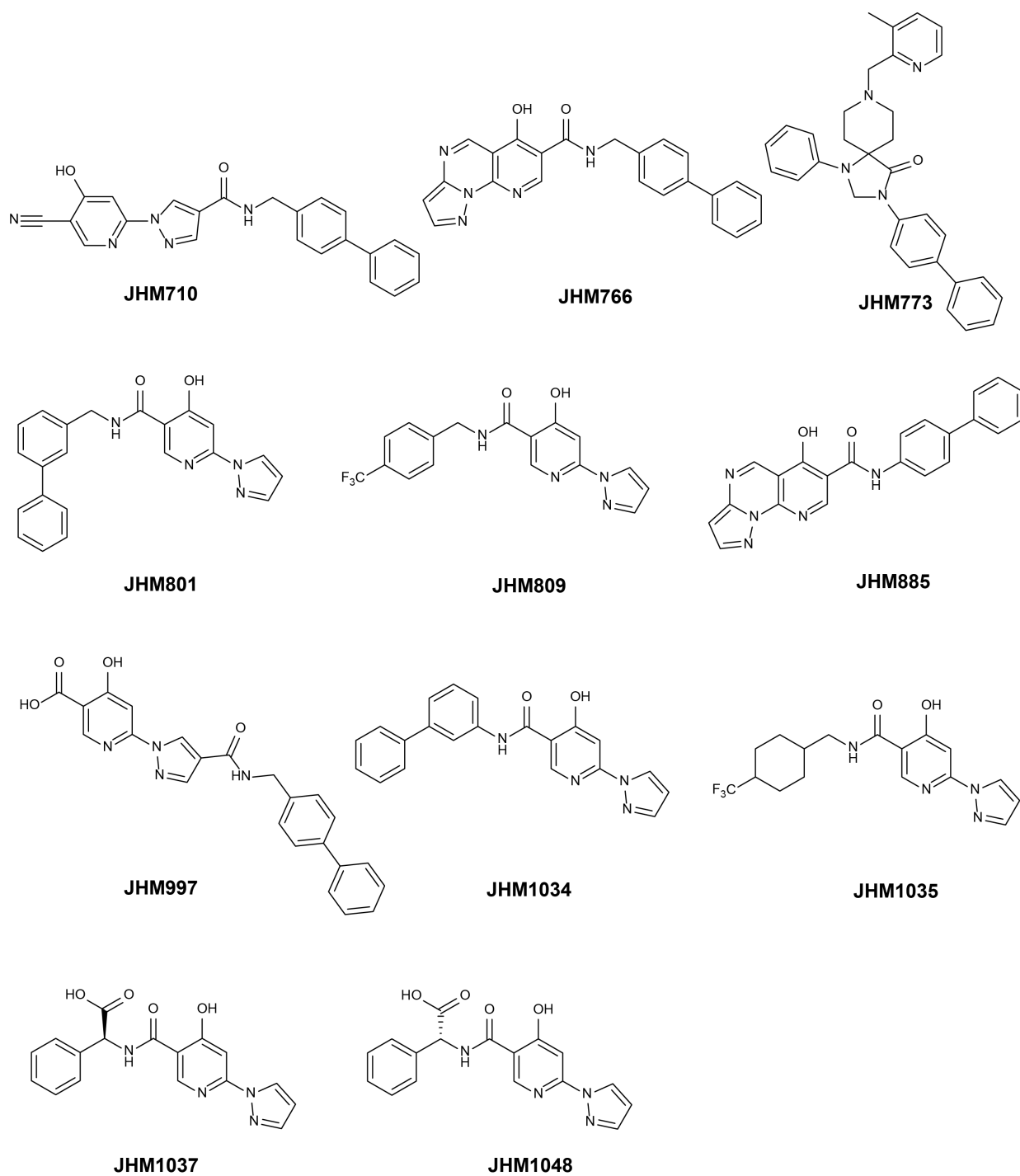


Figure 3.15: Structures of PHD inhibitors (designed and synthesised by James Holt-Martyn, University of Oxford, UK).

Table 3.6 IC₅₀ values determined for activation and inhibition of PHD2 and PHD3. * compounds measured by James Holt-Martyn and Anthony Tumber (University of Oxford, UK, unpublished data).

Compound ID	IC ₅₀ for PHD2 HIF1α Codd (μM) *	IC ₅₀ for PHD3 HIF1α Codd (μM)	IC ₅₀ for PHD3 HIF2α Codd (μM)	IC ₅₀ for PHD3 HIF3α Codd (μM)
Inhibitors				
JHM766	0.31	0.19	0.28	0.19
JHM773	0.51	0.15	0.17	0.11
JHM801	0.06	0.08	0.09	0.07
JHM809	0.06	0.06	0.07	0.05
JHM885	0.15	0.51	0.62	0.41
JHM997	2.23	0.06	0.08	0.05
JHM1034	0.11	0.69	0.61	0.67
JHM1035	0.08	0.07	0.07	0.06
JHM1037	0.59	0.11	0.13	0.10
JHM1048	0.18	0.30	0.34	0.24
JHM154	0.18	0.20		
JHM167	0.50	0.38		
JHM201	0.26	0.34		
JHM204	0.25	0.26		
JHM447	0.50	1.02		
JHM532	0.15	0.13		
JHM642	0.09	0.15		
JHM689	0.32	0.89		
JHM710	0.39	0.31		
Compounds causing increases in PHD2-catalysed HIF1α Codd hydroxylation				
JHM377	61% activation	9.15	9.06	8.77
JHM675	65% activation	inactive		
JHM682	41% activation	inactive		

To investigate potential effects of different HIF α substrates on the outcome of the assays, PHD3 inhibition was additionally tested in the presence of HIF2 α CODD and HIF3 α CODD. However, no substantial differences in the obtained IC₅₀ values were apparent (**Table 3.6**), suggesting that the tested compounds equally inhibit HIF1-3 α CODD binding and/or hydroxylation by PHD3.

Notably, the compounds JHM377, JHM675, and JHM682 that increased PHD2-catalysed HIF1 α CODD hydroxylation, did not cause any increase in HIF1 α CODD hydroxylation by PHD3 (**Table 3.6**). On the contrary, JHM377 potently inhibited PHD3 (IC₅₀ (PHD3, HIF1 α CODD, JHM377) = 9.15 μ M), while JHM675 and JHM682 were (at least) inactive on PHD3. The finding that the compounds that cause increase in PHD2-catalysed HIF1 α CODD hydroxylation, discriminate between PHD2 and PHD3 is interesting from a mechanistic perspective, as it implies that structural differences between the two enzymes might be exploited by these compounds to achieve the observed selectivity.

3.3 Conclusions

HIF prolyl hydroxylases are major targets for the therapeutic manipulation of the HIF pathway. The majority (if not all) of current generation PHD inhibitors (including those used in clinical studies for treatment of aenamia in chronic kidney disease) act by competing with 2OG binding to the enzymes (see *General Introduction*, **Chapter 1.3**).

It was of interest to investigate if the available PHD inhibitors discriminate between different PHD isoforms. In this Chapter, a MALDI-TOF-MS-based PHD1-3 inhibition screen is presented, that allows to comparatively test inhibitors against PHD1, 2, and 3 under identical conditions. The results obtained from the PHD1-3 inhibition screen show that NOG, IOX2, IOX4, FG-2216/IOX3, and the clinically-used PHD inhibitors Molidustat, FG-4592, GSK1278863, Vadadustat and Fluoro-vadadustat all potently inhibit PHD1, 2, and 3. Given that PHD1-3 all use 2OG as a co-factor and 2OG is (likely) bound in a similar manner at the active sites of the enzymes, it is not surprising to find no discrimination between PHD1-3 by the tested 2OG analogue inhibitors (within limits of detection). Combined NMR and crystallographic studies previously showed that some of the tested inhibitors protrude into the substrate-binding pocket of PHD2, thus displacing HIF1 α NODD (in case of Vadadustat, Molidustat, IOX4, FG-4592, GSK1278863) and HIF1 α CODD (in case of FG-4592 and FG-2216/IOX3) (32, 59). However, given that PHD1-3 are all potently inhibited by IOX4, FG-2216, and the clinically-used inhibitors, it seems that the capacity of the inhibitors to displace HIF1 α CODD/NODD does not cause or relate to PHD isoform selectivity.

Emerging evidence suggests disparate roles of PHD1-3 in the HIF pathway, including differences in the substrate selectivity profiles of the enzymes (60-61) and the differential expression of PHD1-3 in various cell types (62-63). The development of PHD isoform-selective inhibitors is therefore of pharmaceutical interest, to achieve a better differentiation of clinical effects.

In the work described in this Chapter, newly designed PHD inhibitors were comparatively screened against PHD2 and PHD3 using a RapidFire-based high-throughput assay. Although the majority of the tested compounds did not exhibit any selectivity between PHD2 and PHD3, four inhibitors were identified (JHM773, JHM885, JHM997, and JHM1037) that showed 5-to 37-fold differences in potency against PHD2 *vs.* PHD3.

Future studies should aim on validating the obtained results, e.g. by AlphaScreen and by cellular inhibition studies. Additionally, it might be of interest to investigate PHD1 inhibition by these compounds. Further, structural and mechanistic studies might be necessary to rationalise the observed differential effects of the four compounds on PHD2 and PHD3.

Most interestingly, the comparative PHD2/3 inhibition screen revealed that the compounds JHM377, JHM675, and JHM682 selectively increased HIF1 α CDD hydroxylation by PHD2, but did not exhibit any similar effect (or even acted as inhibitors) against PHD3. These findings are interesting from a mechanistic point of view, because understanding the mode of action of JHM377, JHM675, and JHM682 on PHD2 vs. PHD3 might provide new strategies for the design of both more potent compounds that increase PHD2-catalysed HIF1 α CDD hydroxylation, and PHD isoform-selective inhibitors. Further mechanistic studies on compounds that increase PHD2-catalysed HIF1 α CDD hydroxylation were conducted by James Holt-Martyn, Anthony Tumber and Tzu-Lan Yeh (University of Oxford, UK, unpublished data). The results of these studies imply that these compounds (to a certain extent) discriminate between HIF1 α CDD and HIF1 α NDD, as it was found that JHM377 and JHM675 more strongly activated PHD2-catalysed hydroxylation of HIF1 α CDD, whereas JHM682 exhibited a stronger effect on HIF1 α NDD. Taken together, the preliminary data obtained so far on compounds that increase PHD2-catalysed HIF1 α CDD hydroxylation, suggest that these compounds might differentially affect substrate binding to PHD2 and PHD3. However, further structural and mechanistic studies on the compounds that increase PHD2-catalysed HIF1 α CDD hydroxylation are necessary to elucidate their exact mode of action.

3.4 References

- (1) Chan, M. C., Holt-Martyn, J. P., Schofield, C. J., & Ratcliffe, P. J. (2016). Pharmacological targeting of the HIF hydroxylases – A new field in medicine development. *Molecular Aspects of Medicine*, 47(Supplement C), 54–75.
- (2) Haase, V. H. (2013). Regulation of erythropoiesis by hypoxia-inducible factors. *Blood Reviews*, 27(1), 41–53.
- (3) Barrett, T. D., Palomino, H. L., Brondstetter, T. I., Kanelakis, K. C., Wu, X., Haug, P. V., ... Shankley, N. P. (2011). Pharmacological Characterization of 1-(5-Chloro-6-(trifluoromethoxy)-1*H*-benzoimidazol-2-yl)-1*H*-pyrazole-4-carboxylic Acid (JNJ-42041935), a Potent and Selective Hypoxia-Inducible Factor Prolyl Hydroxylase Inhibitor. *Molecular Pharmacology*, 79(6), 910–920.
- (4) Barrett, T. D., Palomino, H. L., Brondstetter, T. I., Kanelakis, K. C., Wu, X., Yan, W., ... Rabinowitz, M. H. (2015). Prolyl hydroxylase inhibition corrects functional iron deficiency and inflammation-induced anaemia in rats. *British Journal of Pharmacology*, 172(16), 4078–4088.
- (5) Besarab, A., Provenzano, R., Hertel, J., Zabaneh, R., Klaus, S. J., Lee, T., ... Neff, T. B. (2015). Randomized placebo-controlled dose-ranging and pharmacodynamics study of roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients. *Nephrology Dialysis Transplantation*, 30(10), 1665–1673.
- (6) Flamme, I., Oehme, F., Ellinghaus, P., Jeske, M., Keldenich, J., & Thuss, U. (2014). Mimicking Hypoxia to Treat Anemia: HIF-Stabilizer BAY 85-3934 (Molidustat) Stimulates Erythropoietin Production without Hypertensive Effects. *PLoS ONE*, 9(11), e111838.
- (7) Holdstock, L., Meadowcroft, A. M., Maier, R., Johnson, B. M., Jones, D., Rastogi, A., ... Cobitz, A. R. (2016). Four-Week Studies of Oral Hypoxia-Inducible Factor–Prolyl Hydroxylase Inhibitor GSK1278863 for Treatment of Anemia. *Journal of the American Society of Nephrology : JASN*, 27(4), 1234–1244.
- (8) Hsieh, M. M., Linde, N. S., Wynter, A., Metzger, M., Wong, C., Langsetmo, I., ... Tisdale, J. F. (2007). HIF–prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. *Blood*, 110(6), 2140–2147.
- (9) Besarab, A., Bolton, W. K., Browne, J. K., Egrie, J. C., Nissenson, A. R., Okamoto, D. M., ... Goodkin, D. A. (1998). The Effects of Normal as Compared with Low Hematocrit Values in Patients with Cardiac Disease Who Are Receiving Hemodialysis and Epoetin. *New England Journal of Medicine*, 339(9), 584–590.
- (10) Unger, E. F., Thompson, A. M., Blank, M. J., & Temple, R. (2010). Erythropoiesis-Stimulating Agents — Time for a Reevaluation. *New England Journal of Medicine*, 362(3), 189–192.

-
- (11) Szczech, L. A., Barnhart, H. X., Sapp, S., Felker, G. M., Hernandez, A., Reddan, D., ... Singh, A. K. (2010). A secondary analysis of the CHOIR trial shows that comorbid conditions differentially affect outcomes during anemia treatment. *Kidney International*, 77(3), 239–246.
- (12) McCullough, P.A., Barnhart, H.X., Inrig, J.K., Reddan, D., Sapp, S., Patel, U.D. et al (2013). Cardiovascular toxicity of epoetin-alfa in patients with chronic kidney disease. *The American Journal of Nephrology*, 37(6), 549–558.
- (13) Shah, Y. M., & Xie, L. (2014). Hypoxia-Inducible Factors Link Iron Homeostasis and Erythropoiesis. *Gastroenterology*, 146(3), 630–642.
- (14) Ang, S. O., Chen, H., Hirota, K., Gordeuk, V. R., Jelinek, J., Guan, Y., ... Prchal, J. T. (2002). Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nature Genetics*, 32(4), 614–621.
- (15) Astellas Pharma Inc. (2007). Adverse event of FG-2216 for the treatment of anemia. www.astellas.com/en/corporate/news/pdf/070507_eg.pdf. Accessed 9th October 2017.
- (16) Astellas Pharma Inc. (2008). The FDA accepts the complete response for clinical holds of FG-2216*/FG-4592 for the treatment of anemia. www.astellas.com/en/corporate/news/pdf/080402_eg.pdf. Accessed 9th October 2017.
- (17) Robinson, A., Keely, S., Karhausen, J., Gerich, M. E., Furuta, G. T., & P.Colgan, S. (2008). Mucosal Protection by Hypoxia-Inducible Factor (HIF) Prolyl Hydroxylase Inhibition. *Gastroenterology*, 134(1), 145–155.
- (18) Bernhardt, W. M., Wiesener, M. S., Scigalla, P., Chou, J., Schmieder, R. E., Günzler, V., & Eckardt, K.-U. (2010). Inhibition of Prolyl Hydroxylases Increases Erythropoietin Production in ESRD. *Journal of the American Society of Nephrology : JASN*, 21(12), 2151–2156.
- (19) Besarab, A., Chernyavskaya, E., Motylev, I., Shutov, E., Kumbar, L. M., Gurevich, K., ... Neff, T. B. (2016). Roxadustat (FG-4592): Correction of Anemia in Incident Dialysis Patients. *Journal of the American Society of Nephrology : JASN*, 27(4), 1225–1233.
- (20) Van Welden, S., Selfridge, A. C., & Hindryckx, P. (2017). Intestinal hypoxia and hypoxia-induced signalling as therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol*, 14(10), 596–611.
- (21) Roda, J. M., Wang, Y., Sumner, L. A., Phillips, G. S., Marsh, C. B., & Eubank, T. D. (2012). Stabilization of HIF-2 α induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. *Journal of Immunology (Baltimore, Md. : 1950)*, 189(6), 3168–3177.

- (22) Chan, M. C., Ilott, N. E., Schödel, J., Sims, D., Tumber, A., Lippl, K., ... Schofield, C. J. (2016). Tuning the transcriptional response to hypoxia by inhibiting Hypoxia-inducible Factor (HIF) prolyl and asparaginyl hydroxylases. *Journal of Biological Chemistry*, 291(39).
- (23) Rose, N. R., McDonough, M. A., King, O. N. F., Kawamura, A., & Schofield, C. J. (2011). Inhibition of 2-oxoglutarate dependent oxygenases. *Chemical Society Reviews*, 40(8), 4364–4397.
- (24) Kaule, G., Gunzler, V. (1990). Assay for 2-oxoglutarate decarboxylating enzymes based on the determination of [1-14C]succinate: application to prolyl 4-hydroxylase. *Analytical Biochemistry*, 184(2), 291-297.
- (25) Chowdhury, R., Candela-Lena, J. I., Chan, M. C., Greenald, D. J., Yeoh, K. K., Tian, Y.-M., ... Schofield, C. J. (2013). Selective Small Molecule Probes for the Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases. *ACS Chemical Biology*, 8(7), 1488–1496.
- (26) Hewitson, K. S., Liénard, B. M. R., McDonough, M. A., Clifton, I. J., Butler, D., Soares, A. S., ... Schofield, C. J. (2007). Structural and Mechanistic Studies on the Inhibition of the Hypoxia-inducible Transcription Factor Hydroxylases by Tricarboxylic Acid Cycle Intermediates. *Journal of Biological Chemistry*, 282(5), 3293–3301.
- (27) Lee, S.-H., Moon, J. H., Cho, E. A., Ryu, S.-E., & Lee, M. K. (2008). Monoclonal Antibody-Based Screening Assay for Factor Inhibiting Hypoxia-Inducible Factor Inhibitors. *Journal of Biomolecular Screening*, 13(6), 494–503.
- (28) Oehme, F., Jonghaus, W., Narouz-Ott, L., Huetter, J., & Flamme, I. (2004). A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity. *Analytical Biochemistry*, 330(1), 74–80.
- (29) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (30) McNeill, L. A., Hewitson, K. S., Gleadle, J. M., Horsfall, L. E., Oldham, N. J., Maxwell, P. H., ... Schofield, C. J. (2002). The use of dioxygen by HIF prolyl hydroxylase (PHD1). *Bioorganic & Medicinal Chemistry Letters*, 12(12), 1547–1550.
- (31) Tarhonskaya, H., Chowdhury, R., Leung, I. K. H., Loik, N. D., McCullagh, J. S. O., Claridge, T. D. W., ... Flashman, E. (2014). Investigating the contribution of the active site environment to the slow reaction of hypoxia-inducible factor prolyl hydroxylase domain 2 with oxygen. *Biochemical Journal*, 463(3), 363-372.

- (32) Yeh, T.-L., Leissing, T., Abboud, M. I., Thinnies, C. C., Atasoylu, O., Holt-Martyn, J. P., ... Schofield, C. J. (2017). Molecular and cellular mechanisms of HIF prolyl hydroxylase inhibitors in clinical trials. *Chemical Science*, 8, 7651-7668.
- (33) Chan, M. C., Atasoylu, O., Hodson, E., Tumber, A., Leung, I. K. H., Chowdhury, R., ... Schofield, C. J. (2015). Potent and Selective Triazole-Based Inhibitors of the Hypoxia-Inducible Factor Prolyl-Hydroxylases with Activity in the Murine Brain. *PLoS ONE*, 10(7), e0132004.
- (34) Galdeano, C., Gadd, M. S., Soares, P., Scaffidi, S., Van Molle, I., Birced, I., ... Ciulli, A. (2014). Structure-Guided Design and Optimization of Small Molecules Targeting the Protein-Protein Interaction between the von Hippel-Lindau (VHL) E3 Ubiquitin Ligase and the Hypoxia Inducible Factor (HIF) Alpha Subunit with in Vitro Nanomolar Affinities. *Journal of Medicinal Chemistry*, 57(20), 8657-8663.
- (35) Pollard, P. J., Loenarz, C., Mole, D. R., McDonough, M. A., Gleadle, J. M., Schofield, C. J., & Ratcliffe, P. J. (2008). Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1 α . *Biochemical Journal*, 416(3), 387-394.
- (36) Ellis, L., Hammers, H., & Pili, R. (2009). Targeting tumor angiogenesis with Histone Deacetylase Inhibitors. *Cancer Letters*, 280(2), 145.
- (37) Geng, H., Liu, Q., Xue, C., David, L. L., Beer, T. M., Thomas, G. V., ... Qian, D. Z. (2012). HIF1 α Protein Stability Is Increased by Acetylation at Lysine 709. *The Journal of Biological Chemistry*, 287(42), 35496-35505.
- (38) Qian, D. Z., Kachhap, S. K., Collis, S. J., Verheul, H. M. W., Carducci, M. A., Atadja, P., & Pili, R. (2006). Class II Histone Deacetylases Are Associated with VHL-Independent Regulation of Hypoxia-Inducible Factor 1 α . *Cancer Research*, 66(17), 8814-8821.
- (39) Jeong, J.-W., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., ... Kim, K.-W. (2002). Regulation and Destabilization of HIF-1 α by ARD1-Mediated Acetylation. *Cell*, 111(5), 709-720.
- (40) Kato, H., Tamamizu-Kato, S., & Shibasaki, F. (2004). Histone Deacetylase 7 Associates with Hypoxia-inducible Factor 1 α and Increases Transcriptional Activity. *Journal of Biological Chemistry*, 279(40), 41966-41974.
- (41) Lim, J.-H., Lee, Y.-M., Chun, Y.-S., Chen, J., Kim, J.-E., & Park, J.-W. (2010). Sirtuin 1 Modulates Cellular Responses to Hypoxia by Deacetylating Hypoxia-Inducible Factor 1 α . *Molecular Cell*, 38(6), 864-878.
- (42) Geng, H., Harvey, C. T., Pittsenbarger, J., Liu, Q., Beer, T. M., Xue, C., & Qian, D. Z. (2011). HDAC4 Protein Regulates HIF1 α Protein Lysine Acetylation and Cancer Cell Response to Hypoxia. *The Journal of Biological Chemistry*, 286(44), 38095-38102.

- (43) Sadoul, K., Boyault, C., Pabion, M., & Khochbin, S. (2008). Regulation of protein turnover by acetyltransferases and deacetylases. *Biochimie*, 90(2), 306–312.
- (44) Sanchez, R., & Zhou, M.-M. (2009). The role of human bromodomains in chromatin biology and gene transcription. *Current Opinion in Drug Discovery & Development*, 12(5), 659–665.
- (45) Zengerle, M., Chan, K.-H., & Ciulli, A. (2015). Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4. *ACS Chemical Biology*, 10(8), 1770–1777.
- (46) Choudhry, V. P., & Naithani, R. (2007). Current status of iron overload and chelation with deferasirox. *The Indian Journal of Pediatrics*, 74(8), 759–764.
- (47) P H Yang, L., J Keam, S., & M Keating, G. (2007). Deferasirox: A review of its use in the management of transfusional chronic iron overload. *Drugs* (Vol. 67).
- (48) Poggiali, E., Cassinerio, E., Zanaboni, L., & Cappellini, M. D. (2012). An update on iron chelation therapy. *Blood Transfusion*, 10(4), 411–422.
- (49) Sivakumar, S., Khatiwada, C. P., & Sivasubramanian, J. (2014). Studies the alterations of biochemical and mineral contents in bone tissue of mus musculus due to aluminum toxicity and the protective action of desferrioxamine and deferiprone by FTIR, ICP-OES, SEM and XRD techniques. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 126(Supplement C), 59–67.
- (50) Cho, E. A., Song, H. K., Lee, S.-H., Chung, B. H., Lim, H. M., & Lee, M. K. (2013). Differential in vitro and cellular effects of iron chelators for hypoxia inducible factor hydroxylases. *Journal of Cellular Biochemistry*, 114(4), 864–873.
- (51) Ponka, P. (1999). Cellular iron metabolism. *Kidney International*, 55(Supplement 69), S2–S11.
- (52) Williams, J. W., and Morrison, J. F. (1979). The kinetics of reversible tight-binding inhibition. *Methods Enzymology*, 63, 437–467.
- (53) Schiller, R., Scozzafava, G., Tumber, A., Wickens, J. R., Bush, J. T., Rai, G., ... Kawamura, A. (2014). A Cell-Permeable Ester Derivative of the JmJc Histone Demethylase Inhibitor IOX1. *Chemmedchem*, 9(3), 566–571.
- (54) Nwogu, J. I., Geenen, D., Bean, M., Brenner, M. C., Huang, X., & Buttrick, P. M. (2001). Inhibition of Collagen Synthesis With Prolyl 4-Hydroxylase Inhibitor Improves Left Ventricular Function and Alters the Pattern of Left Ventricular Dilatation After Myocardial Infarction. *Circulation*, 104(18), 2216–2221.
- (55) Sjakste, N., Gutcaits, A., & Kalvinsh, I. (2005). Mildronate: An Antiischemic Drug for Neurological Indications. *CNS Drug Reviews*, 11(2), 151–168.
- (56) Furst D.E. (1998) Meloxicam: selective COX-2 inhibition in clinical practice. In: Vane J., Botting J. (eds) *Selective COX-2 Inhibitors*. Springer, Dordrecht.

- (57) Thinnes, C. C., Tumber, A., Yapp, C., Scozzafava, G., Yeh, T., Chan, M. C., ... Schofield, C. J. (2015). Betti reaction enables efficient synthesis of 8-hydroxyquinoline inhibitors of 2-oxoglutarate oxygenases. *Chemical Communications*, 51(84), 15458–15461.
- (58) Rose, N. R., Woon, E. C. Y., Tumber, A., Walport, L. J., Chowdhury, R., Li, X. S., ... Kawamura, A. (2012). The Plant Growth Regulator Daminozide is a Selective Inhibitor of the Human KDM2/7 Histone Demethylases. *Journal of Medicinal Chemistry*, 55(14), 6639–6643.
- (59) Leung, I. K. H., Flashman, E., Yeoh, K. K., Schofield, C. J., & Claridge, T. D. W. (2010). Using NMR Solvent Water Relaxation to Investigate Metalloenzyme–Ligand Binding Interactions. *Journal of Medicinal Chemistry*, 53(2), 867–875.
- (60) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (61) Huang, J., Zhao, Q., Mooney, S. M., & Lee, F. S. (2002). Sequence Determinants in Hypoxia-inducible Factor-1 α for Hydroxylation by the Prolyl Hydroxylases PHD1, PHD2, and PHD3. *Journal of Biological Chemistry*, 277(42), 39792–39800.
- (62) Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R., & Taubman, M. B. (2002). Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. *Biochemistry and Cell Biology*, 80(4), 421–426.
- (63) Willam, C., Maxwell, P. H., Nichols, L., Lygate, C., Tian, Y. M., Bernhardt, W., ... Pugh, C. W. (2017). HIF prolyl hydroxylases in the rat; organ distribution and changes in expression following hypoxia and coronary artery ligation. *Journal of Molecular and Cellular Cardiology*, 41(1), 68–77.

Chapter 4

Studies on Variants of *HsPHD2* and *HsHIF1 α* CODD

4.1 Introduction

The aim of the work described in this Chapter was to study the role of PHD2 variants in the development of disease. Mutations in the *EGLN1* gene have been associated with, among others, erythrocytosis, a disease state characterised by an augmented red cell mass (at least 125 % increased compared to healthy subjects, (1-2)). The symptoms of erythrocytosis include headaches, tiredness, and high blood pressure, with an increased risk of formation of blood clots (leading to heart attacks and strokes). There are many different causes of erythrocytosis, which are usually classified according to primary or secondary effects (2). Primary erythrocytosis is caused by increased production of red cells in the bone marrow due to an intrinsic bone marrow defect, with the EPO levels being below the normal range (2). By contrast, in secondary erythrocytosis, EPO levels are usually elevated, causing an increase in red cell production. Some types of secondary hereditary erythrocytosis are due to defects in hypoxic genes, leading to increased EPO expression in the patients (2-5). Mutations in the genes encoding PHD2 (*EGLN1*), pVHL, and HIF2 α (*EPAS1*), have been associated with secondary erythrocytosis, as they lead to HIF α stabilisation and, subsequently increased EPO levels (2-8). However, no mutations in the genes encoding for the other PHD and HIF isoforms have been identified in patients with erythrocytosis to date (2).

The first erythrocytosis related mutation in the gene encoding for pVHL was found in a study conducted on 103 individuals with familial erythrocytosis in the Chuvash region in Russia (3, 9). Genetic analysis revealed a homozygous mutation in the VHL gene, R200W, resulting in reduced affinity of the pVHL R200W variant for hydroxylated HIF α , and subsequently increased levels of EPO in the patients (3, 6, 10). In addition, the pVHL R200W variant was shown to have altered affinity for suppression of cytokine signaling 1 (SOCS1), hindering the formation of a heterodimeric pVHL-SOCS1 E3 ubiquitin ligase complex. As a consequence, the ubiquitin-mediated degradation of the EPO receptor (EPO-R) coupled kinase, phosphorylated JAK2 (pJAK2), is prevented (3, 11).

The identification of two distinct mechanisms that underlie the development of erythrocytosis in patients carrying the VHL R200W mutation, illustrates that regulation of erythropoiesis by pVHL occurs at different levels and is not restricted to regulation of EPO production (3). Apart from the VHL R200W mutation, the homozygous mutations P138L and H191D, and multiple heterozygous mutations in VHL have been identified in patients with erythrocytosis (3-4, 12-15).

Numerous erythrocytosis-associated mutations in the EPAS1 gene, encoding for HIF2 α , have been described (3-4, 16-17). Notably, all identified amino acid substitutions in HIF2 α occur close to the target prolyl residue in the CODD of HIF2 α (P531). It was shown that in the erythrocytosis-associated variant HIF2 α G537W, hydroxylation of P531 is impaired, thus leading to stabilisation of HIF2 α during normoxia (17).

Studies conducted with erythrocytosis patients described at least 23 different mutations in the EGLN1 gene, including missense, frameshift, and nonsense mutations (2, 7-8, 18-25). Notably, all identified germline mutations in the EGLN1 gene were found in a heterozygous state, suggesting that partial loss of PHD2 activity is sufficient to produce the erythrocytosis phenotype (2). Studies of EGLN1 mutations *in vitro* and in cells showed varying effects, ranging from mutations causing loss of function of PHD2, to mutations that only exhibit a mild effect on PHD2 activity (2, 21, 26). The first reported EGLN1 mutation, P317R, causes the single amino acid substitution of P317 to arginine in the vicinity of the active site of PHD2, and was found to diminish the activity of PHD2 on HIF1 α and HIF2 α (7, 26). By contrast, the PHD2 variant P200Q exhibits only very mild loss of activity (2, 21). Besides mutations that directly target the catalytic activity of PHD2, there are mutations described that interrupt the interaction between PHD2 and HIF α , in some cases selectively for either HIF1 α NODD or CODD (27). The erythrocytosis-associated PHD2 P317R variant is active on HIF1 α CODD, but does not hydroxylate HIF1 α NODD (7, 27), whereas a PHD2 R396T variant (present in breast carcinoma (28)), efficiently hydroxylates HIF1 α NODD, but is inactive towards HIF1 α CODD (27)). PHD2 binds a number of proteins in the cell, including the peptidyl prolyl cis/trans isomerase FKBP38, which plays a role in PHD2 stability (2, 29-32). Therefore, it is possible that EGLN1 mutations interrupt the interaction between PHD2 and its cellular binding partners, thus affecting the regulation of the HIF system.

Further, mutations in EGLN1 are shown to play a role in the adaptation of the human organism to high altitude. Studies on populations living at high altitude identified single-nucleotide polymorphisms in the EGLN1 gene (33-34). Some of these are located in the noncoding, regulatory regions of EGLN1 and have been associated with decreased haemoglobin levels (35). It is proposed that the decrease in haemoglobin levels observed in the Tibetan population constitutes a mechanism for protection from erythrocytosis as part of the hypoxic adaptation to life at high altitudes (35). Additionally, polymorphisms in the coding region of EGLN1 (D4E, C127S) were identified, and functional studies on the PHD2 D4E C127S variant showed that the substitutions slightly increased the activity of the enzyme (2, 36-37). Another study found that the interaction between PHD2 and p23, a HS90 cochaperone, is diminished in the PHD2 D4E C127S variant, resulting in an impaired PHD2-mediated down-regulation of the HIF pathway (38). Taken together, the genomic studies on populations living at high altitude suggest that multiple mechanisms are involved in hypoxic adaptation, including decreased haemoglobin levels, and up-regulation of the HIF pathway (39).

To further elucidate the role of clinically observed EGLN1 mutations in the development of disease, a selection of PHD2 variants was studied *in vitro*. The chosen PHD2 variants include those found in patients with clinical phenotype of erythrocytosis (PHD2 R312H, PHD2 F366L, Richard V. Wijk, University Medical Center Utrecht, Netherlands and Celeste Bento, Hospitais da Universidade de Coimbra, Portugal, unpublished data), and mutations selected from the Catalogue of Somatic Mutations in Cancer (COSMIC) database (PHD2 D277G, PHD2 G294S, and PHD2 W334S (28)).

The positions of the single amino acid substitutions caused by EGLN1 mutations are depicted in the crystal structure of PHD2 (**Figure 4.1**). The substitutions R312H and F366L were selected for the study due to their vicinity to the active site, whilst G294S and D277G were chosen because these two substitutions are situated in proximity to the substrate binding site. In addition, the W334S substitution was selected based on in-solution NMR data (Rasheduzzaman Chowdhury, University of Oxford, UK, unpublished data) that suggest a possible role of W334 in substrate binding by PHD2.

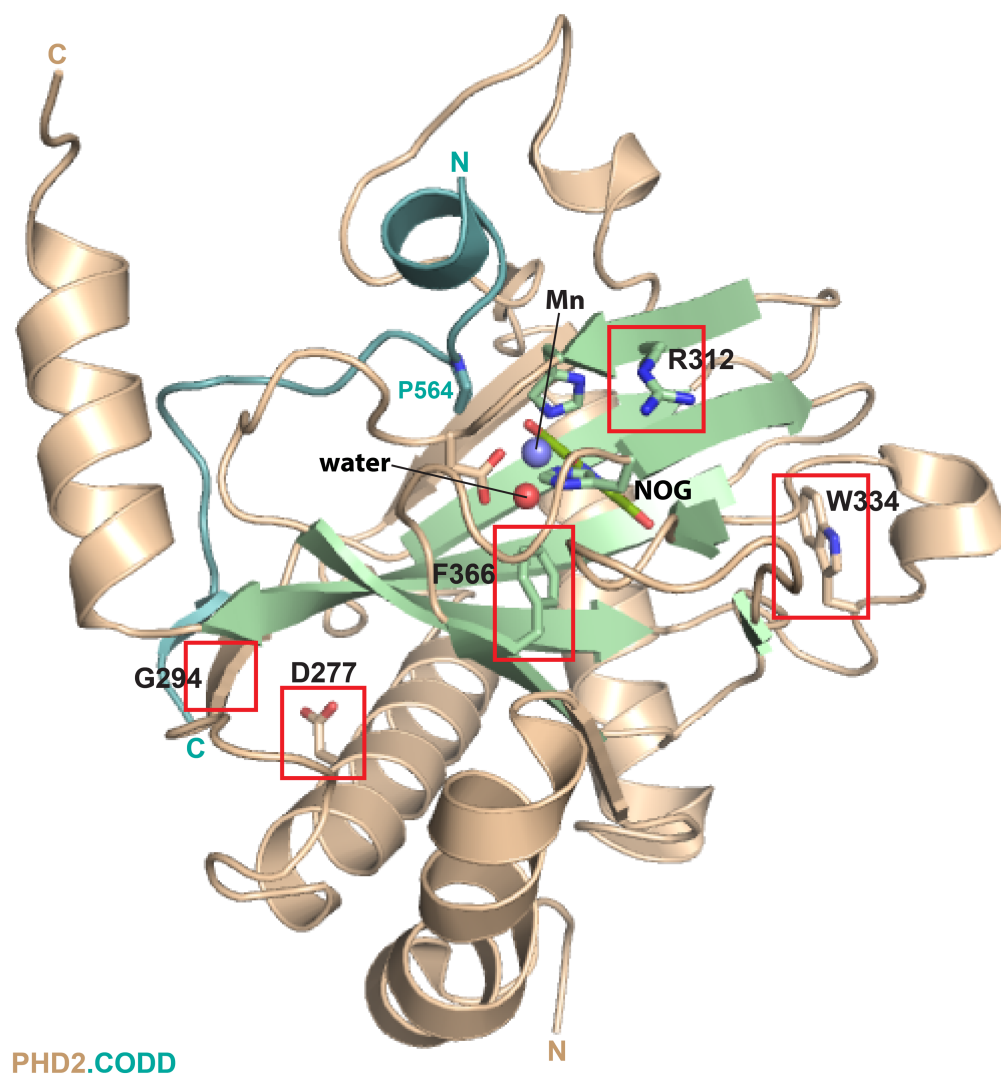


Figure 4.1: Positions of single amino acid substitutions caused by EGLN1 mutations, in the crystal structure of PHD2.CODD (PDB:3HQR, (40)). The substitutions chosen for functional studies include residues in the vicinity of the active site (F366, R312), residues at HIF binding sites (G294, D277), and a residue that potentially affects substrate-binding by PHD2 (W334S).

To investigate the mechanism by which these substitutions affect PHD2 and/or the HIF pathway, functional studies were carried out:

1. The activity of the PHD2 variants against different HIF α substrates was determined.
2. Structural effects of the substitutions were investigated by crystallising the PHD2 variants.
3. The thermal stabilities of the PHD2 variants was examined by circular dichroism (CD) spectroscopy.

Another part of this Chapter focuses on variants of HIF1 α CODD. The aim of the work is to investigate whether single amino acid residues in the sequence of HIF1 α CODD can be substituted by a alanine residue without reducing the turnover of HIF1 α CODD by PHD2. The results provide an insight into the role of different residues in HIF1 α CODD for binding to PHD2, and they help to improve the current understanding of the substrate preferences of PHD2 (and other PHDs).

4.2 Results and Discussion

4.2.1 Activity of PHD2 variant enzymes

Genes encoding for the *N*-terminally truncated tPHD2 variants were prepared by site-directed mutagenesis (*Materials and Methods*, **Chapter 7.1.1.4**). tPHD2 variants (residues 181-426) were produced in *E. coli* and purified by protein chromatography as described in *Materials and Methods*, **Chapter 7.2** and **7.3**, following the established protocol for production of wild-type tPHD2 (41). The identity of each tPHD2 variant was confirmed by ESI-LC-MS (**Figure 4.2**, *Materials and Methods*, **Chapter 7.6.2**).

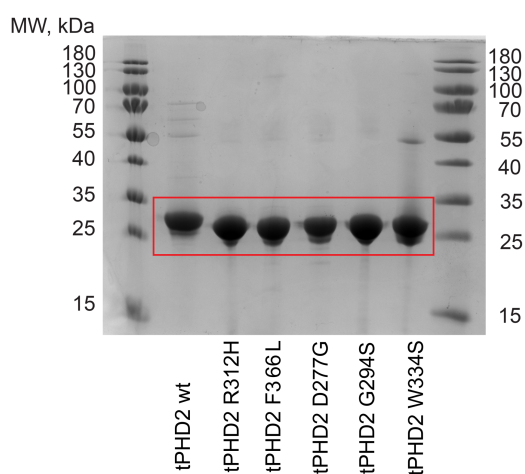
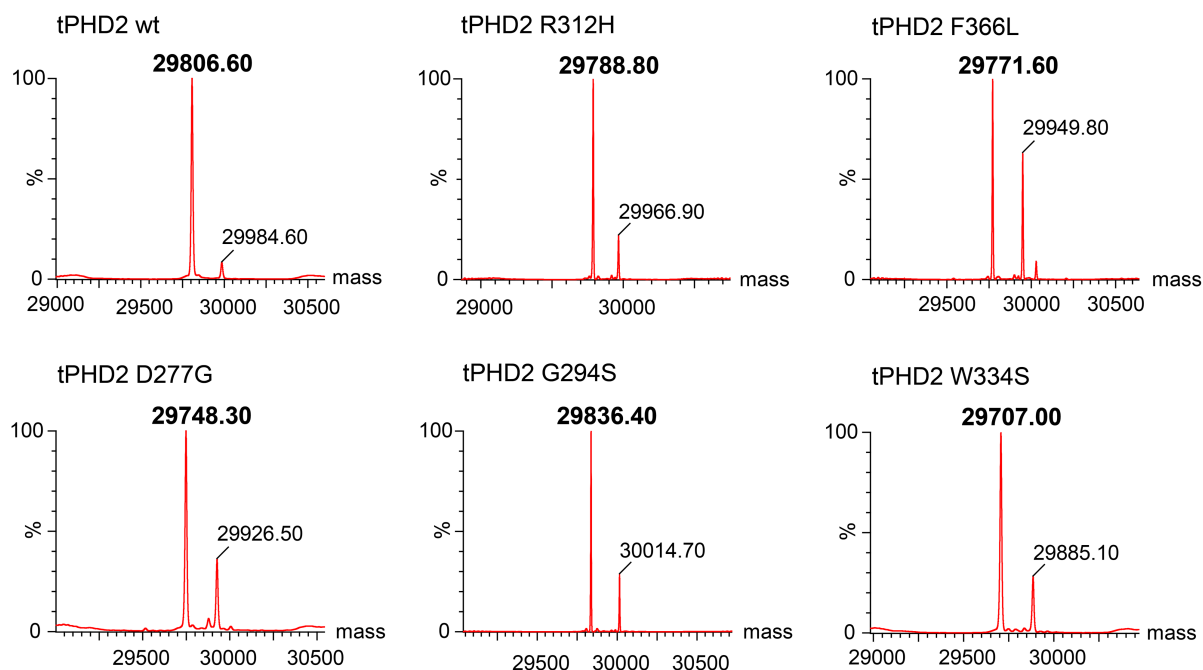
A**B**

Figure 4.2: Purification of tPHD2 variants. (A) SDS-PAGE gel and (B) deconvoluted protein ESI-LC-MS spectra. Affinity purification followed by size-exclusion chromatography yielded tPHD2 variants in approx. 95 % purity. The identity of the purified protein was verified by ESI-LC-MS (main peak in each spectrum, expected MW (g/mol): 29806 (tPHD2 wt), 29787 (tPHD2 R312H), 29772 (tPHD2 F366L), 29748 (tPHD2 D277G), 29836 (tPHD2 G294S), 29707 (tPHD2 W334S)). The second most intense peak observed in the ESI-LC-MS spectra (29984.60 in case of tPHD2 wt) is likely due to post-translational modification in *E.coli* or constitutes a MS artefact.

To assess the activity of the tPHD2 (subsequently referred to as 'PHD2') variants, hydroxylation of 19-20mer HIF α CODD/NODD peptides was studied using ESI-LC-MS based assays (see *Materials and Methods*, **Chapter 7.6.2**). An enzyme to substrate ratio of 1 : 12.5 was chosen, and the reaction was stopped after 10 min incubation at 25 °C.

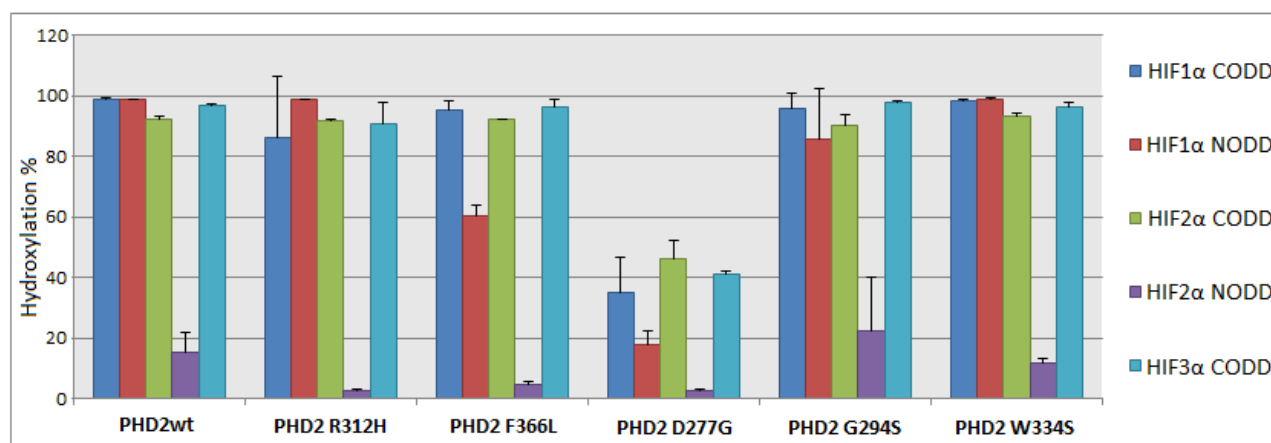


Figure 4.3: Activity of PHD2 variants on their HIF α substrates. Assay conditions: 4 μ M PHD enzyme, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 300 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate, 1 mM dithiothreitol, and 50 μ M 19-20 mer HIF α CODD/NODD-peptides in 50 mM Tris, pH 7.5. Reactions were incubated at 25 °C for 10 min and subsequently quenched with 1 % (v/v) formic acid. Hydroxylation levels were quantified using MassLynx™ V4.0. Non-enzymatic background oxidation was subtracted, and the data was normalised (errors are indicated as standard deviations, n=3).

It was found that all PHD2 variants efficiently catalyse the hydroxylation of HIF1-3 α CODD under the chosen reaction conditions, except for PHD2 D277G, that manifests a strongly reduced activity against its HIF α substrates (**Figure 4.3** and **Figure 4.9**, **Figure 4.10** (*Appendix*, ESI-LC-MS spectra). Amongst the NODD substrates, a clear preference of PHD2 wt and PHD2 variants for HIF1 α NODD over HIF2 α NODD was observed, as the latter is only turned over to 15-20 % under the chosen conditions, and was hardly turned over above background level by PHD2 variants R312H, F366L, and D277G. Whilst PHD2 R312H, PHD2 G294S, and PHD2 W334S hydroxylate HIF1 α NODD as efficiently as PHD2 wt, the two variants PHD2 F366L and PHD2 D277G manifest reduced activity on HIF1 α NODD (~60 % turnover of HIF1 α NODD by PHD2 F366L, ~18 % turnover by PHD2 D277G, **Figure 4.3**).

The results suggest that the tested substitutions in PHD2 differentially affect the catalytic activity of the enzyme variants. Whilst the PHD2 variants G294S and W334S exhibit no effect on the hydroxylation efficiency of PHD2 under the chosen conditions, the activity of PHD2 D277G is strongly reduced on all of its substrates (**Figure 4.3**). More subtle effects were observed for PHD2 F366L and (potentially) PHD2 R312H, as their efficiency towards HIF2 α NODD (and HIF1 α NODD in case of F366L) was decreased when compared to PHD2 wt. Previous studies showed that PHD2 prefers CODD substrates over NODD substrates, resulting in slower hydroxylation kinetics of NODD, compared to CODD (42-43). Therefore, under the chosen assay conditions, it is likely that subtle changes in the activity of PHD2 variants (as compared to PHD2 wt), become more apparent in NODD, than in CODD, hydroxylation levels.

4.2.2 Crystallographic analysis of PHD2 variants

To investigate potential effects of EGLN1 mutations on the structure of PHD2 and on substrate binding, crystallographic experiments were carried out. Previous crystallographic studies on the catalytic domain of PHD2 identified suitable conditions for crystallisation of tPHD2 wt (41). Assuming that the single substitutions in the PHD2 variants will not (strongly) affect crystallisation, the conditions found for tPHD2 wt were chosen as a starting point for crystallising the PHD2 variants.

For that purpose, the PHD2 variants were concentrated to 25 mg/mL (note that the variants were stable in different buffer conditions, as described in *Materials and Methods*, **Chapter 7.3.1**). The enzymes were co-crystallised with 1.5 mM MnCl₂ and 3 mM FG-2216/IOX3 (*Materials and Methods*, **Chapter 7.10.1**). The commercial screens JCSG-Plus (Molecular Dimensions) and Crystal Screen (Hampton Research) were used for crystallisation, as these screens have yielded well-diffracting crystals of tPHD2 wt before. After set up (*Materials and Methods*, **Chapter 7.10.1**), the crystal plates were incubated at 20 °C, and small crystals were usually observed after one day in various well conditions (**Table 4.1**). In order to allow for optimal crystal growth, the plates were usually incubated for 7 days, before large, rod-shaped crystals (**Figure 4.4**) were harvested for data collection (*Materials and Methods*, **Chapter 7.10.2** and **7.10.3**).

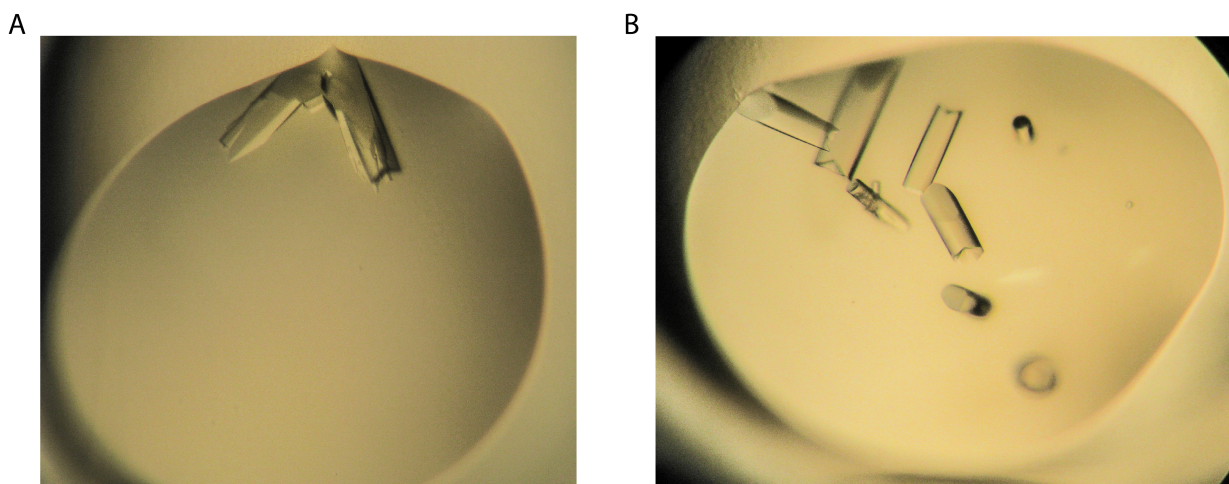


Figure 4.4: Examples of crystals obtained of PHD2 G294S with FG-2216/IOX3 and Mn(II). Conditions: **(A)**, Crystal screen, position D3 (2.0 M ammonium sulfate, 2 % (v/v) polyethylene glycol 400, 0.1 M HEPES sodium, pH 7.5), 1:2 ratio protein:well solution **(B)**, JCSG+ position G11 (2.0 M ammonium sulfate, 0.1 M Bis-Tris, pH 5.5), 1:2 ratio protein:well solution.

Table 4.1 Conditions for crystallisation of PHD2 variants.

PHD2 variant	Screen, position	Well solution	Ratio protein:well solution	Resolution, Å
R312H	Crystal screen, F2	0.2 M potassium sodium tartrate tetrahydrate 0.1 M sodium citrate tribasic dihydrate, pH 5.6 2.0 M ammonium sulfate	1:2	1.4
F366L	Crystal screen, D3	0.1 M HEPES sodium, pH 7.5 2 % (v/v) polyethylene glycol 400 2.0 M ammonium sulfate	1:2	1.6
G294S	Crystal screen, D3	0.1 M HEPES sodium, pH 7.5 2 % (v/v) polyethylene glycol 400 2.0 M ammonium sulfate	1:2	1.4
G294S	Crystal screen, C4	0.2 M sodium acetate trihydrate 0.1 M sodium cacodylate trihydrate, pH 6.5 30 % (w/v) polyethylene glycol 8000	1:2	1.6
W334S	Crystal screen, G9	0.1 M HEPES, pH 7.5 2.0 M ammonium formate	1:2	1.5

The crystals were measured at the Diamond Light Source (Oxfordshire, UK) and X-ray diffraction data up to a resolution of 1.4 - 1.6 Å was obtained. All crystals diffracted in the same space group, the primitive hexagonal space group P6₃. The data sets were processed and refined as described in *Materials and Methods*, **Chapter 7.10.3** (see **Table 4.2** for data collection and refinement statistics).

Table 4.2 Data collection and refinement statistics (refinement under progress).

	PHD2 R312H	PHD2 F366L	PHD2 D277G	PHD2 G294S	PHD2 W334S
Resolution range ($\text{\AA}$) *	55.50-1.42 (1.46-1.42)	56.17-1.56 (1.60-1.56)	48.64-1.39 (1.43-1.39)	95.98-1.57 (1.60-1.57)	55.45-1.51 (1.54-1.51)
Space group	P6 ₃	P6 ₃	P6 ₃	P6 ₃	P6 ₃
Unit cell (a b c, α β γ)	111 111 39.34, 90 90 120	112.35 112.35 40.59, 90 90 120	112.33 112.33 40.58, 90 90 120	110.83 110.83 39.96, 90 90 120	110.90 110.90 40.03, 90 90 120
Total reflections	339415	559582	778396	195012	429229
Unique reflections	52560	41942	59125	39471	44457
Multiplicity *	6.5 (6.1)	13.3 (12.5)	13.2 (13.2)	4.9 (5.0)	9.7 (9.4)
Completeness (%) *	100 (100)	99.9 (99.9)	100 (100)	100 (99.5)	100 (99.1)
Mean $I/\sigma(I)$ *	19.4 (1.4)	15.1 (1.4)	14.0 (1.2)	14.2 (1.4)	15.8 (1.0)
CC(1/2) *	1.0 (0.6)	1.0 (0.6)	1.0 (0.6)	1.0 (0.5)	1.0 (0.6)
R_{work}	0.1701	0.1804	0.1613	0.1655	0.1768
R_{free}	0.1890	0.2050	0.1736	0.1867	0.1977
RMS (bonds)	0.017	0.007	0.019	0.009	0.016
RMS (angles)	1,557	0.963	1.516	1,078	1,424
Ramachandran favoured (%)	97.70	99.08	97.70	98.17	96.71
Ramachandran allowed (%)	2.30	0.92	2.30	1.83	2.78
Ramachandran outliers (%)	0.0	0.0	0.0	0.0	0.47
Rotamer outliers (%)	1.10	1.14	0.0	0.55	1.09
Clashscore	5.71	3.15	2.27	4.35	9.16
Average B-factor ($\text{\AA}^2$)	36.53	34.64	35.57	38.25	39.10
Macromolecules ($\text{\AA}^2$)	35.57	33.36	32.71	37.24	38.64
Ligands ($\text{\AA}^2$)	52.96	57.65	56.00	19.13	20.98

* values for outer shell in parenthesis.

The PHD2 variants R312H, F366L, D277G, G294S, and W334S all co-crystallised with the inhibitor FG-2216/IOX3, the electron density of which is clearly visible in the PHD2 variant structures (**Figure 4.5** and **4.6**). The structures of the PHD2 variants all reveal a similar overall fold to wild-type PHD2, that is the DSBH core and three helices (helices α 1-3) that stabilise the β -sheet core (overall structures not shown).

Investigating the electron density of the substituted residues in each of the PHD2 variant structures clearly shows that the desired residues are present in the structures ($2F_o - F_c$ density map in cyan, **Figure 4.5** and **4.6**). In case of the PHD2 D277G structure, in addition to the density for glycine (G277), density for the aspartate carboxyl is observed (**Figure 4.5**, first row). This is (likely) due to model bias, as the structure of PHD2 D277G was solved using model-based phases (of the wild-type PHD2 structure, PDB: 4BQX, (44)).

The structure of PHD2 D277G suggests that this variant probably manifests altered substrate binding properties compared to the substrate binding modes observed in the PHD2.CODD and PHD2.NODD structures (**Figure 4.5**, first row). In the PHD2.CODD/NODD structures, the sidechain of D277 is facing in direction of the bound substrate. By contrast, in the PHD2 D277G structure, this residue is substituted by a glycine residue, and changes in the sidechain conformations of the adjacent residues R281 and D278 in helix α 3 are observed. In the PHD2.CODD structure, R281 is involved in substrate binding, by forming hydrogen bonds to the CODD residue L574 (note that this residue is substituted by cysteine in the PHD2.NODD structure for cross-linking purposes). However, in the PHD2 D277G structure, two alternative conformations of R281 are visible, one of which is facing away from the substrate (**Figure 4.5**, first row). It is possible that the substitution of D277 to glycine reduces the interaction between the substrate and PHD2 residues in helix α 3. This finding is in accordance with the low activity of PHD2 D277G on HIF α CODD/NODD substrates as observed in time-point assays (**Figure 4.3**).

In the PHD2 G294S structure, the substituted residue S294 is positioned in proximity to where CODD/NODD are bound in the PHD2.CODD/NODD substrate structures (**Figure 4.5**, second row). However, it appears that the substitution of G294 by serine does not induce substantial changes in the structure of PHD2. Notably, PHD2 G294S does not manifest any reduction in its enzymatic activity as determined in time-point assays (**Figure 4.3**). It is therefore likely that the substitution does not directly affect substrate binding or turnover (at least not to an extent that can be observed *in vitro* under the used assay conditions).

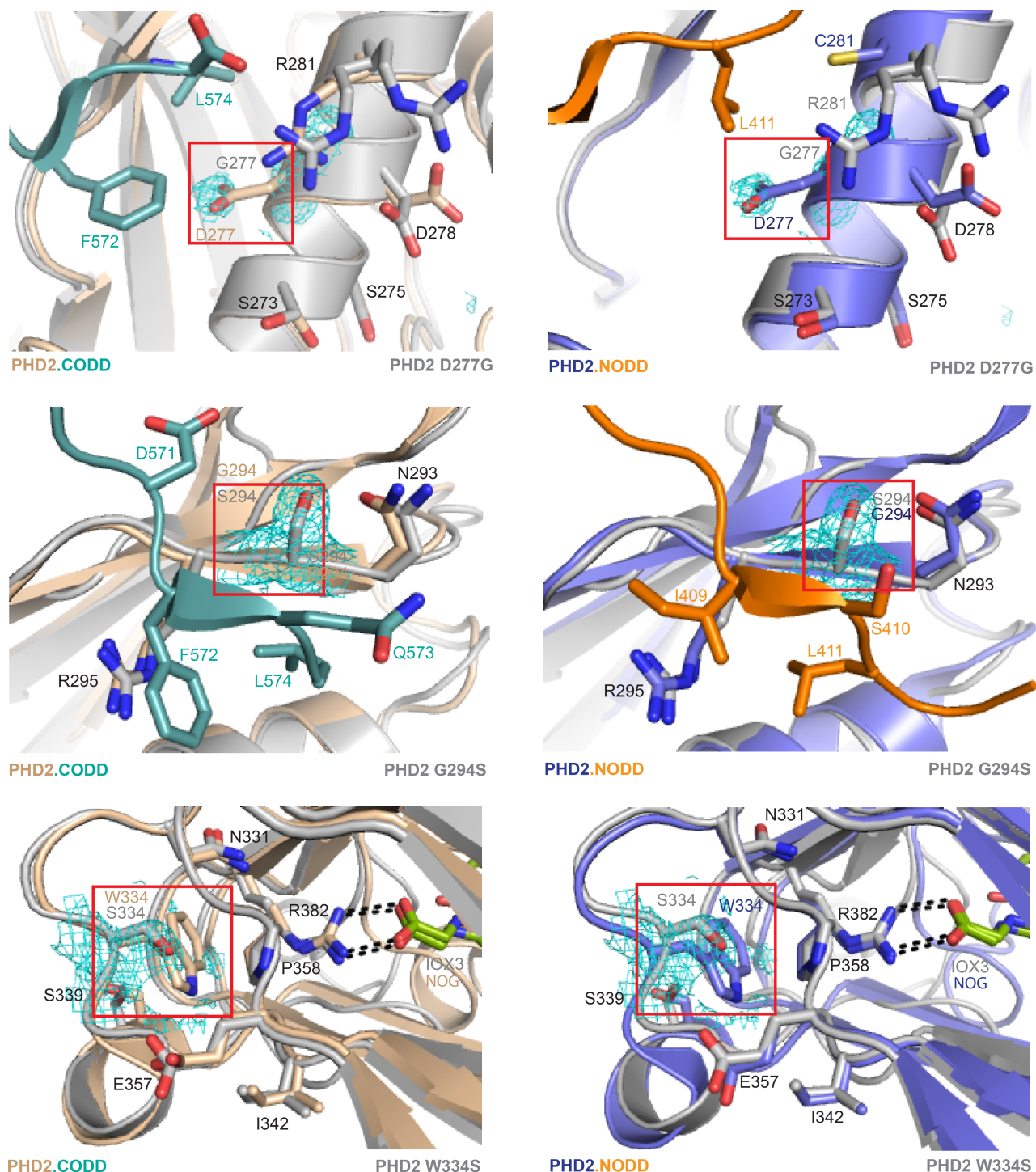


Figure 4.5: Views from the clinically observed PHD2 variant crystal structures, PHD2 D277G, G294S, and W334S, superimposed with the wild-type PHD2.CODD (PDB: 3HQ R, (40)) and PHD2.NODD (PDB: 5L9V, (27)) complex structures. Electron density maps of the mutated residues are displayed in cyan ($2F_o - F_c$, contoured to 1σ).

In a similar manner, when comparing the PHD2.CODD/NODD and PHD2 W334 structures, no effect of the W334S substitution on the structure of PHD2 is observed (**Figure 4.5**, third row). This includes residue R382, the crucial 2OG binding residue, which is located close to the mutated site, and which forms hydrogen bonds to the 2OG substitute FG-2216/IOX3 in the PHD2 W334S structure. Again, the crystallographic findings are in accordance with the observed unaltered activity of PHD2 W334S on its HIF α substrates, compared to wild-type PHD2 (**Figure 4.3**).

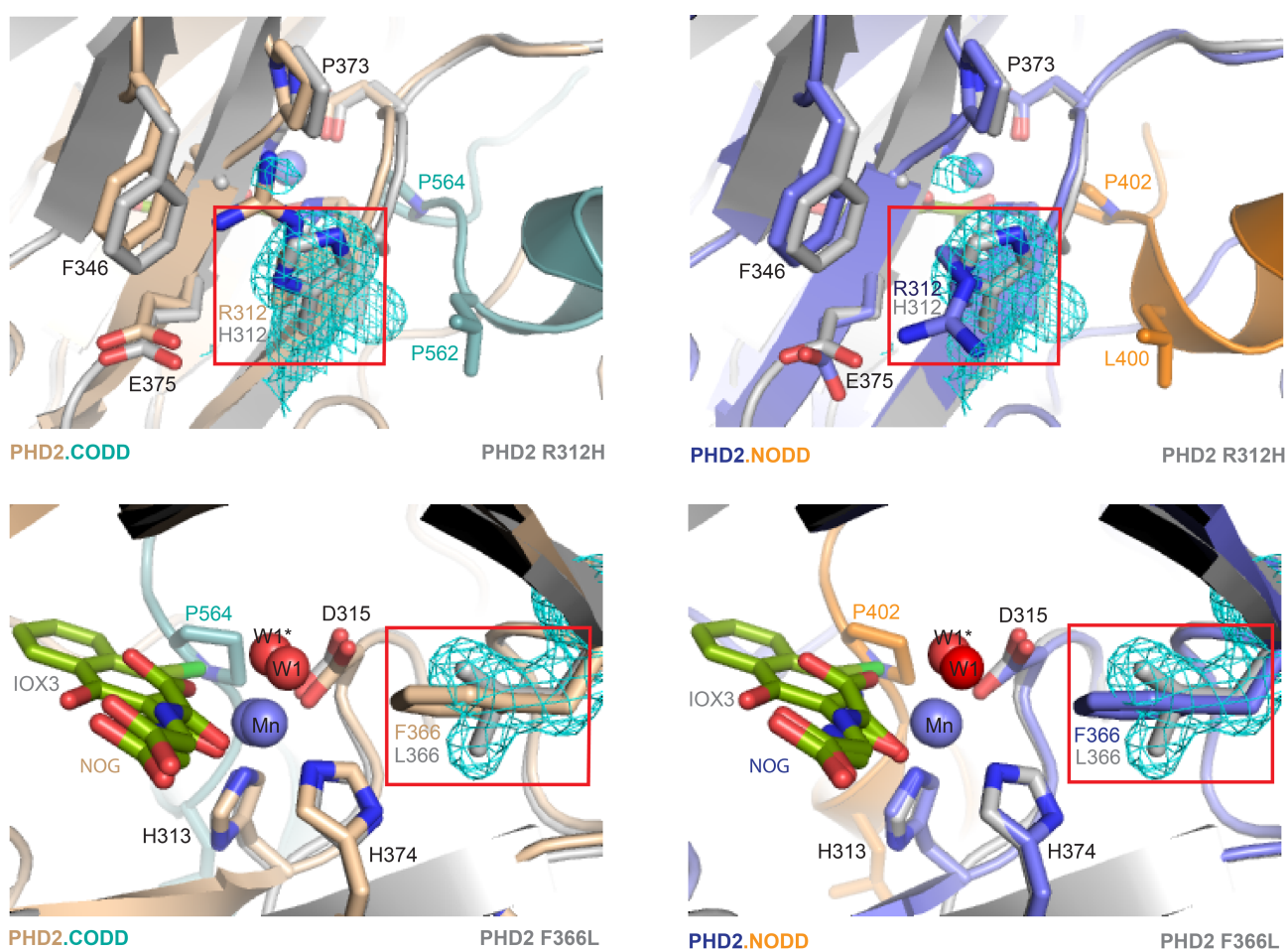


Figure 4.6: Views from the PHD2 R312H and F366L crystal structures, superimposed with the wild-type PHD2.CODD (PDB: 3HQR, (40)) and PHD2.NODD (PDB: 5L9V, (27)) complex structures. Electron density maps of the mutated residues are displayed in cyan ($2F_o - F_c$, contoured to 1σ); W1 corresponds to the metal-coordinated water in the PHD2.CODD/NODD structures, W1* corresponds to the respective water in the PHD2 F366L structure.

Comparison of the crystal structures obtained for erythrocytosis-associated PHD2 variants R312H and F3366L with the PHD2.CODD/NODD substrate complex structures, reveals no major structural changes in PHD2 upon introduction of the respective substitutions (**Figure 4.6**). Both substitutions are located in the proximity of the active site, with R312H additionally being positioned close to the substrate binding site (**Figure 4.6**, first row). Given that F366L is positioned next to the metal-coordinated water molecule that is proposed to play a role in the observed slow reaction of Fe(II) with oxygen (40-41, 45), it was hypothesised that the substitution might alter the water and oxygen binding properties of PHD2. In fact, the water molecule in the PHD2 F366L structure (W1*) is located 0.8 Å and 0.6 Å apart from the respective water molecules (W1) in the PHD2.CODD and PHD2.NODD complex structures (**Figure 4.6**, second row); for comparison, the distance between W1 in the PHD2.CODD structure and W1 in the PHD2.NODD structure is 0.2 Å. It is possible that the small shift in the position of the metal-coordinated water molecule is related to the slight decrease in activity as observed in the time-point assays, yet further studies (i.e. kinetic studies on the oxygen dependence of the reaction catalysed by PHD2 F366L) are necessary to clarify if this is the case.

4.2.3 Thermal stability of PHD2 variants

To investigate if the substitutions in the PHD2 variants R312H, F366L, D277G, G294S, and W334S affect the overall stability of the enzyme, circular dichroism (CD) spectroscopy was performed. First, a CD spectrum of wild-type PHD2 was obtained (**Figure 4.7 A**) to check the experimental set-up and to find a suitable wavelength for further experiments. The thermal stability of wild-type PHD2 and its variants was then studied using a temperature gradient (*Materials and Methods, Chapter 7.9*).

The following melting temperatures were obtained: T_m (PHD2 wt) = 49.5 ± 0.2 °C, T_m (PHD2 R312H) = 50.0 ± 0.2 °C, T_m (PHD2 F366L) = 47.2 ± 0.3 °C, T_m (PHD2 D277G) = 49.4 ± 0.1 °C, T_m (PHD2 G294S) = 47.2 ± 0.3 °C, and T_m (PHD2 W334S) = 51.6 ± 0.1 °C (**Figure 4.7 B**). The data suggest that two substitutions, F366L and G294S, manifest a modestly destabilising effect on PHD2, whereas for the other substitutions no substantial change in enzyme stability was observed. It is possible that the decrease in enzyme stability in case of PHD2 F366L and G294S plays a role in the development of clinical phenotypes as observed for patients who carry these mutations.

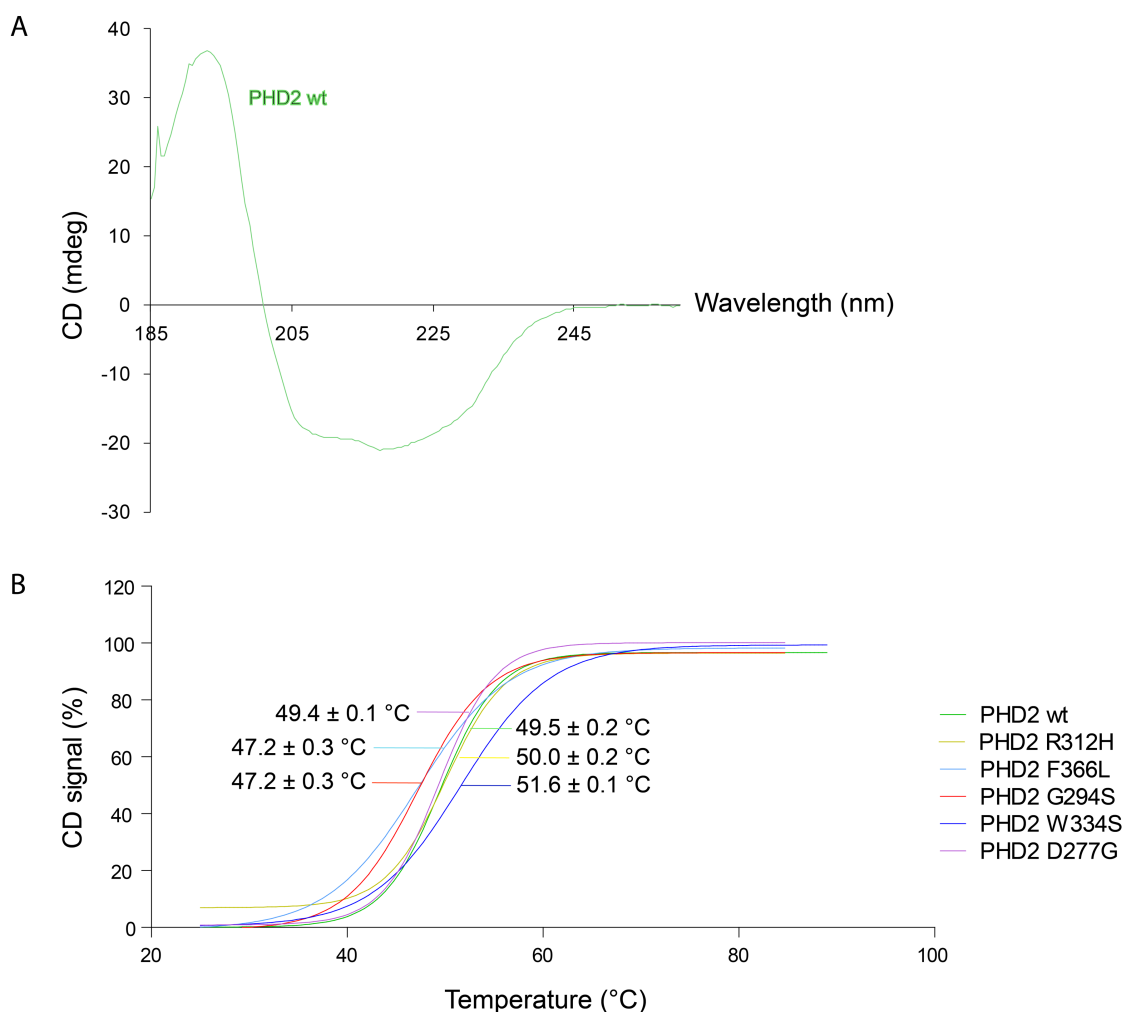


Figure 4.7: Thermal stability of wild-type PHD2 and its variants as determined by CD spectroscopy. (A) CD spectrum of wild-type PHD2 at a concentration of 0.3 mg/mL in 10 mM sodium phosphate, pH 7.5. Data from three independent scans was averaged and smoothed. **(B)** Thermal stability of wild-type PHD2 and PHD2 variants was determined by measuring the ellipticity at 215 nm (θ_{215}) over a temperature gradient (25 to 90 °C). Data from three independent measurements was averaged and normalised (errors represent standard errors, n=3).

4.2.4 Activity of PHD2 on HIF1 α CODD variants

To investigate the role of different residues in HIF1 α CODD for binding to PHD2 and subsequent PHD2-catalysed hydroxylation, HIF1 α CODD peptides containing single (or triple) alanine substitutions were tested for turnover by PHD2 in a MALDI-TOF-MS based hydroxylation assay (for assay conditions, see *Materials and Methods*, **Chapter 7.6.1**). The results of the assay are shown in **Figure 4.8** (peptide sequences in *Appendix*, **Chapter 4.4**).

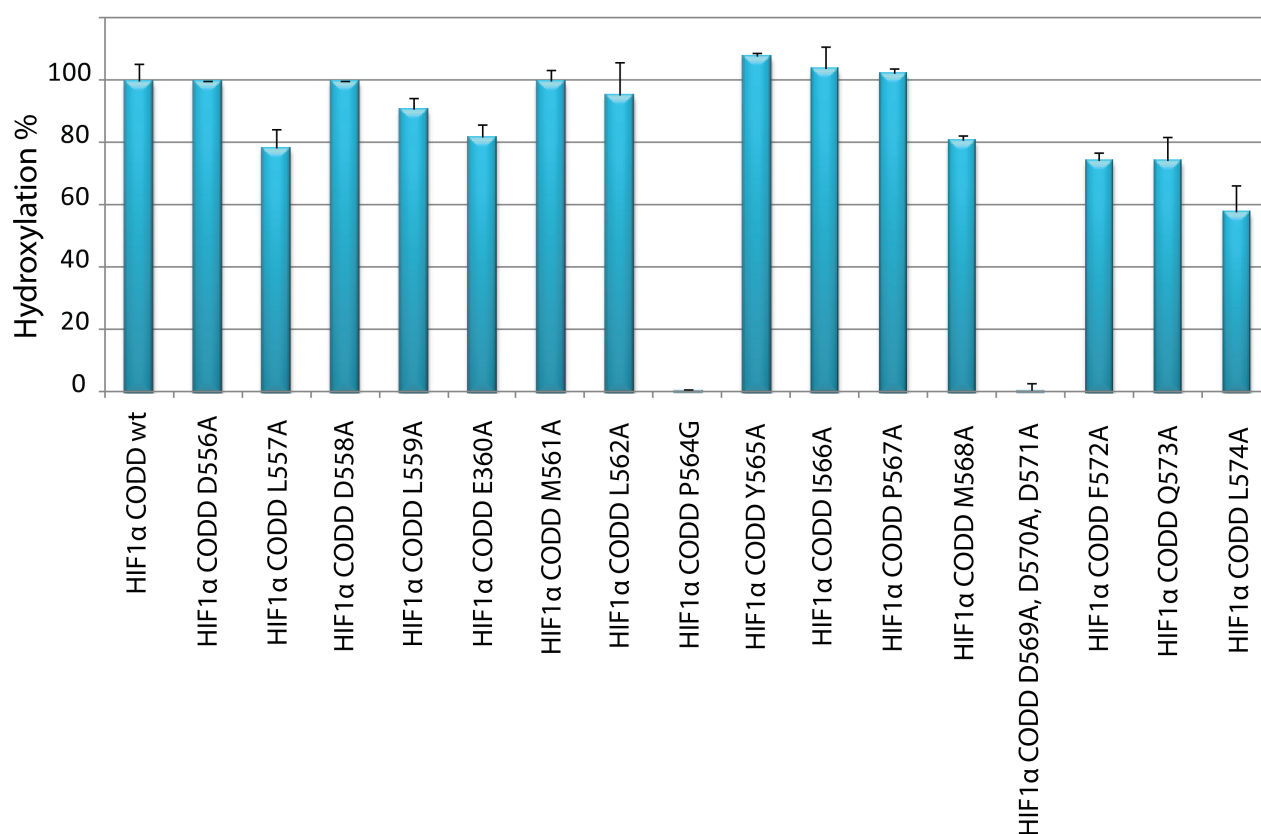


Figure 4.8: Hydroxylation of HIF1 α CODD variant peptides by PHD2. Conditions: 4 μ M PHD2, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 60 μ M 2-oxoglutarate disodium salt, 300 μ M sodium L-ascorbate and 50 μ M HIF1 α CODD variant peptides, in 50 mM Tris, pH 7.5. The samples were incubated at 25 °C, quenched with 1 % (v/v), and subsequently analysed by MALDI-TOF-MS. Hydroxylation levels were determined using MassLynx™ V4.1, non-enzymatic background oxidation was subtracted, and data were normalised. The data represents the average of three independent measurements and errors are depicted as standard deviations (n=3).

PHD2 is active on all of the tested HIF1 α CODD variants, except for HIF1 α CODD P564G and HIF1 α CODD D569A, D570A, D571A. As expected, no hydroxylation was observed when the target proline residue P564 was substituted by glycine, indicating a strong selectivity of PHD2 for prolyl residues. Substituting the three consecutive aspartate residues D569-D571 with alanine completely abolished hydroxylation of the HIF1 α CODD variant by PHD2. Notably, single alanine substitutions at the C-terminus of HIF1 α CODD (residues F572-L574) decreased the hydroxylation levels by >20 % (>40 % in case of HIF1 α CODD L574A). Taken together, the results suggest that PHD2 accepts single alanine substituted HIF1 α CODD variants as substrates (although in some cases with decreased activity towards the variants when compared to HIF1 α CODD wt). This is in agreement with presence of five HIF1-3 α CODD/NODD substrates in humans, all of which differ in their sequences.

4.3 Conclusions

In previous studies, germline mutations in the *EGLN1* gene encoding for PHD2 were observed in patients with erythrocytosis. Functional analyses of erythrocytosis-associated PHD2 variants revealed differential effects of single amino acid substitutions on PHD2, ranging from mildly decreased catalytic activity as observed for the PHD2 P200Q variant (2, 21), to diminished activity as is the case for PHD2 P317R (7, 26). Notably, some PHD2 variants exhibit HIF1 α NODD- or HIF1 α CODD-selective hydroxylation profiles (27). However, in many cases the mechanism by which erythrocytosis-associated *EGLN1* mutations affect the HIF pathway and/or cause the observed disease phenotypes is not well understood. Therefore, it was of interest to further elucidate the effect of clinically-observed *EGLN1* mutations and the resultant PHD2 variants in structural and functional studies.

The PHD2 R312H and F366L variants that were observed in patients with erythrocytosis (Richard V. Wijk, University Medical Center Utrecht, Netherlands and Celeste Bento, Hospitais da Universidade de Coimbra, Portugal, unpublished data), both retained full activity on CODD substrates in the time-point assay when compared to wild-type PHD2, yet the activity of the two variants towards HIF2 α NODD (and HIF1 α NODD in case of PHD2 F366) was reduced (**Figure 4.3**). The finding of a mildly reduced activity of the PHD2 variants, caused by the R312H and F366L substitutions, might (partially) explain the observed phenotype in erythrocytosis patients, however, it is difficult to predict if the subtle decrease of HIF2 α NODD hydroxylation sufficiently up-regulates HIF2 α in patients to cause erythrocytosis. Notably, PHD2 F366L exhibited reduced thermal stability as determined by CD spectroscopy (T_m (PHD2 F366L) = 47.2 ± 0.3 °C vs. T_m (PHD2 wt) = 49.5 ± 0.2 °C, **Figure 4.7 B**), a property that might possibly contribute to the development of erythrocytosis. The crystal structure of PHD2 F366L revealed a slight shift of the metal-coordinated water molecule (**Figure 4.6**, 0.8 Å and 0.6 Å shift respective to the water molecules in the PHD2.CODD and PHD2.NODD complex structures). However, further studies are necessary to establish if and how the positioning of the metal-bound water molecule affects the overall activity and kinetics of PHD2.

A number of *EGLN1* mutations associated with tumour development have been described (2, 46). In a patient with erythrocytosis and recurrent paraganglioma, mutation of H374 in PHD2 to arginine was found (46). However, the exact mechanism of paraganglioma development and the (potential) role of PHD2 in the oncogenic process remains to be elucidated (2).

By contrast, a PHD2 R396T variant present in breast carcinoma exhibits altered substrate selectivity, implying a potential functional role of the mutation in up-regulation of the HIF pathway and tumour development (27-28). In this context, it was of interest to conduct further functional and structural studies on cancer-associated mutations in the coding regions of EGLN1.

Activity analyses of the three chosen cancer-associated PHD2 variants (COSMIC data base, (28)), revealed that HIF α hydroxylation rates were unaffected by the substitutions in PHD2 G294S and PHD2 W334S variants when compared to wild-type PHD2 (**Figure 4.3**). However, the PHD2 D277G variant exhibited strongly reduced activity against all HIF α substrates. Additionally, the crystal structure of PHD2 D277G revealed changes in the sidechain conformations of residues R281 and D278 in helix α 3 relative to the wild-type PHD2.CODD/NODD complex structures (**Figure 4.5**). Judging from the position of the helix α 3 close to the bound substrate, it is possible that the observed changes in the PHD2 D277G structure affect substrate binding by the enzyme. Taken together, the finding on PHD2 D277G suggest that the substitution causes functional changes in PHD2, which might lead to up-regulation of the HIF-pathway in patients carrying the mutation. Since the D277G mutation was found in patients with biliary tract cancer (28), further studies on a potential role of the PHD2 D277G variant in tumour development might be of interest. Although no reduction in the activity of the PHD2 G294S variant relative to wild-type PHD2 was detected, it was found that the G294S substitution decreased the thermal stability of the PHD2 variant (T_m (PHD2 G294S) = 47.2 ± 0.3 °C vs. (PHD2 wt) = 49.5 ± 0.2 °C, **Figure 4.7 B**). In case of the PHD2 W334S variant, no functional effect of the substitution on PHD2 was observed. However, it cannot be excluded that the W334S substitution affects the activity and/or stability of PHD2 *via* other mechanisms, e.g. by interrupting interactions between PHD2 and cellular binding partners.

Time-point assays conducted with wild-type PHD2 and single or triple alanine mutated HIF1 α CODD variants were performed (**Figure 4.8**). The results of the study suggest that PHD2 accepts single alanine substituted HIF1 α CODD variants as substrates, yet in some cases a decrease in activity towards the variants when compared to wild-type HIF1 α CODD were observed. Single alanine substitutions at the C-terminus of HIF1 α CODD tended to affect hydroxylation rates more strongly than single alanine substitutions at the N-terminus. The study therefore provides insights into the substrate selectivity profile of PHD2 and the role of different residues within HIF1 α CODD for efficient binding to PHD2.

4.4 References

- (1) Pearson, T. C., Guthrie, D. L., Simpson, J., Chinn, S., Barosi, G., Ferrant, A., ... Najean, Y. (1995). Interpretation of measured red cell mass and plasma volume in adults: Expert Panel on Radionuclides of the International Council for Standardization in Haematology. *British Journal of Haematology*, 89(4), 748–756.
- (2) Gardie, B., Percy, M. J., Hoogewijs, D., Chowdhury, R., Bento, C., Arsenault, P. R., ... Lee, F. S. (2014). The role of PHD2 mutations in the pathogenesis of erythrocytosis. *Hypoxia*, 2, 71–90.
- (3) Franke, K., Gassmann, M., & Wielockx, B. (2013). Erythrocytosis: the HIF pathway in control. *Blood*, 122(7), 1122–1128.
- (4) Lee, F. S., & Percy, M. J. (2011). The HIF Pathway and Erythrocytosis. *Annual Review of Pathology: Mechanisms of Disease*, 6(1), 165–192.
- (5) Semenza, G. L. (2009). Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. *Blood*, 114(10), 2015–2019.
- (6) Ang, S. O., Chen, H., Gordeuk, V. R., Sergueeva, A. I., Polyakova, L. A., Miasnikova, G. Y., ... Prchal, J. T. (2002). Endemic Polycythemia in Russia: Mutation in the VHL Gene. *Blood Cells, Molecules, and Diseases*, 28(1), 57–62.
- (7) Percy, M. J., Zhao, Q., Flores, A., Harrison, C., Lappin, T. R. J., Maxwell, P. H., ... Lee, F. S. (2006). A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(3), 654–659.
- (8) Bento, C., Percy, M. J., Gardie, B., Maia, T. M., van Wijk, R., Perrotta, S., ... Ugo, V. (2014). Genetic Basis of Congenital Erythrocytosis: Mutation Update and Online Databases. *Human Mutation*, 35(1), 15–26.
- (9) Sergeyeva, A., Gordeuk, V. R., Tokarev, Y. N., Sokol, L., Prchal, J. F., & Prchal, J. T. (1997). Congenital Polycythemia in Chuvashia. *Blood*, 89(6), 2148–2154.
- (10) Ang, S. O., Chen, H., Hirota, K., Gordeuk, V. R., Jelinek, J., Guan, Y., ... Prchal, J. T. (2002). Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nature Genetics*, 32(4), 614–621.
- (11) Russell, R. C., Sufan, R. I., Zhou, B., Heir, P., Bunda, S., Sybingco, S. S., ... Ohh, M. (2011). LOSS OF JAK2 REGULATION VIA VHL-SOCS1 E3 UBIQUITIN HETEROCOMPLEX UNDERLIES CHUVASH POLYCYTHEMIA. *Nature Medicine*, 17(7), 845–853.

- (12) Bond, J., Gale, D. P., Connor, T., Adams, S., de Boer, J., Gascoyne, D. M., ... Ancliff, P. J. (2011). Dysregulation of the HIF pathway due to VHL mutation causing severe erythrocytosis and pulmonary arterial hypertension. *Blood*, 117(13), 3699-3701.
- (13) Lanikova, L., Lorenzo, F., Yang, C., Vankayalapati, H., Drachtman, R., Divoky, V., & Prchal, J. T. (2013). Novel homozygous VHL mutation in exon 2 is associated with congenital polycythemia but not with cancer. *Blood*, 121(19), 3918-3924.
- (14) Tomasic, N. L., Piterkova, L., Huff, C., Bilic, E., Yoon, D., Miasnikova, G. Y., ... Prchal, J. T. (2013). The phenotype of polycythemia due to Croatian homozygous VHL H191D mutation is different from that of Chuvash polycythemia VHL R200W. *Haematologica*, 98(4), 560-567.
- (15) Pastore, Y., Jedlickova, K., Guan, Y., Liu, E., Fahner, J., Hasle, H., ... Prchal, J. T. (2003). Mutations of von Hippel-Lindau Tumor-Suppressor Gene and Congenital Polycythemia. *The American Journal of Human Genetics*, 73(2), 412-419.
- (16) Percy, M. J., Furlow, P. W., Lucas, G. S., Li, X., Lappin, T. R. J., McMullin, M. F., & Lee, F. S. (2008). A Gain-of-Function Mutation in the HIF2A Gene in Familial Erythrocytosis. *New England Journal of Medicine*, 358(2), 162-168.
- (17) Percy, M. J., Chung, Y. J., Harrison, C., Mercieca, J., Hoffbrand, A. V., Dinardo, C. L., ... Lee, F. S. (2012). Two new mutations in the HIF2A gene associated with erythrocytosis(). *American Journal of Hematology*, 87(4), 439-442.
- (18) Albiero, E., Ruggeri, M., Fortuna, S., Finotto, S., Bernardi, M., Madeo, D., & Rodeghiero, F. (2012). Isolated erythrocytosis: study of 67 patients and identification of three novel germ-line mutations in the prolyl hydroxylase domain protein 2 (PHD2) gene. *Haematologica*, 97(1), 123-127.
- (19) Bento, C., Almeida, H., Maia, T. M., Relvas, L., Oliveira, A. C., Rossi, C., ... Ribeiro, M. L. (2013). Molecular study of congenital erythrocytosis in 70 unrelated patients revealed a potential causal mutation in less than half of the cases (Where is/are the missing gene(s)?). *European Journal of Haematology*, 91(4), 361-368.
- (20) Ladroue, C., Carcenac, R., Leporrier, M., Gad, S., Le Hello, C., Galateau-Salle, F., ... Gardie, B. (2008). PHD2 Mutation and Congenital Erythrocytosis with Paraganglioma. *New England Journal of Medicine*, 359(25), 2685-2692.
- (21) Ladroue, C., Hoogewijs, D., Gad, S., Carcenac, R., Storti, F., Barrois, M., ... Gardie, B. (2012). Distinct deregulation of the hypoxia inducible factor by PHD2 mutants identified in germline DNA of patients with polycythemia. *Haematologica*, 97(1), 9-14.
- (22) Percy, M. J., Furlow, P. W., Beer, P. A., Lappin, T. R. J., McMullin, M. F., & Lee, F. S. (2007). A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. *Blood*, 110(6), 2193-2196.

- (23) Al-Sheikh, M., Moradkhani, K., Lopez, M., Wajcman, H., & Pr  hu, C. (2008). Disturbance in the HIF-1 α pathway associated with erythrocytosis: Further evidences brought by frameshift and nonsense mutations in the prolyl hydroxylase domain protein 2 (PHD2) gene. *Blood Cells, Molecules, and Diseases*, 40(2), 160–165.
- (24) Jang, J.-H., Seo, J. Y., Jang, J., Jung, C. W., Lee, K.-O., Kim, S.-H., & Kim, H.-J. (2014). Hereditary gene mutations in Korean patients with isolated erythrocytosis. *Annals of Hematology*, 93(6), 931–935.
- (25) Albiero, E., Ruggeri, M., Fortuna, S., Bernardi, M., Finotto, S., Madeo, D., & Rodeghiero, F. (2011). Analysis of the oxygen sensing pathway genes in familial chronic myeloproliferative neoplasms and identification of a novel EGLN1 germ-line mutation. *British Journal of Haematology*, 153(3), 405–408.
- (26) Pappalardi, M. B., Martin, J. D., Jiang, Y., Burns, M. C., Zhao, H., Ho, T., ... Luo, L. (2008). Biochemical Characterization of Human Prolyl Hydroxylase Domain Protein 2 Variants Associated with Erythrocytosis. *Biochemistry*, 47(43), 11165–11167.
- (27) Chowdhury, R., Leung, I. K. H., Tian, Y.-M., Abboud, M. I., Ge, W., Domene, C., ... Schofield, C. J. (2016). Structural basis for oxygen degradation domain selectivity of the HIF prolyl hydroxylases. *Nature Communications*, 7, 12673.
- (28) Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., ... Wooster, R. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *British Journal of Cancer*, 91, 355 – 358.
- (29) Huo, Z., Ye, J.-C., Chen, J., Lin, X., Zhou, Z.-N., Xu, X.-R., ... Li, J. (2012). Prolyl hydroxylase domain protein 2 regulates the intracellular cyclic AMP level in cardiomyocytes through its interaction with phosphodiesterase 4D. *Biochemical and Biophysical Research Communications*, 427(1), 73–79.
- (30) Song, D., Li, L.-S., Heaton-Johnson, K. J., Arsenault, P. R., Master, S. R., & Lee, F. S. (2013). Prolyl Hydroxylase Domain Protein 2 (PHD2) Binds a Pro-Xaa-Leu-Glu Motif, Linking It to the Heat Shock Protein 90 Pathway. *The Journal of Biological Chemistry*, 288(14), 9662–9674.
- (31) Vogel, S., Wottawa, M., Farhat, K., Zieseniss, A., Schnelle, M., Le-Huu, S., ... Katschinski, D. M. (2010). Prolyl Hydroxylase Domain (PHD) 2 Affects Cell Migration and F-actin Formation via RhoA/Rho-associated Kinase-dependent Cofilin Phosphorylation. *The Journal of Biological Chemistry*, 285(44), 33756–33763.
- (32) Barth, S., Nesper, J., Hasgall, P. A., Wirthner, R., Nytko, K. J., Edlich, F., ... Camenisch, G. (2007). The Peptidyl Prolyl cis/trans Isomerase FKBP38 Determines Hypoxia-Inducible Transcription Factor Prolyl-4-Hydroxylase PHD2 Protein Stability. *Molecular and Cellular Biology*, 27(10), 3758–3768.
- (33) Yi, X., Liang, Y., Huerta-Sanchez, E., Jin, X., Cuo, Z. X. P., Pool, J. E., ... Wang, J. (2010). Sequencing of Fifty Human Exomes Reveals Adaptation to High Altitude. *Science (New York, N.Y.)*, 329(5987), 75–78.

- (34) Bigham, A., Bauchet, M., Pinto, D., Mao, X., Akey, J. M., Mei, R., ... Shriver, M. D. (2010). Identifying Signatures of Natural Selection in Tibetan and Andean Populations Using Dense Genome Scan Data. *PLoS Genetics*, 6(9), e1001116.
- (35) Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., ... Ge, R. (2010). Genetic Evidence for High-Altitude Adaptation in Tibet. *Science*, 329(5987), 72-75.
- (36) Lorenzo FR, Huff CD, Myllymaki M, et al. EGLN1 variant associated with the Tibetan adaptation in high altitude; *Keystone Symposia 2014. Sensing and signaling of hypoxia : interfaces with biology and medicine*; 2014.
- (37) Petousi N, Sciesielski LK, Ratcliffe PJ, Robbins PA, Masson N. Investigation of the functional significance of two coding variants found in Tibetans; *Keystone Symposia 2014. Sensing and signaling of hypoxia: interfaces with biology and medicine*; 2014.
- (38) Song, D., Li, L., Arsenault, P. R., Tan, Q., Bigham, A. W., Heaton-Johnson, K. J., ... Lee, F. S. (2014). Defective Tibetan PHD2 Binding to p23 Links High Altitude Adaption to Altered Oxygen Sensing. *The Journal of Biological Chemistry*, 289(21), 14656–14665.
- (39) Petousi, N., & Robbins, P. A. (2014). Human adaptation to the hypoxia of high altitude: the Tibetan paradigm from the pregenomic to the postgenomic era. *Journal of Applied Physiology*, 116(7), 875–884.
- (40) Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., ... Schofield, C. J. (2017). Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*, 17(7), 981–989.
- (41) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (42) Flashman, E., Bagg, E. A. L., Chowdhury, R., Mecinović, J., Loenarz, C., McDonough, M. A., ... Schofield, C. J. (2008). Kinetic Rationale for Selectivity toward N- and C-terminal Oxygen-dependent Degradation Domain Substrates Mediated by a Loop Region of Hypoxia-Inducible Factor Prolyl Hydroxylases. *Journal of Biological Chemistry*, 283(7), 3808–3815.
- (43) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (44) Chowdhury, R., Candela-Lena, J. I., Chan, M. C., Greenald, D. J., Yeoh, K. K., Tian, Y.-M., ... Schofield, C. J. (2013). Selective Small Molecule Probes for the Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases. *ACS Chemical Biology*, 8(7), 1488–1496.

- (45) Flashman, E., Hoffart, L. M., Hamed, R. B., Bollinger, J. M., Krebs, C., & Schofield, C. J. (2010). Evidence for the Slow Reaction of Hypoxia-Inducible Factor Prolyl Hydroxylase 2 with Oxygen. *The FEBS Journal*, 277(19), 4089–4099.
- (46) Ladroue, C., Carcenac, R., Leporrier, M., Gad, S., Le Hello, C., Galateau-Salle, F., ... Gardie, B. (2008). PHD2 Mutation and Congenital Erythrocytosis with Paraganglioma. *New England Journal of Medicine*, 359(25), 2685–2692.

4.5 Appendix

Table 4.3 Sequences of HIF1 α CODD variant peptides. Hydroxylation sites are bold, underlined.

HIF1 α CODD variant	Sequence	MW g/mol
wt	DLDLEMLA <u>P</u> YIPMDDDFQL	2254.6
D556A	ALDLEMLA <u>P</u> YIPMDDDFQL	2210.5
L557A	DADLEMLA <u>P</u> YIPMDDDFQL	2212.4
D558A	DLALEMLA <u>P</u> YIPMDDDFQL	2210.5
L559A	DLDAEMLA <u>P</u> YIPMDDDFQL	2212.4
E560A	DLDLAMLA <u>P</u> YIPMDDDFQL	2196.5
M561A	DLDLEAL <u>A</u> PYIPMDDDFQL	2194.4
L562A	DLDLEMA <u>A</u> PYIPMDDDFQL	2212.4
P564G	DLDLEMLAAYIPMDDDFQL	2228.5
Y565A	DLDLEMLA <u>P</u> AIIPMDDDFQL	2162.4
I566A	DLDLEMLA <u>P</u> YAPMDDDFQL	2212.4
P567A	DLDLEMLA <u>P</u> YIAMDDDFQL	2228.5
M568A	DLDLEMLA <u>P</u> YIPADDDFQL	2194.4
D569A, D570A, D571A	DLDLEMLA <u>P</u> YIPMAAAFQL	2122.5
F572A	DLDLEMLA <u>P</u> YIPMDDDAQL	2178.4
Q573A	DLDLEMLA <u>P</u> YIPMDDDFAL	2197.5
L574A	DLDLEMLA <u>P</u> YIPMDDDFQA	2212.4

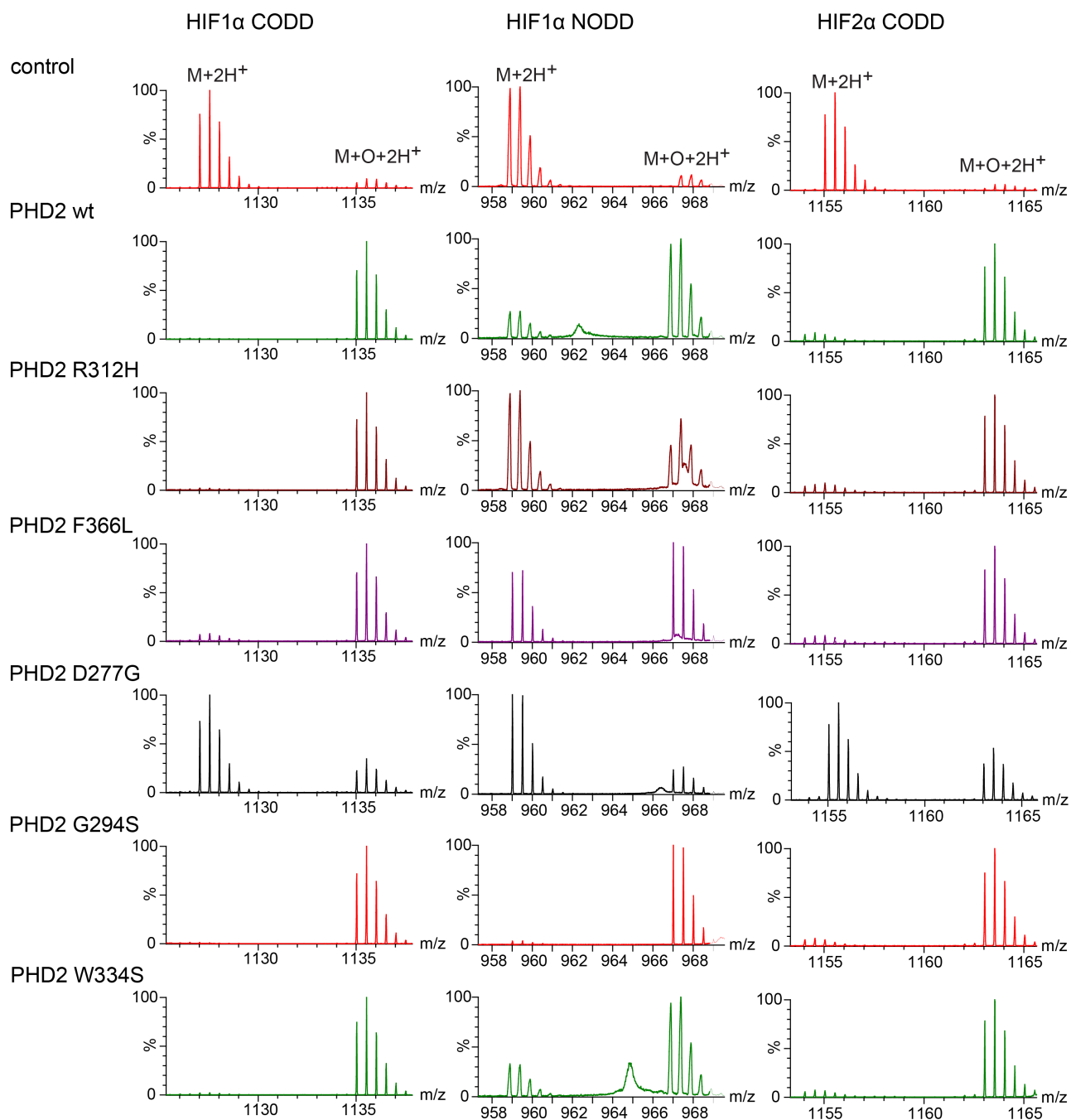


Figure 4.9: Activity of PHD2 variants on their HIF α substrates - ESI-LC-MS spectra. For assay conditions, see legend of Figure 4.3.

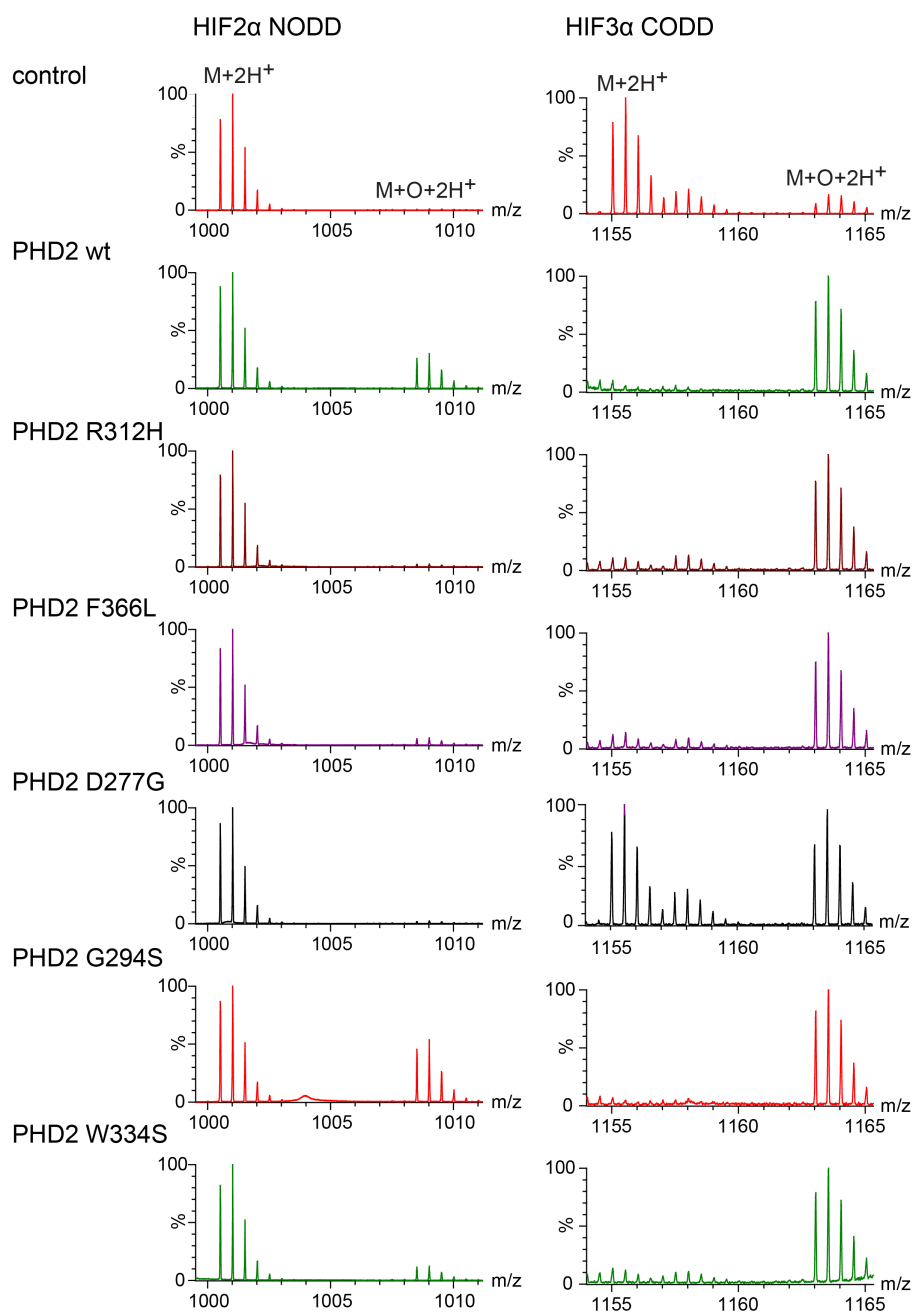


Figure 4.10: Activity of PHD2 variants on their HIF α substrates - ESI-LC-MS spectra. For assay conditions, see legend of **Figure 4.3**.

Chapter 5

Biochemical Studies on the HIF Prolyl Hydroxylase from *Trichoplax adhaerens*

5.1 Introduction

Bioinformatic analyses of metazoan genomes ranging from humans to the simplest animal *T. adhaerens*, have revealed the conservation of a minimal HIF α ODD/PHD/pVHL system in all animals, with at least one copy of HIF α (containing at least one ODD), and one copy of a MYND-type finger containing PHD present in all animal genomes (1). Given the position of *T. adhaerens* in evolution of animals, as the single existent member of placozoa, the study of the HIF system in this organism is of interest.

Candidates for HIF α and HIF β genes in the *T. adhaerens* genome were identified by bioinformatic search for characteristic domains in HIF, such as basic helix-loop-helix (bHLH) motives and Per-Arnt-Sim (PAS) domains (1). The identified single ODD in *T. adhaerens* (*TaHIF α* ODD) was found to differ substantially from human sequences (15 % identity to *HsHIF1 α* NODD, 35 % identity to *HsHIF1 α* CODD) (1). Notably, *TaHIF α* ODD lacks the consensus LXXLAP motif present in humans and many other animals. A functional HIF system in *T. adhaerens* was demonstrated by identifying HIF target genes (e.g. fructose-biphosphate aldolase and pyruvate dehydrogenase kinase), that were up-regulated by exposure of *T. adhaerens* to hypoxia or treatment of the placozoan with DMOG (1).

Analysis of the *T. adhaerens* genome revealed a single putative PHD homologue (*TaPHD*), that, like human PHD2 (*HsPHD2*), contains a MYND-type finger domain (see sequence alignment in **Figure 5.1**). *TaPHD* shows a high sequence similarity to *HsPHDs* in its oxygenase domain, yet the *N*-terminal domain largely differs from the human PHD sequences. Conserved features between *TaPHD* and *HsPHDs* include the β 2/ β 3-finger-loop finger loop that is crucial for substrate binding in human PHDs, and the catalytic triad of residues (H209_{*TaPHD*}, D211_{*TaPHD*}, and H270_{*TaPHD*}) that coordinate Fe(II) in the active site.

Recombinantly-expressed *Ta*PHD was used to demonstrate *Ta*PHD catalysed hydroxylation of prolyl residue P486 in the *Ta*HIF α ODD, which promoted binding of *Ta*HIF α ODD to human pVHL (1). Biochemical analysis of *Ta*PHD revealed conserved properties between *Ta*PHD and human PHDs, including the use of 2OG and Fe(II) as co-factors, stereoselective production of *trans*-4-hydroxyproline under incorporation of molecular oxygen, and formation of a stable *Ta*PHD.Fe(II).2OG complex in the absence of substrate (half-life > 24 h) (1). However, further biochemical analyses are necessary to assess to which extent the oxygen sensing properties of human PHDs are conserved in *Ta*PHD. Notably, it was found that *Ta*PHD catalyses the hydroxylation of human HIF1 α CODD, but does not accept human HIF1 α NODD, suggesting a substrate preference similar to that of human PHD3 (1-3).

Crystal structures of *Ta*PHD with and without its peptidic substrate (*Ta*ODD) bound, have been solved by Anna Boleininger, University of Oxford, UK, and Michael McDonough, University of Oxford, UK (4). The structures provide insights into catalytic properties and the substrate binding mode of *Ta*PHD. Comparison of these features in *Ta*PHD with other prolyl-4-hydroxylases, including human PHDs, may help to gain an understanding of the evolution of oxygen sensing HIF prolyl hydroxylases. Crystal structures of the enzyme-substrate complex of the prolyl-4-hydroxylase from *Chlamydomonas reinhardtii* (*Cr*P4H), a member of the collagen-prolyl-4-hydroxylase-subfamily (5), and the prokaryotic PHD homolog PPHD from *Pseudomonas putida*, have been reported (5-6). Both *Cr*P4H and PPHD act on non-HIF α substrates, i.e. *Cr*P4H catalyses the hydroxylation of the proline residue P6 in a proline-rich (Ser-Pro)₅ substrate (5), and PPHD hydroxylates the proline residue P54 in the translation elongation factor EF-Tu (6). Sequence alignment of *Ta*PHD and human PHDs with *C. reinhardtii* *Cr*P4H and *P. putida* PPHD (**Figure 5.1**) reveals conservation of the 2OG-oxygenase domain (E90_{*Ta*PHD}-F287_{*Ta*PHD}) among the HIF and non-HIF prolyl-4-hydroxylases, whereas the *N*-terminal region of the enzymes is less well conserved. This observation is consistent with the important role of the 2OG-oxygenase domain in enzyme catalysis.

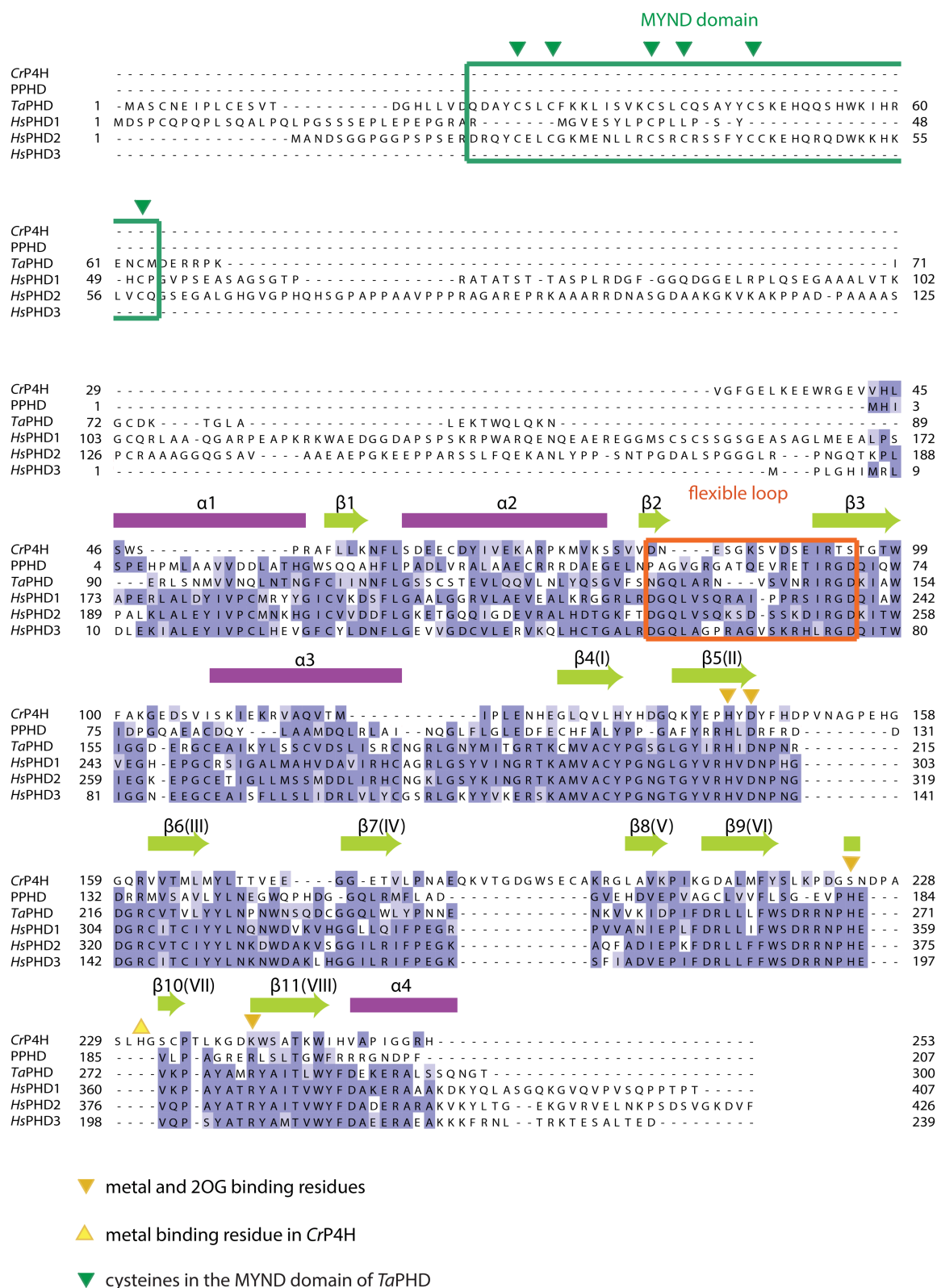


Figure 5.1: Sequence comparison of human PHD isoforms 1-3 with *TaPHD*, *CrP4H* and *Pseudomonas putida* PPHD. Sequences corresponding to the myeloid, Nervy, and DEAF-1 (MYND) motif and the flexible β 2/ β 3-finger-loop are boxed, α -helices are marked purple, and β -sheets are labelled with green arrows. The alignment was generated using Clustal Omega (7) and Jalview 2.9.0b2 (8).

Taken together, the major aims of the work described in this Chapter are:

1. In-depth analysis of the crystal structures obtained from *Ta*PHD with and without bound substrate, and comparison with enzyme-substrate structures of other prolyl-4-hydroxylases (human PHDs, *Cr*P4H, and PPHD), with focus on substrate-binding modes and key structural features necessary for oxygen sensing.
2. Comparative biochemical studies with *Ta*PHD and *Hs*PHD2, to investigate to which extent the unique kinetic properties of *Hs*PHD2 that are central to its oxygen sensing role, are conserved between the two enzymes.

5.2 Results and Discussion

5.2.1 Comparative analyses of *Ta*PHD crystal structures

5.2.1.1 Crystal structures of *Ta*PHD with and without bound substrate

In order to investigate the extent to which the structural features necessary for oxygen sensing are conserved between *Hs*PHD2 and *Ta*PHD, a crystal structure of *Ta*PHD in complex with non-reactive substitutes for Fe(II) and 2OG (Mn(II) and the inhibitor FG2216/IOX3) was obtained (Anna Boleininger, University of Oxford, UK, (4)). A *Ta*PHD construct (aa 64-300, *Ta*PHD^{t64}) lacking the *N*-terminal MYND domain was used for crystallisation, as the shorter construct expressed substantially well in *E. coli*, by contrast to a full-length *Ta*PHD construct, for which only low expression levels were achieved (4). The structure of *Ta*PHD^{Δ64} was solved to 1.2 Å resolution by molecular replacement, using *Hs*PHD2 (PDB: 2G19, (9)) as search model (4). To gain insight into the substrate-binding mode of *Ta*PHD, *Ta*PHD^{t64} was co-crystallised with a 21mer fragment of *Ta*HIFα ODD (*Ta*ODD, residues E477-L497), Mn(II) and NOG (4). The *Ta*PHD.*Ta*ODD.Mn.NOG structure was solved to a resolution of 1.3 Å by molecular replacement (search model: *Ta*PHD without *Ta*ODD, (4)).

The overall architecture of *Ta*PHD is shown in **Figure 5.2 A**. The enzyme contains the conserved double-stranded β-helix core-fold (DSBH), which is present in all characterised 2OG dependent oxygenases (10). As with *Hs*PHD2, the major β-sheet core is stabilised by three α-helices (α1-3). In the substrate complex structure (*Ta*PHD.*Ta*ODD), eighteen residues of *Ta*ODD (A480_{*Ta*ODD}-L497_{*Ta*ODD}, **Figure 5.2 B**) are visible. The overall folds of the substrate-unbound *Ta*PHD and the *Ta*PHD.*Ta*ODD complex are very similar, as judged by root-mean-square deviation (RMSD) of atomic positions in the two structures (RMSD for all Cα of *Ta*PHD_{C73-S295}.*Ta*ODD - *Ta*PHD_{C73-S295} = 0.15 Å). Differences between the *Ta*PHD and *Ta*PHD.*Ta*ODD structures are found in the loop regions of the enzyme. The β2/β3-finger-loop, which is disordered in the substrate-unbound *Ta*PHD structure (missing residues Q137_{*Ta*PHD}-R146_{*Ta*PHD}), is ordered and makes extensive contacts with the substrate in the *Ta*PHD.*Ta*ODD structure (**Figure 5.2 A and B**). By contrast, the β7(VI)/β8(V)-loop residue N245_{*Ta*PHD} is not apparent in the *Ta*PHD.*Ta*ODD structure, unlike in the substrate-unbound *Ta*PHD structure, in which the complete β7(VI)/β8(V)-loop is observed.

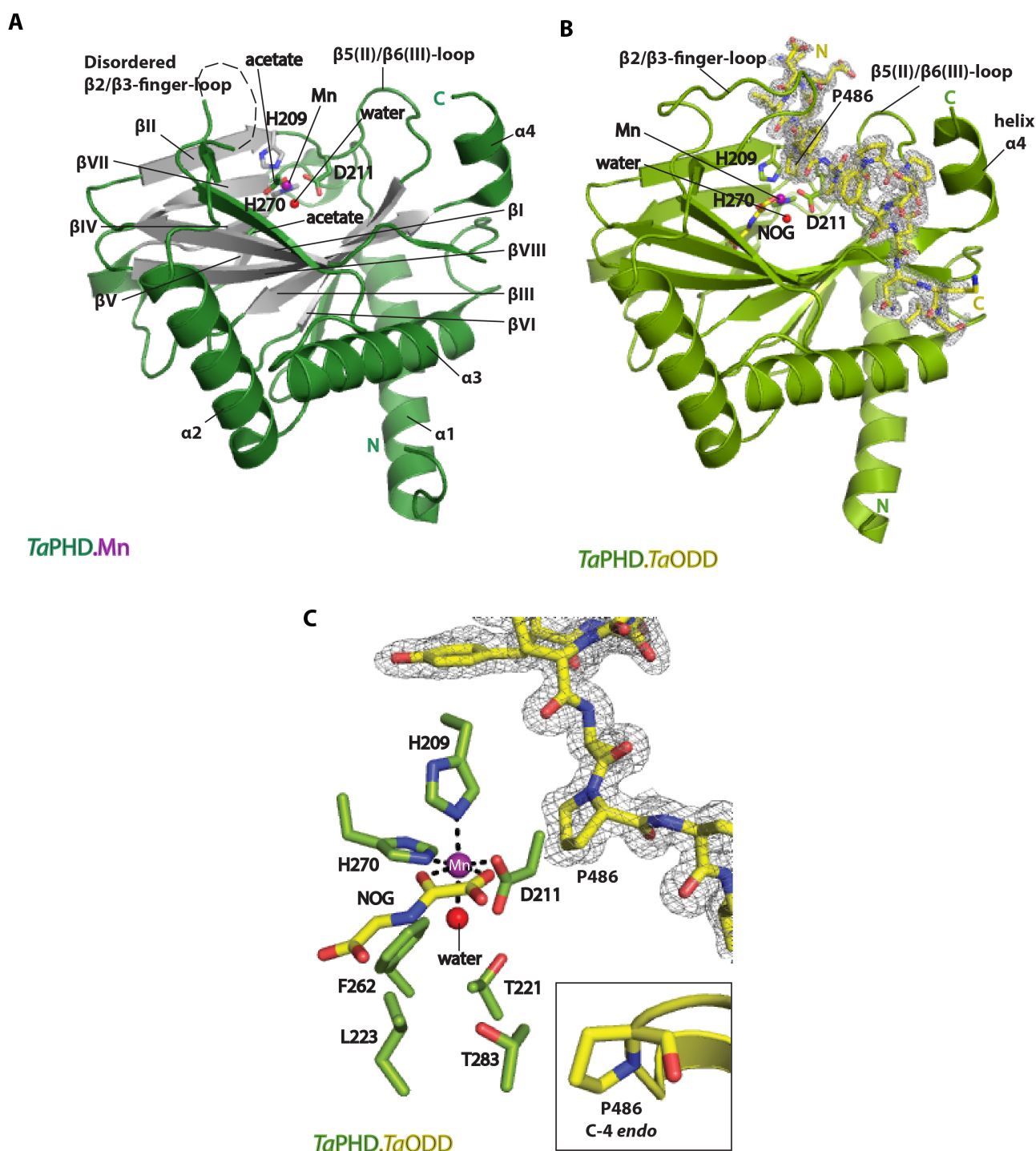


Figure 5.2: Views from crystal structures of *TaPHD* without substrate bound and in complex with a fragment of its substrate (*TaPHD.TaODD*). (A) Secondary structural elements in *TaPHD* comprise four α helices and ten β strands of which eight form the double-stranded β -helix core fold (DSBH, grey, Roman numerals). (B) Overall binding mode of *TaODD* to *TaPHD* in the *TaPHD.TaODD* complex structure showing the 2Fo-Fc electron density mask of the peptidic substrate (contoured to 1.0 σ). (C) Active site close-up of *TaPHD.TaODD* reveals that the P486_{*TaODD*} C-4-methylene adopts an *endo*-conformation (2Fo-Fc density, contoured to 1.0 σ).

The active site pocket of *Ta*PHD, enclosed by the major and minor β -sheets of the DSBH, deeply embeds the metal ion and the 2OG analogue NOG (**Figure 5.2 B and C**). The highly buried nature of the 2OG binding site in the *Ta*PHD structure likely contributes to the formation of the stable *Ta*PHD.Fe.2OG complex, in a similar manner as observed before in structures of *Hs*PHD2 (9, 12).

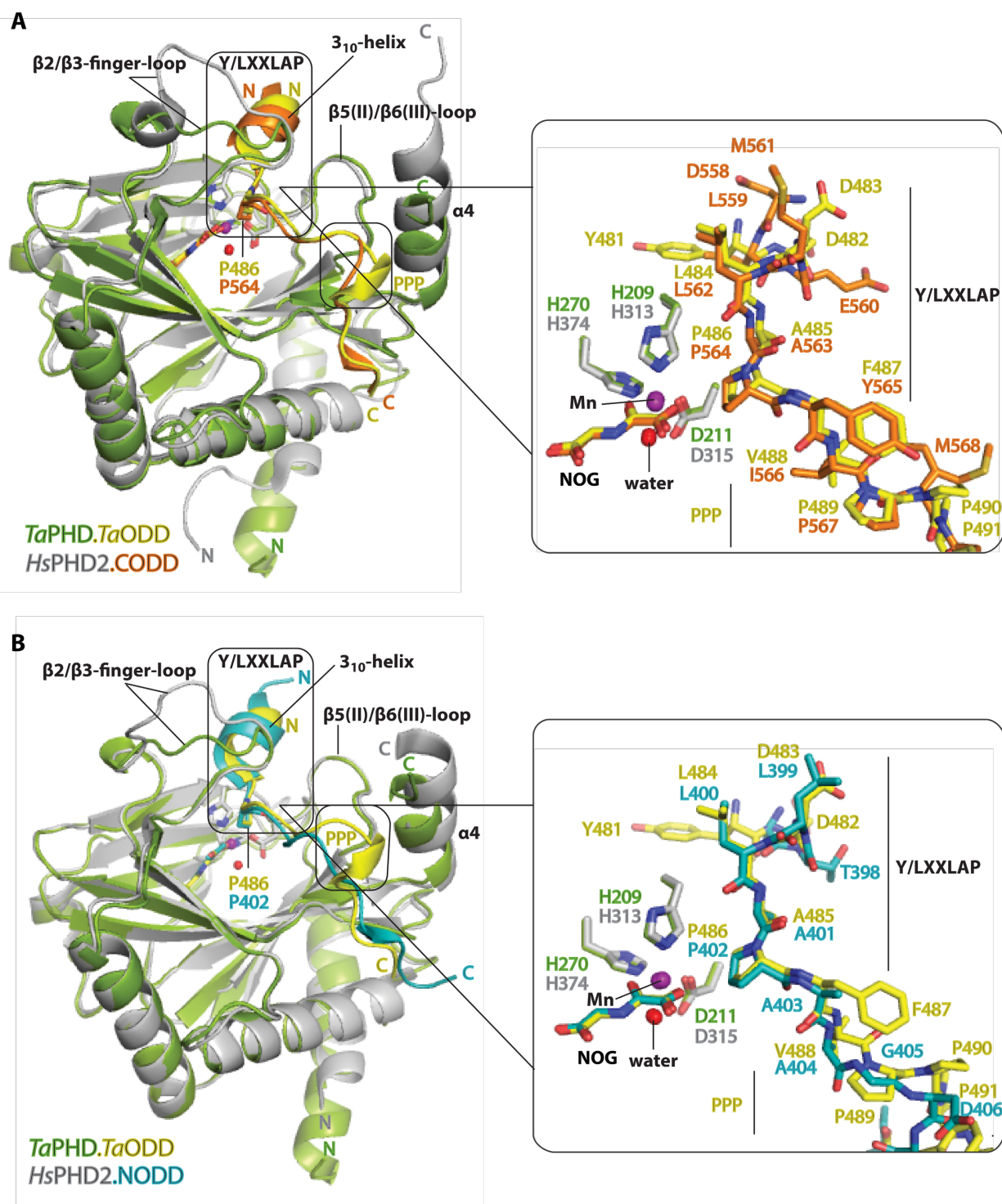
In the *Ta*PHD structure without substrate bound, the Mn(II) is octahedrally coordinated by the conserved triad of residues (H209_{*Ta*PHD}, D211_{*Ta*PHD}, and H270_{*Ta*PHD}), an acetate ion and two water molecules (11). The 6-coordinate state of the metal is retained in the *Ta*PHD.*Ta*ODD.Mn.NOG complex, including the triad of metal-coordinating residues, and an ordered, metal-bound water molecule (W1) that is, in addition to the metal coordination, stabilised via hydrogen bonding with the metal-ligating side chain of D211_{*Ta*PHD} (**Figure 5.2 C**). The observation of a well-ordered metal bound water in both *Ta*PHD and *Hs*PHD2 (9, 12), is important from a hypoxia sensing perspective, because displacement of this water from the metal is required for oxygen to bind, and is proposed to be rate limiting in *Hs*PHD2 catalysis (9, 12-13). In the *Ta*PHD.*Ta*ODD structure, NOG coordinates to the metal ion in a bidentate manner and interacts with R279_{*Ta*PHD} by its C5 carboxylate. The 1-carboxylate of NOG is positioned at the coordination site closest to the hydroxylation target, P486_{*Ta*ODD}, making the remaining metal coordination site (occupied by W1) less accessible, and thus apparently hindering oxygen binding to the metal ion.

Taken together, the active site arrangement observed in the *Ta*PHD structures exhibits a very high degree of conservation to the reported *Hs*PHD2 structures (9, 12, 14), implying that core features necessary for the oxygen sensing role of the enzymes are conserved between *Ta*PHD and *Hs*PHD2.

5.2.1.2 Comparison of *Ta*PHD.*Ta*ODD and *Hs*PHD2.CODD/NODD structures

The *Ta*PHD.*Ta*ODD enzyme-substrate structure was compared with crystal structures of *Hs*PHD2 in complex with its substrates *Hs*HIF1 α CODD (PDB: 3HQR, (12)) and *Hs*HIF1 α NODD (PDB: 5L9V, (14)). A high degree of conservation in the substrate binding modes between *Ta*PHD and *Hs*PHD2 was revealed, as both enzymes employ the active site containing groove, the β 2/ β 3-finger-loop, and the C-terminal α 4-helix for substrate binding (**Figure 5.3 A** and **5.3 B**). The *Ta*ODD peptide binds to *Ta*PHD in an extended form, and residues N-terminal to the target proline form a 3_{10} -helix. The target proline in *Ta*ODD and its N-terminally flanking residues form a YXXLAP motif, which differs by one residue (L $\rightarrow$ Y) from the consensus LXXLAP prolyl hydroxylation site present in all human ODDs.

Binding and positioning of the hydroxylation target P486_{*Ta*ODD} directly adjacent to the metal in the catalytic centre is highly similar to that observed in *Hs*PHD2.CODD and *Hs*PHD2.NODD complex structures (**Figure 5.3 A** and **5.3 B**). Several residues in the β 2/ β 3-finger-loop region of *Ta*PHD (Q137_{*Ta*PHD}, L138_{*Ta*PHD}, and A139_{*Ta*PHD}, corresponding to Q239_{*Hs*PHD2}, L240_{*Hs*PHD2}, and V241_{*Hs*PHD2}) form H-bonds and hydrophobic contacts with *Ta*ODD (P486_{*Ta*ODD}, F487_{*Ta*ODD}, A485_{*Ta*ODD}, corresponding to P564_{*Hs*HIF1 α} CODD, A563_{*Hs*HIF1 α} CODD, Y565_{*Hs*HIF1 α} CODD and P402_{*Hs*HIF1 α} NODD, A401_{*Hs*HIF1 α} NODD, A403_{*Hs*HIF1 α} NODD, respectively). The hydroxylation target proline is further positioned via hydrophobic and polar contacts with residues in the β II(β 5)/ β III(β 6) and in the C-terminal strand β VIII(β 11) (see *Appendix*, **Table 5.2**). Notably, the conformations of the substrate target prolyl rings at the *Ta*PHD and *Hs*PHD2 active sites are nearly identical (**Figure 5.3 A** and **5.3 B**), as the substrate proline pyrrolidine rings in *Ta*ODD and *Hs*CODD/NODD all adopt a C-4 *endo*-conformation, with the 4*R* C-H bond that is cleaved during hydroxylation, positioned close to the metal centre.



In the human PHDs, the $\beta 2/\beta 3$ -finger-loop plays a significant role in substrate recognition and in determining NODD/CODD selectivity (12, 14-15). In a similar manner, the $\beta 2/\beta 3$ -finger-loop in the *Ta*PHD.*Ta*ODD complex constitutes one of the major substrate interaction sites, yet it is three residues shorter than in *Hs*PHD2, between residues N141_{*Ta*PHD} and V142_{*Ta*PHD}. As a consequence, and by contrast to *Hs*PHD2, the $\beta 2/\beta 3$ -finger-loop in the *Ta*PHD.*Ta*ODD structure is more condensed and stabilised by intramolecular interactions between loop residues, including backbone-backbone H-bonds and salt bridges between side chains (see **Figure 5.3** and **5.5**). The observed differences in the $\beta 2/\beta 3$ -finger-loop might reflect the fact that there is a single HIF α isoform with one ODD in *T. adhaerens*, which contrasts with the situation in humans where there are three HIF α isoforms, with both a *N*-terminal and a *C*-terminal ODD in case of HIF1 α and HIF2 α . It is likely that binding of multiple HIF substrates to *Hs*PHD2 requires a higher degree of flexibility in the $\beta 2/\beta 3$ -finger-loop, compared to the more rigid $\beta 2/\beta 3$ -finger-loop in *Ta*PHD.

In the *Ta*PHD.*Ta*ODD and *Hs*PHD2.CODD structures a salt-bridge between an arginine residue in the *C*-terminal $\alpha 4$ -helix and an aspartate residue in the *C*-terminus of the respective peptide (R292_{*Ta*PHD}/D494_{*Ta*ODD}, R396_{*Hs*PHD2}/D571_{*Hs*HIF1 α} CODD) is formed. By contrast, in the *Hs*PHD2.NODD structure, the *Hs*HIF1 α NODD-peptide is positioned further from the *C*-terminal $\alpha 4$ -helix, and interacts with the *C*-terminal region of *Hs*PHD2 solely via hydrophobic contacts (**Figure 5.3 B**, (14)). Unlike the *Hs*HIF1 α CODD/NODD peptides, *Ta*ODD contains a Pro-Pro-Pro motif (P489-491_{*Ta*ODD}), which is *C*-terminal to the target proline P486_{*Ta*PHD} residue (**Figure 5.3**). The region adjacent to the Pro-Pro-Pro motif adopts a short 3_{10} -helix, and constitutes the interaction surface with the *C*-terminal $\alpha 4$ -helix of *Ta*PHD.

In a broad sense, the interactions between *Ta*PHD and *Ta*ODD apparently more closely resemble those of *Hs*PHD2 and *Hs*HIF1 α CODD, rather than those of *Hs*PHD2 and *Hs*HIF1 α NODD. Given the likely relatively rigid nature of the Pro-Pro-Pro motif in *Ta*ODD, it also appears that *Ta*ODD binds to *Ta*PHD in a less flexible manner than does *Hs*HIF1 α NODD to *Hs*PHD2, and to a lesser extent, *Hs*HIF1 α CODD. These differences may further reflect the evolved roles of the *Hs*PHDs in accepting multiple substrates (NODDs/CODDs of HIF α isoforms), compared to *Ta*PHD.

5.2.1.3 Comparison with enzyme-substrate structures of other prolyl-4-hydroxylases

The *Ta*PHD.*Ta*ODD structure was further compared with crystal structures of *Cr*P4H in complex with a proline-rich (Ser-Pro)₅ substrate (PDB: 3GZE, (5)), and PPHD bound to its substrate EF-Tu (PDB: 4IW3, (6)).

Analysis of the structural conservation between *Ta*PHD and *Hs*PHD2 reveals a high overall similarity as judged by RMSD of atomic positions in the respective structures (**Figure 5.3**, RMSD for all C α of *Ta*PHD_{C73-S295}.*Ta*ODD - *Hs*PHD2_{Q184-K408}.CODD = 0.54 Å, and RMSD for all C α of *Ta*PHD_{C73-S295}.*Ta*ODD - *Hs*PHD2_{P189-Y403}.NODD = 0.39 Å). In addition, it was found that *Ta*PHD shares a stronger structural conservation with PPHD (**Figure 5.4 A**, RMSD for all C α of *Ta*PHD_{C73-S295}.*Ta*ODD - PPHD_{H7-F207}.EF-Tu = 0.92 Å), than with *Cr*P4H (**Figure 5.4 B**, RMSD for all C α of *Ta*PHD_{C73-S295}.*Ta*ODD - *Cr*P4H_{W38-G250}.(Ser-Pro)₅ = 2.20 Å), suggesting a closer evolutionary relationship between HIF hydroxylases and PPHD than to the algal collagen prolyl hydroxylase *Cr*P4H. The structural similarity between PPHD and *Hs*PHD2 (RMSD for all C α of *Hs*PHD2_{Q184-K408}.CODD - PPHD_{H7-F207}.EF-Tu = 1.57 Å) is less than for PPHD and *Ta*PHD, which might indicate a closer evolutionary relationship between the prokaryotic PPHD and *Ta*PHD, than between human *Hs*PHD2 and PPHD.

The PHDs and the other two prolyl-4-hydroxylases adopt strikingly similar substrate-binding features, as they all employ a flexible β 2/ β 3-finger-loop (G136_{*Ta*PHD}-D150_{*Ta*PHD}, G238_{*Hs*PHD2}-D254_{*Hs*PHD2}, S77_{*Cr*P4H}-S95_{*Cr*P4H}, A53_{PPHD}-D70_{PPHD}) that undergoes a major conformational change upon substrate binding and helps to enclose the target proline of the substrate in the active site cleft (**Figure 5.4** and **5.5**). In *Ta*PHD, *Hs*PHD2 and PPHD, the C-terminus interacts with the substrate (**Figure 5.3** and **5.4 A**); it is unknown if this is the case for *Cr*P4H, since the shorter peptide substrate used in the *Cr*P4H.(Ser-Pro)₅ structure appears not to reach to the C-terminus (**Figure 5.4 B**, (5)). Whilst *Ta*ODD, *Hs*HIF1 α CODD and *Hs*HIF1 α NODD are predicted to be disordered in solution and form a partial helical structure (3₁₀-helix), when bound to enzyme (**Figure 5.3**), the EF-Tu switch loop retains loop secondary structure upon binding to PPHD (**Figure 5.4 A**), albeit after a very large conformational change (6).

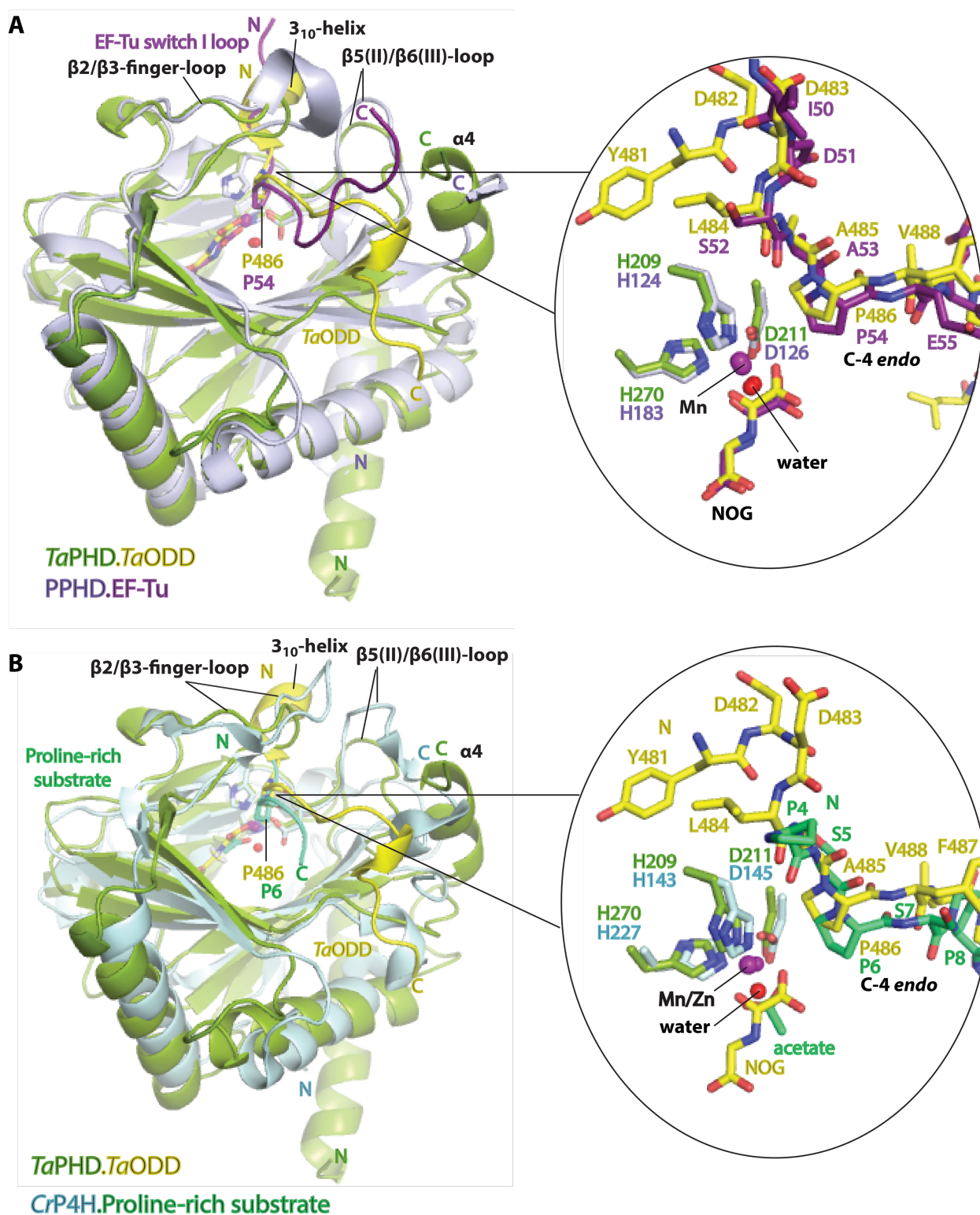


Figure 5.4: Conserved modes of prolyl-substrate binding in TaPHD, CrP4H and *Pseudomonas putida* PPHD. Overall superimposition and active site close-up of the TaPHD.TaODD complex with (A) *Pseudomonas putida* PPHD in complex with EF-Tu (PDB:4IW3, (6)), and (B) CrP4H (PDB:3GZE, (5)) in complex with a proline-rich peptidic substrate.

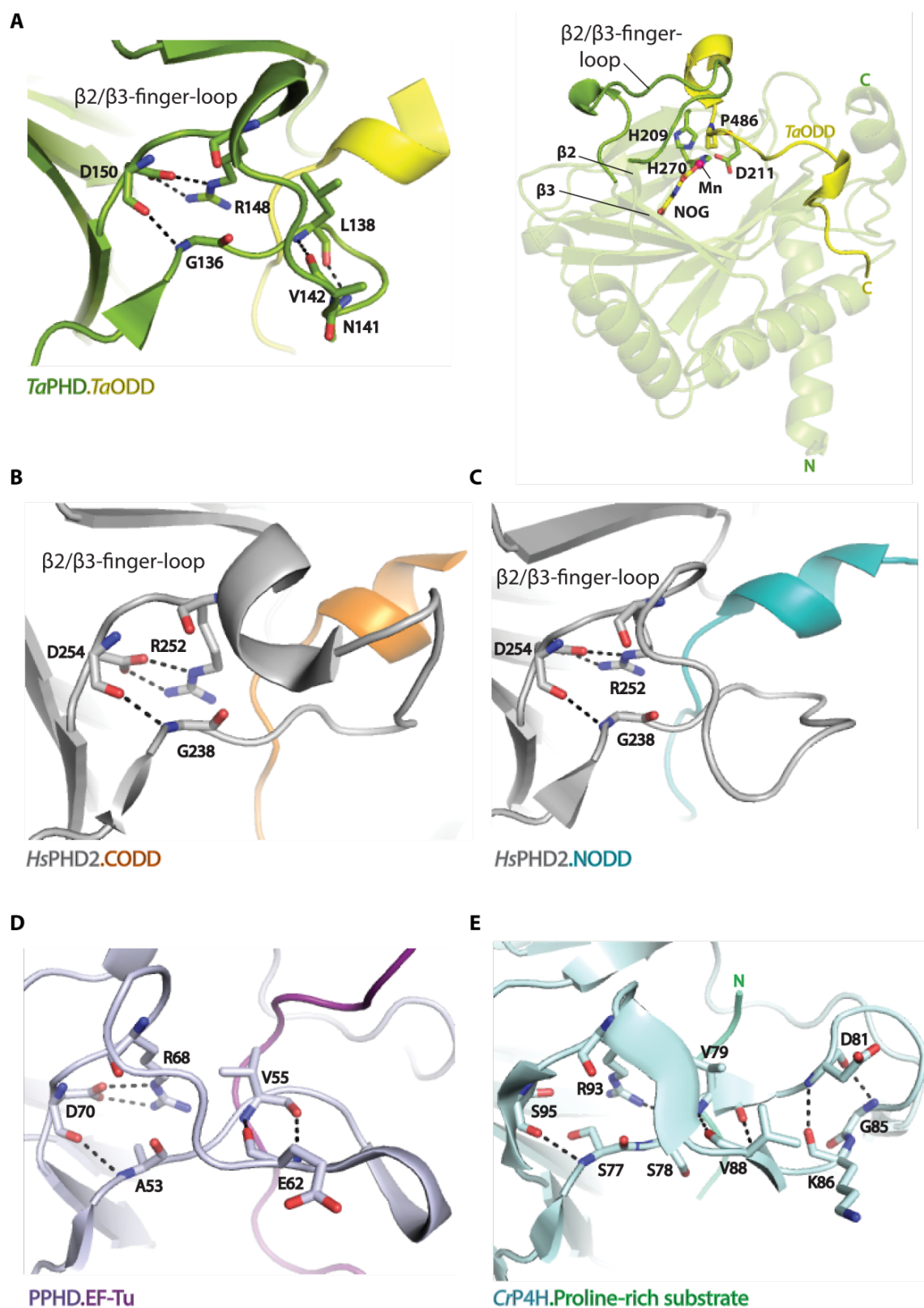


Figure 5.5: Views of the $\beta 2/\beta 3$ -finger-loop in the (A) *TaPHD.TaODD*, (B) *HsPHD2.CODD* (PDB:3HQR, (12)), (C) *HsPHD2.NODD* (PDB:5L9V, (14)), (D) *PPHD.EF-Tu*, (PDB:4IW3, (6)), and (E) *CrP4H.proline-rich substrate* (PDB:3GZE, (5)) structures. Hydrogen bonding and electrostatic interactions between loop-residues are displayed.

Comparison of the binding mode of the prolyl-4-hydroxylase substrates in the active site reveals a high degree of conservation (**Figure 5.3, 5.4, and 5.6**). The substrates all show a similar orientation towards the active site residues and the metal centre, and the target prolyl residues all adopt the C-4 *endo*-conformation. Similar to the HIF prolyl-4-hydroxylases *Ta*PHD and *Hs*PHD2, PPHD and *Cr*P4H both employ a HXD...H motif for metal-binding in the active site (H143_{*Cr*P4H}, D145_{*Cr*P4H}, H227_{*Cr*P4H}, and H124_{PPHD}, D126_{PPHD}, H183_{PPHD}). In PPHD, the metal ion is octahedrally coordinated and one of the coordination sites is occupied by a water molecule (**Figure 5.6 D**), demonstrating evolutionary conservation in the active site arrangement between the HIF prolyl-4-hydroxylases in animals and the bacterial non-HIF prolyl-4-hydroxylase PPHD. By contrast, in the *Cr*P4H structure, the zinc ion (used for crystallisation) is tetrahedrally coordinated, which leaves no free coordination site for water binding to the metal ion (**Figure 5.6 E**). The presence of zinc, instead of iron, in the active site of *Cr*P4H limits the degree to which the structure can be interpreted in regard to the common features that enable oxygen sensing by the PHDs, such as positioning of 2OG and the presence of a metal-coordinated water molecule.

All in all, structure-based comparisons reveal a high degree of conservation in the substrate binding modes of *T. adhaerens* and human PHDs, with their prokaryotic PHD-homolog PPHD and *C. reinhardtii* P4H, a member of the collagen-prolyl-4-hydroxylase-subfamily, which is related to the PHD subfamily VIII (10).

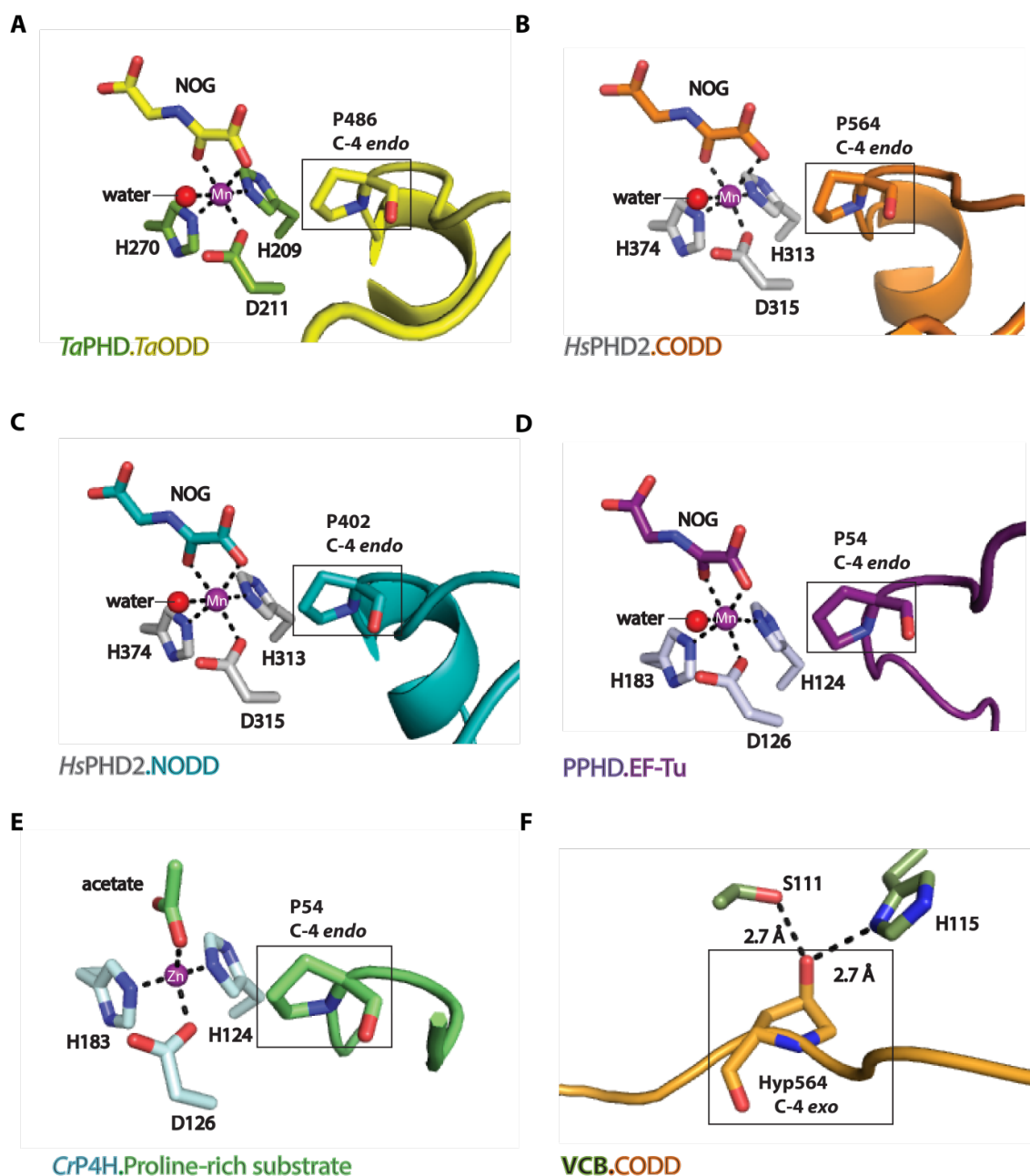


Figure 5.6: Active site details and conformation of the target prolyl-residues in the enzyme-substrate complex structures of *TaPHD*, *HsPHD2*, *PPHD*, and *CrP4H*. (A) *TaPHD.TaODD*, (B) *HsPHD2.CODD* (PDB:3HQR, (12)), (C) *HsPHD2.NODD* (PDB:5L9V, (14)), (D) *PPHD.EF-Tu*, (PDB:4IW3, (6)) and (E) *CrP4H.proline-rich substrate* (PDB:3GZE, (5)) complex structures. Note that in the enzyme-substrate complexes, the proline C-4-methylene that is hydroxylated, adopts the *endo*-conformation. (F) By contrast, Hyp564 in *HsHIF1α* CODD adopts the C-4 *exo*-conformation when bound to the VCB complex (PDB:1LM8, (16)). In (A)-(D), *N*-oxalylglycine (NOG) acts as a 2OG analogue. Note that the Zn (substituting for Fe) in the *CrP4H* active site is tetrahedrally coordinated, with an acetate binding instead of the 2OG (NOG) cosubstrate.

5.2.2 Comparative biochemical studies on *Ta*PHD and *Hs*PHD2

5.2.2.1 Production and purification of *Ta*PHD

The catalytic domain of *T. adhaerens* PHD (*Ta*PHD⁶⁴⁻³⁰⁰, hereafter: *Ta*PHD) was recombinantly produced in *E. coli* and purified using affinity chromatography, followed by size-exclusion chromatography, as described in *Materials and Methods*, **Chapter 7.3**, and as reported in (1, 4). The initial yield of purified *Ta*PHD from a 8 L large-scale culture was low (< 10 mg), therefore the buffer conditions for lysis and affinity purification were optimised. A substantial increase in protein yield (> 25 mg per 8 L culture) was achieved by adding DTT to the lysis buffer at a final concentration of 5 mM, and 5 % glycerol to every buffer used in lysis and purification. The purity of the protein was assessed by SDS-PAGE (> 90 % final purity, **Figure 5.7 A**) and the molecular weight of the enzyme was confirmed by LC-MS (**Figure 5.7 B**).

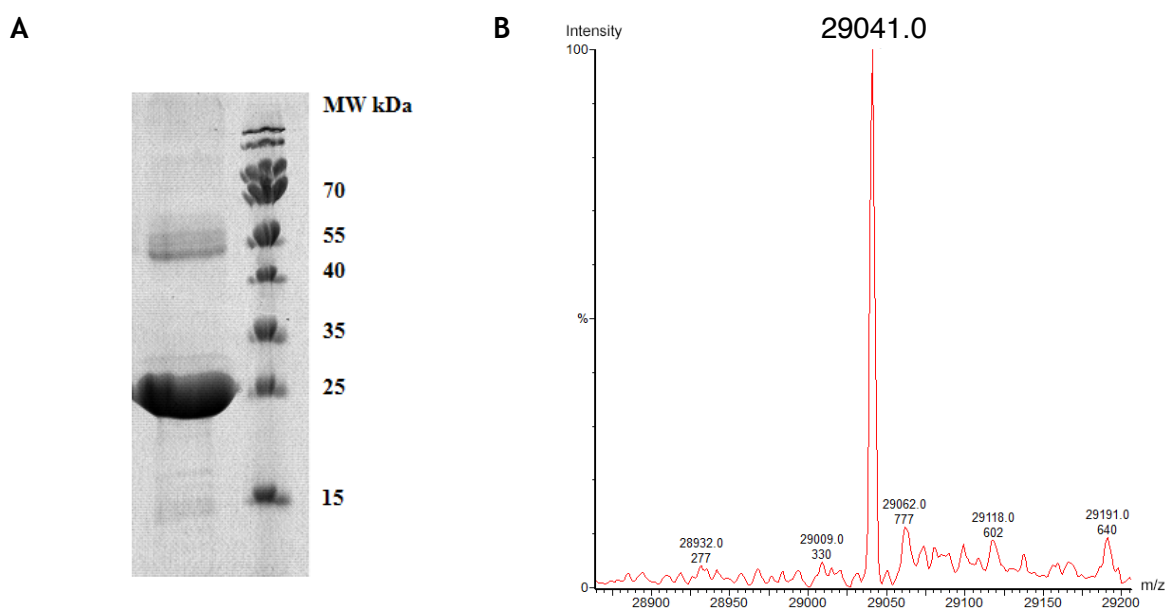


Figure 5.7: Purification of *Ta*PHD⁶⁴⁻³⁰⁰. (A) SDS-PAGE gel of purified *Ta*PHD⁶⁴⁻³⁰⁰ (MW= 29.2 kDa). (B) ESI-LC-MS spectrum (positive ion mode) of *Ta*PHD⁶⁴⁻³⁰⁰. A peak corresponding to the mass of *Ta*PHD⁶⁴⁻³⁰⁰ minus the *N*-terminal methionine residue (MW, expected = 29042 Da) is observed.

5.2.2.2 Steady-state kinetic studies on *Ta*PHD and *Hs*PHD2

Previous studies have shown that *N*-terminally truncated *Ta*PHD catalyses prolyl hydroxylation of both the *Ta*ODD 25mer and the human HIF1 α CODD and HIF1 α NODD 19mer peptide substrates, with the latter being hydroxylated less efficiently (1). Alignment of *Ta*ODD with human HIF α CODD/NODD sequences and substrates of PHD-like enzymes in *Dictyostelium discoideum* (*Dd*Skp1) and *Pseudomonas putida* (*Pp*EF-Tu) reveal that the *Ta*ODD sequence manifests the highest degree of sequence similarity to human HIF1 α -HIF3 α CODD (**Figure 5.8**). Moreover, whilst a NODD like sequence is (apparently) present in all vertebrates, it is not present in some non-vertebrates, e.g. *Placozoa* and *Cnidaria*, suggesting that the CODD sequence might have evolved prior to that of NODD (1). To further investigate the substrate preferences of *Ta*PHD, its affinity for *Ta*ODD 25mer, human HIF sequences, and its co-substrate 2OG was determined in steady-state kinetic studies, and compared to the truncated human PHD isoform 2 (*t*PHD2, hereafter *Hs*PHD2).

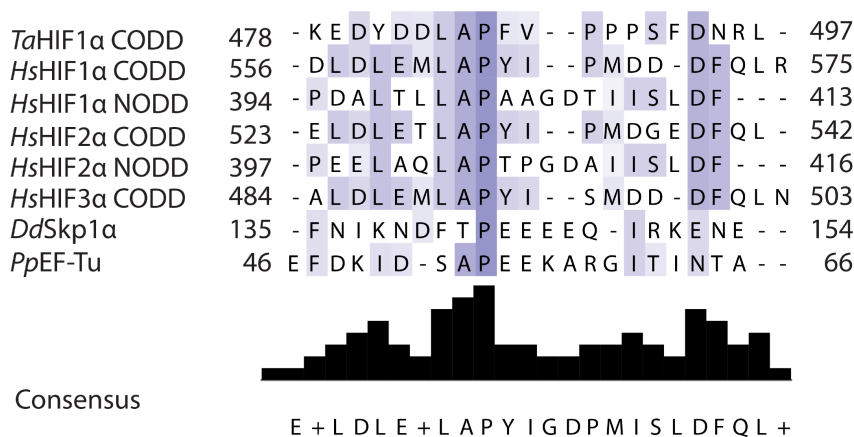


Figure 5.8: Sequence alignment of *Ta*HIF α , *Hs*HIF α , and substrates of PHD-like enzymes in *Dictyostelium discoideum* (*Dd*Skp1) and *Pseudomonas putida* (*Pp*EF-Tu).

Steady-state kinetic parameters (K_m , k_{cat}) for HIF peptide substrates and 2OG were determined under the optimised assay conditions described in *Materials and Methods*, **Chapter 7.6.1**, and peptide hydroxylation levels were analysed by MALDI-TOF-MS (**Figure 5.9**, **Table 5.1**).

Consistent with previous reports (1), *Ta*PHD efficiently catalysed the hydroxylation of its natural substrate *Ta*ODD and human HIF1 α CODD, whilst *Hs*HIF1 α NODD was not turned over above background levels by *Ta*PHD under the conditions/limits of detection of these assays). The observed affinity of *Ta*PHD for its natural substrate *Ta*ODD, and *Hs*HIF1 α CODD was very similar, as judged by *K_m* values (*K_m*(*Ta*ODD, *Ta*PHD) = 11.5 $\pm$ 1.8 μ M, *K_m*(*Hs*HIF1 α CODD, *Ta*PHD) = 15.1 $\pm$ 2.2 μ M). However, its catalytic efficiency as judged by *k_{cat}* is lower with the unnatural human CODD sequence (*k_{cat}* (*Hs*HIF1 α CODD, *Ta*PHD) = 0.012 $\pm$ 0.001 s⁻¹, *k_{cat}* (*Ta*ODD, *Ta*PHD) = 0.026 $\pm$ 0.001 s⁻¹). *Hs*PHD2, by comparison, manifests a decreased affinity for the *Ta*ODD (*K_m*(*Hs*HIF1 α CODD, *Hs*PHD2) = 10.8 $\pm$ 1.9 μ M, *K_m*(*Ta*ODD, *Hs*PHD2) = 40.5 $\pm$ 4.9 μ M). *Hs*PHD2, however, catalysed hydroxylation of *Hs*HIF1 α CODD and *Ta*ODD with comparable efficiency (*k_{cat}* (*Hs*HIF1 α CODD, *Hs*PHD2) = 0.027 $\pm$ 0.001 s⁻¹, *k_{cat}* (*Ta*ODD, *Hs*PHD2) = 0.035 $\pm$ 0.002 s⁻¹).

Notably, the affinity of 2OG for *Ta*PHD (*K_m*(2OG, *Ta*PHD) = 1.9 $\pm$ 0.3 μ M) is higher than for *Hs*PHD2 (*K_m*(2OG, *Hs*PHD2) = 15.8 $\pm$ 2.6 μ M), as judged by *K_m* comparison. The higher affinity of 2OG for *Ta*PHD is in accord with the observation of formation of a stable *Ta*PHD.Fe.2OG complex, with a half-life > 24 h (1). It is also consistent with the crystallographic finding of a highly buried 2OG-binding pocket at the active site of *Ta*PHD.

The kinetic parameters reported here for the affinity of *Hs*HIF1 α CODD and 2OG for *Hs*PHD2 are in agreement with previous studies ((17), *K_m*(*Hs*HIF1 α CODD, *Hs*PHD2) = 9 $\pm$ 3 μ M), *K_m*(2OG, *Hs*PHD2) = 13 $\pm$ 2 μ M)).

Table 5.1 Steady-state kinetic parameters of *Ta*PHD and *Hs*PHD2.

	<i>Ta</i>PHD	<i>Hs</i>PHD2
<i>K_m</i> (<i>Ta</i> ODD), μ M	11.5 $\pm$ 1.8	40.5 $\pm$ 4.9
<i>k_{cat}</i> (<i>Ta</i> ODD), s ⁻¹	0.026 $\pm$ 0.001	0.035 $\pm$ 0.00
<i>K_m</i> (<i>Hs</i> HIF1 α CODD), μ M	15.1 $\pm$ 2.2	10.8 $\pm$ 1.9
<i>k_{cat}</i> (<i>Hs</i> HIF1 α CODD), s ⁻¹	0.012 $\pm$ 0.001	0.027 $\pm$ 0.001
<i>K_m</i> (2OG), μ M	1.9 $\pm$ 0.3	15.8 $\pm$ 2.6

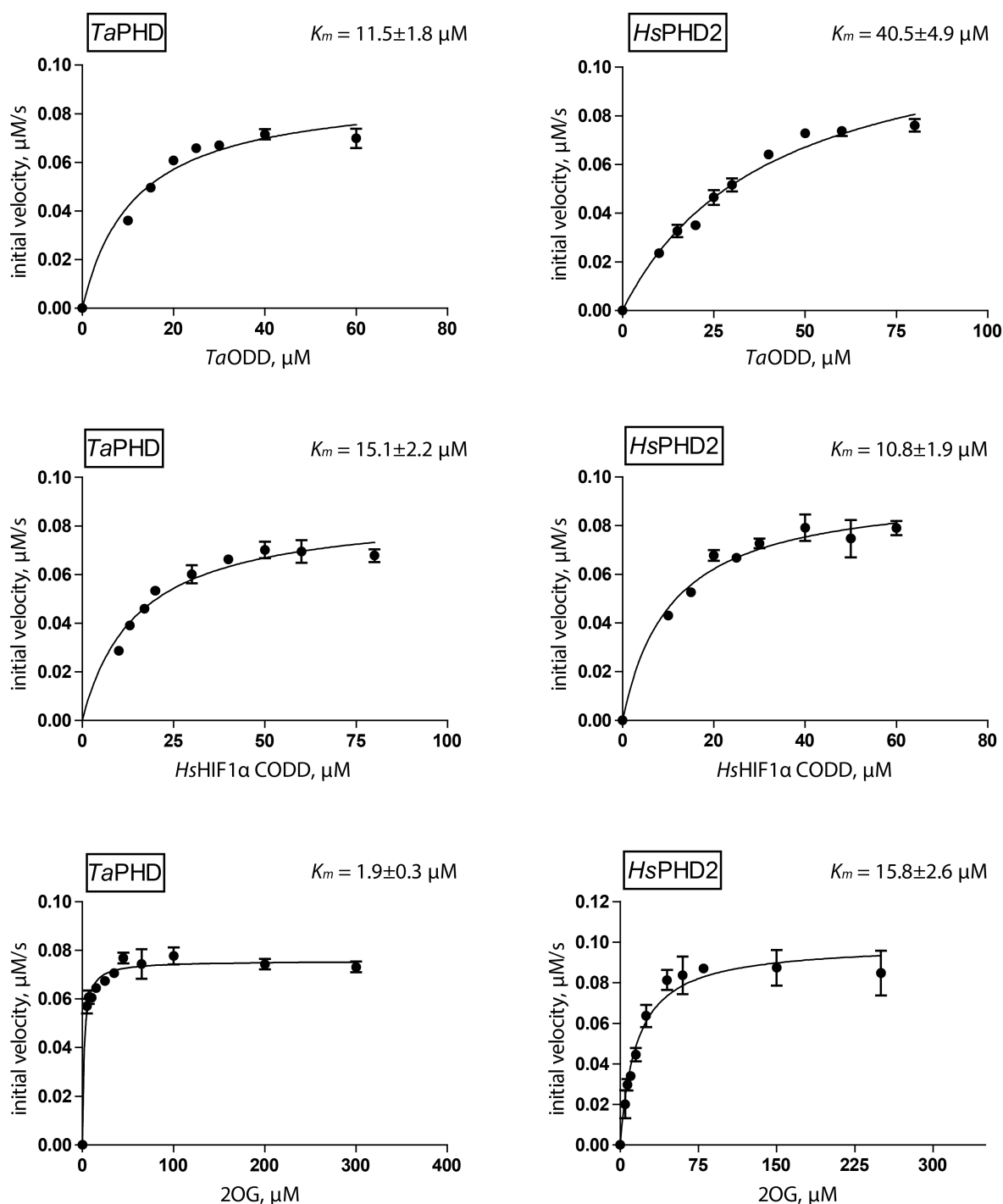


Figure 5.9: Comparison of kinetic parameters for *TaPHD* and *HsPHD2*. Conditions: 3.5 μM *HsPHD2* or *TaPHD*, 100 μM *Hs*HIF1α CODD 19mer peptide or 100 μM *Ta*HIFα ODD peptide, 50 μM (NH₄)₂Fe(II)(SO₄)₂, 4 mM sodium L-ascorbate and 300 μM 2-oxoglutarate disodium salt in 50 mM Tris, pH 7.5. Initial rates were determined by varying the concentration of the respective peptide or 2OG. Peptide hydroxylation was analysed by MALDI-TOF-MS, non-enzymatic methionine oxidation was subtracted, and the data were fitted with the Michaelis-Menten equation using GraphPad Prism (errors are indicated as standard deviations, n=3).

5.2.2.3 Substrate binding studies on *Ta*PHD

To investigate the extent to which *Ta*PHD can bind *Hs*HIF1 α NODD and CODD, 1D CLIP HSQC NMR binding studies with *Hs*HIF1 α CODD and *Hs*HIF1 α NODD peptides labelled with [^{13}C] at the hydroxylation target prolyl residues were performed. The [^{13}C]-labelled ‘reporter’ *Hs*HIF1 α CODD/NODD peptides were incubated with 2OG in the presence and absence of *Ta*PHD at a 1:1 ratio of enzyme:ODD, as described in *Materials and Methods*, **Chapter 7.7.2**). The decrease of the reporter signal upon binding of the peptide to the enzyme was measured to investigate the relative binding strength (**Figure 5.10**, supplementary spectra in *Appendix*, **Figure 5.13**). The results revealed that *Ta*PHD strongly discriminates between binding of *Hs*HIF1 α CODD and *Hs*HIF1 α NODD, as ~ 40 % of the *Hs*HIF1 α NODD peptide remained unbound, whilst *Hs*HIF1 α CODD was apparently completely bound to *Ta*PHD. The finding that *Ta*PHD binds *Hs*HIF1 α NODD less efficiently than *Hs*HIF1 α NODD is in agreement with the observed low turnover of *Hs*HIF1 α NODD by the enzyme.

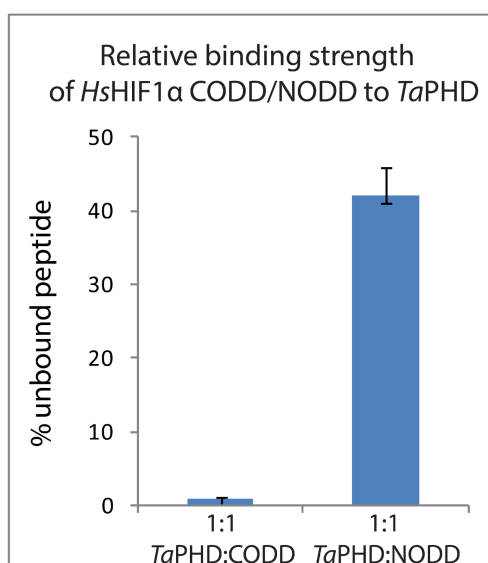


Figure 5.10: 1D CLIP HSQC [^{13}C]- *Hs*HIF1 α CODD and [^{13}C]- *Hs*HIF1 α NODD displacement experiments. Conditions: 50 μM [^{13}C]-proline *Hs*HIF1 α CODD/NODD), 50 μM *Ta*PHD, 50 μM 2-oxoglutarate disodium salt in 50 mM Tris- D_{11} , pH 7.5, 10 % D_2O and 90 % H_2O . (errors are indicated as standard deviations, $n=3$).

5.2.2.4 Monitoring the coupled and uncoupled 2OG turnover of *Ta*PHD and *Hs*PHD2

The extent to which 2OG dependent oxygenases catalyse the uncoupled (i.e. in absence of substrate) turnover of 2OG to succinate and CO₂ varies (11). In the case of the HIF hydroxylases, 2OG decarboxylation by *Hs*PHD2 is strongly coupled to CODD hydroxylation, whilst FIH catalyses 2OG decarboxylation in the absence of a substrate to a higher extent (13). The findings are supported by the observation of an (especially) stable *Hs*PHD2.Fe.2OG complex (17).

To directly compare the extent of uncoupled turnover in *Ta*PHD and *Hs*PHD2, succinate formation and 2OG depletion were monitored by ¹H-NMR in the presence and absence of their respective natural substrates (**Figure 5.11**, supplementary spectra in *Appendix*, **Figure 5.14**, *Materials and Methods*, **Chapter 7.7.1**). The results demonstrate that the rate of uncoupled turnover is very low for both *Ta*PHD⁶⁴⁻³⁰⁰ and *Hs*PHD2¹⁸¹⁻⁴²⁶; 2OG decarboxylation is strongly increased in presence of substrate peptide, implying conservation of important kinetic properties between *Hs*PHD2 and *Ta*PHD. Overall, these results support the proposal that the strong binding of Fe(II) and 2OG, and the formation of a stable PHD.Fe.2OG in the absence of HIF α substrates, are conserved properties of the PHDs in humans and *T. adhaerens*.

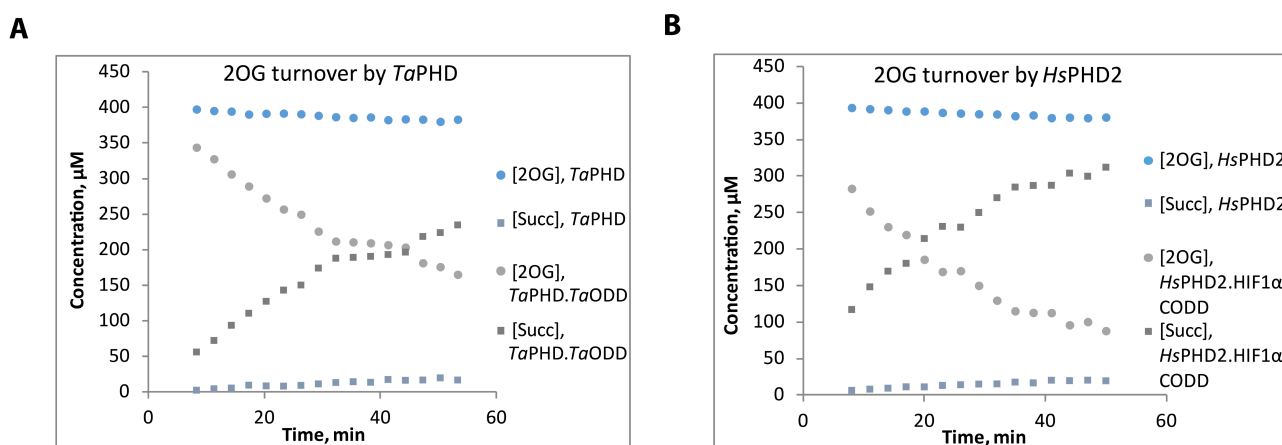


Figure 5.11: Comparison of coupled and uncoupled (i.e. in the absence of substrate) 2OG turnover by (A) *Ta*PHD and (B) *Hs*PHD2. 2OG turnover was monitored by ¹H CPMG NMR, conditions: 20 μ M *Ta*PHD or *Hs*PHD2, 125 μ M (NH₄)₂Fe(II)(SO₄)₂, 1 mM sodium L-ascorbate, 500 μ M *Hs*HIF1 α CODD or *Ta*HIF α ODD substrate where necessary, 400 μ M 2-oxoglutarate disodium salt, in 50 mM Tris-D₁₁, pH 7.5, 10 % D₂O and 90 % H₂O; [Succ]=Succinate.

5.2.2.5 Steady-state oxygen dependence of *Ta*PHD

In order for the PHDs to act as hypoxia sensors in cells, their activity must be limited by oxygen availability. This role is proposed to be reflected in the slow reaction of the *Hs*PHD2.CODD/NODD.Fe.2OG complex with oxygen (13, 17), and manifests in the unusually high K_m value of *Hs*PHD2 for oxygen ($K_m(\text{O}_2, \text{PHD}) > 400 \mu\text{M}$), when compared to other 2OG oxygenases (2, 17-18). To investigate the O_2 dependence of the *Ta*PHD-catalysed reaction, steady-state kinetic studies, using reported experimental conditions for *Hs*PHD2 (17), were conducted (see *Materials and Methods*, **Chapter 7.6.1**). A solution of *Ta*ODD 25mer peptide was pre-equilibrated in sealed glass vials under different O_2/N_2 ratios by use of mass-flow controllers, prior to addition of co-factors and *Ta*PHD enzyme. Initial rates of *Ta*ODD hydroxylation at varied O_2 concentrations were determined by quenching the assay mixture at a defined time point in the linear range of enzyme activity, and subsequent analysis by MALDI-TOF-MS.

No saturation of O_2 was reached for the *Ta*PHD-catalysed reaction with *Ta*ODD at 60 % O_2 level, resulting in a $K_m(\text{O}_2, \text{TaPHD}) > 400 \mu\text{M}$ (**Figure 5.12 A**). This result suggests that *Ta*PHD has the potential to react to small changes in atmospheric O_2 partial pressure and could act as an effective oxygen sensor, indicating that the core biochemical features of *Hs*PHD2 in the human oxygen sensing machinery are conserved in *Ta*PHD.

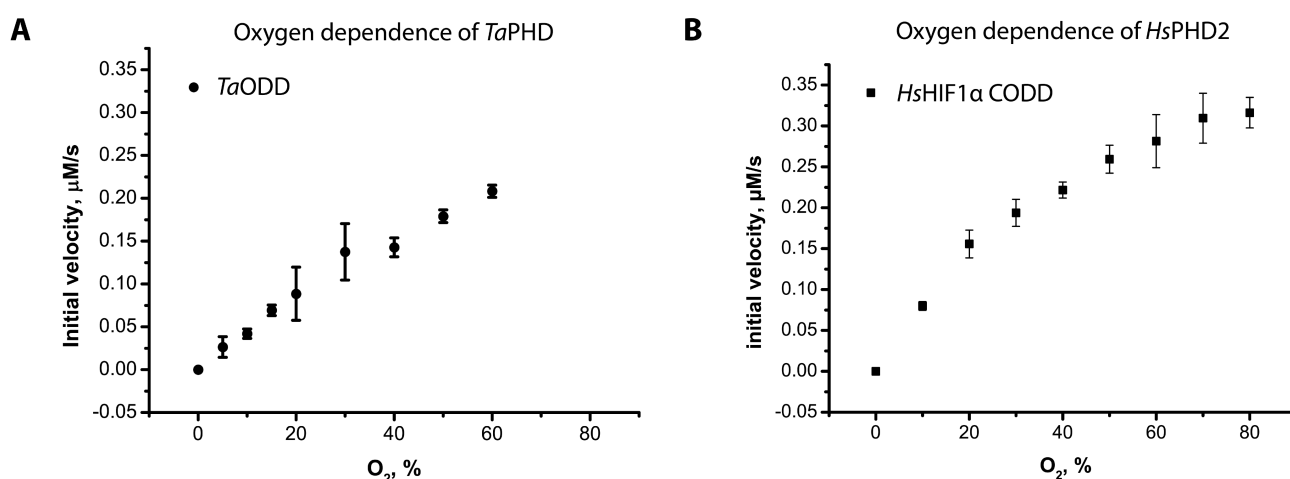


Figure 5.12: Steady-state O_2 -dependence of (A) *Ta*PHD and (B) *Hs*PHD2 (published in (17)).

Conditions: 4 μM *Ta*PHD/*Hs*PHD2, 100 μM *Ta*HIF α ODD/*Hs*HIF1 α CODD, 50 μM $(\text{NH}_4)_2\text{Fe}(\text{II})(\text{SO}_4)_2$, 300 μM 2-oxoglutarate disodium salt and 4mM sodium L-ascorbate, in 50 mM Tris, pH 7.5 were incubated at 37°C under different % O_2 . The extent of hydroxylation was analysed by MALDI-TOF-MS (errors are indicated as standard deviations, $n=3$).

Another unusual kinetic feature of *Hs*PHD2 is manifested in its relatively slow reaction with oxygen, compared to other 2-oxoglutarate oxygenases, as determined by stopped flow/UV-visible spectroscopy experiments (13, 17). It was aimed to conduct a similar study on *Ta*PHD, to underpin the characteristic kinetic properties of both *Hs*PHD2 and *Ta*PHD. However, neither *Ta*PHD⁶⁴⁻³⁰⁰, nor other tested constructs *Ta*PHD¹⁻³⁰⁰ and *Ta*PHD⁷⁹⁻³⁰⁰, could reach a sufficiently high protein concentration for the study, likely due to aggregation of the protein.

5.3 Conclusions

The development of hypoxia sensing mechanisms likely played a major role in the evolution of (complex) aerobic organisms, that rely on a steady oxygen supply for their energy metabolism (19). In higher animals, hypoxia sensing is achieved through the HIF pathway, that requires a functional HIF α -PHD-pVHL triad. Bioinformatic analyses revealed that the HIF α -PHD-pVHL triad exists in all animals ranging from humans to the simplest existent animal *T. adhaerens*, yet have not provided evidence for HIF transcription factors beyond animals (1). By contrast, PHD like enzymes are much more widely distributed than animals (1, 6, 20).

The aim of the study presented in this Chapter was to explore the biological distribution of hypoxia/oxygen sensing HIF prolyl hydroxylases among animals. Further, the structural relationship between HIF prolyl hydroxylases, an early evolutionary homolog in *P. putida*, and a member of the pro-collagen prolyl hydroxylase family from *C. reinhardtii* was investigated.

Central to the hypoxia sensing ability of the human PHDs is, that their activity in cells can be limited by oxygen availability (17, 21). It is proposed that the slow reaction with oxygen at the active site Fe(II) (as observed for *Hs*PHD2, (13)), plays a substantial role in the hypoxia sensing ability of the enzymes. Additionally, unusually high *K_m* values of *Hs*PHD2 for oxygen have been observed (13, 17). By contrast, there is evidence that the human PHDs are relatively less sensitive to changes in iron or 2OG availability (22). The combined structural and biochemical studies presented in this Chapter provide strong evidence that these apparently special properties of the human PHDs (especially *Hs*PHD2) are conserved in the *Ta*PHD. Crucially, the structural elements which enable the unusually slow reaction of *Hs*PHD2 with oxygen (12-13), (as well as tight 2OG/Fe(II) binding and low substrate uncoupled turnover), were observed in the *Ta*PHD crystal structures (**Figure 5.2**). Comparative kinetic studies revealed that *Ta*PHD, like *Hs*PHD2, is highly sensitive to changes in oxygen concentrations (**Figure 5.12**), yet is substantially less sensitive to changes in 2OG levels. Indeed, the affinity of 2OG for *Ta*PHD is 8-fold higher than for *Hs*PHD2 as judged by *K_m* values (**Table 5.1**), suggesting an exceptional stability of the *Ta*PHD.Fe.2OG complex. This proposal is further supported by the finding of a low rate of uncoupled 2OG turnover by *Ta*PHD in the absence of its prime substrate (**Figure 5.11**). Since humans and *T. adhaerens* are at opposite ends of animal evolution, it seems possible that PHDs in organisms between them will have similar kinetic properties, that will enable them to efficiently sense hypoxia in cells.

Comparison of the ODD binding modes in *Ta*PHD and *Hs*PHD2 revealed that the elements involved in substrate binding, in particular the $\beta 2/\beta 3$ -finger-loop and the C-terminal helix, are conserved between the two enzymes (**Figure 5.3**). In addition, it was found that the interactions between *Ta*PHD and *Ta*ODD more closely resemble those of *Hs*PHD2 and *Hs*HIF1 α CODD, rather than those of *Hs*PHD2 and *Hs*HIF1 α NODD (**Figure 5.3**). This finding is interesting from an evolutionary point of view, since bioinformatic analyses suggest that CODD-like sequences are more widely distributed among lower (non-bilateral) animals, than NODD-like sequences, leading to the proposal that CODD evolved before NODD (see *General Introduction*, **Chapter 1.5**, **Figure 1.14** (1)).

Structural comparison of *T. adhaerens* and human PHDs with prolyl-4-hydroxylases from *P. putida* and *C. reinhardtii*, revealed a high degree of conservation in the overall folds of the catalytic domains, including with respect to the major substrate binding elements, such as the $\beta 2/\beta 3$ -finger-loop (**Figure 5.4** and **5.5**). In the active site, the target prolyl residues of the HIF and non-HIF substrates all adopt the C-4 *endo*-conformation (**Figure 5.6**). Crucially, major features that enable the oxygen sensing capability of PHDs are conserved in *P. putida* PPHD (**Figure 5.4 A**). This finding is in agreement with the proposed role of PPHD as an early evolutionary homolog of HIF prolyl hydroxylases (6). To further explore the evolution of oxygen sensing HIF prolyl hydroxylases in the future, the study of the PHD-like enzyme in *M. brevicollis* is of great interest. *M. brevicollis* constitutes a key organisms, as it is closely related to animals, but lacks the HIF transcription factors that are essential for hypoxia sensing in animals. In this respect, the identification of the substrate of the PHD-like enzyme in *M. brevicollis* could provide an important link for understanding the evolution of oxygen sensing prolyl hydroxylases.

5.4 References

- (1) Loenarz, C., Coleman, M. L., Boleining, A., Schierwater, B., Holland, P. W. H., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Reports*, 12(1), 63–70.
- (2) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (3) Huang, J., Zhao, Q., Mooney, S. M., & Lee, F. S. (2002). Sequence Determinants in Hypoxia-inducible Factor-1 α for Hydroxylation by the Prolyl Hydroxylases PHD1, PHD2, and PHD3. *Journal of Biological Chemistry*, 277(42), 39792–39800.
- (4) Anna Boleining, (2012). Evolution of PHDs as oxygen sensors: mechanistic and structural studies of the PHD of *Trichoplax adhaerens*, the simplest animal, and mechanistic studies of a PHD-like enzyme of the protist *Monosiga brevicollis*. DPhil. University of Oxford.
- (5) Koski, M. K., Hieta, R., Hirsilä, M., Rönkä, A., Myllyharju, J., & Wierenga, R. K. (2009). The Crystal Structure of an Algal Prolyl 4-Hydroxylase Complexed with a Proline-rich Peptide Reveals a Novel Buried Tripeptide Binding Motif. *The Journal of Biological Chemistry*, 284(37), 25290–25301.
- (6) Scotti, J. S., Leung, I. K. H., Ge, W., Bentley, M. A., Paps, J., Kramer, H. B., ... Schofield, C. J. (2014). Human oxygen sensing may have origins in prokaryotic elongation factor Tu prolyl-hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America*, 111(37), 13331–13336.
- (7) Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7, 539.
- (8) Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25(9), 1189–1191.
- (9) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (10) Aik, W. S., Chowdhury, R., Clifton, I. J., Hopkinson, R. J., Leissing, T., McDonough, M. A., ... Walport, L. J. (2015). CHAPTER 2 Introduction to Structural Studies on 2-Oxoglutarate-Dependent Oxygenases and Related Enzymes. In *2-Oxoglutarate-Dependent Oxygenases* (pp. 59–94). The Royal Society of Chemistry.

- (11) Hausinger, R. P. (2004). Fe(II)/ α -Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 39(1), 21–68.
- (12) Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., ... Schofield, C. J. (2009). Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*, 17(7), 981–989.
- (13) Flashman, E., Hoffart, L. M., Hamed, R. B., Bollinger, J. M., Krebs, C., & Schofield, C. J. (2010). Evidence for the Slow Reaction of Hypoxia-Inducible Factor Prolyl Hydroxylase 2 with Oxygen. *The FEBS Journal*, 277(19), 4089–4099.
- (14) Chowdhury, R., Leung, I. K. H., Tian, Y.-M., Abboud, M. I., Ge, W., Domene, C., ... Schofield, C. J. (2016). Structural basis for oxygen degradation domain selectivity of the HIF prolyl hydroxylases. *Nature Communications*, 7, 12673.
- (15) Flashman, E., Bagg, E. A. L., Chowdhury, R., Mecinović, J., Loenarz, C., McDonough, M. A., ... Schofield, C. J. (2008). Kinetic Rationale for Selectivity toward N- and C-terminal Oxygen-dependent Degradation Domain Substrates Mediated by a Loop Region of Hypoxia-Inducible Factor Prolyl Hydroxylases. *Journal of Biological Chemistry*, 283(7), 3808–3815.
- (16) Min, J.-H., Yang, H., Ivan, M., Gertler, F., Kaelin, W. G., & Pavletich, N. P. (2002). Structure of an HIF-1 α -pVHL Complex: Hydroxyproline Recognition in Signaling. *Science*, 296(5574), 1886–1889.
- (17) Tarhonskaya, H., Chowdhury, R., Leung, I. K. H., Loik, N. D., McCullagh, J. S. O., Claridge, T. D. W., ... Flashman, E. (2014). Investigating the contribution of the active site environment to the slow reaction of hypoxia-inducible factor prolyl hydroxylase domain 2 with oxygen. *Biochemical Journal*, 463(3), 363–372.
- (18) Ehrismann, D., Flashman, E., Genn, D. N., Mathioudakis, N., Hewitson, K. S., Ratcliffe, P. J., & Schofield, C. J. (2007). Studies on the activity of the hypoxia-inducible-factor hydroxylases using an oxygen consumption assay. *Biochemical Journal*, 401(Pt 1), 227–234.
- (19) Taylor, C. T., & McElwain, J. C. (2010). Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans. *Physiology*, 25(5), 272–279.
- (20) van der Wel, H., Ercan, A., & West, C. M. (2005). The Skp1 Prolyl Hydroxylase from Dictyostelium Is Related to the Hypoxia-inducible Factor- α Class of Animal Prolyl 4-Hydroxylases. *Journal of Biological Chemistry*, 280(15), 14645–14655.
- (21) Tian, Y.-M., Yeoh, K. K., Lee, M. K., Eriksson, T., Kessler, B. M., Kramer, H. B., ... Ratcliffe, P. J. (2011). Differential Sensitivity of Hypoxia Inducible Factor Hydroxylation Sites to Hypoxia and Hydroxylase Inhibitors. *The Journal of Biological Chemistry*, 286(15), 13041–13051.

- (22) McNeill, L. A., Flashman, E., Buck, M. R. G., Hewitson, K. S., Clifton, I. J., Jeschke, G., ... Schofield, C. J. (2005). Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. *Molecular BioSystems*, 1(4), 321–324.

5.5 Appendix

Table 5.2 Hydrogen bonds and hydrophobic interactions between *Ta*PHD and *Ta*ODD, as observed in the *Ta*PHD.*Ta*ODD structure, compared to *Hs*PHD2.CODD (PDB:3HQR) and *Hs*PHD2.NODD (PDB:5L9V) structures (hydrophobic interactions were defined with a cut-off at 4 Å distance).

<i>Ta</i> PHD. <i>Ta</i> ODD		<i>Hs</i> PHD2.CODD		<i>Hs</i> PHD2.NODD		Structural region in <i>Ta</i> PHD/ <i>Hs</i> PHD2
Protein	Substrate	Protein	Substrate	Protein	Substrate	
Gln137	Phe487*†	Gln239	Tyr565*†	Gln239	Ala403*†	β2/β3
		Leu240	Tyr565*	Leu240	Pro402*	β2/β3
Ala139	Ala485*† Phe487*	Val241	Ala563*†	Val241	Thr398* Ala401*† Pro402*	β2/β3
		Ser242	Glu560*†	Ser242	Thr398*† Leu399*	β2/β3
		Lys244	Met561*	Lys244	Leu399*	β2/β3
Asn141	Phe487*					β2/β3
Ile147	Leu484*	Ile251	Leu562*	Ile251	Leu400*	β2/β3
H2O- Arg148NH	Phe487†	H2O- Arg252NH	Tyr565†	H2O- Arg252NH	Ala403†	β2/β3
Trp154	Phe487* Pro489*	Trp258	Ile566* Pro567*			β3
Asp173	Leu497*			Asp277	Leu411*	α3
		Ile280	Leu574*			α3
		Arg281	Leu574†	Cys281 ^{mutated}	Cys412 ^X	α3
Ile188	Leu497*	Ile292	Leu574*			α3/β4
Thr189	Leu497*†	Asn293	Leu574*†	Asn293	Leu411*†	α3/β4
Gly190	Leu497*	Gly294	Leu574*	Gly294	Ile408* Leu411*	α3/β4
Arg191	Asn495*† Leu497*	Arg295	Phe572† Leu574*	Arg295	Ile409*†	α3/β4
Thr192	Val488*					α3/β4
Lys193	Phe493*	Lys297	Asp570*	Lys297	Thr407* Ile409*	α3/β4
Tyr206	Leu484*† Pro486*†	Tyr310	Leu562† Pro564†	Tyr310	Leu400* † Pro402*	β5

<i>Ta</i> PHD. <i>Ta</i> ODD		<i>Hs</i> PHD2.CODD		<i>Hs</i> PHD2.NODD		Structural region in <i>Ta</i> PHD/ <i>Hs</i> PHD2
Protein	Substrate	Protein	Substrate	Protein	Substrate	
Ile207	Leu484*	Val311	Leu562*	Val311	Leu400*	β5
Arg208	Tyr481*†					β5
His209	Tyr481*	His313	Leu562*	His313	Leu400*	β5
	Leu484*		Pro564*		Pro402*	
	Pro486*					
Ile210	Tyr481*	Val314	Leu559*			β5
Asp211	Pro486*	Asp315	Pro564*	Asp315	Pro402*	β5
Pro213	Tyr481*	Pro317	Leu559*	Cys317 ^{mutated}	Cys397 [×]	β5/β6
	Ala485*		Ala563*			
Asp216	Val488*					β5/β6
Arg218	Pro486†	Arg322	Pro564†	Arg322	Pro402†	β5/β6
	Val488*		Ile566*		Ala404*	
Arg266	Asp482†	Arg370	Leu559*	Arg370	Asp395†	β9/β10
Trp285	Pro486*	Trp389	Pro564*	Trp389	Pro402*	β11
			Ile566*			
Tyr286	Leu497*	Tyr390	Leu574*	Tyr390	Leu411*	β11
Phe287	Val488*	Phe391	Ile566*	Phe391	Ile408*	β11/α4
	Asp494*					
Arg292	Pro489†	Arg396	Pro567†	Arg396	Ile408*	α4
	Asp494†		Met568†			
			Asp571†			
Ser295	Pro491*					α4
Ser296	Pro491*	Lys400	Asp571†			α4
		Tyr403	Met568*			
		Leu404	Met568*			

* Hydrophobic interaction.

† Hydrogen bond.

× Disulfide bond.

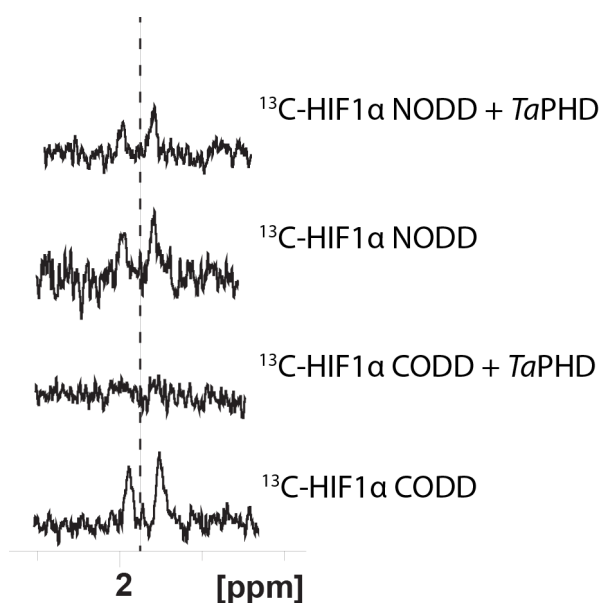


Figure 5.13: 1D CLIP HSQC ^{13}C - *HsHIF1α* CODD and ^{13}C - *HsHIF1α* NODD displacement experiments - supplementary spectra. For assay conditions, see legend of Figure 5.10.

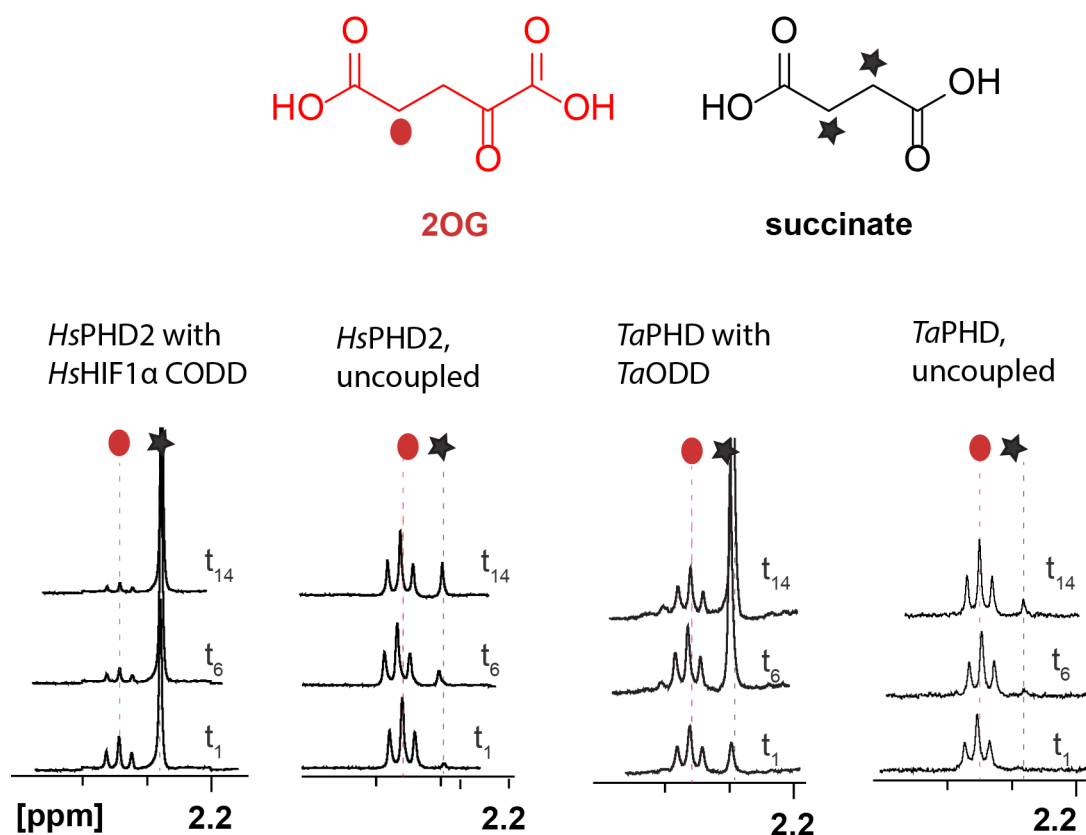


Figure 5.14: Comparison of coupled and uncoupled (i.e. in the absence of substrate) 2OG turnover by *TaPHD* and *HsPHD2* - supplementary spectra. For assay conditions, see legend of Figure 5.11.

Chapter 6

Interaction Studies between HsPHD2 and HsSkp1

6.1 Introduction

The S-phase kinase associated protein 1 (Skp1) is a crucial component of the oxygen sensing pathways in the amoeba *Dictyostelium discoideum* and the protozoan *Toxoplasma gondii* (see *General Introduction*, **Chapter 1.5**, (1)). Genome analyses on the phylogenetic distribution of Skp1 modification genes indicate that other protists and pathogens may also employ a Skp1-mediated mechanism to sense changes in oxygen levels, as genes encoding for PhyA and the glucose N-acetyltransferase Gnt1 are present in all major protist phyla (1).

Skp1 is part of the E3 Skp1-cullin 1-F-box protein (SCF) ubiquitin complex, that regulates the degradation of a large number of substrates *via* poly-ubiquitination (**Figure 6.1**, (1-2)). Recognition of the substrates involves members of the F-box protein (FBP) family, that serve as substrate adaptors (1-2). The primary role of Skp1 in the E3 SCF ubiquitin complex is to bind the F-box proteins, thus mediating substrate binding to the complex (1-2).

Studies in *D. discoideum* suggest that prolyl hydroxylation and subsequent glycosylation regulates FBP binding to Skp1, as two distinct FBPs (FbxD and FbxA) were identified that preferentially associate with glycosylated Skp1 compared to unmodified Skp1 (**Figure 6.1**, (1, 3)). Therefore, it is possible that the PhyA-mediated oxygen-dependent modification of Skp1 has an impact on the cell proteome in *D. discoideum* *via* regulating the degradation of E3 SCF ubiquitin complex substrate proteins (1, 3).

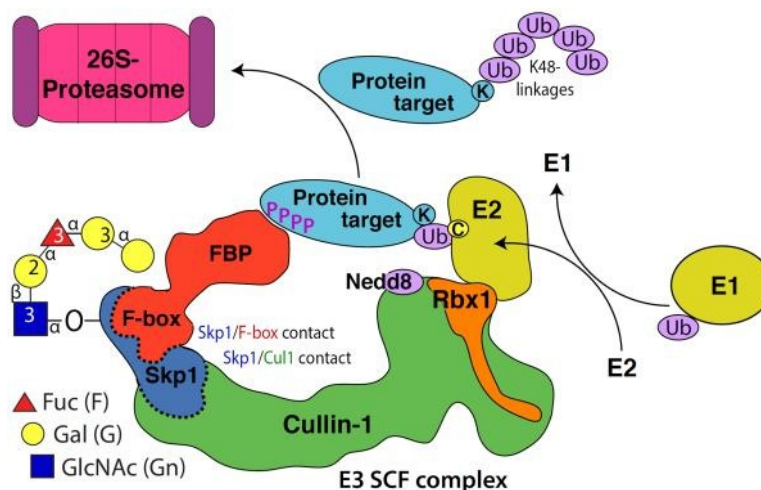


Figure 6.1: Composition of the E3 Skp1-cullin 1-F-box protein (SCF) ubiquitin complex (figure adapted from (1)). Protein acronyms: S-phase kinase associated protein 1 (Skp1), RING box protein 1 (Rbx1), F-box protein (FBP), Cullin box 1 (Cullin-1/ Cul 1), E1 ubiquitin-activating enzyme (E1), E2 ubiquitin-conjugating enzyme (E2), E3 ubiquitin ligase (E3). Modification acronyms: neural-precursor-cell-expressed developmentally down-regulated 8 (Nedd8), ubiquitin (Ub), fucose (Fuc), galactose (Gal), *N*-acetylglucosamine (GlcNAc). Skp1 hydroxylation and subsequent glycosylation likely regulates substrate recognition by the SCF complex in *D. discoideum*. Skp1 modifications occur near its C-terminus, and are proposed to promote interactions of FBPs with Skp1 (1, 3).

Skp1 is highly conserved between *T. gondii*, *D. discoideum*, and animals (**Figure 6.2**). The target proline residue for *DdPhyA*- and *TgPhA*-catalysed hydroxylation (P143 in *D. discoideum*, P148 in *T. gondii* (4-5)) is present in *T. adhaerens* and *D. melanogaster*, however, the corresponding proline residue is substituted by glutamate in most higher animals, including humans (**Figure 6.2**). The finding of a proline to glutamate mutation event that took place in the evolution of higher animals, implies that Skp1 from these organisms (likely) do not constitute substrates for prolyl-4-hydroxylation by PHDs or PHD-related enzymes. Consistent with this proposal, no hydroxylation of the human Skp1 (*HsSkp1*) by either human PHD2 (*HsPHD2*), or *Dd/TgPhyA* was observed in an ESI-LC-MS-based assay, conducted with recombinantly produced *HsPHD2*, *DdPhyA*, *TgPhyA*, and *HsSkp1* proteins (Tongri Liu, University of Oxford, UK, unpublished data).

<i>Toxoplasma gondii</i> Skp1	1	MSKERMGDARKVTLVSSQEGDEFDVDIEVASMSALIKTMVEEDSD	55
<i>Dictyostelium discoideum</i> Skp1	1	MSLVKLESSDEKVFIEKEIACMSVTIKNMIEDIGES	47
<i>Trichoplax adhaerens</i> Skp1	1	MDNKGNDSDKIILRSSDGFIEHKVDIKVAKMSATIKTMLEDLGVEV	57
<i>Drosophila melanogaster</i> Skp1	1	MPSIKLQSSDEEIFDTDIQIAKCSGTIKTMLEDGGMEDDE	50
<i>Homo sapiens</i> Skp1	1	MPSIKLQSSDGEIFEVDVEIAKQSVTIKTMLEDLGMDDEGDDDPVPLPNVN	51
<i>Xenopus laevis</i> Skp1	1	MPSIKLQSSDGEIFEVDVEIAKQSVTIKTMLEDLGMDDEGDDDPVPLPNVN	51
<i>Rattus norvegicus</i> Skp1	1	MPTIKLQSSDGEIFEVDVEIAKQSVTIKTMLEDLGMDDEGDDDPVPLPNVN	51
<i>Toxoplasma gondii</i> Skp1	56	TCILKKIIEYCEHHNNPPEEIPKPLKSSNLAEEVSEWDYQFINENSDQKILFALILAA	114
<i>Dictyostelium discoideum</i> Skp1	48	STILEKVLDDYCRHHQHPSPPQGGDDKKDEKRLDDIPPYDRDFCK	103
<i>Trichoplax adhaerens</i> Skp1	58	NAILELVVKKWAEHHKDDPPPPDDDDIREKRTDDIPVWDQEFLLK	113
<i>Drosophila melanogaster</i> Skp1	51	STILRKVLTWAHYHKDDPPQPTEDDESKEKRTDDISWDADFLK	106
<i>Homo sapiens</i> Skp1	52	AAILKKVIQWCTHHKDDPPPPEDDENKEKRTDDIPVWDQEFLLK	107
<i>Xenopus laevis</i> Skp1	52	AAILKKVIQWCTHHKDDPPPPEDDENKEKRTDDIPVWDQEFLLK	107
<i>Rattus norvegicus</i> Skp1	52	AAILKKVIQWCTHHKDDPPPPEDDENKEKRTDDIPVWDQEFLLK	107
<i>Toxoplasma gondii</i> Skp1	115	NYLNIKPLLDLSVAKVATMIKAKTPEEIRRFNIVNDFTPEEEAQVREENKWCEDA	170
<i>Dictyostelium discoideum</i> Skp1	104	NYLDIKPLLDVTCTVANMIRGKTPEEIRKIFNIKNDFTPEEEQIRKENEWCEDKGGN	162
<i>Trichoplax adhaerens</i> Skp1	114	NYLDIKGLLDVCTVANMIKGTPEEIRRTFNINKNDFTPEEEAQVRKENEWCEEK	169
<i>Drosophila melanogaster</i> Skp1	107	NYLDIKGLLELTCTVANMIKGTPEEIRKTFNIKNDFTPEEEAQVRKENEWCEEK	162
<i>Homo sapiens</i> Skp1	108	NYLDIKGLLDVTCTVANMIKGTPEEIRKTFNIKNDFTPEEEAQVRKENQWCEEK	163
<i>Xenopus laevis</i> Skp1	108	NYLDIKGLLDVTCTVANMIKGTPEEIRKTFNIKNDFTPEEEAQVRKENQWCEEK	163
<i>Rattus norvegicus</i> Skp1	108	NYLDIKGLLDVTCTVANMIKGTPEEIRKTFNIKNDFTPEEEAQVRKENQWCEEK	163

Figure 6.2: Comparison of Skp1 sequences from different organisms. The target proline residue for *Dd*PhyA- and *Tg*PhyA-catalysed hydroxylation is marked with a red box. The alignment was generated using Clustal Omega (6) and Jalview 2.9.0b2 (7).

Sequence comparisons of *Dd*/*Tg*PhyA with human and *T. adhaerens* HIF prolyl hydroxylases (**Figure 6.3**) reveal the conservation of crucial residues for catalytic activity (H191_{*Dd*PhyA}/H183_{*Tg*PhyA}, D193_{*Dd*PhyA}/D185_{*Tg*PhyA}, and H266_{*Dd*PhyA}/H242_{*Tg*PhyA}) and 2OG binding (R276_{*Dd*PhyA}/R252_{*Tg*PhyA}) in the 2OG-oxygenase domain of the PhyAs and PHDs. Given the (likely) evolutionary relationship between *Dd*/*Tg*PhyA and HIF prolyl hydroxylases in animals (see *General Introduction*, **Chapter 1.5**), and the high overall sequence conservation of Skp1 from protists to higher animals, it was hypothesised that *Hs*PHDs may be capable of interacting with *Hs*Skp1. Based on this hypothesis, the aims of the work described in this Chapter were:

1. Biochemical analysis of potential interactions between Skp1 and *Hs*PHDs.
2. Investigations on a potential *Hs*Skp1-*Hs*PHD2 interaction in human cells, by conducting immunoprecipitation experiments and hypoxia response element (HRE) luciferase reporter assays.

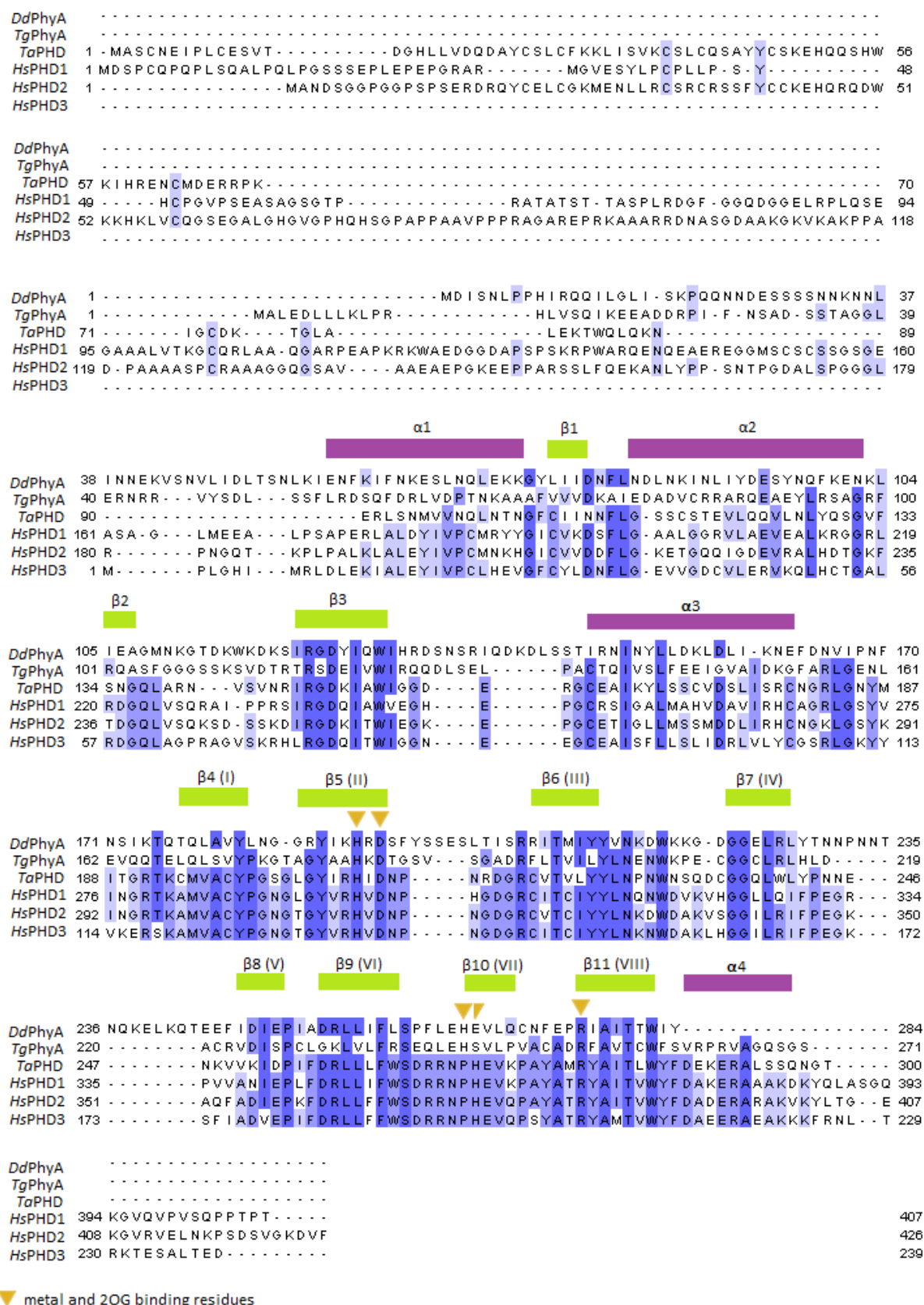


Figure 6.3: Sequence comparison of *Dd*Skp1, *Tg*Skp1, *Ta*PHD and human PHD1-3. α -Helices are marked purple, and β -sheets are labelled with green arrows. The alignment was generated using Clustal Omega (6) and Jalview 2.9.0b2 (7).

6.2 Results and Discussion

6.2.1 Competition of *Hs*Skp1 with HIF α hydroxylation by *Hs*PHD2 and *Hs*PHD3

To investigate if Skp1 competes with HIF1 α CODD/NODD for binding to PHDs, competition experiments with *Hs*PHD2 and *Hs*PHD3 (tPHD2 and PHD3) were performed. Full-length *Hs*Skp1, *Dd*Skp1, and *Tg*Skp1 protein was kindly provided for the experiment by Tongri Liu (University of Oxford, UK). The same assay conditions as described in **Chapter 2.2.2** and *Materials and Methods*, **Chapter 7.6.2**, were used. A HIF1 α CODD/NODD : Skp1 ratio of 1 : 6 was chosen in the initial competition experiments. Hydroxylation levels in the presence and absence of Skp1 were determined by ESI-LC-MS as described (*Materials and Methods*, **Chapter 7.6.2**).

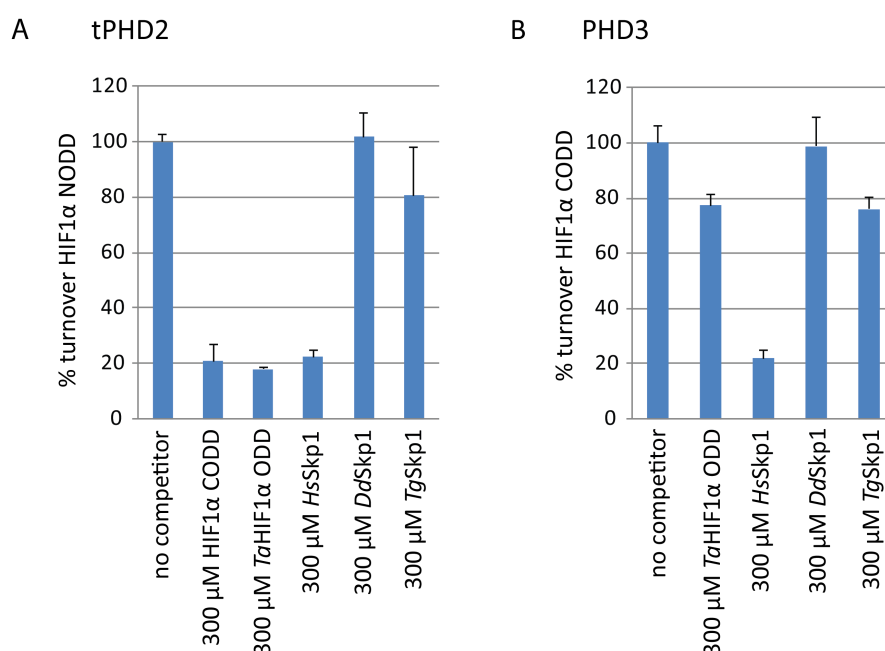


Figure 6.4: Competition of Skp1 with HIF1 α CODD/NODD hydroxylation by tPHD2 and PHD3. Full-length Skp1 proteins were screened for their ability to reduce PHD-catalysed HIF-hydroxylation levels. **(A)** HIF1 α NODD hydroxylation by tPHD2, **(B)** HIF1 α CODD hydroxylation by PHD3. Conditions: 2 μ M tPHD2 or PHD3, 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 300 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate, 50 μ M HIF1 α CODD/NODD, and 300 μ M *Hs*/*Dd*/*Tg*Skp1 or 300 μ M HIF1 α CODD/*Ta*HIF1 α ODD in 50 mM Tris, pH 7.5. The samples were quenched with 1% (v/v) FA at a time point in the linear region of enzyme activity, and analysed by electrospray ionization liquid chromatography–mass spectrometry (ESI-LC-MS). Hydroxylation levels were quantified using MassLynx™ V4.1, non-enzymatic background oxidation was subtracted, and data was normalised. Errors are depicted as standard deviations (n=3).

The results of the competition experiments are presented in **Figure 6.4** (see *Appendix*, **Figure 6.8**, for ESI-LC-MS spectra). As expected, HIF1 α NODD hydroxylation by tPHD2 was decreased to $\leq 20\%$ in the two control samples containing 300 μM HIF1 α CODD/*Ta*HIF1 α ODD (**Figure 6.4 A**). Similarly, tPHD2-catalysed HIF1 α NODD hydroxylation levels were decreased to $\sim 20\%$ in the presence of 300 μM *Hs*Skp1, when compared to samples without competitor. By contrast, *Dd*Skp1 did not affect tPHD2-catalysed HIF1 α NODD hydroxylation, and *Tg*Skp1 only caused a decrease to $\sim 80\%$ hydroxylation when compared to samples without competitor. Comparable results were obtained for PHD3-catalysed HIF1 α CODD hydroxylation levels (**Figure 6.4 B**). The strongest effect was observed in the presence of 300 μM *Hs*Skp1 (decrease in hydroxylation levels to $\sim 20\%$ compared to samples without competitor). Notably, PHD3-catalysed HIF1 α CODD hydroxylation levels were only slightly decreased in the presence of *Ta*HIF1 α ODD (hydroxylation levels $\sim 80\%$ compared to samples without competitor), suggesting that *Hs*Skp1, more strongly than *Ta*HIF1 α ODD, competes with HIF1 α CODD hydroxylation by PHD3. However, it is possible that the observed differences between *Hs*Skp1 and *Ta*HIF1 α ODD in terms of competition with HIF1 α CODD hydroxylation are due to different lengths of the substrates (full-length *Hs*Skp1 protein substrate vs. 25mer *Ta*HIF1 α ODD peptidic substrate).

6.2.2 HRE luciferase reporter assay

Following up on the results from *in vitro* competition experiments, it was investigated if *HsSkp1* is capable of stabilising HIF α in a cellular environment. For this aim, *HsSkp1* (hereafter: Skp1) was overexpressed in HeLa cells, and the transcriptional activity of HIF α was determined using a hypoxia response element (HRE) luciferase reporter assay.

Luciferase reporter assays are commonly used tools for quantifying the transcriptional activity of proteins of interest (8). A reporter construct consisting of a quantifiable reporter gene (luciferase/Luc) fused to an upstream promotor or enhancer sequence of interest (such as HRE), is transiently transfected into a cellular system (8). Binding of the protein of interest (such as HIF α) to the promotor or enhancer sequence activates the transcription of the luciferase gene (8). The luciferase reporter is quantified by measuring the bioluminescence signal resulting from the reaction of luciferase with its substrate luciferin (9).

In order to overexpress Skp1 in HeLa cells, the DNA sequence of Skp1 was first cloned into a pcDNA3 vector as described in *Materials and Methods*, **Chapter 7.1.1.3**. A C-terminal FLAG-tag was attached to the Skp1 sequence to make Skp1 detectable by Anti-FLAG antibodies.

HeLa cells were grown to ~50% confluence before they were transiently transfected with a carbonic anhydrase 9 (CA9) hypoxia response element (HRE) luciferase reporter construct (10), β -galactosidase (β gal) in a pCMV vector (for correcting variable transfection efficiencies between samples), and FLAG-tagged or untagged Skp1 in pcDNA3 (**Table 6.1**, *Materials and Methods*, **Chapter 7.11.2 and 7.11.3**). Control cells were transfected with a simian virus 40 (SV40) luciferase reporter construct instead of the CA9 HRE luciferase reporter construct.

Table 6.1 Experimental setup of HRE luciferase reporter assays.

Sample name	Transfected with	Additives added
Empty vector (EV) control	CA9 HRE luciferase reporter construct pCMV-βgal pcDNA3	-
Skp1	CA9 HRE luciferase reporter construct pCMV-βgal pcDNA3-Skp1	-
Skp1-FLAG	CA9 HRE luciferase reporter construct pCMV-βgal pcDNA3-Skp1-FLAG	-
EV + DMSO	CA9 HRE luciferase reporter construct pCMV-βgal pcDNA3	1 mM DMSO

Transfected HeLa cells were grown for 24 h at normoxia or 1 % O₂ (hypoxia). Subsequently, the cells were harvested, lysed and the luciferase and β-galactosidase activity in samples of cellular supernatant was quantified as described (*Materials and Methods*, **Chapter 7.11.3**, (9, 11)). In order to correct for variable transfection efficiencies between different samples, the ratio of luciferase : β-galactosidase signals was determined.

The results of the HRE luciferase reporter assays are presented in **Figure 6.5**. The transcriptional activity of HIFα as determined from the luciferase reporter signal was strongly increased in all samples under hypoxic conditions (~ 2 x 10⁶ relative luminescence units (RLU)), when compared to normoxia (signal below background level). By contrast, in the SV40 luciferase control samples, no substantial difference between samples incubated under normoxic vs hypoxic conditions was observed.

The maximum transcriptional activity of HIFα was obtained in samples containing the PHD inhibitor DMSO, and stabilisation of HIFα by DMSO was observed under hypoxic and normoxic conditions.

Although hypoxia and DMSO clearly induced HIFα transcriptional activity, no such effect was found upon overexpressing FLAG-tagged or untagged Skp1, as the luciferase signal is unchanged by Skp1 overexpression both under hypoxic and normoxic conditions, when compared to empty vector (EV) control samples. Therefore, the results of the HRE luciferase reporter assays imply that Skp1 does not stabilise HIFα in HeLa cells under the used assay conditions.

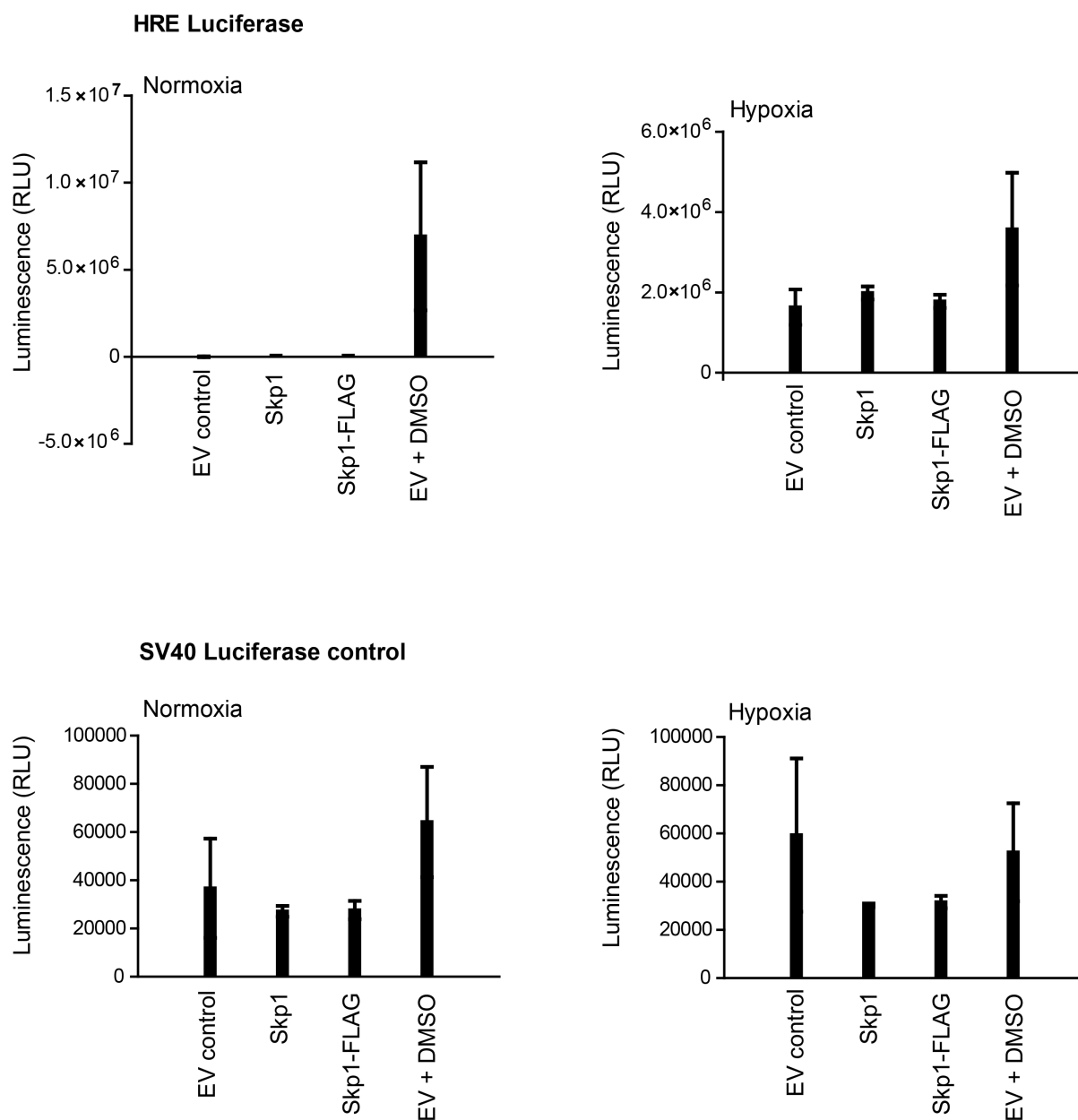


Figure 6.5: HRE luciferase reporter assays. HeLa cells were transfected with CA9 HRE luciferase reporter construct, β gal-pCMV, and FLAG-tagged/untagged Skp1 as described in **Table 6.1**, incubated at normoxia or hypoxia (1 % O₂) for 24 h, harvested and lysed. Control samples were transfected with a SV40 luciferase reporter construct. The plotted signal corresponds to the ratio of luciferase : β -galactosidase signal of the respective samples, measured in relative luminescence units (RLU).

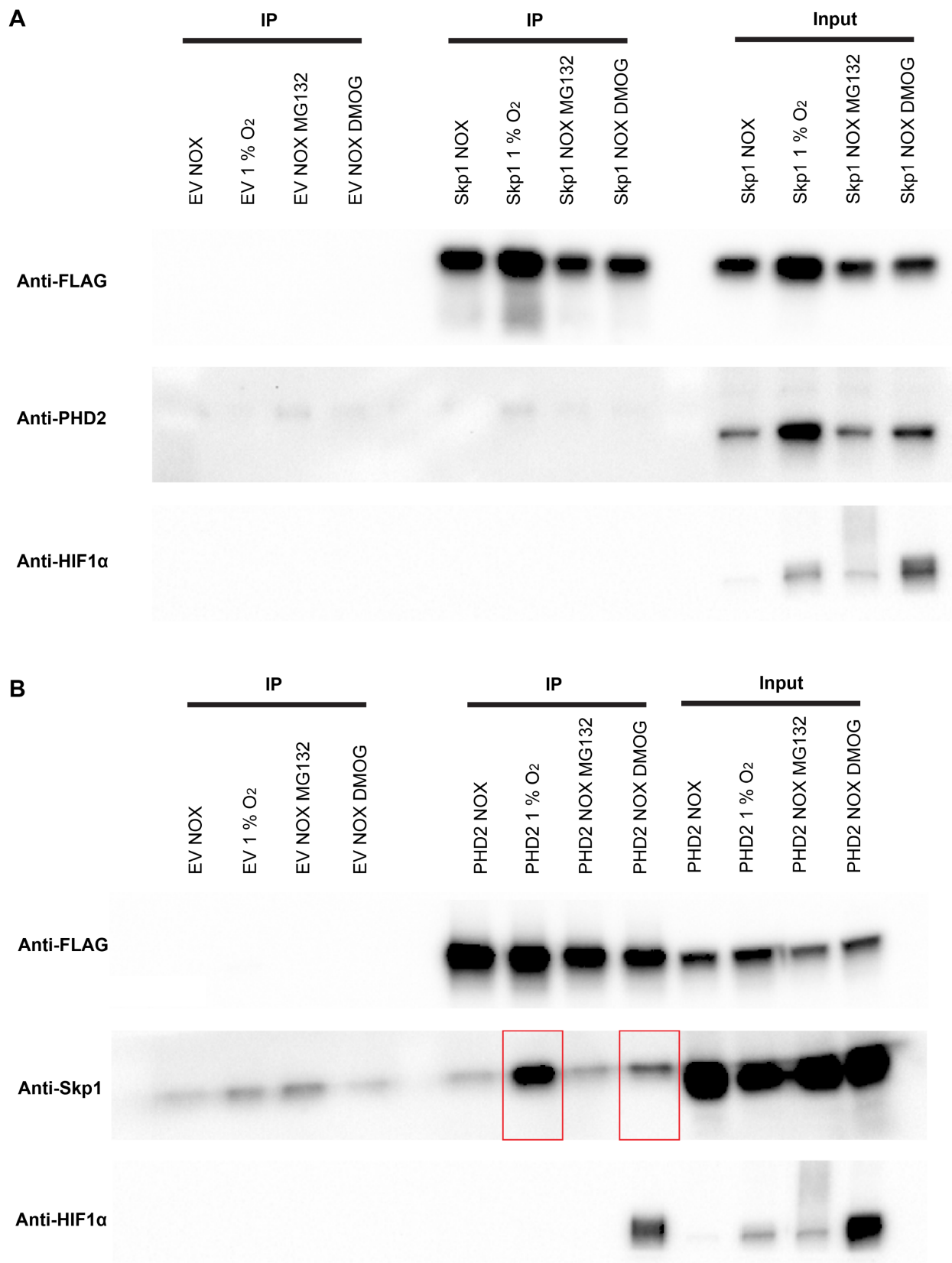
6.2.3 Immunoprecipitation experiments

It was then investigated if Skp1 and PHD2, which is the most important PHD isoform for oxygen homeostasis (12), interact with each other in the cellular environment. For that purpose, overexpressed FLAG-tagged Skp1 and FLAG-tagged PHD2 were immunoprecipitated from HeLa cells using an anti-FLAG antibody on magnetic beads as described in *Materials and Methods*, **Chapter 7.11.4**. Samples from immunoprecipitations were subsequently analysed by Western Blotting.

The results of the immunoprecipitation (IP) experiments are presented in **Figure 6.6**. Probing the Western Blot with anti-FLAG antibodies showed that PHD2-FLAG (**Figure 6.6 B**, first row) and Skp1-FLAG (**Figure 6.6 A**, first row) were successfully overexpressed in all samples of correspondingly transfected cells. Skp1 overexpression was further confirmed using a anti-Skp1 antibody (**Figure 6.6 C**, first row).

Antibody-mediated detection of HIF1 α showed that HIF1 α was stabilised under hypoxic conditions, and in samples of cells treated with DMOG or the proteasome inhibitor MG132 (**Figure 6.6 A, B**, third row, and **C**, second row). HIF1 α co-immunoprecipitated with PHD2-FLAG from cells that were treated with DMOG under normoxic conditions (**Figure 6.6 B**, third row), due to strong HIF1 α stabilisation by the PHD inhibitor DMOG.

Samples from cells overexpressing Skp1-FLAG were probed with an anti-PHD2 antibody (**Figure 6.6 A**, second row), showing that PHD2 was not present in Skp1-FLAG-IPs. However, when samples from cells overexpressing PHD2-FLAG (**Figure 6.6 B**, second row) were treated with an anti-Skp1 antibody, a strong band became visible in the IP of cells grown at 1 % O₂, and a faint band was visible in the IP of cells treated with DMSO under normoxia. This result suggests that PHD2-FLAG and Skp1 interact with each other in a cellular environment under hypoxic conditions, or (potentially) when cells are treated with DMOG, yet no such interaction is observed under normoxic conditions or in cells treated with MG132. The finding that an interaction between PHD2-FLAG and Skp1 was observed in IPs from cells overexpressing PHD2-FLAG, yet no interaction between Skp1-FLAG and PHD2 was apparent in IPs from cells overexpressing Skp1-FLAG, is possibly due to the C-terminal FLAG-tag of Skp1, that might block binding of PHD2 to Skp1-FLAG.



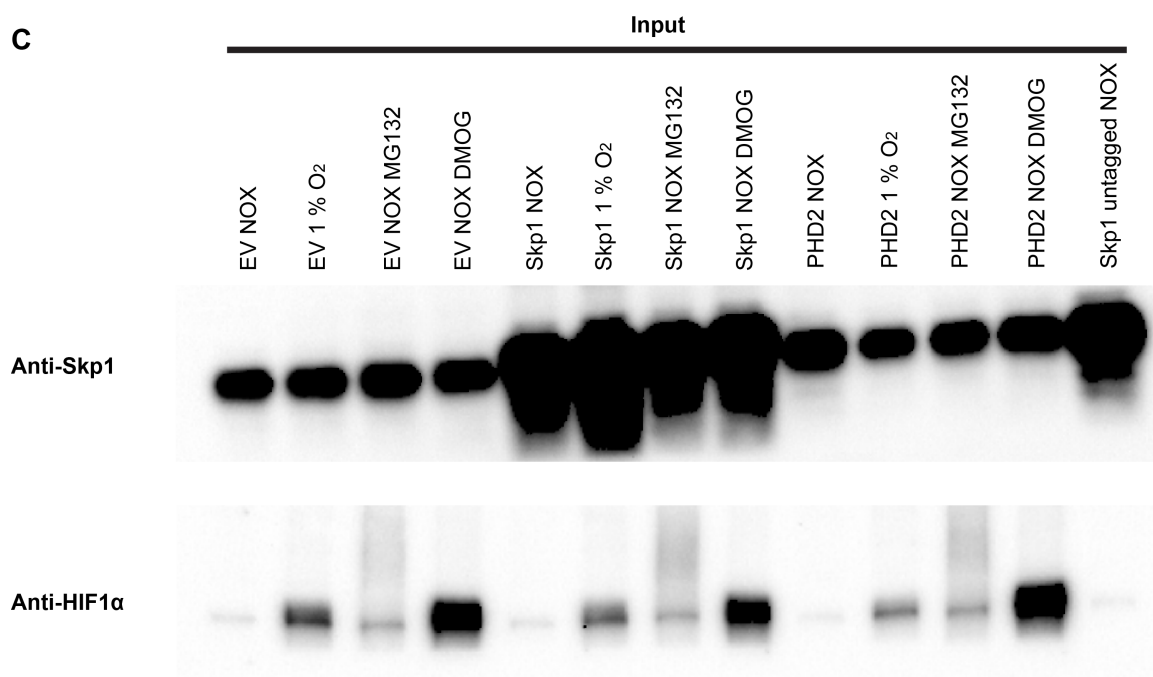
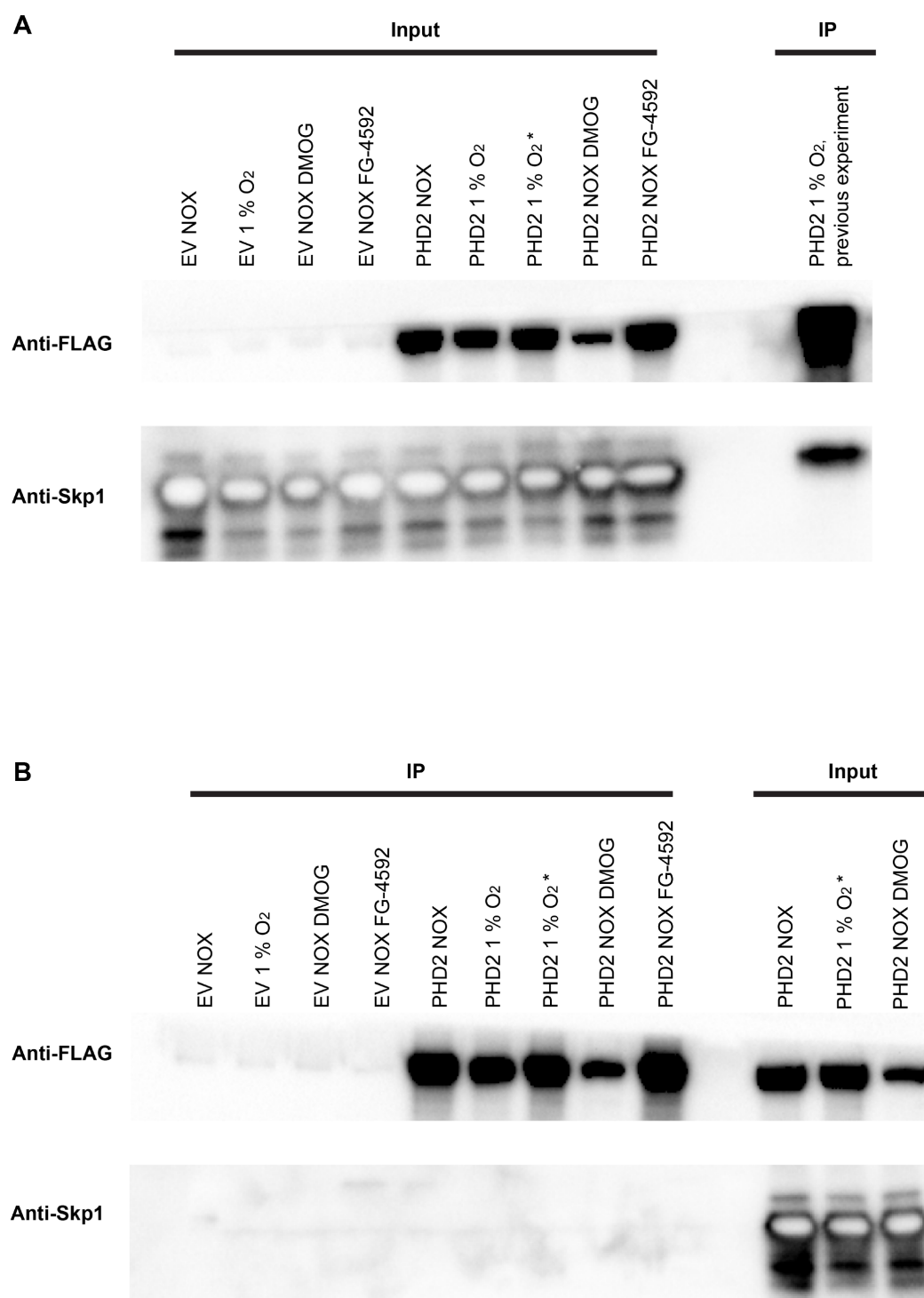


Figure 6.6: Western Blots of immunoprecipitation experiments. (A) FLAG-tagged Skp1, and (B) FLAG-tagged PHD2 were overexpressed in HeLa cells for 24 h at normoxia or hypoxia (1 % O₂). Input: supernatant of lysed cells, IP: samples from immunoprecipitations using Anti-FLAG M2 Magnetic beads, EV: cells transfected with empty vector. (C) Input samples. Results indicating an interaction between PHD2-FLAG and Skp1-FLAG under hypoxic conditions, or after treatment with DMOG, are highlighted in a red box.

Efforts were undertaken to reproduce the observed interaction between PHD2-FLAG and Skp1 twice, yet in both experiments, no such interaction was found (**Figure 6.7 B**, second row), indicating that the interaction between PHD2-FLAG and Skp1 might be of a transient nature. Close examination of the band detected by an anti-SKP1 antibody in the IP from cells overexpressing PHD2-FLAG in the previous experiment, suggests that the band is positioned slightly above bands that correspond to Skp1 in input samples (**Figure 6.7 A**, second row). This finding might be due to a post-translational modification of Skp1 that shifts the band of Skp1 to a higher molecular weight. However, it also is possible that the shift in the band indicates unspecific labeling with the anti-Skp1 antibody.



* harvested at 1 % O₂ inside hypoxic chamber

Figure 6.7: Western Blots of immunoprecipitation experiments (third repeat). FLAG-tagged PHD2 was overexpressed in HeLa cells for 24 h at normoxia or hypoxia (1 % O₂). **(A)** Input samples: supernatant of lysed cells, **(B)** samples from immunoprecipitations (IP), using Anti-FLAG M2 Magnetic beads.

6.3 Conclusions

In the unicellular amoeba *Dictyostelium discoideum* and the protozoan *Toxoplasma gondii*, oxygen-dependent culmination (in case of *D. discoideum*) and parasite growth (in case of *T. gondii*) are regulated by the prolyl-4-hydroxylases *DdPhyA* and *TgPhyA*, via hydroxylation of their substrates *DdSkp1* and *TgSkp1* (1, 4-5). As a consequence of the (likely) evolutionary relationship between prolyl-4-hydroxylases in *D. discoideum* and *T. gondii*, and HIF prolyl hydroxylases in animals, it was of interest to investigate whether, throughout evolution, HIF prolyl hydroxylases retained their ability to interact with Skp1.

The results obtained from competition experiments show that *HsSkp1* competes with HIF1 α NODD hydroxylation by *HsPHD2*, and HIF1 α CODD hydroxylation by *HsPHD3*, suggesting that *in vitro*, human PHDs are capable of interacting with Skp1. However, the results from studies in HeLa cells using HRE luciferase reporter assays indicate that overexpression of Skp1 has no stabilising effect on HIF α . In cells, Skp1 is part of the SCF complex, whereas the competition experiments were performed using isolated Skp1 protein. Therefore, it is possible that PHD2 interacts with isolated Skp1, but in the cellular environment, the interaction site might be blocked due to formation of the SCF complex.

The potential cellular interaction between PHD2 and Skp1 was further investigated by immunoprecipitating FLAG-tagged PHD2 or FLAG-tagged Skp1 from HeLa cells. However, the results obtained from IPs were non-conclusive, as it was found in one experiment that Skp1 co-immunoprecipitated with FLAG-tagged PHD2, yet this result could not be reproduced in subsequent experiments. Possible ways of interpreting the obtained results are:

- The interaction between Skp1 and PHD2 might be of a transient nature, or only observed under certain cellular conditions (e.g. in certain phases of the cell cycle).
- It is possible that a post-translational modification of Skp1 is necessary for interaction with PHD2 in the cellular environment.
- The band detected by use of anti-Skp1 antibody, in IPs from cells overexpressing FLAG-PHD2, might be due to unspecific binding of the anti-Skp1 antibody to the Western Blot membrane or to other proteins.

Using immunofluorescence microscopy, it was shown by Freed *et al.* that in mammalian cells, Skp1 is localised both in the cytoplasm and the nucleus, where Skp1 co-localises to among others, interphase and mitotic centrosomes (13). Intracellular localisation studies showed that PHD2 is localised mostly in the cytoplasm, whereas PHD1 is predominately localised in the nucleus, and PHD3 is found in both compartments (14). Given the results of these studies, it is possible that Skp1 interacts with any of the three PHD isoforms in cells, and future experiments should aim to investigate whether Skp1 (preferentially) binds PHD1, 2, or 3. In future studies, immunoprecipitation experiments should be assisted by mass spectrometric methods, such as LC-MS/MS, to identify co-immunoprecipitated proteins unambiguously. Overall, future studies should aim to elucidate the nature of the interaction between Skp1 and PHD2/3, and investigate a potential biological relevance of the interaction.

6.4 References

- (1) West, C. M., & Blader, I. J. (2015). Oxygen Sensing by Protozoans: How They Catch Their Breath. *Current Opinion in Microbiology*, 26, 41–47.
- (2) Skaar, J. R., Pagan, J. K., & Pagano, M. (2013). Mechanisms and function of substrate recruitment by F-box proteins. *Nature Reviews. Molecular Cell Biology*, 14(6), 10.1038/nrm3582.
- (3) Sheikh, M. O., Xu, Y., van der Wel, H., Walden, P., Hartson, S. D., & West, C. M. (2015). Glycosylation of Skp1 Promotes Formation of Skp1–Cullin-1–F-box Protein Complexes in Dictyostelium. *Molecular & Cellular Proteomics : MCP*, 14(1), 66–80.
- (4) West, C. M., Wang, Z. A., & van der Wel, H. (2010). A cytoplasmic prolyl hydroxylation and glycosylation pathway modifies Skp1 and regulates O(2)-dependent development in Dictyostelium. *Biochimica et Biophysica Acta*, 1800(2), 160–171.
- (5) Xu, Y., Brown, K. M., Wang, Z. A., van der Wel, H., Teygong, C., Zhang, D., ... West, C. M. (2012). The Skp1 Protein from Toxoplasma Is Modified by a Cytoplasmic Prolyl 4-Hydroxylase Associated with Oxygen Sensing in the Social Amoeba Dictyostelium. *The Journal of Biological Chemistry*, 287(30), 25098–25110.
- (6) Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7, 539.
- (7) Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25(9), 1189–1191.
- (8) Doran, D. M., Kulkarni-Datar, K., Cool, D. R., & Brown, T. L. (2011). Hypoxia Activates Constitutive Luciferase Reporter Constructs. *Biochimie*, 93(2), 361–368.
- (9) Promega. Luciferase Assay System. Technical Bulletin TB281 (2015). Retrieved from: <https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/luciferase-assay-system-protocol.pdf>, 14.09.2017.
- (10) Wykoff, C. C., Beasley, N. J. P., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., ... Harris, A. L. (2000). Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases. *Cancer Research*, 60(24), 7075–7083.
- (11) Thermo Scientific. Mammalian β -Galactosidase Assay Kit. Instructions 0861.3. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011390_Mammalian_BetaGalactosidase_Asy_UG.pdf, 14.09.2017.
- (12) Takeda, K., Cowan, A., & Fong, G.-H. (2007). Essential Role for Prolyl Hydroxylase Domain Protein 2 in Oxygen Homeostasis of the Adult Vascular System. *Circulation*, 116(7), 774–781.

- (13) Freed, E., Lacey, K. R., Huie, P., Lyapina, S. A., Deshaies, R. J., Stearns, T., & Jackson, P. K. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. *Genes & Development*, 13(17), 2242–2257.
- (14) Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., ... Fandrey, J. (2003). Intracellular localisation of human HIF-1 α hydroxylases: implications for oxygen sensing. *Journal of Cell Science*, 116(7), 1319-1326.

6.5 Appendix

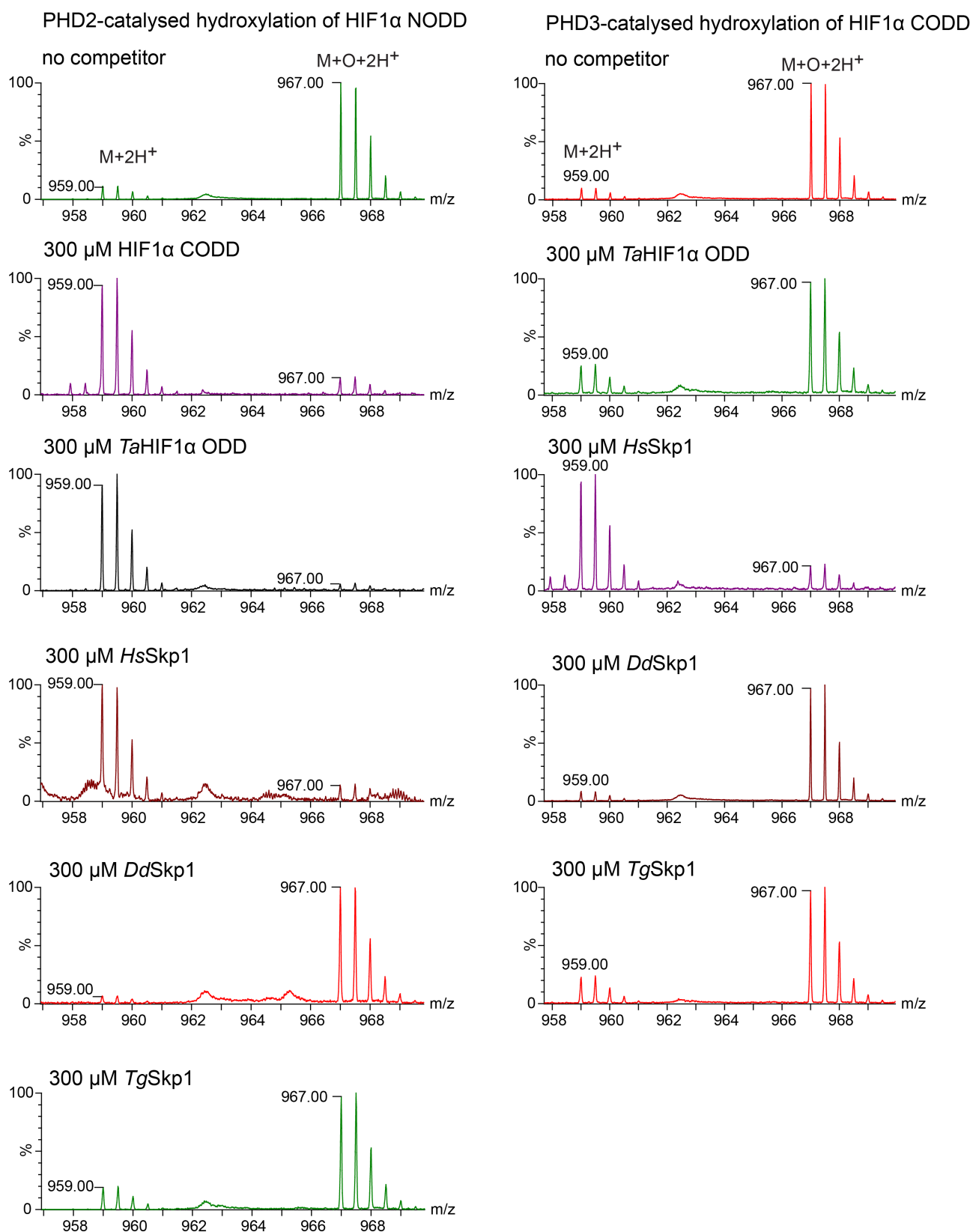


Figure 6.8: Competition of Skp1 with HIF1 α CODD/NODD hydroxylation by tPHD2 and PHD3 - ESI-LC-MS spectra. For assay conditions, see legend of Figure 6.4.

Chapter 7

Materials and Methods

All chemicals, unless otherwise stated, were obtained from Sigma Aldrich. Components of culture media (bacto tryptone, yeast extract, agar, sodium chloride) were from OXOID or Fisher Chemicals. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was from Apollo Limited, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel staining solutions (Instant Blue™) were from Expedition. Restriction enzymes, polymerase chain reaction (PCR) reagents, and competent cells (NEB 10-beta and BL21(DE3)) were from New England Biolabs. Oligonucleotides were from Sigma-Aldrich. Peptides were obtained from either ChinaPeptides, GLBiochem, Severn Biotech, or synthesised in-house.

All buffers and water were filtered through a Millipore Elix 10 system (22 μ m filter, subsequently referred to as 'Milli-Q'), and water used for DNA applications was sterilised by autoclaving (referred to as 'ddH₂O').

7.1 Molecular biology

7.1.1 Cloning

7.1.1.1 Overview of plasmids and primers used in the work

Table 7.1 Plasmids used and their origins.

Name of construct	Construct length	Vector	Tag	Source
tPHD2	181-426	pET-28a	His ₆	Rasheduzzaman Chowdhury, University of Oxford, UK
PHD3	full-length	pNH-TrxT	His ₆ -thioredoxin	Cloning
PHD3	full-length	pNIC28	His ₆	Cloning
PHD1	full-length	pMAL-c5X	Maltose-binding-protein	Kirsty Hewitson, University of Oxford, UK
TaPHD	64-300	pET-28a	His ₆	Christoph Loenarz, Universität Freiburg, Germany
tPHD2 R312H	181-426	pET-28a	His ₆	Site-directed mutagenesis
tPHD2 F366L	181-426	pET-28a	His ₆	Site-directed mutagenesis
tPHD2 D277G	181-426	pET-28a	His ₆	Site-directed mutagenesis
tPHD2 G294S	181-426	pET-28a	His ₆	Site-directed mutagenesis
tPHD2 W334S	181-426	pET-28a	His ₆	Site-directed mutagenesis
tPHD2 L279P	181-426	pET-28a	His ₆	Rasheduzzaman Chowdhury, University of Oxford, UK
HsSkp1	full-length	pcDNA3	untagged	Cloning
HsSkp1-FLAG	full-length	pcDNA3	FLAG (C-terminal)	Cloning
ADRB2	330-413	pGEX-6P-1	Glutathione-S-transferase	Cloning
PKM2	full-length	pGEX-6P-1	Glutathione-S-transferase	Cloning
ACTB	full-length	pGEX-6P-1	Glutathione-S-transferase	Cloning
HIF1 α	530-698	pGEX-6P-1	Glutathione-S-transferase	Christoph Loenarz, Universität Freiburg, Germany

Table 7.2 Primers. BamHI restriction site (orange), NotI restriction site (green) and LIC-sequence (purple), mutagenesis sites (red).

Construct	Primer sequence (5'-3')
PHD3	F TACTTCCAATCCATGCCGCT R TATCCACCTTTACTGTTAATCTTCGG
tPHD2 R312H	F GGCAATGG AACGGGTAT GTACATCATG TTGATAATCC R GGATTATCAA CATGATGTAC ATAACCCGTT CCATTGCC
tPHD2 F366L	F CCCAAATTTGATAGACTGCTGTTTTTATGGTCTGACCGTCGC R GCGACGGTCAGACCATAAAACAGCAGTCTATCAAATTTGGG
tPHD2 D277G	F GCTCATGAGCAGCATGGGTGACCTGATA CGCC R GGCATATCAGGTCACCATGCTGCTCATGAGC
tPHD2 G294S	F GGGCAGCTACAAAATCAATTCCCGGACGAAAGCCATGG R CCATGGCTTTCGTCCGGGAATTGATTTTGTAGCTGCC
tPHD2 W334S	F GTATATATTATCTTAATAAAGACTCGGATGCCAAGGTAAGTGG R CCACTTACCTTGGCATCCGAGTCTTTATTAAGATAATATATAC
HsSkp1	F ATAGGATCCATGCCTTCAATTAAGTTGCAGA R ATAGCGGCCGCTCACTTCTCTTCACACCACTGGTTC
HsSkp1-FLAG	F ATAGGATCCATGCCTTCAATTAAGTTGCAGA R ATAGCGGCCGCTCACTTGTCTCATCGTCTTTGTAGTCCT TCTCTTCACACCACTGGTTC
ADRB2	F ATAGGATCCATGCCGGACTTCCGTATC R TAAGCGGCCGCTTACAGCAGAGAATCATTCTGTG
PKM2	F ATAGGATCCATGAGCAAACCGCATAGC R ATAGCGGCCGCTTACGGCACCGGGACAAC
ACTB	F TAAGGATCCATGGACGATGATATCGCCG R ATAGCGGCCGCTTAGAAGCATTTGCGGTGAA

7.1.1.2 Ligation independent cloning (LIC)

For ligation independent cloning, the procedure in (1) was optimised.

Polymerase chain reaction (PCR)

Codon-optimised genes from GenScript were prepared according to the manufacturer's manual and diluted to an end concentration of 10-20 ng/ μ L. Primers were from Sigma Aldrich. Before use, the primers were resuspended in sterile Milli-Q water to a concentration of 10 μ M. The PCR was set up as depicted in **Table 7.3** and run in a Thermocycler (Techne Genius, Scientific Laboratory Supplies) with the cycling parameters given in **Table 7.4**.

Table 7.3 PCR sample preparation.

Reagent	Volume
Q5 Reaction Buffer (5x)	10 μ L
dNTPs (10mM)	1 μ L
Forward Primer (10 μ M)	2.5 μ L
Reverse Primer (10 μ M)	2.5 μ L
Template DNA (10-20 ng/ μ L)	5 μ L
Q5 High Fidelity DNA Polymerase	0.5 μ L
ddH ₂ O	28.5 μ L
Σ	50 μ L

Table 7.4 PCR program.

Segment	# Cycles	Temperature (°C)	Time (sec)
Initial Denaturation	1	98	30
Denaturation	35	98	10
Annealing	35	62-69*	30
Extension	35	72	60
Final Extension	1	72	120
Final Hold	1	4	

*The annealing temperature was determined with the T_m calculator from New England Biolabs.

A sample of the PCR products (5 μL) was analysed on a 2 % agarose gel. The PCR products (50 μL) were treated with 1 μL DpnI and incubated for 1 h at 37 °C. Subsequently, the PCR products were purified with a column-based PCR purification kit (TScientific) and eluted in 20 μL ddH₂O.

T4 polymerase treatment of PCR products

The PCR products were treated with T4 polymerase as shown in **Table 7.5** and incubated at room temperature (rt) for 30 min, then at 75 °C for 20 min. The reaction mix was stored at 4 °C until further use.

Table 7.5 T4 polymerase treatment of PCR products.

Reagent	Volume
ddH ₂ O	4.5 μL
NEBuffer2 (10x)	2 μL
dCTP(100 mM)	0.5 μL
Dithiothreitol (DTT, 100 mM)	1 μL
Bovine serum albumin (BSA, 2 mg/mL)	1 μL
T4 DNA Polymerase (3000 U/mL)	1 μL
PCR product (>10 ng/ μL)	10 μL
Σ	20 μL

Vector preparation

Empty LIC-plasmids (pNH-TrxT or pNIC28) were cut with the restriction enzyme BsaI. The reaction mixture (**Table 7.6**) was incubated for 2.5 h at 50 °C. Subsequently, the DNA was separated using a 1 % agarose gel (**Chapter 7.1.1.5**), extracted from the gel (TScientific Gel Extraction Kit) and eluted in 20 μL ddH₂O.

Table 7.6 Treatment of empty vectors with BsaI.

Reagent	Volume
NEBuffer3 (10x)	10 μL
BSA (10 mg/mL)	1 μL
Empty LIC-plasmid	x μL (total 5 μg)
BsaI (10 000 U/mL)	4 μL
ddH ₂ O	top up
Σ	100 μL

Generation of cohesive vector ends

The empty vectors were treated with T4 Polymerase (**Table 7.7**) and incubated at rt for 30 min, then at 75 °C for 20 min. The reaction mix was stored on ice until further use.

Table 7.7 T4 polymerase treatment of empty vectors.

Reagent	Volume
ddH ₂ O	4.5 μL
Empty LIC-plasmid	10 μL
NEBuffer2 (10x)	2 μL
dGTP (100 mM)	0.5 μL
DTT (100 mM)	1 μL
BSA (2 mg/mL)	1 μL
T4 DNA Polymerase (3000 U/mL)	1 μL
Σ	20 μL

Annealing and transformation

To anneal the cleaved vectors and T4-treated inserts, 2 μ L of insert DNA (~100 ng) was added to 1 μ L vector and incubated on ice for 10 min. The mix was transformed into NEB-10 β competent cells following the protocol in **Chapter 7.2.1**. The transformed bacteria were grown on agar plates containing 25 % sterile sucrose and the respective antibiotic. Overnight cultures of single colonies were grown, DNA was extracted by Miniprep and sequenced using the Source Bioscience Sequencing service.

7.1.1.3 Classic cloning

All steps were optimised using a protocol obtained from Christoph Loenarz (Universität Freiburg, Germany).

Polymerase chain reaction (PCR)

The PCR was set up in a volume of 2x 100 μ L and run as described in **Chapter 7.1.1.2**. 5 μ l samples of PCR products were analysed on a 2 % agarose gel. The two 100 μ L PCR reactions were combined on a PCR purification column (TScientific), purified and eluted in 20 μ L ddH₂O.

Double digestion of vector and insert

Empty vectors and insert DNA were cut with the respective restriction enzymes (**Table 7.2**). Reaction mixtures (2x 20 μ L per insert and vector, **Table 7.8**) were incubated for 4 h at 37 °C. Subsequently, the DNA was separated using a 1 % agarose gel (**Chapter 7.1.1.5**), extracted from the gel (TScientific Gel Extraction Kit) and eluted in 20 μ L ddH₂O.

Table 7.8 Double digestion conditions.

Reagent	Volume, vector digestion	Volume, insert digestion
Insert DNA		x μ L (total 2 μ g)
Empty plasmid	x μ L (total 1 μ g)	
ddH ₂ O	top up	top up
NEBuffer4 (10x)	2 μ L	2 μ L
BSA (10 mg/mL)	1 μ L	1 μ L
Restriction enzyme 1 (20 000 U/mL)	1 μ L	2 μ L
Restriction enzyme 2 (20 000 U/mL)	1 μ L	2 μ L
Σ	20 μ L	20 μ L

T4-ligase reaction

The T4-ligase reaction was set up as described in **Table 7.9** and incubated overnight at 18 °C.

Table 7.9 T4-ligase reaction.

Reagent	Volume
Insert DNA	12 μ L
Cleaved vector	5 μ L
T4-Ligase Buffer (10x)	2 μ L
T4-Ligase (400 000 U/mL)	1 μ L
Σ	20 μ L

The ligated vectors were transformed into NEB-10 β competent cells according to the standard procedure in **Chapter 7.2.1**. Single colonies were grown in overnight cultures (**Chapter 7.2.3**), extracted with a Plasmid Miniprep Kit (TScientific) and sequenced by Source Bioscience.

7.1.1.4 Site-directed mutagenesis

Site-directed mutagenesis was carried out following the procedure in the QuikChange™ site-directed mutagenesis protocol (Revision C, Agilent Technologies, (2)). Primers (typically 30mers) were designed according to the protocol with the desired mutation in the middle and GC-rich ends for optimal annealing. The following PCR protocol was used to insert the mutation and amplify the full vector (**Table 7.10** and **7.11**).

Table 7.10 PCR condition for site-directed mutagenesis.

Reagent	Volume
<i>Pfu</i> Turbo reaction buffer (10x)	2.5 μ L
DNA template (30 ng/ μ L)	1 μ L
Forward Primer (10 μ M)	0.5 μ L
Reverse Primer (10 μ M)	0.5 μ L
dNTPs (10 mM)	0.5 μ L
ddH ₂ O	20 μ L
<i>Pfu</i> Turbo DNA polymerase	0.5 μ L
Σ	25.5 μ L

Table 7.11 PCR program for site-directed mutagenesis.

Segment	# Cycles	Temperature (°C)	Time (sec)
Initial Denaturation	1	95	120
Denaturation	16	98	30
Annealing	16	62-69	40
Extension	16	72	60 sec/kb plasmid
Final Extension	1	72	300
Final Hold	1	4	

The amplified DNA was treated with 1 μ L Dpn1 (10 U/ μ L) at 37 °C for 1 hour to digest parental DNA. The samples were transformed into NEB-10 β competent cells following the protocol in **Chapter 7.2.1**.

7.1.1.5 Agarose gel electrophoresis

Agarose gels (1-2%) were prepared by heating 0.6-1.2 g agarose in 60 mL TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1mM ethylenediaminetetraacetic acid (EDTA)) in a SuperWave 750 microwave (Tecnolec) until dissolved. The mix was allowed to cool down to 60 °C, before addition of 9 µL SYBR Safe™ DNA gel stain (Invitrogen). Gels were cast in a H2-SET gel tray (Anachem) and allowed to cool down to room temperature for 20 min. DNA Loading Buffer (NEB) was added to the samples before loading them onto the gel. The gels were run on a Bio-Rad system at 120 V for approximately 35 min. DNA bands were visualised by UV light illumination using a Bio Rad gel imaging system. Samples were run against a GeneRuler 1kb DNA Ladder (TScientific) for molecular weight determinations.

7.2 Microbiological techniques

7.2.1 Transformation into bacteria

Plasmid DNA was transformed into *E. coli* NEB-10 β or BL21(DE3) competent cells according to the following protocol. In pre-cooled Eppendorf tubes, the respective vector DNA (1 μ L for transformation into NEB-10 β or 0.5 μ L for BL21(DE3)) was added. Bacterial cells (50 μ L of NEB-10 β or 20 μ L BL21(DE3)) were thawed on ice and added to the vector DNA. After incubating on ice for 20 min (NEB-10 β) or 5 min (BL21(DE3)), the bacteria were heat shocked at 42°C for 30 s (NEB-10 β) or 45 s (BL21(DE3)), and left to rest on ice for 2 min. The 3-fold volume of SOC-medium (NEB) was added to the bacteria, and the dispersion was incubated at 37°C for 1 h. Subsequently, the bacteria were spread onto LB-agar plates (10 g NaCl, 5 g yeast extract, 10 g tryptone, 15 g agar per 1 L medium) with relevant antibiotic (1/1000 v/v of 30 mg/mL kanamycin and/or 100 mg/mL ampicillin) and allowed to grow at 37°C overnight.

7.2.2 Glycerol stocks

The respective bacterial starter culture (1.2 mL) was pelleted in a cryotube and the cells were resuspended in 350 μ L of 2TY Growth Medium (5 g NaCl, 10 g yeast extract, and 16 g bactotryptone per 1 L medium, kindly prepared and autoclaved by Mr Herminio Manso Jubier). 350 μ L Glycerol Stock Buffer (65% glycerol, 0.1 M MgSO₄, 0.025 M Tris pH 7.6, autoclaved, stored at 4 °C) was added to the cell suspension and the glycerol stock was shortly mixed by vortexing. Glycerol stocks were stored at -80 °C.

7.2.3 Starter cultures

For protein expression, all plasmids were transformed into BL21(DE3) cells. 100 mL of 2TY growth medium (kindly prepared and autoclaved by Mr Herminio Manso Jubier) were treated with the respective antibiotics (1/1000 v/v of 30 mg/mL kanamycin, and/or 100 mg/mL ampicillin), and inoculated with either a single colony from a freshly prepared agar plate or a small amount of a glycerol stock. The bacteria were allowed to grow in an incubator shaker overnight at 37 °C and 180 rpm.

7.2.4 Expression trials

2-TY growth medium (100 mL) were inoculated with the appropriate antibiotic (1/1000 v/v of 30 mg/mL kanamycin, and/or 100 mg/mL ampicillin) and 10 mL starter culture. At OD₆₀₀ 0.6-0.8, the cultures were separated into incubators at different temperatures (typically 18, 28, and 37 °C), and each induced by adding different concentrations of isopropyl-β-D-1-thiogalactopyranoside (IPTG, typically 0.05 mM and 0.5 mM). After incubating the cultures for 4 h, 1.5 mL samples were taken, and the cultures were continued to grow overnight. A sample of each culture (1.5 mL) was pelleted, and the cells were resuspended in 0.3 mL Binding Buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM imidazole) per OD₆₀₀ 1.2. The cells were lysed by sonification (1 min, 30 % amplitude, 4s intervals followed by 4s pauses), and cell debris was removed by centrifugation. 10 μL of supernatant and whole cell samples were analyzed by SDS-PAGE as described in **Chapter 7.3.5**.

7.2.5 Large-scale protein production

Large-scale cultures were grown in 2TY growth medium containing the respective antibiotics (1/1000 v/v of 30 mg/mL kanamycin, and/or 100 mg/mL ampicillin, co-expression cultures contained only kanamycin). The cultures were inoculated with 1/100 v/v of starter culture and the bacteria were grown at 37 °C, 180 rpm until an OD₆₀₀ of 0.6-0.8 was reached, and induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG). In some cases, the cultures were allowed to grow to a higher density, in order to improve the protein yield. The expression conditions were optimised for each protein construct (**Table 7.12**). Cells were harvested by centrifuging at 8000 rpm in a Beckman Avanti J-26S XP centrifuge, at 4 °C, for 8 min.

Table 7.12 Optimised conditions for protein production.

Name of construct	Expression conditions
tPHD2	0.5 mM IPTG 4 h, 28 °C
PHD3	Culture grown to OD ₆₀₀ 1.2 0.05 mM IPTG O/N, 18 °C
PHD1	0.5 mM IPTG 4 h, 37 °C
TaPHD	0.5 mM IPTG 4 h, 28 °C, then O/N, 18 °C
tPHD2 R312H	0.5 mM IPTG O/N, 18 °C
tPHD2 F366L	0.5 mM IPTG O/N, 18 °C
tPHD2 D277G	0.5 mM IPTG O/N, 18 °C
tPHD2 G294S	0.5 mM IPTG O/N, 18 °C
tPHD2 W334S	0.5 mM IPTG O/N, 18 °C
tPHD2 L279P	0.5 mM IPTG 4 h, 28 °C
ADRB2, co-expressed with PHD3	0.5 mM IPTG O/N at 18 °C
PKM2, co-expressed with PHD3	0.5 mM IPTG O/N at 18 °C
ACTB, co-expressed with PHD3	0.5 mM IPTG O/N at 18 °C
HIF1α	0.5 mM IPTG O/N at 18 °C
HIF1α, co-expressed with tPHD2	0.5 mM IPTG O/N at 18 °C
HIF1α, co-expressed with PHD3	0.5 mM IPTG O/N at 18 °C

7.2.6 Cell lysis

Cell pellets were resuspended in 5 mL Binding Buffer per 1 g cell pellet (buffer composition in **Table 7.13**). DnaseI, a cOmplete, EDTA-free Protease inhibitor tablet (Roche), MgCl₂ (end concentration of 20 mM), and 5 mM dithiothreitol (DTT) in case of PHD3 and *Ta*PHD, were added, and the cells were fully resuspended by stirring at 4° C for 20-30 min. Cells were lysed on ice by sonication (Sonics Vibracell VCX750 Ultrasonic Cell Disruptor) at 60% amplitude in 8 steps of 45 s, each followed by a rest of 45 s. The supernatant was separated from the cell debris via centrifugation (Beckman Avanti J-26S XP, 15 500 rpm, 10 min, 24 500 rpm, 20 min, 4°C) and filtered through a 0.45 µm syringe filter (Sartorius Stedim Biotech) before it was loaded onto an appropriate column.

7.3 Protein purification

7.3.1 Purification strategies

Proteins were purified at 4 °C using an ÄKTA fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). All buffers were prepared from Milli-Q-H₂O, filtered through a 0.2 µm Millipore filter, and degassed before use. All stated pH values correspond to a buffer temperature of 4 °C.

Table 7.13 gives an overview of the different purification strategies used for the preparation of proteins. GST-tagged proteins were purified in one step by GST-affinity chromatography, whereas His₆-tagged and MBP-tagged proteins were purified by Ni-NTA or amylose affinity chromatography, followed by size-exclusion chromatography. Due to its instability on size-exclusion columns, PHD3 was purified in only one step by Ni-NTA affinity chromatography. Cleavage of the *N*-terminal His₆-thioredoxin tag from PHD3 resulted in loss of activity. It was therefore decided to leave the His₆-thioredoxin tag on PHD3.

Samples of the eluted proteins were analysed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The desired protein fractions were concentrated in centrifugation filters (Amicon) with the respective molecular weight cut-off. Protein concentrations were determined by measuring A₂₈₀ in each sample using an ND-1000 Nanodrop spectrophotometer (TScientific). All proteins were analysed by ESI-LC-MS (**Chapter 7.6.2** and **7.6.4**).

Table 7.13 Protein purification strategies.

Protein construct	Purification strategy	Buffers	Gradient (% Elution Buffer)
tPHD2 tPHD2 R312H tPHD2 F366L tPHD2 D277G tPHD2 G394S tPHD2 W344S tPHD2 L296P	1. <i>Ni-NTA affinity chromatography</i>	<u>Binding Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM imidazole) <u>Wash Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 60 mM imidazole) <u>Elution Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 M imidazole) tPHD2 variants: 5 % glycerol added to every buffer	5 CV, 40 %, 4.00 {base} 3 CV, 100 %, 2.00 {base} 5 CV, 0 %, 0.00 {base}
	2. <i>Thrombin-cleavage</i>		
	3. <i>Size-exclusion chromatography (SEC)</i>	<u>SEC Buffer</u> (50 mM Tris-HCl pH 7.5) tPHD2 R312H/F366L: 5% glycerol and 100 mM NaCl added	
PHD3	1. <i>Ni-NTA affinity chromatography</i>	<u>Binding Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM imidazole, 5% glycerol) <u>Wash Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 60 mM imidazole, 5% glycerol) <u>Elution Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 M imidazole, 5% glycerol)	5 CV, 40 %, 4.00 {base} 3 CV, 100 %, 2.00 {base} 5 CV, 0 %, 0.00 {base}
	2. <i>Buffer exchange</i>	<u>Storage Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5% glycerol)	
TaPHD	1. <i>Ni-NTA affinity chromatography</i>	<u>Binding Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM imidazole, 5% glycerol) <u>Wash Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 60 mM imidazole, 5% glycerol) <u>Elution Buffer</u> (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 M imidazole, 5% glycerol)	1 CV, 10 %, 1.00 {base} 1 CV, 15 %, 1.00 {base} 1 CV, 20 %, 1.00 {base} 4 CV, 40 %, 2.00 {base} 2 CV, 100 %, 0.00 {base}
	2. <i>Size-exclusion chromatography (SEC)</i>	<u>SEC buffer</u> (500 mM NaCl, 100 mM Tris-HCl pH 7.5, 5% glycerol)	

Protein construct	Purification strategy	Buffers	Gradient (% Elution Buffer)
PHD1	1. <i>Amylose affinity chromatography</i>	<u>Amylose-Binding Buffer</u> (200 mM NaCl, 20 mM Tris-HCl pH 7.4) <u>Amylose-Elution Buffer</u> (200 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM maltose)	
	2. <i>Size-exclusion chromatography (SEC)</i>	<u>SEC buffer</u> (100 mM NaCl, 100 mM Tris-HCl pH 7.5, 5% glycerol)	
ADRB2 PKM2 ACTB HIF1α	1. <i>Glutathione-S-transferase (GST) affinity chromatography</i>	<u>GST-Binding Buffer</u> (200 mM NaCl, 50 mM Tris-HCl, pH 7.5) <u>GST-Elution Buffer</u> (200 mM NaCl, 50 mM Tris-HCl 20 mM reduced glutathione (added shortly before use), pH 7.5)	

7.3.2 Protein chromatography

Glutathione-S-transferase (GST) affinity chromatography

A 5 mL Protino GST/4B column (Fisher Scientific) was washed with 5 CV Milli-Q H₂O, equilibrated with 5 CV GST-Binding Buffer (**Table 7.13**), loaded with the protein sample, washed with 10 CV GST-Binding Buffer, and eluted in 5 mL fractions with 10 CV GST-Elution Buffer (**Table 7.13**). The column was cleaned after each usage to avoid cross-contamination, using 2 CV of 6 M guanidine hydrochloride, 5 CV Milli-Q H₂O, 3 CV of 70 % ethanol, and 5 CV Milli-Q H₂O.

Ni-NTA affinity chromatography

A 5 mL HisTrap™ FF crude column (GE Healthcare) was washed with 5 CV Milli-Q H₂O, charged with 3 CV Charge Buffer (50 mM NiSO₄), equilibrated with 5 CV Binding Buffer (**Table 7.13**), and loaded with protein sample. The column was first washed with 11 CV Binding Buffer, followed by a second wash with 8 CV Wash Buffer (**Table 7.13**), to remove any unspecific binding proteins from the column. The protein of interest was then eluted in 5 mL fractions by applying a gradient of Wash Buffer and Elution Buffer (**Table 7.13**). The gradient was optimised for each protein (**Table 7.13**). The column was cleaned after each usage with 5 CV Milli-Q H₂O, followed by 2 CV Strip Buffer (500 mM NaCl, 20 mM Tris-HCl, 100 mM EDTA, pH 7.9), and 5 CV Milli-Q H₂O.

Amylose affinity chromatography

A 25 mL MCS amylose column was equilibrated with 5 CV Amylose-Binding Buffer (**Table 7.13**), and loaded with protein sample. The column was then washed with 12 CV Amylose-Binding Buffer, and the protein of interest was then eluted in 5 mL fractions with 4 CV Amylose-Elution Buffer (**Table 7.13**). The column was regenerated after each usage with 3 CV Milli-Q H₂O, followed by 3 CV 0.1 % SDS, 1 CV Milli-Q H₂O, and 3 CV Amylose-Binding Buffer.

Size-exclusion chromatography (SEC)

Proteins were further purified via size-exclusion chromatography on a Superdex 75 300 mL column (Amershan Pharmacia Biosciences). The column was washed with 0.6 CV Milli-Q H₂O, equilibrated with 1.1 CV SEC Buffer (**Table 7.13**), and the protein sample was loaded using a 2 mL loop. The protein of interest was eluted in 5 mL fractions by applying 1.0 CV SEC Buffer, and the column was cleaned with 1.1 CV Milli-Q H₂O, 1.0 CV 0.5 M NaOH, and 2 CV Milli-Q H₂O.

7.3.3 Protease cleavage

TEV cleavage

The His₆-thioredoxin-tag at the *N*-terminus of fusion proteins in pNH-TrxT vectors was cleaved by treating the protein with TEV protease (kindly provided by Christoph Loenarz, Universität Freiburg, Germany). The salt concentration in the buffer was increased to 1 M NaCl, in order to avoid precipitation. 20 µL of TEV protease was added to 1 mg of protein, the mix was incubated overnight at 4 °C, and then resolved by size exclusion chromatography.

Thrombin cleavage

The His₆-tag at the *N*-terminus of a fusion protein in pET-28a was cleaved by treating with restriction grade thrombin (Novagen) according to the standard manufacturer's instructions (3). Thrombin (1 U) was added to 1 mg of protein, the thrombin protein mixture was incubated overnight at 4 °C, and then resolved by size exclusion chromatography.

7.3.4 Buffer exchange

Proteins were buffer-exchanged using PD-10 columns (GE Healthcare) and following the instructions in the manual.

7.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels with the following composition (**Table 7.14**) were prepared using the Bio-Rad Mini-Protean Tetra Electrophoresis System from 2x SDS-PAGE stacking gel buffer (250 mM Tris-HCl pH 6.8, 0.2 % (w/v) SDS) and 2x SDS-PAGE separating gel buffer (750 mM Tris-HCl, pH 8.8, 0.2% (w/v) SDS).

Table 7.14 Composition of SDS-PAGE gels.

Component	Reagent	Volume
Separating gel	30 % acrylamide	2.5 mL
	2x SDS-PAGE separating buffer	2.5 mL
	tetramethylethylenediamine (TEMED)	10 μ L
	25 % (w/v) APS	30 μ L
Stacking gel	30 % acrylamide	0.3 mL
	Milli-Q H ₂ O	0.7 mL
	2x SDS-PAGE separating buffer	1 mL
	TEMED	5 μ L
	25 % (w/v) APS	15 μ L

Samples were prepared by adding 10 μ L of 2x loading buffer (**Table 7.15**) to 10 μ L samples, and the proteins in the sample were further denatured at 95 °C for 5 min, before loaded onto the gel. Electrophoresis was conducted in SDS-PAGE running buffer (**Table 7.15**) at a constant voltage of 180 V. The gels were stained with a quick stain solution (Instant Blue TM, Expedition).

Table 7.15 Composition of SDS-PAGE buffers.

Component	Reagent	Volume/ Mass
10x SDS-Page running buffer	Tris base	30.2 g
	Glycine	144 g
	10 % (w/v) SDS	100 mL
	Milli-Q H ₂ O	900 mL
2x SDS-Page loading buffer	Tris-HCl 0.5 M, pH 6.8	
	Bromphenol blue	0.2g
	10 % (w/v) SDS	20 mL
	Glycerol	12 mL
	β -mercaptoethanol	5 mL
	Milli-Q H ₂ O	top up to 100 mL

7.4 Peptide synthesis

MultiPep synthesis

Peptides were prepared in a 1-5 mg scale on a MultiPep RS (Intavis) peptide synthesizer by solid phase peptide synthesis. Peptides were cleaved from the Tantagel S RAM (RAPP Polymere) resin, and protection groups were removed by incubating for 3 h at rt with 0.5 to 1.0 mL of a cleavage cocktail containing 97.5% trifluoroacetic acid, and 2.5% triisopropylsilane. Cold ether (4 mL) was added to each peptide and the precipitate was pelleted by centrifuging for 10 min at 4000 rpm (Beckman Allegra X-30R centrifuge). The pellets were dried under vacuum, resuspended in 1 mL of water, and freeze-dried. The peptides were resuspended in 1 mL of water and analyzed by MALDI-TOF-MS or LC-MS.

Large-scale synthesis

Peptides were prepared in 5-30 mg scale using a Liberty Blue™ automated microwave peptide synthesiser (CEM). The peptides were prepared using Fluorenyl-methyloxycarbonyl (Fmoc) rink amide MBHA resin (CS Bio), *N*α-Fmoc-protected amino acids (Polymer labs), and the activators hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) at a ratio of 1:1 in DMF. Peptides were first washed with DMF, then with DCM, and were cleaved by incubating for 3 h with a cleavage mix containing trifluoroacetic acid (TFA, 92.5 %), triisopropylsilane (2.5 %), 1,3-dimethoxybenzene (2.5%) and water (2.5%). The cleaved peptides were precipitated by adding 30-40 mL of cold ether and centrifuged at 2000 rpm (Beckman Allegra X-30R centrifuge), at 4 °C for 5 min. The peptide pellets were dried on air, resuspended in ~15 mL water, lyophilised and purified (**Chapter 7.5**).

For synthesis of prolyl-hydroxylated peptides, 4-hydroxyproline (Novabiochem) was used instead of the unmodified proline, and peptides were synthesised as described above.

7.5 Peptide purification by HPLC

Peptides were purified by high pressure liquid chromatography (HPLC). First, the peptides were dissolved in water with max. 30 % acetonitrile if necessary, and applied onto a SunFire™ Prep C18 column (Waters). A suitable gradient of H₂O:Acetonitrile was used to separate the samples and fractions containing the desired peptides were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or liquid chromatography–mass spectrometry (LC-MS). The desired fractions were pooled and lyophilised.

7.6 Mass spectrometry

7.6.1 MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) assays were performed using a Waters Micromass MALDI micro MX™ mass spectrometer. Samples were prepared by diluting 1:1 with acetonitrile and mixing of 1 µL sample with 1 µL of α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (1 mg CHCA in 100 µL 50 % acetonitrile, 0.1 % trifluoroacetic acid). The mix (1 µL) was spotted directly onto a 96-spot MALDI plate (Waters) and the sample was run in the positive ion reflectron mode under standard calibration with a laser energy of 160-320 V. Instrument control and data processing was conducted with MassLynx™ V4.1. The following standard assay conditions were used, unless otherwise stated.

Endpoint hydroxylation assays

Endpoint hydroxylation assays were carried out using the following assay conditions: 4 µM enzyme, 50 µM substrate, 50 µM (NH₄)₂Fe(II)(SO₄)₂, 4 mM sodium L-ascorbate, 300 µM 2-oxoglutarate disodium salt, 1 mM dithiothreitol (DTT) in 50 mM Tris, pH 7.5. The reactions were incubated at 25 °C and quenched with 1 % formic acid (v/v) at a time point in the linear region. Hydroxylation levels were quantified using MassLynx™ V4.0 and non-enzymatic background oxidation was subtracted.

Steady-state kinetics

Steady-state kinetics were performed via a modified version of the reported procedure (4), using the following conditions: 3.5 µM *Hs*PHD2 or *Ta*PHD, 100 µM *Hs*HIF1 α CODD (DLDLEMLA**P**YIPMDDDFQL-NH₂) or *Ta*HIF α ODD substrate (PINEKEDYDDL**A**PFVPPPSFDNRLY-NH₂), 50 µM (NH₄)₂Fe(II)(SO₄)₂, 4 mM sodium L-ascorbate and 300 µM 2-oxoglutarate disodium salt in 50 mM Tris, pH 7.5. The reactions were incubated at 25 °C and quenched with 1 % (v/v) formic acid at various time points. Initial rates of the enzymatic reaction were determined by varying the concentration of one of the substrates/co-substrates (*Hs*HIF1 α CODD, *Ta*ODD or 2OG) and maintaining fixed saturating concentrations of the other component. Hydroxylation levels were quantified using MassLynx™ V4.0. *K_m* and *k_{cat}* values were determined using GraphPad Prism.

Steady-state oxygen-dependence studies

Steady-state oxygen-dependence studies were performed as described in (4), with the assay conditions in **Table 7.16**.

Table 7.16 Assay conditions for steady-state oxygen-dependence studies.

Solution	Reagent	Final concentration	Final volume
peptide solution	peptide substrate 50 mM Tris pH 7.5	100 μ M	94 μ L
cofactor solution 1	sodium L-ascorbate 2OG	4 mM 300 μ M	2 μ L
cofactor solution 2	Fe(II)	50 μ M	2 μ L
enzyme solution	enzyme	3.5 μ M	2 μ L

Mass flow controllers (Brooks Instrument) were used to obtain the desired O₂:N₂ ratios (typically 0, 5, 10, 15, 20, 30, 40, 50, and 60 % O₂). peptide solutions (94 μ L) in sealed glass vials were equilibrated for 10 min at 37 °C with the respective O₂:N₂ ratio. Sodium L-ascorbate/2-oxoglutarate disodium salt (2 μ L), 2 μ L (NH₄)₂Fe(II)(SO₄)₂ and 2 μ L enzyme solutions were added using gas-tight Hamilton syringes, the reaction mix was incubated at 37 °C, and quenched with a final concentration of 1 % formic acid at a time point in the linear region of the reaction. Ratios of hydroxylated and unhydroxylated peptides were analysed by MALDI-TOF-MS and non-enzymatic background oxidation was subtracted.

Inhibition Assays

Inhibition assays were performed as reported in (5). Dose responses were assessed by incubation of the respective 1 μ M PHD enzyme with increasing concentrations of inhibitor (0.03 μ M, 0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 100 μ M) in the presence of 50 μ M (NH₄)₂Fe(II)(SO₄)₂, 10 μ M 2-oxoglutarate disodium salt, 4 mM sodium L-ascorbate and 10 μ M 19mer CODD-peptide (sequence: DLDLEMLAPYIPMDDDFQL) in 50 mM Tris, pH 7.5 at 37 °C. To allow for the inhibitor to bind to PHD, the inhibitor was first incubated 5 min together with the enzyme, and the reaction was started by adding a mix containing co-factors and substrate. Reactions were quenched with formic acid (1 % v/v) at a time-point within the linear region of enzymatic activity. Hydroxylation levels were quantified using MassLynx™ V4.0, non-enzymatic background oxidation was subtracted, and IC₅₀ values were determined with GraphPad Prism.

7.6.2 ESI-LC-MS

Electrospray-ionization liquid chromatography–mass spectrometry (ESI-LC-MS) assays were performed using an ACQUITY Xevo G2-S QToF mass spectrometer (Waters). Assay samples were directly transferred to Chromecol 300 μ L fixed insert vials (TScientific), or first diluted to the desired concentration. The samples were separated on a ProSwift™ RP-15 Analytical column (TScientific) using a 10 min separation method with a gradient of solution A (Milli-Q H₂O + 0.1 % formic acid) and solution B (acetonitrile + 0.1 % formic acid) under standard calibration. Instrument control and data processing was conducted with MassLynx™ V4.1. The following standard assay conditions were used, unless otherwise stated.

Peptide hydroxylation assays

PHD1-3 peptide hydroxylation assays were carried out using the assay conditions in **Table 7.17**. PHD1 and 2 were incubated at an enzyme:substrate ratio of 1:25, and PHD3 was incubated at a ratio of 1:10, to reach complete hydroxylation of the control HIF1 α CODD peptide. The reactions were incubated at 37 °C for 4 h, and quenched with 1 % formic acid (v/v). Hydroxylated substrates were identified using MassLynx™ V4.1.

Table 7.17 Conditions for PHD1-3 peptide hydroxylation assays.

Reagent	Final concentration
PHD1-3	2 μ M (10 μ M in PHD3 assays)
peptide substrate	50 μ M (100 μ M in PHD3 assays)
sodium L-ascorbate	300 μ M
2OG	300 μ M
Fe(II)	50 μ M

Competition experiments

Peptide competition assays were performed using the assay conditions in **Table 7.18**. The reactions were incubated at 37 °C, and quenched by adding a 1:1 volume of acetonitrile and freezing in liquid nitrogen, at a time point in the linear region of enzymatic activity. Hydroxylation levels were quantified using MassLynx™ V4.1, and normalised to samples containing no competitive peptide.

Table 7.18 Assay conditions for competition assays.

Reagent	Final concentration
tPHD2/PHD3 ^{fl}	2 µM
HIF1α CODD/NODD	50 µM
competitive peptide	500 µM
sodium L-ascorbate	4 mM
2OG	300 µM
Fe(II)	50 µM

7.6.3 RapidFire-based enzymatic assays

High-throughput screening was performed using a RapidFire RF360 Agilent6530 QTOF system (Agilent), using a C4 cartridge (Agilent). Samples were prepared in 96 deep well plates (Agilent) and 384 well polypropylene micro-plates (Greiner Bio-One Ltd). Instrument control was conducted with MasshunterTOF Version B.05.01 (Agilent), and data was processed using RapidFire Integrator Version 4.0 (Agilent). The following standard assay conditions were used, unless otherwise stated.

Time-courses

Time-course assays were performed with varying enzyme and substrate concentrations, using the following assay conditions: 10 µM (NH₄)₂Fe(II)(SO₄)₂, 10 µM sodium L-ascorbate, 10 µM 2-oxoglutarate disodium salt, 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine (TCEP, in case of PHD3). The reactions were incubated at 25 °C, and hydroxylation levels were determined at various time points.

Active site titrations

Active site titration experiments were set up using the following assay conditions: 150 nM enzyme (tPHD2/PHD3), 5 μ M HIF1 α CODD (in case of tPHD2), 2 μ M HIF1 α CODD (in case of PHD3^{fl}), 0-500 nM inhibitor, 10 μ M (NH₄)₂Fe(II)(SO₄)₂, 10 μ M sodium L-ascorbate, 10 μ M 2-oxoglutarate disodium salt, 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM TCEP (in case of PHD3). Inhibitors at varying concentrations (at least 6 different concentrations in a suitable range) were first incubated with PHD enzyme for 25 min, to allow for complete binding to the enzyme. The reaction then was started by addition of a substrate-co-factor mix, incubated at 25 °C, and hydroxylation levels were determined at various time points in the linear region. Initial rates of hydroxylation were determined using RapidFire Integrator, and the active site concentration was calculated from the Morrison Plot (6), using GraphPad Prism.

Inhibitor screens

Inhibitor screens were performed as detailed below (7). All screens were set up in 384 well plates using an Echo 550 Acoustic Dispenser (Labcyte). Dose responses were assessed by incubation of 100 nM active PHD enzyme (as determined by active site titration), with increasing concentrations of inhibitor in the presence of 5 μ M HIF1 α CODD (in case of tPHD2), 2 μ M HIF1 α CODD (in case of PHD3), 10 μ M (NH₄)₂Fe(II)(SO₄)₂, 10 μ M sodium L-ascorbate, 10 μ M 2-oxoglutarate disodium salt, 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM TCEP (in case of PHD3). To allow for the inhibitor to bind to PHD, the inhibitor was first incubated 30 min together with the enzyme, and the reaction was started by adding a mix containing co-factors and substrate. Reactions were quenched with formic acid (1 % v/v) at a time-point within the linear region of enzymatic activity. Hydroxylation levels were quantified using RapidFire Integrator, and IC₅₀ values were determined with GraphPad Prism.

7.6.4 Protein ESI-LC-MS

Samples of purified protein batches were analysed on a LCT Premier ESI-TOF Mass Spectrometer (Waters), and the data was processed using Mass Lynx V4.0.

7.6.5 Proteomics

7.6.5.1 Sample preparation

Samples of GST-affinity purified proteins were prepared according to the proteomics protocol of the Kessler Lab Proteomics Group (Target Discovery Institute, University of Oxford, (8)).

Methanol/chloroform extraction

The protein sample (1 mg) was first adjusted to a volume of 200 μ L in GST Binding Buffer (**Chapter 7.3.1, Table 7.13**). The protein was extracted by adding 600 μ L methanol, 150 μ L chloroform and vortexing. Milli-Q H₂O (450 μ L) was added and the sample was vortexed again. The sample was centrifuged at rt for 1 min and the upper phase was taken off without disrupting the precipitated proteins at the interface. Methanol (450 μ L) was added and the sample was vortexed, followed by centrifugation at rt for 2 min, and the supernatant was removed from the pelleted protein.

In-solution digestion

Urea (100 μ L, 6M) was added to the protein pellet, the sample was vortexed and subsequently sonicated 2 min. Dithiothreitol (DTT, 5 μ L, 200 mM) in 0.1 M Tris, pH 7.8 was added, the sample was vortexed again and incubated for 30-60 min at rt. Iodoacetamide (20 μ L, 200 mM) in 0.1 M Tris, pH 7.8 was added, the sample was vortexed, and incubated for 30-60 min at rt. After adding 20 μ L of 200 mM DTT in 0.1 M Tris, pH 7.8, the sample was vortexed, incubated for an additional 30-60 min at rt, and diluted with 775 μ L of Milli-Q H₂O (followed by vortexing). To digest the sample, trypsin was added in a 1:50 ratio regarding the total amount of protein in the sample (1 mg) and the sample was softly mixed and incubated overnight at 37 °C. The reaction was stopped by adding concentrated acetic acid until pH < 6.

SEP-PAK C18 purification

Digested samples were de-salted and purified over a Sep-Pack® Light C18 cartridge (Waters) using HPLC-grade solvents. The column was flushed with 5 mL of solution B (65% acetonitrile, 35% MilliQ water, 0.1% formic acid) and equilibrated with 10 mL of solution A (98% MilliQ water, 2% acetonitrile, 0.1% formic acid). The sample was then slowly applied onto the Sep-Pack column and the column was then washed with 10 mL of solution A. The sample was eluted with 2 x 1 mL of solution B and the peptides were dried down by vacuum centrifugation.

7.6.5.2 LC-MS/MS analysis

Samples were subjected to LC-MS/MS analysis on a service basis provided by Roman Fischer, University of Oxford, UK. LC-MS/MS data was analysed by Roman Fischer using the Mascot database (Matrix Science Ltd).

7.7 NMR experiments

7.7.1 2OG turnover monitoring by ^1H CPMG NMR experiments

2OG turnover to succinate was monitored by ^1H Carr-Purcell-Meiboom-Gill (CPMG) experiments (9); typical experimental parameters for CPMG NMR spectroscopy were as follows: total echo time, 40 ms; acquisition time, 2.72 s; relaxation delay, 2 s; number of transients, 64. The PROJECT-CPMG sequence ($90^\circ\text{x}-[\tau-180^\circ\text{y}-\tau-90^\circ\text{y}-\tau-180^\circ\text{y}-\tau]n-\text{acq}$) was applied (10). Water suppression was achieved by pre-saturation. Data were processed using Bruker 3.1 software with a line broadening of 0.3 Hz. Assay mixtures contained 20 μM enzyme, 125 μM $(\text{NH}_4)_2\text{Fe(II)(SO}_4)_2$, 1 mM sodium L-ascorbate, 500 μM substrate (where necessary), 400 μM 2-oxoglutarate disodium salt, in 10 % D_2O and 90 % H_2O , 50 mM Tris- D_{11} , pH 7.5.

7.7.2 ^{13}C - HIF1 α CODD and ^{13}C - HIF1 α NODD displacement experiments

^{13}C -HIF1 α CODD and ^{13}C -HIF1 α NODD displacement experiments were carried out as described in (7). The 1D CLIP HSQC displacement experiments were conducted using a Bruker AV700 NMR instrument. 3 mm MATCH NMR tubes were used (Cortecnet). All experiments were performed at 298 K. The reporter HIF1 α CODD/NODD peptide (DLDLEMLAPYIPMDDDFQL-NH₂/DALTLAPAAGDTIISLDF-NH₂) was ^{13}C labelled on all carbons in its proline ring. The CLIP-HSQC sequence was used for 1D HSQC experiments (without ^{13}C decoupling). A relaxation delay of 2 s was applied. The $^1J_{\text{CH}}$ was set to 160 Hz. A 6.8 ms Q3.1000 180 degree pulse was used and selective irradiation was applied at the selective chemical shift. Data were processed with Bruker 3.1 software. Prior to Fourier transformation, data were multiplied with a Gaussian-Lorentzian function with a line broadening of 3 Hz.

Comparison of HIF1 α CODD/NODD binding to TaPHD

Assay mixtures contained 50 μM ^{13}C -proline HIF1 α CODD/NODD, 50 μM TaPHD (where necessary), 50 μM 2-oxoglutarate disodium salt buffered with 50 mM Tris- D_{11} , pH 7.5, in 10 % D_2O and 90 % H_2O .

Non-HIF substrate binding to tPHD2

Assay mixtures contained 50 μM ^{13}C -proline HIF1 α CODD/NODD, 50 μM tPHD2 (where necessary), 400 μM Zn(II), 800 μM non-HIF substrate peptide (were necessary), 50 μM 2-oxoglutarate disodium salt buffered with 50 mM Tris-D₁₁, pH 7.5, in 10 % D₂O and 90 % H₂O.

7.8 Fluorescence-polarisation displacement assays

The fluorescence-polarisation displacement assays were set up in 384 well plates (Coming 3575) as shown in **Table 7.19** (11). Serial dilutions of the competitive inhibitor (1 μ M - 1 mM) were prepared in 100 % DMSO. The plate was shaken for 1 min, centrifuged, and fluorescence-polarisation was measured on a PHERAstar FS reader (excitation 485 nm, emission 520 nm).

Table 7.19 Assay conditions for steady-state oxygen-dependence studies.

Reagent	Volume per well
competitive inhibitor (1 μ M - 1 mM)	2 μ L
VCB (1 μ M)	9 μ L
FAM-DEALAHypYIPD (278 nM)	9 μ L
Σ	20 μ L

The degree of displacement of the fluorescence-labelled HIF1 α -peptide (FAM-DEALAHypYIPD) peptide by a competitive inhibitor was determined as follows:

Maximum polarisation (or minimum displacement) was determined in wells containing DMSO, VCB, and FAM-DEALAHypYIPD. Minimum polarisation (or maximum displacement) was determined from wells containing DMSO and FAM-DEALAHypYIPD. The degree of displacement of FAM-DEALAHypYIPD from the VCB complex by the competitive binder was then determined by measuring the difference in fluorescence-polarisation (mP) between samples containing VCB and FAM-DEALAHypYIPD (maximum polarisation, minimum displacement), and samples containing the competitive binder.

Compound dose-response curves were fitted using GraphPad Prism and IC₅₀ values were calculated using one-site fitting log IC₅₀ models. K_d values for the competitive inhibitor-VCB complex were calculated from the IC₅₀ values as described in (11).

7.9 CD spectroscopy

CD spectroscopy experiments were performed using a Chirascan CD/Fluorimeter spectrometer (Applied Photophysics) using an optimised version of the protocol in (4). Data were recorded at 0.3 mg/mL enzyme concentration in 10 mM sodium phosphate, pH 7.5, in a quartz cuvette (1 mm path-length). CD spectra were measured at 25 °C in a wavelength range of 260 to 185 nm at 0.5 nm intervals, 0.5 scans/s. Spectra were baseline-corrected by subtracting the buffer spectrum, and data from three independent scans was averaged. Thermal stability measurements were conducted under the same conditions, by measuring the ellipticity at 215 nm (θ_{220}) at temperatures ranging from 25 to 90 °C, ramping: 2 °C/min, 12 scans/s. The data from 3 different scans was averaged, the signal was normalised, and then fitted to a boltzmann sigmoidal curve using GraphPad Prism.

7.10 Crystallography

7.10.1 Crystallisation trials

Commercial crystallisation screens Crystal Screen HT (Hampton Research), and JCSG+ (Molecular Dimensions) were used to screen for suitable crystallisation conditions. tPHD2 variants were incubated with MnCl₂ and FG-2216/IOX3 (unreactive substitutes for Fe and 2OG) for 45 min on ice as described in **Table 7.20**.

Table 7.20 Crystallisation of tPHD2 variants.

Reagent	Final concentration
tPHD2 variant (in SEC buffer, Table 7.13)	25 mg/mL
MnCl ₂ (100 mM stock in 50 mM Tris pH 7.5)	1.5 mM
FG2216 (100 mM stock in 100 % DMSO)	3 mM

The sample was centrifuged for 5 min at 4 °C, 14 K, to separate off any precipitate. The crystallisation screens were set up in sitting drop plates (Hampton Research), using an automated liquid dispenser (PHENIX). 3 different protein concentrations per plate were screened (reservoir:protein = 1:2, 1:1, 2:1).

7.10.2 Crystal harvesting

Crystals were harvested with appropriate loops (Hampton Research), and cryo-cooled in liquid nitrogen. The cryo-protectant contained 25 % glycerol and 75 % of the respective reservoir solution.

7.10.3 Data collection and structure solution

All data sets were collected on single crystals at 100 K at the Diamond synchrotron. The structures were solved by molecular replacement using PHASER (12) with tPHD2 wt as search model (PDB: 4BQX, (13)). Structure refinement was carried out in iterative cycles using PHENIX (14) and model building with COOT (15), until no further improvement of R_{free} could be achieved.

7.11 Human cell culture

All procedures were carried out under sterile conditions in a Class II Biological Safety Cabiner (Nuair Nu 437-400E). *H. Sapiens* HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) containing 4 mM L-glutamine and 10 % fetal bovine serum (FBS) at 37 °C unless otherwise described.

7.11.1 Cell passaging

HeLa cell cultures (20 mL) were grown in cell culture bottles and passaged every 3 days following the procedure described below. Trypsin and fresh media was pre-warmed in a water bath at 37 °C. First, the media was removed completely from the cells, and the cells were washed with 10 mL phosphate-buffered saline (PBS). The cells were trypsinised from the bottle by adding 3 mL trypsin (0.25 %, Gibco), and incubated at 37 °C, until the cells were released from the surface of the bottle. Cells were resuspended in 7 mL fresh media by pipetting, 1 mL of resuspended cells were left in the bottle, and fresh media was added up to 20 mL. The remaining 9 mL trypsinised cells were used to seed new plates at desired cell densities. For hypoxia response element (HRE) luciferase reporter assays, cells were seeded into 24 well plates at 1×10^5 cells/well. For immunoprecipitation experiments, cells were seeded into 6 cm dishes at 0.8×10^6 cells/dish.

7.11.2 Transient transfection

HeLa cells were grown to ~ 50 % confluence before they were transiently transfected using GeneJuice (Novagen), following the procedure in the manual (GeneJuice Transfection Reagent, Novagen, (16)). For transfection of 24 well plates, 0.5 µg total DNA per well, and for transfection of 6 cm dishes 4 µg total DNA, were used. GeneJuice (3 µL per 1 µg DNA) were diluted in 50 µL Opti-MEM reduced serum medium (TScientific), mixed thoroughly by vortexing, and incubated for 5 min at rt. The desired amount of DNA was added, the solution was mixed gently by pipetting, and incubated 10 min at rt, before drop-wise addition to the cells.

7.11.3 HRE luciferase assay

Cells were plated into 24 well plates as described above one day prior to transfection. The cells were transiently transfected (see **Chapter 7.11.2**) with 50 ng carbonic anhydrase 9 (CA9) hypoxia response element (HRE) luciferase reporter construct (17), 50 ng pCMV- β gal (Promega), and 400 ng of the desired construct for over-expression. Control cells were transfected with 50 ng simian virus 40 (SV40) luciferase reporter construct instead of CA9 HRE luciferase reporter construct. Dimethyloxalyl glycine (DMOG, 100 mM) in PBS was added to the cells to a final concentration of 1 mM were necessary, and cells were grown for 24 h at normoxia or 1 % O₂ in hypoxic chambers.

The next steps were carried out following the procedures in the Luciferase Assay System Manual (Promega, (18)) and the Mammalian β -Galactosidase Assay Kit Manual (TScientific, (19)).

Cell harvest

For cell harvest, the growth medium was carefully removed, the cells were rinsed with PBS, and vacuum-dried. 100 μ L of pre-warmed Reporter Lysis Buffer (Promega) was added into each well, and the plates were rocked to ensure full coverage of the cells with the lysis buffer. Lysis was completed by freezing the cells at -80 °C for > 30 min. The cells were thawed at 4 °C, and shortly at rt, the lysed cells were transferred to an eppendorf tube, and centrifuged for 5 min at 13 K, 4°C. 20 μ L of the supernatant were transferred to 96 well plates (transparent plate for β -galactosidas Assay, white plate for luciferase assay). Reporter Lysis Buffer (20 μ L) was plated for normalisation.

Luciferase assay

For determination of the luciferase activity, 100 μ L Luciferase Assay Reagent (Promega) was added into each well, and the plate was incubated for 5 min at rt (darkened workspace). The luciferase signal was measured using a PHERAstar FS reader.

β-galactosidase Assay

Transfection efficiency in each sample was determined by measuring the β-galactosidase activity. β-Galactosidase Assay Reagent (20 μL, TScientific) was added to 20 μL sample in each well of the 96 well plate. The plate was covered and incubated for 30 min at 37 °C. The β-galactosidase signal was measured at 405 nm using a PHERAstar FS reader. Saturated samples (OD₄₀₅ > 1.0) were diluted and remeasured. The ratio of luciferase : β-galactosidase signals was determined in order to correct for variable transfection efficiencies in the cell lysates.

7.11.4 Immunoprecipitation experiments

Cells were plated into 6 cm dishes as described above one day prior to transfection. The cells were transiently transfected (**Chapter 7.11.2**) using 4 μg of the desired over-expression construct, and the cells were grown for 24 h at normoxia or different levels of hypoxia. Where necessary, 100 mM DMOG in PBS was added to the cells to a final concentration of 1 mM 16 h prior to cell harvest, and 100 mM of the protease inhibitor MG132 was added to a final concentration of 1 mM 6 h prior to cell harvest.

Cell harvest

For cell harvest, the growth medium was completely removed, the cells were washed with PBS (~5 mL per 6 cm dish), and vacuum-dried. Pre-chilled Jie's buffer (1 mL, 50 mM NaCl, 10 mM Tris HCl pH 7.4, 5 mM MgCl₂, 0.5 % (v/v) tergitol type NP40), containing Complete EDTA-free protease inhibitor cocktail (Roche) was added to the cells, the lysed cells were transferred to eppendorf tubes and centrifuged 15 min at 4 °C, 13 K rpm. The supernatant (800 μL) was used for immunoprecipitation experiments, and 100 μL samples were kept as control samples for western blotting.

Immunoprecipitation

Anti-FLAG M2 Magnetic beads (Sigma Aldrich, 20 μ L per sample) were washed 5 times with 70 μ L of pre-chilled Jie's buffer, before 800 μ L of the cell lysate was added. The mix was incubated on a rotor overnight at 4 °C. The beads were washed 5 times with 800 μ L Jie's buffer, and proteins were eluted from the beads by addition of 100 μ L 3x FLAG peptide (200 μ g/mL, Sigma Aldrich) in PBS, and subsequent incubation for 5 min at 800 rpm. Proteins in the supernatant were detected by SDS-PAGE separation, followed by western blotting.

Western blotting

Protein samples were separated by SDS-PAGE electrophoresis on gradient gels (NuSEP) at 25 mA per gel. The gel was blotted onto a membrane (pre-soaked in MeOH, washed with Milli-Q H₂O, and equilibrated in Transfer Buffer (**Table 7.21**) for 10 min), for 50 min at a constant voltage of 120 V. The membrane was blocked by incubating 30 min in Blocking Buffer (5 % milk in PBS/Tween, **Table 7.21**), washed with Milli-Q H₂O, and primary antibodies at appropriate dilutions in 10 mL Blocking Buffer were added (**Table 7.22**). The membrane was incubated overnight, washed 3 times with PBS/Tween, and 10 mL secondary antibody (or FLAG-antibody) solutions were added. After incubating on a shaker for 1 h, the membrane was washed 3 times with PBS/Tween, and bands were visualised by incubating 3 min with 1 mL of a detection reagent (SuperSignal™ Chemiluminescent HRP Substrates, TScientific). The western blots were analysed by using a Bio Rad gel imaging system.

Table 7.21 Western blotting buffers.

Component	Reagent	Volume/ Mass
20x Transfer Buffer	Tris base	24.2 g
	Glycine	150 g
	Milli-Q H ₂ O	top up to 1 L
1x Transfer Buffer	MeOH	100 mL
	20x Transfer Buffer	50 mL
	20 % (w/v) SDS	0.25 mL
	Milli-Q H ₂ O	top up to 1 L
10x PBS/Tween	Tween-20	10 mL
	NaCl	50 g
	KCl	2 g
	Na ₂ HPO ₄ x 7 H ₂ O	11.5 g
	KH ₂ PO ₄	2 g
	Milli-Q H ₂ O	top up to 1 L

Table 7.22 Primary and secondary antibodies.

Primary	Purified Mouse Anti-Human HIF-1 α antibody (Transduction Laboratories)
	Anti-PHD2 antibody (Novus Biologicals)
	Anti-p19 ^{Skp1} monoclonal antibody (Transduction Laboratories)
	Monoclonal Anti-FLAG (R) M2-peroxidase (HRP) antibody (Sigma Aldrich)
Secondary	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP antibody (Invitrogen)

7.12 References

- (1) Gileadi, O., Burgess-Brown, N. A., Colebrook, S. M., Berridge, G., Savitsky, P., Smee, C. E. A., ... Pantic, N. H. (2008). High Throughput Production of Recombinant Human Proteins for Crystallography BT - Structural Proteomics: High-Throughput Methods. In B. Kobe, M. Guss, & T. Huber (Eds.) (pp. 221–246). Totowa, NJ: Humana Press.
- (2) Agilent Technologies. QuikChange™ Site-Directed Mutagenesis Kit. Intruction Manual (Revision C). Retrieved from: <http://www.chem-agilent.com/pdf/strata/200518.pdf>, 14.09.2017.
- (3) Novagen. Thrombin Kits. User Protocol TB188 Rev. C 0311JN (2011). Retrieved from: http://www.merckmillipore.com/GB/en/product/Thrombin-Restriction-Grade,EMD_BIO-69671?ReferrerURL=https%3A%2F%2Fwww.google.co.uk%2F#anchor_USP, 14.09.2017.
- (4) Tarhonskaya, H., Chowdhury, R., Leung, I. K. H., Loik, N. D., McCullagh, J. S. O., Claridge, T. D. W., ... Flashman, E. (2014). Investigating the contribution of the active site environment to the slow reaction of hypoxia-inducible factor prolyl hydroxylase domain 2 with oxygen. *Biochemical Journal*, 463(3), 363–372.
- (5) Chan, M. C., Ilott, N. E., Schödel, J., Sims, D., Tumber, A., Lippl, K., ... Schofield, C. J. (2016). Tuning the transcriptional response to hypoxia by inhibiting Hypoxia-inducible Factor (HIF) prolyl and asparaginyl hydroxylases. *Journal of Biological Chemistry*, 291(39).
- (6) Williams, J. W., and Morrison, J. F. (1979). The kinetics of reversible tight-binding inhibition. *Methods Enzymology*, 63, 437–467.
- (7) Yeh, T.-L., Leissing, T., Abboud, M. I., Thinnies, C. C., Atasoylu, O., Holt-Martyn, J. P., ... Schofield, C. J. (2017). Molecular and cellular mechanisms of HIF prolyl hydroxylase inhibitors in clinical trials. *Chemical Science*, 8, 7651–7668.
- (8) Kessler Lab, IN-SOLUTION DIGESTION. Guidelines for sample preparation. Retrieved from: https://www.tdi.ox.ac.uk/_asset/file/solution.pdf, 20.02.2018.
- (9) Carr, H. Y., & Purcell, E. M. (1954). Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments. *Physical Review*, 94(3), 630–638.
- (10) Aguilar, J. A., Nilsson, M., Bodenhausen, G., & Morris, G. A. (2012). Spin echo NMR spectra without J modulation. *Chemical Communications*, 48(6), 811–813.
- (11) Van Molle, I., Thomann, A., Buckley, D. L., So, E. C., Lang, S., Crews, C. M., & Ciulli, A. (2012). Dissecting Fragment-Based Lead Discovery at the von Hippel-Lindau Protein:Hypoxia Inducible Factor 1 α Protein-Protein Interface. *Chemistry & Biology*, 19(10), 1300–1312.
- (12) McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., & Read, R. J. (2007). Phaser crystallographic software. *Journal of Applied Crystallography*, 40(Pt 4), 658–674.

-
- (13) Chowdhury, R., Candela-Lena, J. I., Chan, M. C., Greenald, D. J., Yeoh, K. K., Tian, Y.-M., ... Schofield, C. J. (2013). Selective Small Molecule Probes for the Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases. *ACS Chemical Biology*, 8(7), 1488–1496.
- (14) Adams, P. D., Afonine, P. V, Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., ... Zwart, P. H. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica Section D: Biological Crystallography*, 66(Pt 2), 213–221.
- (15) Emsley, P., & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallographica Section D*, 60(12 Part 1), 2126–2132.
- (16) Novagen. GeneJuice® Transfection Reagent. User Protocol TB289 (2012). Retrieved from: http://www.merckmillipore.com/GB/en/product/GeneJuice-Transfection-Reagent,EMD_BIO-70967?ReferrerURL=https%3A%2F%2Fwww.google.co.uk%2F#anchor_USP, 14.09.2017.
- (17) Wykoff, C. C., Beasley, N. J. P., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., ... Harris, A. L. (2000). Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases. *Cancer Research*, 60(24), 7075-7083.
- (18) Promega. Luciferase Assay System. Technical Bulletin TB281 (2015). Retrieved from: <https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/luciferase-assay-system-protocol.pdf>, 14.09.2017.
- (19) Thermo Scientific. Mammalian β -Galactosidase Assay Kit. Instructions 0861.3. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011390_Mammalian_BetaGalactosidase_Asy_UG.pdf, 14.09.2017.

Chapter 8

Summary and Conclusions

The development of oxygen sensing mechanisms likely played a crucial role in the evolution of aerobic organisms, that rely on a steady oxygen supply for their energy metabolism (1). In humans and other animals, the response to hypoxia is critically regulated by the HIF pathway and involves HIF prolyl hydroxylases (PHDs) that act as oxygen sensors. Extensive studies conducted on the human PHDs, above all on the most important of the three PHD isoforms, PHD2 (2-3), have informed on the enzymatic mechanism of prolyl hydroxylation employed by PHD2 (and likely other PHDs); unique kinetic characteristics of PHD2 necessary for its role as an oxygen sensor have been identified (4-7). Further, structural studies on PHD2-HIF1 α CODD/NODD enzyme-substrate complexes have provided insights into substrate binding and ODD selectivity by PHD2 (8-9). Moreover, the potential of the HIF pathway for therapeutic manipulation is being exploited, with PHD inhibitors currently in late-stage clinical trials for treatment of anaemia (10).

The first part of the work described in this thesis (**Chapters 2-4**), focused on remaining open questions of medicinal relevance, regarding the human HIF prolyl hydroxylases. Emerging evidence in the literature suggests that PHDs are not selective for their well established HIF substrates, but accept additional substrates other than HIFs (see **Chapter 2, Table 2.1**). In the light of current clinical studies on PHD inhibitors for treatment of anaemia, and potential implications of the proposed promiscuous nature of PHDs on clinical effects of PHD inhibitors, the work in **Chapter 2** was directed towards investigating the validity of reported non-HIF substrates. In a systematic approach, all reported hydroxylation sites were probed for reaction with PHD1-3 at the peptide level using a ESI-LC-MS-based assay, and a selection of potential non-HIF substrates was analysed at the protein level by use of LC-MS/MS. In parallel, extensive hydroxylation studies using non-HIF substrates produced by *in vitro* transcription and translation (IVTT) were conducted by collaborators (Ya-Min Tian, Matthew Cockman, and Peter Ratcliffe, Nuffield Department of Medicine, University of Oxford, UK, and Francis Crick Institute, UK). Despite the usage of different experimental approaches and various techniques, such as mass spectrometry and monitoring of 2OG turnover by NMR, no evidence was found for PHD-catalysed hydroxylation of non-HIF substrates.

However, from the results obtained in these studies, it cannot be excluded that PHD-catalysed hydroxylation of non-HIF substrates occurs *in vivo*, potentially mediated by cellular interaction partners or post-translational modifications. Nevertheless, the results of the studies clearly show that the set of techniques that is currently used in the field for defining prolyl hydroxylation sites in proteins needs to be reconsidered, as none of the reported prolyl hydroxylation sites could be validated in the study presented here, even when identical peptide sequences were tested in similar experimental set ups as reported in the literature. It is suggested that a standard approach for the unambiguous annotation of prolyl hydroxylation sites should combine MS/MS analysis and amino acid analysis, to avoid that non-enzymatic oxidations such as methionine oxidations are wrongly assigned on prolyl residues. Additionally, a clear proof of the enzyme-dependence of the reaction needs to be provided, as non-enzymatic prolyl hydroxylations might occur within the cell, or during sample preparation for mass spectrometric analysis.

By contrast to PHDs, the results obtained from VCB-binding studies revealed a (likely) promiscuous nature of VCB. Several non-HIF peptides were capable of binding to VCB, including peptides corresponding to sequences of serine/threonine kinase 1 (Akt1), in a hydroxyproline dependent manner. The results of the studies raise the possibility that VCB might accept additional binding partners other than HIFs in cells. Indeed, hydroxylation-dependent interactions between α -chains of collagen IV and pVHL in cells have been reported (11-12). Hydroxylations of prolines in X-Pro-Gly sequences in collagens are catalysed by the collagen prolyl-4-hydroxylases (P4Hs), which are members of the 2OG-oxygenase family, and related to the HIF prolyl hydroxylases (13-17). The collagen P4Hs play a crucial role in the biosynthesis of collagens, as 4-hydroxyproline residues are essential for the collagen triple helix formation (15-16). The finding of a (potential) promiscuous nature of VCB implies a possible new mechanism for regulating HIF α degradation, via competition of alternative substrates with HIF α binding to VCB. In the future, cellular studies should aim at confirming the biological relevance of the VCB interaction partners that were identified in the work described in this thesis.

From a medicinal chemistry perspective, the results from the VCB binding studies are interesting, as they provide information about the substrate binding preferences of VCB. In future studies, the identified criteria for peptides capable of binding to VCB, can be exploited for the design of new VCB inhibitors, including, but not limited to, cyclic peptide inhibitors.

A major aim of the work presented in **Chapter 3** was to investigate if currently available PHD inhibitors are capable of discriminating between PHD isoforms. Considering the differential expression profiles of PHD1-3 and differences in ODD selectivity, by use of PHD isoform-selective inhibitors a more specific therapeutic effect might be achieved than by inhibitors that are equally active on all three PHD isoforms (2-3, 10, 18-19).

A MALDI-TOF-MS-based inhibition assay with PHD1-3 was established, and a RapidFire-based high-throughput inhibition assay with PHD2-3, featuring improved sensitivity when compared to MALDI-TOF-MS, was developed. Applying these assays, current-generation PHD inhibitors and 2OG oxygenase inhibitors were tested against different PHD isoforms. The results revealed that none of the tested inhibitors, including those used in clinical trials, was capable of discriminating between PHD1-3. Further studies were carried out on a series of new PHD inhibitors, in order to investigate if it is possible to develop PHD isoform selective inhibitors. By comparably testing the PHD inhibitors against PHD2 and PHD3, it was found that compounds of the series (JHM377, JHM675, and JHM682) selectively increased HIF1 α CDD hydroxylation by PHD2, but did not exhibit the same effect on PHD3 (or even inhibited PHD3 with an IC₅₀ in the low micromolar range in case of JHM377). Therefore, a proof of concept was provided that small molecule inhibitors can be designed that discriminate between PHD2 and PHD3. Future work should include structural and mechanistic studies on JHM377, JHM675, and JHM682, to elucidate the mode of action of these compounds. Cellular studies could inform whether the apparent PHD isoform selectivity observed by using isolated enzymes manifests in cells.

Emerging evidence suggests a role of PHD2 variants in the adaptation of humans and animals to high altitude (20-22) and in the development of disease, such as erythrocytosis and cancer (23). The work in **Chapter 4** was directed towards establishing a functional relationship between clinically-observed mutations in EGLN1 and disease phenotypes.

The PHD2 R312H and F366L variants were found in patients with erythrocytosis (Richard V. Wijk, University Medical Center Utrecht, Netherlands and Celeste Bento, Hospitais da Universidade de Coimbra, Portugal, unpublished data). Activity analysis of the two variants using time-point-assays revealed reduced activity of PHD2 R312H and F366L towards HIF2 α NODD (and HIF1 α NODD in case of PHD2 F366) when compared to PHD2 wt, and PHD2 F366L exhibited a reduced thermal stability as determined by CD spectroscopy.

A crystal structure obtained of PHD2 F366L revealed a slight shift of the metal-coordinated water molecule, when compared to the positioning of the respective water molecules in the PHD2.CODD and PHD2.NODD complex structures. It is possible that these altered properties of the PHD2 R312H and F366L variants contribute to the development of erythrocytosis via up-regulation of HIF α . Analyses of the three cancer-associated PHD2 variants PHD2 G294S, W334S and D277G (24) revealed that the activity of PHD2 D277G towards HIF α was strongly reduced compared to PHD2 wt, whereas for PHD2 G294S and PHD2 W334S, the activity was unaffected by the respective mutations. Moreover, a crystal structure of PHD2 D277G suggests changes in the sidechain conformations of residues in helix α 3 relative to PHD2.CODD/NODD complex structures, that might affect substrate binding by PHD2 D277G. Overall, the findings on PHD2 D277G suggest that the mutation causes functional changes in PHD2, that have the potential to up-regulate the HIF-pathway, providing a possible link between the PHD2 D277G variant and tumour development in patients carrying the mutation.

The HIF α -PHD-pVHL triad that is required for hypoxia sensing *via* the HIF pathway, is found in all animals ranging from humans to the simplest existent animal *T. adhaerens*; however no evidence of HIF beyond animals was provided to date (25). By contrast, PHD-like enzymes are present in various organisms outside of *Animalia*, such as in *Dictyostelium discoideum*, where the prolyl-4-hydroxylase DdPhyA regulates oxygen-dependent culmination by hydroxylating Skp1, and in *Pseudomonas*, where the PHD-related enzyme PPHD catalyses the hydroxylation of EF-Tu (26-28). The second part of the work described in this thesis (**Chapters 5-6**), focused on the evolution of the HIF pathway in animals, and investigates links between human HIF prolyl hydroxylases and Skp1-mediated oxygen sensing mechanisms.

The aim of the study presented in **Chapter 5** was to investigate if the HIF prolyl hydroxylase in *T. adhaerens* (TaPHD) is capable of sensing hypoxia in a similar manner as human PHD2 (HsPHD2). Combined structural and biochemical studies provide strong evidence that the special kinetic properties of HsPHD2 are conserved in TaPHD, including unusually high *K_m* values for oxygen of HsPHD2 and TaPHD, low substrate uncoupled 2OG turnover, and structural elements in the active sites of HsPHD2 and TaPHD, which are thought to enable the unusually slow reaction of HsPHD2 with oxygen (5, 7-8). A high degree of conservation between HsPHD2 and TaPHD was found when comparing the ODD binding modes in TaPHD and HsPHD2, as both enzymes employ the β 2/ β 3-finger-loop and the C-terminal α 4-helix for substrate binding.

The finding of such a high degree of conservation between *Hs*PHD2 and *Ta*PHD is remarkable, as it suggests that a functional hypoxia sensing mechanism is already in place in *T. adhaerens*, a simple, multicellular placozoan lacking the internal structure of higher animals. Therefore, the results of the study presented here emphasise the importance of the development of hypoxia sensing mechanisms as a prerequisite for the evolution of higher animals. Considering that humans and *T. adhaerens* are at opposite ends of animal evolution, it is possible that oxygen sensing HIF prolyl hydroxylases exist in all animals. Future work should aim to investigate if this is the case, by studying PHDs from different animal *phyla* and classes. The study of PHDs from *Ornithurae* (birds), *Craniata* (fishes), or *Reptilia* (e.g. snakes), might be of particular interest, as these animals possibly exhibit special requirements on their hypoxia-sensing mechanisms, dictated by the oxygen level (and changes thereof) in their natural habitats.

In **Chapter 6**, it was investigated if human PHDs are capable of interacting with Skp1. The results from competition experiments show that *in vitro*, *Hs*Skp1 competes with HIF1 α NODD hydroxylation by *Hs*PHD2, and HIF1 α CODD hydroxylation by *Hs*PHD3. However, in cellular studies it was observed that overexpressing *Hs*Skp1 did not stabilise HIF α , as determined using HRE luciferase reporter assays. It was further investigated, if *Hs*PHD2 and *Hs*Skp1 bind to each other in the cellular environment, yet immunoprecipitation experiments from HeLa cells were non-conclusive. Future studies should aim to clarify if an interaction of biological relevance exists between *Hs*PHD2 and *Hs*Skp1.

The finding that both *Hs*Skp1 (ortholog of *Dd*/*Tg*Skp1) and the transcription factor *e*EF2 (ortholog of EF-G in *Pseudomonas*) compete with HIF1 α binding to PHDs *in vitro* is of interest from an evolutionary perspective, as it emphasises evolutionary links between human PHDs and the prolyl hydroxylases from *D. discoideum*, *T. gondii*, and *Pseudomonas* (although the PHD-related enzyme in *Pseudomonas* accepts a different transcription factor, EF-Tu).

References

- (1) Taylor, C. T., & McElwain, J. C. (2010). Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans. *Physiology*, 25(5), 272–279.
- (2) Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R., & Taubman, M. B. (2002). Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. *Biochemistry and Cell Biology*, 80(4), 421–426.
- (3) Willam, C., Maxwell, P. H., Nichols, L., Lygate, C., Tian, Y. M., Bernhardt, W., ... Pugh, C. W. (2017). HIF prolyl hydroxylases in the rat; organ distribution and changes in expression following hypoxia and coronary artery ligation. *Journal of Molecular and Cellular Cardiology*, 41(1), 68–77.
- (4) Hausinger, R. P. (2004). Fe(II)/ α -Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 39(1), 21–68.
- (5) Flashman, E., Hoffart, L. M., Hamed, R. B., Bollinger, J. M., Krebs, C., & Schofield, C. J. (2010). Evidence for the Slow Reaction of Hypoxia-Inducible Factor Prolyl Hydroxylase 2 with Oxygen. *The FEBS Journal*, 277(19), 4089–4099.
- (6) Tarhonskaya, H., Chowdhury, R., Leung, I. K. H., Loik, N. D., McCullagh, J. S. O., Claridge, T. D. W., ... Flashman, E. (2014). Investigating the contribution of the active site environment to the slow reaction of hypoxia-inducible factor prolyl hydroxylase domain 2 with oxygen. *Biochemical Journal*, 463(3), 363–372.
- (7) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Liénard, B. M. R., ... Schofield, C. J. (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9814–9819.
- (8) Chowdhury, R., McDonough, M. A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K. S., ... Schofield, C. J. (2009). Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases. *Structure*, 17(7), 981–989.
- (9) Chowdhury, R., Leung, I. K. H., Tian, Y.-M., Abboud, M. I., Ge, W., Domene, C., ... Schofield, C. J. (2016). Structural basis for oxygen degradation domain selectivity of the HIF prolyl hydroxylases. *Nature Communications*, 7, 12673.
- (10) Chan, M. C., Holt-Martyn, J. P., Schofield, C. J., & Ratcliffe, P. J. (2016). Pharmacological targeting of the HIF hydroxylases – A new field in medicine development. *Molecular Aspects of Medicine*, 47(Supplement C), 54–75.

-
- (11) Grosfeld, A., Stolze, I. P., Cockman, M. E., Pugh, C. W., Edelman, M., Kessler, B., ... Masson, N. (2007). Interaction of Hydroxylated Collagen IV with the von Hippel-Lindau Tumor Suppressor. *Journal of Biological Chemistry*, 282(18), 13264–13269.
- (12) Kurban, G., Duplan, E., Ramlal, N., Hudon, V., Sado, Y., Ninomiya, Y., & Pause, A. (2007). Collagen matrix assembly is driven by the interaction of von Hippel–Lindau tumor suppressor protein with hydroxylated collagen IV alpha 2. *Oncogene*, 27, 1004.
- (13) Kivirikko, K. I., & Pihlajaniemi, T. (2006). Collagen Hydroxylases and the Protein Disulfide Isomerase Subunit of Prolyl 4-Hydroxylases. In *Advances in Enzymology and Related Areas of Molecular Biology* (pp. 325–398). John Wiley & Sons, Inc.
- (14) Kivirikko, K. I., & Myllyharju, J. (1998). Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biology*, 16(7), 357–368.
- (15) Myllyharju, J. (2003). Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biology*, 22(1), 15–24.
- (16) Myllyharju, J., & Kivirikko, K. I. (2001). Collagens and collagen-related diseases. *Annals of Medicine*, 33(1), 7–21.
- (17) Aik, W. S., Chowdhury, R., Clifton, I. J., Hopkinson, R. J., Leissing, T., McDonough, M. A., ... Walport, L. J. (2015). CHAPTER 2 Introduction to Structural Studies on 2-Oxoglutarate-Dependent Oxygenases and Related Enzymes. In *2-Oxoglutarate-Dependent Oxygenases* (pp. 59–94). The Royal Society of Chemistry.
- (18) Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., & Myllyharju, J. (2003). Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 278(33), 30772–30780.
- (19) Huang, J., Zhao, Q., Mooney, S. M., & Lee, F. S. (2002). Sequence Determinants in Hypoxia-inducible Factor-1 α for Hydroxylation by the Prolyl Hydroxylases PHD1, PHD2, and PHD3. *Journal of Biological Chemistry*, 277(42), 39792–39800.
- (20) Yi, X., Liang, Y., Huerta-Sanchez, E., Jin, X., Cuo, Z. X. P., Pool, J. E., ... Wang, J. (2010). Sequencing of Fifty Human Exomes Reveals Adaptation to High Altitude. *Science (New York, N.Y.)*, 329(5987), 75–78.
- (21) Biggam, A., Bauchet, M., Pinto, D., Mao, X., Akey, J. M., Mei, R., ... Shriver, M. D. (2010). Identifying Signatures of Natural Selection in Tibetan and Andean Populations Using Dense Genome Scan Data. *PLoS Genetics*, 6(9), e1001116.
- (22) Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., ... Ge, R. (2010). Genetic Evidence for High-Altitude Adaptation in Tibet. *Science*, 329(5987), 72–75.

-
- (23) Gardie, B., Percy, M. J., Hoogewijs, D., Chowdhury, R., Bento, C., Arsenault, P. R., ... Lee, F. S. (2014). The role of PHD2 mutations in the pathogenesis of erythrocytosis. *Hypoxia*, 2, 71–90.
- (24) Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., ... Wooster, R. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *British Journal of Cancer*, 91, 355 – 358.
- (25) Loenarz, C., Coleman, M. L., Boleininger, A., Schierwater, B., Holland, P. W. H., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Reports*, 12(1), 63–70.
- (26) Scotti, J. S., Leung, I. K. H., Ge, W., Bentley, M. A., Paps, J., Kramer, H. B., ... Schofield, C. J. (2014). Human oxygen sensing may have origins in prokaryotic elongation factor Tu prolyl-hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America*, 111(37), 13331–13336.
- (27) West, C. M., Wang, Z. A., & van der Wel, H. (2010). A cytoplasmic prolyl hydroxylation and glycosylation pathway modifies Skp1 and regulates O(2)-dependent development in *Dictyostelium*. *Biochimica et Biophysica Acta*, 1800(2), 160–171.
- (28) Xu, Y., Wang, Z. A., Green, R. S., & West, C. M. (2012). Role of the Skp1 prolyl-hydroxylation/glycosylation pathway in oxygen dependent submerged development of *Dictyostelium*. *BMC Developmental Biology*, 12, 31.

Permissions for Reprints

Figure 1.13: Evolution of life. (A) Changes in atmospheric oxygen levels and their impact on evolution of complex life forms. (Figure adapted from Taylor, C. T., & McElwain, J. C. (2010). Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans. *Physiology*, 25(5), 272-279.).



RightsLink®

Home

Create Account

Help



Title: Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans

Author: Cormac T. Taylor, Jennifer C. McElwain

Publication: *Physiology*

Publisher: The American Physiological Society

Date: Oct 1, 2010

Copyright © 2010, The American Physiological Society

LOGIN

If you're a **copyright.com** user, you can login to RightsLink using your copyright.com credentials. Already a **RightsLink** user or want to [learn more?](#)

Permission Not Required

Permission is not required for this type of use.

BACK

CLOSE WINDOW

Copyright © 2018 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement.](#) [Terms and Conditions.](#) Comments? We would like to hear from you. E-mail us at customercare@copyright.com

Figure 1.14: Bioinformatic analysis of the HIF pathway. (Figure adapted from Loenarz, C., Coleman, M. L., Boleininger, A., Schierwater, B., Holland, P. W. H., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. EMBO Reports, 12(1), 63–70.).

Figure 1.17: Model of the O₂-dependent, Skp1-mediated development of the slime mold *Dictyostelium discoideum*. (Figure adopted from Xu, Y., Wang, Z. A., Green, R. S., & West, C. M. (2012). Role of the Skp1 prolyl-hydroxylation/glycosylation pathway in oxygen dependent submerged development of Dictyostelium. BMC Developmental Biology, 12, 31.).

Licence: <https://creativecommons.org/licenses/by/4.0/legalcode>

Figure 6.1: Composition of the E3 Skp1-cullin 1-F-box protein (SCF) ubiquitin complex.

(Figure adapted from West, C. M., & Blader, I. J. (2015). Oxygen Sensing by Protozoans: How They Catch Their Breath. *Current Opinion in Microbiology*, 26, 41–47.).

ELSEVIER LICENSE TERMS AND CONDITIONS

Feb 21, 2018

This Agreement between University of Oxford -- Kerstin Lippl ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4293761319963
License date	Feb 21, 2018
Licensed Content Publisher	Elsevier
Licensed Content Publication	Current Opinion in Microbiology
Licensed Content Title	Oxygen sensing by protozoans: how they catch their breath
Licensed Content Author	Christopher M West,Ira J Blader
Licensed Content Date	Aug 1, 2015
Licensed Content Volume	26
Licensed Content Issue	n/a
Licensed Content Pages	7
Start Page	41
End Page	47
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 2
Title of your thesis/dissertation	Structural, Biochemical, and Inhibition Studies on Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases
Expected completion date	Mar 2018
Estimated size (number of pages)	250
Requestor Location	University of Oxford Chemistry Research Laboratory 12 Mansfield Road Oxford, Oxfordshire OX1 3TA United Kingdom Attn: University of Oxford
Publisher Tax ID	GB 494 6272 12
Total	0.00 GBP

[Terms and Conditions](#)

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions

established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of

any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. **Revocation:** Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website:** The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com>; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at <http://www.elsevier.com>. All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
 - via their non-commercial person homepage or blog
 - by updating a preprint in arXiv or RePEc with the accepted manuscript

- via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
- directly by providing copies to their students or to research collaborators for their personal use
- for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
 - via non-commercial hosting platforms such as their institutional repository
 - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a [CrossMark logo](#), the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's [posting policy](#) for further information.

18. For book authors the following clauses are applicable in addition to the above:

Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our [open access license policy](#) for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by/4.0>.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-sa/4.0>.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-nd/4.0>. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

**JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS**

Feb 21, 2018

This Agreement between University of Oxford -- Kerstin Lippl ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4293770457985
License date	Feb 21, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	EMBO Reports
Licensed Content Title	The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, <i>Trichoplax adhaerens</i>
Licensed Content Author	Christoph Loenarz, Mathew L Coleman, Anna Boleininger, Bernd Schierwater, Peter W H Holland, Peter J Ratcliffe, Christopher J Schofield
Licensed Content Date	Nov 26, 2010
Licensed Content Pages	8
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 4
Will you be translating?	No
Title of your thesis / dissertation	Structural, Biochemical, and Inhibition Studies on Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases
Expected completion date	Mar 2018
Expected size (number of pages)	250
Requestor Location	University of Oxford Chemistry Research Laboratory 12 Mansfield Road Oxford, Oxfordshire OX1 3TA United Kingdom Attn: University of Oxford
Publisher Tax ID	EU826007151
Total	0.00 GBP
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that

you opened your RightsLink account (these are available at any time at <http://myaccount.copyright.com>).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order**, is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. **For STM Signatory Publishers clearing permission under the terms of the [STM Permissions Guidelines](#) only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts**, You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto
- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES

ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The [Creative Commons Attribution License \(CC-BY\)](#) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The [Creative Commons Attribution Non-Commercial \(CC-BY-NC\) License](#) permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The [Creative Commons Attribution Non-Commercial-NoDerivs License](#) (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library

<http://olabout.wiley.com/WileyCDA/Section/id-410895.html>

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.