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The dual role of HIF hydroxylase PHD3 in cancer cell survival

by

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To Halla

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ABSTRACT

Most advanced tumours face periods of reduced oxygen availability i.e. hypoxia. During these periods tumour cells undergo adaptive changes enabling their survival under adverse conditions. In cancer hypoxia-induced cellular changes cause tumour progression, hinder cancer treatment and are indicative of poor prognosis.

Within cells the main regulator of hypoxic responses is the hypoxia-inducible factor (HIF). HIF governs the expression of over a hundred hypoxia-inducible genes that regulate a number of cellular functions such as angiogenesis, glucose metabolism and cell migration. Therefore the activity of HIF must be tightly governed. HIF is regulated by a family of prolyl hydroxylase enzymes, PHDs, which mark HIF for destruction in normoxia. Under hypoxic conditions PHDs lose much of their enzymatic activity as they need molecular oxygen as a cofactor. Out of the three PHDs (PHD1, 2 and 3) PHD2 has been considered to be the main HIF-1 regulator in normoxic conditions. PHD3 on the other hand shows the most robust induction in response to oxygen deprivation and it has been implied as the main HIF-1 regulator under prolonged hypoxia.

SQSTM1/p62 (p62) is an adaptor protein that functions through its binding motifs to bring together proteins in order to regulate signal transduction. In non-stressed situations p62 levels are kept low but its expression has been reported to be upregulated in many cancers. It has a definitive role as an autophagy receptor and as such it serves a key function in cancer cell survival decisions.

In my thesis work I evaluated the significance of PHD3 in cancer cell and tumour biology. My results revealed that PHD3 has a dual role in cancer cell fate. First, I demonstrated that PHD3 forms subcellular protein aggregates in oxygenated carcinoma cells and that this aggregation promotes apoptosis induction in a subset of cancer cells. In these aggregates an adaptor protein SQSTM1/p62 interacts with PHD3 and in so doing regulates PHD3 expression. SQSTM1/p62 expression is needed to keep PHD3 levels low in normoxic conditions. Its levels rapidly decrease in response to hypoxia allowing PHD3 protein levels to be upregulated and the protein to be diffusely expressed throughout the cell. The interaction between PHD3 and SQSTM1/p62 limits the ability of PHD3 to function on its hydroxylation target protein HIF-1alpha. Second, the results indicate that when PHD3 is upregulated under hypoxia it protects cancer cells by allowing cell cycle to proceed from G1 to S-phase. My data demonstrates that PHD3 may either cause cell death or protect the cells depending on its expression pattern and the oxygen availability of tumours

KEYWORDS: hypoxia, hypoxia-inducible factor, prolyl hydroxylase, cancer

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HIF hydroksylaasi PHD3:n roolit syöpäsolujen selviytymisessä
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TIIVISTELMÄ

Useimmat kasvaimet ovat ajoittain hypoksisia eli niiden happipitoisuus on alentunut. Välttääkseen solukuoleman kasvainsolut sopeuttavat toimintojaan siten, että ne kykenevät selviytymään muuntuneissa olosuhteissa. Nämä syöpäsolujen muutoksen johtavat alentuneeseen hoitovasteeseen ja huonoon ennusteeseen.

Solujen hapentunnistusreitistön pääasiallinen säätelijä on hypoksia-indusoituva faktori 1 (HIF-1). Tämä transkriptiotekijä säätelee yli sadan hypoksiavastegeenin ilmentymistä ja siten HIF-1: n säätely on erittäin tärkeää sellaisissa toiminnoissa kuten angiogeneesissä, metaboliassa ja migraatiossa. HIF-1:n aktiivisuutta säätelevät prolyylihydroksylaasientsyymit, PHD:t. Normaalissa happitasossa eli normoksiassa PHD:t ovat aktiivisia ja negatiivisesti säätelevät HIF-1:n ekspressiota. Siirryttäessä hypoksiaan näiden happiriippuvaisten entsyymien aktiivisuus vähenee huomattavasti mahdollistaen HIF-1:n toiminnan.

Kolmesta PHD entsyymistä (PHD1, 2 ja 3) PHD2 on tärkein HIF-1 –säätelijä normoksiassa. PHD3 on voimakkaimmin hypoksia-indusoituva ja sen ajatellaan toimivan HIF-1:n säätelijänä pitkittyneessä hypoksiassa.

SQSTM1/p62 (p62) on adaptoriproteiini joka toimii lukuisissa signaalinvälitysreitistöissä. Erilaisten sitoutumiskohtiensa ansiosta se kykenee tuomaan yhteen eri proteiineja mahdollistaen näiden väliset interaktiot. Normaalioloissa p62 –tasot ovat soluissa matalat, mutta useissa syövissä sen tasojen on osoitettu olevan koholla. Eräs tärkeimmistä p62:n rooleista on toimia autofagireseptorina ja näin ollen se vaikuttaa oleellisesti syöpäsolujen kasvuun.

Tässä väitöskirjatyössä on selvitetty PHD3:n roolia syöpäsolujen kasvussa. Tulokset osoittavat, että PHD3 ekspressoituu normoksisissa syöpäsoluissa aggregaateissa. Tämän aggregaation osoitettiin osassa syöpäsoluja johtavan ohjelmoituun solukuolemaan eli apoptoosiin. PHD3:n osoitettiin interaktoivan näissä aggregaateissa p62:n kanssa ja että p62 säätelee PHD3:n proteiinitasoja normoksisissa karsinoomasoluissa. p62 –tasojen osoitettiin myös olevan riippuvaisia hapesta, sillä siirryttäessä hypoksiaan p62-proteiinitasot laskivat nopeasti mahdollistaen PHD3 –tasojen nousun ja vapautumisen aggregaateista. PHD3-p62 –interaktion näytettiin rajoittavan PHD3:n aktiivisuutta HIF-1:tä kohtaan.

AVAINSANAT: hypoksia, hypoksia-indusoituva faktori, prolyylihydroksylaasi, syöpä

TABLE OF CONTENTS

ABSTRACT ... **4**

TIIVISTELMÄ ... **5**

ABBREVIATIONS ... **9**

LIST OF ORIGINAL PUBLICATIONS ... **12**

1. INTRODUCTION ... **13**

2. REVIEW OF LITERATURE ... **14**

2.1 Life with oxygen ... 14
2.2 Normal oxygen demand of the body ... 14
2.3 Hypoxia ... 15
2.3.1 Altered oxygen demand – transformation to malignancy ... 17
2.3.2 Hypoxia in cancer ... 17
2.4 The hypoxia-inducible factor (HIF) system ... 19
2.4.1 HIF isoforms ... 20
2.4.2 Structure of HIFs ... 21
2.4.3 Regulation ... 22
2.4.4 Functions of HIF ... 25
2.5 Von Hippel-Lindau protein (pVHL) ... 27
2.6 2-oxoglutarate and iron(II)-dependent family of dioxygenases ... 28
2.7 Prolyl 4-hydroxylases ... 28
2.7.1 Targets of PHDs ... 31
2.7.2 Structure and general function of PHDs ... 32
2.7.3 PHD1 ... 34
2.7.4 PHD2 ... 34
2.7.5 PHD3 ... 36
2.7.5.1 General ... 36
2.7.5.2 Expression and localization of PHD3 ... 37
2.7.5.3 Regulation of PHD3 ... 38
2.7.5.4 Function and targets of PHD3 ... 40
2.7.6 Factor Inhibiting HIF (FIH) ... 43
2.8 p62 ... 43
2.8.1 Structure of p62 ... 44
2.8.1.1 PB1 domain ... 45
2.8.1.2 ZZ domain ... 45
2.8.1.3 TRAF6-binding domain ... 46
2.8.1.4 NLS and NES ... 46
2.8.1.5 PEST sequence ... 46

2.8.1.6 UBA domain 46
2.8.1.7 LIR and KIR domains 47
2.8.2 Function of p62 47
2.8.3 Regulation of p62 48
2.8.4 p62 in disease 49
2.8.5 p62 and cancer 50
2.9 Eukaryotic intracellular protein clearance 52
2.9.1 Autophagy 52
2.9.1.1 p62 and autophagy 53

3. AIMS OF THE STUDY 55

4. MATERIALS AND METHODS 56

5. RESULTS 59

5.1 PHD3 forms subcellular bodies in oxygen and hydroxylase activity dependent manner (I) 59
5.2 PHD3 bodies colocalize with proteasomal components (I) 60
5.3 PHD3 bodies associated with apoptosis in normoxic cancer cells (I) 61
5.4 SQSTM1/p62 –expression is dependent on oxygen availability (II) 61
5.5 Oxygen depency of p62 is VHL- and HIF –independent and does not mimick PHD inhibition (II) 62
5.6 Hypoxia enhances autophagy-mediated clearance of p62 (II) 62
5.7 p62 has a role in hypoxia-induced ERK phosphorylation (II) 63
5.8 PHD3 is upregulated in HNSCC tumours (III) 63
5.9 PHD3 is needed for HNSCC growth in hypoxia independent of its hydroxylase activity (III) 63
5.10 Hypoxia-induced G1-S block is enhaced by the inhibition of PHD3 (III) 64
5.11 Hyperphosphorylation of retinoblastoma protein is reduced in PHD3 depleted hypoxic cells (III) 64
5.12 PHD3 is expressed in aggresome-like structures in normoxic carcinoma cells where it colocalizes and interacts with p62 (IV) 64
5.13 Aggregation of PHD3 is induced by p62 (IV) 65
5.14 PHD3 protein levels are regulated by p62 (IV) 66
5.15 The biological outcome of PHD3 regulation by p62 is the restriction in HIF-1α and PHD3 interaction (IV) 66

6. DISCUSSION 67

6.1 Cell cycle regulation by PHD3 under hypoxia 67
6.2 PHD3 aggregation 67
6.3 p62 regulation by oxygen availability 69
6.4 The connection between PHD3 and p62 70

6.5 Implications of PHD3 in neurodegenerative disorders 73
6.6 PHD3 in cancer..74

7. SUMMARY 75

8. ACKNOWLEDGEMENTS 76

9. REFERENCES 78

10. ORIGINAL PUBLICATIONS 97

ABBREVIATIONS

2-OG	2-oxoglutarate
ALS	amyotrophic lateral sclerosis
AP-1	activator protein 1
aPKC	atypical protein kinase C
ARD	ankyrin repeat domain
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF4	activating transcription factor 4
Atg	autophagy-related protein
b2Ar	beta2-adrenergic receptor
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
bHLH	basic helix-loop-helix
BNIP3	Bcl2/adenovirus E1B 19kDa protein -interacting protein 3
BRCA1	breast cancer 1, early onset
cAMP	cyclic adenosine monophosphate
CBP	creb-binding protein
RCC	renal cell carcinoma
Cdk1	cyclin-dependent kinase 1
CREB	cAMP responsive element binding protein
CTAD	C-terminal transactivation domain
DMOG	dimethyloxalyl glycine
EGFP	enhanced green fluorescence protein
EGL-9	egg-laying abnormal 9
EGLN	egg-laying abnormal protein homologue
EIT-6	Estrogen-induced tag 6
EPAS1	endothelial PAS domain protein 1
EPO	erythropoietin
ERK	extracellular signal-regulated protein kinase
FADH	flavin adenine dinucleotide, reduced form
FAK	focal adhesion kinase
FGF-2	fibroblast growth factor 2
FIH	factor inhibiting HIF
FKBP38	FK506-binding protein 38
FRAP	fluorescence recovery after photobleaching
FTD	frontotemporal dementia
GABARAP	gamma-aminobutyric acid receptor-associated protein
GLUT	glucose transporter
HIF	hypoxia inducible factor
HK	hexokinase
HMOX	heme oxygenase-1
HNSCC	head and neck squamous cell carcinoma
HPH	HIF prolyl hydroxylase
HRE	hypoxia-responsive element

Hsp90	heat shock protein 90
htt	huntingtin
iBMK	immortal baby mouse kidney
IDH	isocitrate dehydrogenase
IGF	insulin-like growth factor
IkB	inhibitor of NF-kB
IKKbeta	IkappaB kinase beta
IL-1	interleukin 1
ING4	inhibitor of growth family, member 4
IRAK1	Interleukin-1 receptor-associated kinase 1
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
KIF1B	kinesin family member 1B
KIR	KEAP1 interacting region
LC3	Light chain 3
LDHA	lactate dehydrogenase A
LIR	LC3 –interacting region
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase
MEK	mitogen/extracellular-signal-regulated kinase kinase 5
MEKK 3	mitogen-activated protein/ERK kinase kinase 3
MORG1	mitogen-activated protein kinase organizer 1
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
MYND	myeloid translocation protein 8
NAD	nicotinamide adenine dinucleotide, oxidized form
NBR1	neighbor of BRCA1 gene 1
NES	nuclear exit signal
NF1	nuclear factor 1
NF-E2	Erythroid transcription activator nuclear factor erythroid-derived 2
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	neurofibrillary tangles
NGF	neural growth factor
NLS	nuclear localization signal
NO	nitric oxide
Nrf2	Nuclear factor erythroid 2 –related factor 2
NTAD	N-terminal transactivation domain
ODDD	oxygen dependent degradation domain
OS-9	Osteosarcoma amplified 9
P4H-TM	transmembrane prolyl 4-hydroxylase
PARP	poly ADP ribose polymerase
PAS	Per-Arnt-Sim
Pax2	paired box gene 2
PB1	Phox and Bem1
PDB	Paget's disease of bone

PDGF	platelet-derived growth factor
PDK1	pyruvate dehydrogenase kinase 1
PEST	proline, glutamate,serine,threonine
PHD	prolyl hydroxylase domain containing protein
PI3K	phosphoinositide 3-kinase
PK-M2	pyruvate kinase M2
PLA	proximity ligation assay
PML	promyelocytic leukemia nuclear bodies
polyQ	polyglutamine
PPAR	peroxisome proliferator-activated receptor
PPP	pentose phosphate pathway
PRP19	pre-mRNA processing factor 19
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
RANK	Receptor Activator of Nuclear Factor κ B
RB1CC1	RB1-inducible coiled-coil protein 1
RCC	renal clear cell carcinoma
RHEB	Ras-homolog enriched in brain
RING	really interesting new gene
RIP1	receptor-interacting protein 1
ROS	reactive oxygen species
Rpt1	regulatory protein T-lymphocyte-1
SDH	succinate dehydrogenase
SIAH	seven in absentia homolog
SIRT	sirtuin
SKP1	S-phase kinase-associated protein 1
SLC	solute carrier family
SOD	superoxidise dismutase
SP-1	specificity protein 1
SQSTM1	sequestesome 1
TCA cycle	tricarboxylic acid cycle
TGFbeta	transforming growth factor beta
TRAF6	TNF receptor-associated factor 6
TRiC	TCP-1 Ring complex
TrkA	tyrosine kinase A
TrkB	tyrosine kinase B
TrkC	tyrosine kinase C
TSC	tuberous sclerosis complex
UBA	ubiquitin associated-domain
UPR	unfolded protein response
UPS	ubiquiting-proteasome system
V-ATPse	vacuolar type H^+ ATPase
VEGF	vascular endothelial growth factor
pVHL	von Hippel Lindau protein
ZIP2	PKC-zeta-interacting protein 2
ZIP3	PKC-zeta-interacting protein 3

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (referred to with Roman numerals in the text):

I Rantanen K, Pursiheimo J, Högel H, Himanen V, Metzen E and Jaakkola PM. Prolyl hydroxylase PHD3 activates oxygen- dependent protein aggregation. **Molecular Biology of the Cell** 2008 May 19(5):2231-40.

II Pursiheimo J-P, Rantanen K, Heikkinen PT, Johansen T, Jaakkola PM 2009 Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. **Oncogene**. Jan 22; 28(3):334-44

III Högel H*, Rantanen K*, and Jaakkola P 2011Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. **PLoS One** Nov 1 *(*equal contribution)*

IV Rantanen K*, Pursiheimo J-P*, Högel H. and Jaakkola PM. Normoxic PHD3 activity is attenuated by p62/SQSTM1 mediated aggregate sequestration and enhanced degradation. *(Submitted, *equal contribution)*

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1. INTRODUCTION

During cancer progression most tumours face periods of poor oxygenation. Due to rapid growth pace of tumour mass the vasculature providing tumour cells with nutrients and oxygen quickly become inadequate. Fluctuating areas of hypoxia form within the tumour thus requiring cells to acquire adaptive changes for instance in angiogenesis and metabolism in order to survive.

On cellular level a large proportion of these changes are governed by a group of transcription factors that mediate the primary transcriptional responses to hypoxic stress. These so called hypoxia-inducible factors (HIFs) are basic helix-loop-helix-PER-ARNT-SIM (PAS) proteins composing of a labile α subunit and a stable β subunit. HIF-α –subunit is rapidly degraded in normoxic conditions thus inhibiting the activity of the HIF complex. When α and β subunits come together for instance during hypoxic exposure they bind the hypoxia-responsive elements (HREs) of their target genes and activate their transcription. HIFs are mainly regulated on post-translational level by a family of prolyl 4-hydroxylase enzymes (PHDs). PHDs use molecular oxygen as a co-substrate, the lack of which causes their inactivation. Modification and stabilization of HIF by these enzymes leads to an elevation in HIF-α protein levels allowing also their transcriptional activity to increase. This modification is possible in the presence of oxygen as the enzymatic activity of the PHD enzymes is highly dependent on molecular oxygen.

Three PHD paralogues have been described to date, namely PHD1, PHD2 and PHD3. PHDs hydroxylate HIF-α at two proline residues thereby earmarking it for recognition by the von Hippel-Lindau protein (pVHL). pVHL then directs HIF-α for ubiquitylation and subsequent proteasomal degradation. PHDs are considered as the key regulators of HIF activity and thus main oxygen sensors of cells. Structurally they harbor highly conserved sites for 2-oxoglutarate and ferrous iron binding but their expression and function differ among tissues. Also their subcellular localization, substrate specificity and regulation are unique for each individual isoform.

PHD3 is itself a target for HIF and it is strongly upregulated in hypoxia. As the increased PHD3 expression in hypoxia is sufficient to restore some of its enzymatic activity, PHD3 is thought to function in a feedback loop to suppress HIF under prolonged hypoxia. What is the precise role of PHD3 in hypoxia signalling and in overall cancer cell growth remains to be further elucidated.

2. REVIEW OF LITERATURE

2.1 Life with oxygen

Oxygen has had a profound role in shaping all life on earth. Atmospheric oxygen level in the Archaean period was less than 1 % but as the level of oxygen started to rise quite rapidly about 2.2 billion years ago, Earth faced its first “pollution” crisis. As oxygen is a powerful degrader of organic compounds, organisms were faced with having to develop cellular level biochemical mechanisms for rendering oxygen harmless. One of these survival methods, oxidative phosphorylation, was efficient in producing large amounts of energy for the cell. This made it the survival method of choice for most eukaryotes.

In order to use the surrounding oxygen, eukaryotes must be able to extract oxygen from atmosphere and deliver it to the cells needing it. Atmospheric oxygen needs to be transported from air to tissues and cells in the body. Being a gas, oxygen moves from areas of high pressure to areas of low pressure. Air around us has a total pressure of 760 mmHg (standard atmosphere, atm) or 101 kPa and at sea level it consists of 21% of oxygen, 78% of nitrogen, 0.9% argon, 0.038% CO_2 and trace amounts of other gases like helium. About 1% of air is water vapour (Treacher and Leach 1998).

When air is inspired it first travels to trachea. There it is warmed up and humified by the upper respiratory tract. The humidity is due to water vapour which itself exerts a partial pressure. At the body temperature of 37°C the pressure of the tracheal water vapour is 47 mmHg. When this is taken into account the partial pressure of oxygen in the trachea while breathing goes down to 150 mmHg. From trachea air travels to alveoli of the lungs. From alveoli oxygen is delivered to blood stream and this process is continuously affected by two processes: first the removal of oxygen by pulmonary capillaries and second its supply by alveolar ventilation *i.e.* breathing. Due to this exchange the partial pressure of alveolar oxygen has now fallen to 100 mmHg. Pulmonary capillaries surround the alveoli and there oxygen diffuses from high-pressure alveoli to an area of lower pressure, blood, in the pulmonary capillaries. Once blood gets oxygenated, it moves into pulmonary veins which in turn return to heart’s left side to be pumped to tissues. Blood circulates in the body and then returns to the heart and further to lungs via pulmonary arteries which have low partial pressure of approximately 40 mmHg. Most of the oxygen delivered to the tissues is carried by haemoglobin but also plasma carries small amounts. If saturated, each gram of haemoglobin can carry 1.31 ml of oxygen, so every litre of blood (at Hb concentration of 15 g/dl and over 100 mmHg PO_2) can carry about 200 ml of oxygen. In comparison, at this PO_2 only about 3 ml of oxygen will dissolve into a litre of plasma (Treacher and Leach 1998).

2.2 Normal oxygen demand of the body

It is difficult to correctly define ”normal” oxygenation levels within an organism. Condition termed “normoxia” covers oxygenation status anywhere between 2% and 9% of oxygen and in organs like the thymus oxygen levels reach hypoxic levels of 1% of oxygen (Figure

1) (Hale et al. 2002). Oxygen consumption of a conscious resting person is approximately 250 ml of oxygen every minute and so every minute about 25% of arterial oxygen is used. Here the saturation of haemoglobin is about 70%. Usually more oxygen is being delivered to cells and tissues than they actually use. During high oxygen consumption like in exercise the increased demand for oxygen is satisfied by increased cardiac output. If cardiac output is for some reason low cells and tissues will face inadequate availability of oxygen. In these situations compensatory changes in oxygen delivery and usage are needed. Tissues can extract more oxygen from haemoglobin which makes the saturation of venous blood fall below 70%. At certain point the reduction in saturation can no longer be compensated with increased oxygen extraction inevitably leading to anaerobic metabolism and lactic acidosis. Although some cells can, for a limited time and unefficiently, produce energy without oxygen (anaerobic metabolism), most cells can survive only if oxygen is continually supplied. Especially sensitive are the cells of the heart and brain. Normal cells within the body are under tight growth control and changes in their environment, such as reduced oxygen supply, leads to rapid adjustment in cells life. Sometimes cells acquire such capabilities that they no longer need as much oxygen as their wild type counterparts. Their genome may change in such a way that they become insensitive to normal growth control. These changes manifest as malignant, unrestricted growth of cells and can eventually lead to cancer in a process called transformation (Vanderkooi et al. 1991).

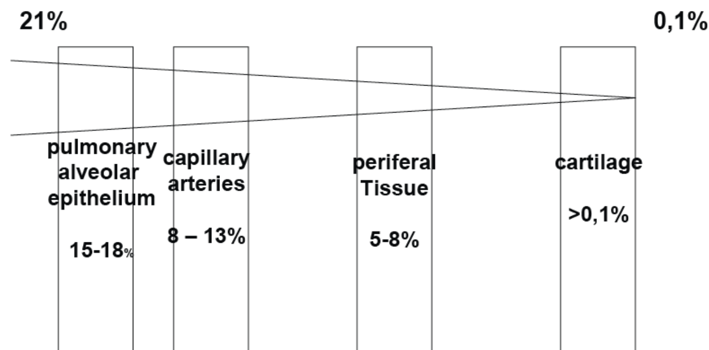

Figure 1. pO_2 in tissues

2.3 Hypoxia

Hypoxia is a state where oxygen availability to cells, tissues or to an organism is reduced. Generally hypoxia is defined to be below 1% of oxygen whereas normal oxygen tension in tissues varies between 2% and 9% (approximately 40 mmHg) and as such it is rather a relative term than an exact measure. And, similarly, the occurance of hypoxia within the body varies. It can occur continuously in cases where an individual goes to high altitudes or intermittently as with individuals suffering from sleep apnea, a condition where a transient airway obstruction blocks oxygen uptake. Hypoxia can also be classified as systemic when suffering from lung disease where all cells in the body are subjected to hypoxia, or local as in the case of myocardial ischemia often occuring in patients with coronary artery disease.

During development hypoxia is a necessary phenomenon. Normal mammalian embryo develops in a hypoxic environment during gestation as oxygen concentration in the uterus is between 1% - 5% (pO_2 0,5 – 30 mmHg) (Fischer and Bavister 1993; Okazaki and Maltepe 2006). In a developing mouse embryo hypoxic conditions prevail until the maternal and fetal blood interface around midgestation (Pringle et al. 2007) and even after this many cell types still reside in hypoxic regions such as the developing heart, gut and skeleton as the developing vasculature is unable to keep up with the pace of development. However, the system that uses hypoxia to sculp the formation of an embryo is very limited when it comes to the ability of responding to non-physiological hypoxia. Enhanced or spatially extended hypoxic condititions, either induced or as consequence of placental insufficiency (for instance due to loss of *retinoblastoma* gene), have been shown to lead to extensive developmental defects (Choi et al. 2005a; Loder et al. 2000; Ream et al. 2008; Sparrow et al. 2012)

In mammals O_2 concentration gradient functions as a signal that guides proper development. Balance between cell growth and vascularization creates a differential gene expression driving force and thereby guides cell behaviour. It has been shown that the development of the cardiovascular-pulmonary system is dependent on physiological hypoxia (Simon and Keith 2008; Simon et al. 2008). Also for vascularization hypoxia is an absolute requirement. In tissue morphogenesis the timing of hypoxic exposure modulates development and differentiation. For example myocardium development is highly dependent on correct genetic responses to hypoxia as has been shown with embryos with dysfunctional oxygen-sensing machinery (Simon and Keith 2008). Also in adult organisms hypoxia is needed as it creates specific niches for specific differentiation processes. Hypoxia keeps stem cells and multipotent progenitor cells in an undifferentiated state (Simon and Keith 2008). Bone marrow is hypoxic and occupied by hematopoietic stem cells (Parmar et al. 2007).

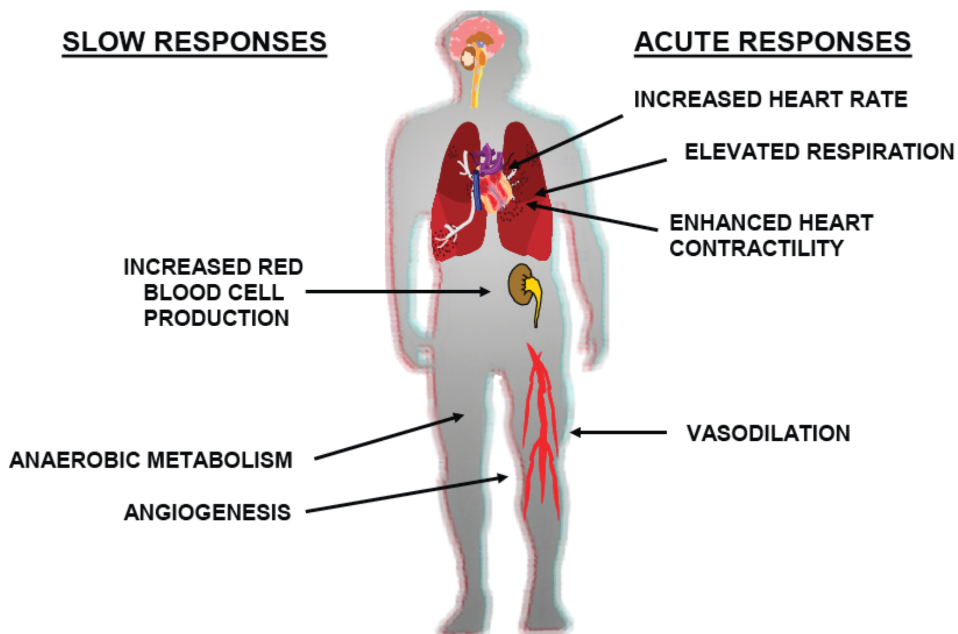

Figure 2. Physiological responses to hypoxia

On cellular level hypoxia is a significant stress as cells need oxygen to create energy. Oxygen deprivation leads to a reduction in oxidative phosphorylation, increased lactate production and inappropriate accumulation of free radicals mounting the stress elicited by hypoxia. Therefore cells must activate adaptive responses to match metabolic, redox and bioenergetic demands to low O_2 supply. On protein level these responses to hypoxia can be divided into acute phase and chronic ones. Acute phase responses are transient and rapid modifying existing proteins posttranslationally. In chronic phase changes are more durable and occur with delay (Figure 2). These changes include altered gene transcription and protein synthesis. All the above mentioned changes enable cells to temporarily arrest cell cycle at G1/S interface, secrete survival and proangiogenic factors and reduce energy consumption response to hypoxia. Many signalling pathways account for these adaptations including mammalian target of rapamycin (mTOR) signalling, unfolded protein responses and hypoxia-inducible factor–pathway.

2.3.1 Altered oxygen demand – transformation to malignancy

If aberrant cells overcome regulatory pathways limiting their number they start to progress towards malignancy. Transformation of cells is not a single phased phenomenon but a multistage cascade of events starting from few genetic changes, mutations that then in a Darwinian process confer some kind of a growth advantage to its carrier. Eventually the cells with defects in their regulatory system are disrupted from their inherent homeostasis. It is not known how many changes are needed for this transformation or whether these changes are due to cell-autonomous events and how many are dependent on extracellular cues. It can only be concluded that as these pro-growth changes follow one another normal human cells start to progress to become cancerous cells (Foulds 1954; Nowell 1976). However, few main alterations dictating cells malignant growth can be listed (Hanahan and Weinberg 2000, 2011). Induction of angiogenesis, cell death resistance, sustained cell death signalling, growth suppressor evasion, activation of metastasis and invasion and replicative immortality are characteristics that lead to development of complex neoplastic disease. Acquiring all these traits results in selection of a cell population the proliferation and self-renewal capacity of which is deregulated and is successfull in competing for growth-promoting niches. This population of cells is inhibited from differentiating but promoted for invasion and prolonged lifespan. It also is capable of avoiding both programmed cell death (apoptosis, anoikis, senescence) and that imposed by innate and adaptive immune responses as well as programmed cell removal. This rapidly growing cell mass forms a tumour (Hanahan and Weinberg 2000).

2.3.2 Hypoxia in cancer

Hypoxia plays a critical role in many pathological processes such as stroke, ischemia and cancer. Cancer is a generic term for a group of diseases characterized by an uncontrolled growth of cells that can affect almost any part of the body. These neoplasms have a tendency to grow beyond their usual boundaries and invade and metastasize to distant sites. Cancer arises when the regulatory circuitry that works to guide cells with normal proliferation and homeostasis malfunctions. It is not clear how many of these circuitries must be working properly for a cell to maintain normal growth restrictions or how many

defaults in them are sufficient to alter cell growth barriers. Nevertheless, at some point these mistakes exceed cells capacity to repair and correct them leading to inevitable transformation towards malignancy. When cancer has arisen, affected tissue often encounters situations where the delivery of oxygen is well below the demand. This bears big clinical implications because most conventional cancer therapies rely in the presence of oxygen in order to be functional.

Over 50 years ago it was noted that human tumours grow in solid rods around blood vessels surrounded by stroma (Thomlinson and Gray 1995). It was also observed that tumour cells that were located more than 180 µm away from blood vessels were necrotic, a distance that is similar to the calculated distance oxygen diffuses as it passes from capillaries to cells. Already at the time it was suggested that there must be a steep falling gradient in oxygen tension between the periphery and the center of these tumour cords. This was known to bear importance during radiotherapy of these tumours as it was already a well known fact that cells facing a complete lack of oxygen (anoxia) cells where less damaged by radiation than well oxygenated one (Gray et al. 1953). Ample evidence shows that solid tumours often encounter hypoxic stress. Heterologously distributed areas of hypoxia are a characteristic feature of advanced tumours. Main pathogenetic mechanisms underlying the emergence of hypoxic areas are the abnormal structure and function of tumour microvasculature, the adverse diffusion geometry and therapy-induced anaemia (reduced capability of blood to transport O_2) (Lowe and Lin 2000).

Initially tumour hypoxia forms because rapidly proliferating tumour cells outgrow their vascular network limiting O_2 diffusion within the tumour (Figure 3). The arisen hypoxia soon induces neoangiogenesis to these areas. However, the vessels

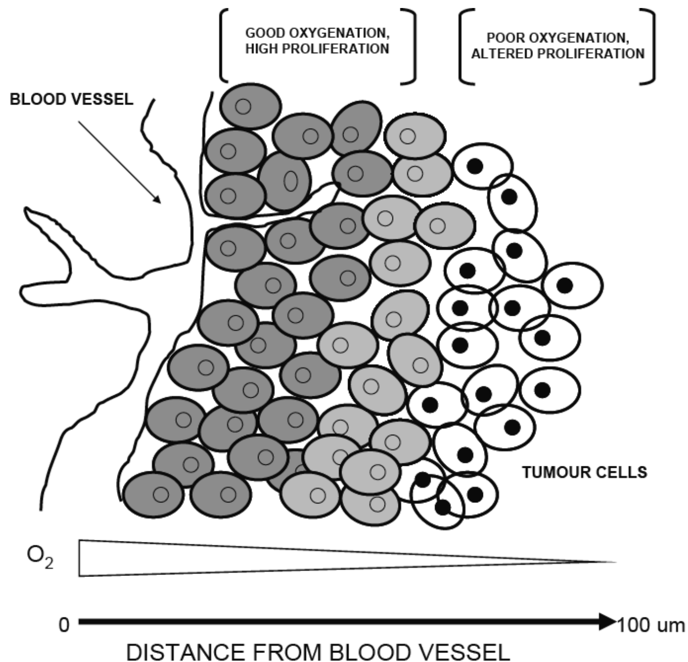

Figure 3. Oxygen gradient in a tumour

of tumours are structurally and functionally abnormal and even tumours that have high density of vasculature still harbour areas of decreased oxygenation (Folkman 1971). Therefore this highly abnormal microvasculature is unable to rectify the oxygen deficit leading to fluctuating levels of oxygen. Oxygen partial pressure below 10 mmHg is considered to associate with poor prognosis. In breast cancer the mean PO_2 is 10 mmHg as compared to normal breast tissue where normal PO_2 is above 60 mmHg (Vaupel et al. 2004).

2.4 The hypoxia-inducible factor (HIF) system

Lowered oxygen tension or hypoxia is a powerful cancer progression driving force. In mammals the primary cellular response to lowered oxygen tension is governed by the transcriptional activation of HIF. The molecular mechanism behind cellular adaptation to hypoxia began to be elucidated when the striking response of hematopoietic growth hormone erythropoietin (EPO) caught the attention of researchers. When the content of oxygen carried by haemoglobin is reduced like in the case of anaemia or when in high altitudes, the production of EPO in renal interstitial fibroblasts is rapidly upregulated. This upregulation of both EPO mRNA and protein induces an eryhtropoietic response that increases blood oxygen transport. Followed by this the enhancer area of EPO was studied for protein – DNA interactions. This led to identification of a protein complex that bound to the hypoxia-responsive element (HRE) within the *EPO* promoter only under hypoxia. It was designated as HIF-1 (Semenza and Wang 1992). Since then HIF has been shown to function as a HRE recognizing factor for over a hundred genes under hypoxia and it is now recognized that all nucleated cells within an organism sense and respond, in one way or another, to reduced oxygen availability (Semenza 2011).

HIF functions as a heterodimer consisting of two subunits, namely HIF-α and HIF-β. HIF-1α is highly labile in the presence of oxygen whereas HIF-1β is constitutively expressed (Wang et al. 1995). The α/β complex binds to the HRE consensus site – RCGTG- and regulates the expression of genes in response to varying oxygen levels. Since the discovery of HIF-1 subsequent database searches identified HIF-2α which similarly to HIF-1α dimerizes with HIF-1β and is oxygen regulated (Figure 4) (Appelhoff et al. 2004; Ema et al. 1997; Tian et al. 1997; Wiesener et al. 1998).

HIF-1 influences the transcription of several hundred messenger RNAs (mRNAs). Either increasing or decreasing in transcription, these mRNAs are HIF-1 –dependently regulated, but HIF-1 binding is detected only at genes with increased expression. The decrease in transcription is indirectly regulated by HIF-1 via transcriptional repressors and microRNAs (Semenza 2010). HIF target genes function in many levels of cell survival under reduced oxygen availability. Some of these target genes promote survival by enhancing proliferation (*insulin-like growth factor 2*, IGF-binding protein 1, 2 and 3). Some enable cells to adapt to lowered oxygen concentration through increasing glycolysis and glucose uptake (*lactate dehydrogenase, glucose transporter 1*). Others work by providing enhanced oxygen transport to hypoxic areas through increased

angiogenesis and vasomotor control (*endothelin-1*, *vascular endothelial growth factor)* or by promoting red blood cell maturation *(transferrin*, *EPO*).

Although many HIF target genes work in favour of cell survival, some have also been shown to induce apoptosis in situations like prolonged chronic hypoxia (Carmeliet et al. 1998). This is the case with HIF-1 target gene *Bnip3* which is shown to be overexpressed in human tumours, especially in the perinecrotic regions. It seems that during prolonged hypoxia the cytoprotective effects of HIF-1 are not sufficient, but it is still a surprise that, under these circumstances, HIF-1 in fact seems to function as an initiator of apoptosis. Bnip3 is a pro-apoptotic member of the Bcl-2 family of proteins and hypoxia has been shown to increase its levels yet in HIF-1 deficient cells this accumulation does not occur. Also *vice versa*, *Bnip3* is expressed even under normoxia in cells deficient with pVHL (Sowter et al. 2001). In general, in addition to controlling erythropoiesis, HIF-responsive genes function in almost all biological processes within a cell: cell growth and survival, metal transport, glycolysis, mitochondrial function and angiogenesis and vascular tone.

From the above mentioned studies it can be concluded that the hypoxia-response pathway is strongly dependent on HIF-1 and that HIF-1 has a pivotal role in the outcome of many disease states such as myocardial ischemia and tumourigenesis (Semenza 2000). Undoubtly HIF has earned its epithet of being the master regulator of hypoxia response pathways. Similarly it is clear that other transcriptional systems such as NF-κB, ATFs and p53 are equally and overlappingly important in responding to ambient oxygen levels (Blais et al. 2004; Cummins et al. 2007; Graeber et al. 1994; Schmedtje and Ji 1998; Zampetaki et al. 2004). These systems may be favored under different stimuli than those predominated by HIF-1. It has been shown that 16h exposure to 1% oxygen is adequate in activating HREs whereas NF-κB pathway is preferred during intermittent hypoxia and reoxygenation and ATF signalling is used possibly in absolute anoxia (Ameri et al. 2007; Ameri et al. 2004; Cummins et al. 2007).

2.4.1 HIF isoforms

Caenorhabditis elegans and *Drosophila melanogaster* have a HIF-system similar to mammals with HIF-1α isoforms termed hif-1 and Similar. HIF-1β isoforms are termed as aha-1 and Tango, respectively (Bacon et al. 1998; Jiang et al. 2001; Lavista-Llanos et al. 2002; Sonnenfeld et al. 1997) Mammalian genome contains three HIF-α genes: HIF-1α, HIF-2α (also known as EPAS1) and HIF-3α (also known as IPAS), of which HIF-1α is the best studied isoform (Tian et al. 1997). For HIF-1β three differently spliced isoforms exist: Arnt1, Arnt2 and Arnt3 (Zagórska and Dulak 2004). HIF-α genes share a similar domain structure but it is likely that they have non-overlapping physiological roles.

HIF-1 and HIF-2 are particularly critical for the hypoxia response in that they are able to dimerize with HIF-1β. HIF-1α is expressed widely in tissues whereas HIF-2α and HIF-3α are more restricted in their expression including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells and cells of myeloid origin (Bertout et al. 2008). HIF-1α is known to regulate more than 100 genes and it

has been proposed that in endothelial setting more than 2% of human genes are HIF-1α responsive either directly or indirectly (Manalo et al. 2005). HIF-2α is more restricted in its expression (Hu et al. 2003; Makino et al. 2001; Warnecke et al. 2004).

HIF-3α is the least studied isoform of the three. HIF-3α exists as multiple splice variants some of which have been shown to act as dominant negative inhibitors of HIF-1α or HIF-2α. The locus of HIF-3α in the human genome is subject to extensive alternative splicing and a number of different variants of this isoform are known. It has recently been described that full-length splice variants of human HIF-1α1, 2 and 4 are widely expressed across human tissues and cell line. In addition to these, splice variants HIF-3α 7, 8, 9 and 10 are also frequently expressed (Pasanen et al. 2010). Of the known HIF-3α isoforms HIF-3α1 differs from HIF-1α and HIF-2α in lacking the C-terminal transactivation domain and in that its ODDD domain contains only one conserved proline (Pro564) instead of the "usual" two. It has been shown that human HIF-3α1 is able to dimerize with HIF-β. It also is able to induce the expression of HRE reporter when overexpressed with HIF-β, but these activities are much weaker than those seen with HIF-1α and HIF-2α. Under condition where HIF-β is the limiting factor HIF-3α1 suppresses the activation potential of HIF-1α and HIF-2α. This has been suggested to be due to the competitive binding of HIF-3α1 to HIF-2α (Mole et al. 2009).

2.4.2 Structure of HIFs

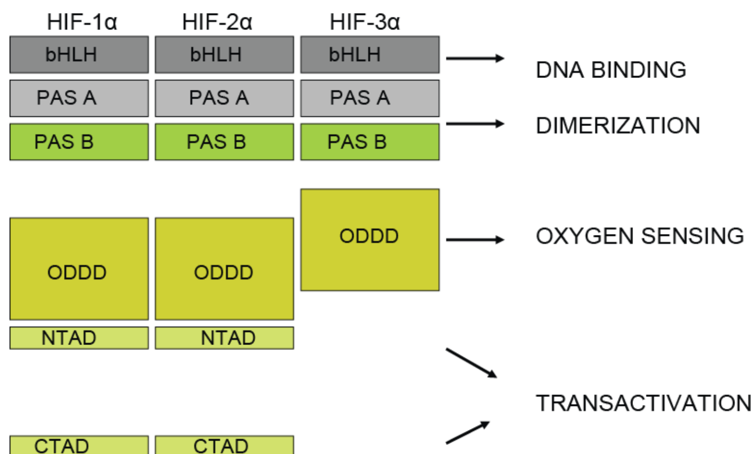

Figure 4. HIF 1 isoform structures and their functions. Abbreviations: (bHLH) basic helix-loop-helix, (PAS) Per-Arnt-Sim, (ODDD) oxygen-dependent degradation domain, (NTAD) N-terminal transactivation domain, (CTAD) C-terminal transactivation domain.

HIF-1α ja HIF-2α are 48% identical to each other in their amino acid composition and are quite similar in their protein structure (Figure 4). HIFs consist of two subunits, an alpha subunit (120 kD in size) and a beta subunit (91–94 kD), both members of the bHLH and PAS domain-containing transcription factor family (Wang et al. 1995).These types of transcription factors are obligate dimers usually forming a heterodimer which binds to DNA at certain promoter elements. After binding they recruit coactivators

through their unstructured transcriptional activation domain located at the C-terminus and subsequently regulate transcriptional responses to diverse stimuli.

bHLH is a structural motif of approximately 60 amino acids which is composed of a DNA-binding region, a variable loop region and two alpha-helices bordering it (Ferré-D'Amaré et al. 1993). bHLH domain is able promote dimerization even between different family members. First they homo-or heterodimerize with other bHLH molecules and following this they target conserved motifs called E-boxes (CANNTG). This binding leads to either activation or inhibition of transcription (Murre et al. 1989).

PAS domain is a structure which can bind to and sense exogenous small molecules. They can build complex assemblies of transcription factors and coactivators by using multiple interfaces of these modular domains (Partch and Gardner 2010).

2.4.3 Regulation

The hypoxic activation of HIF is influenced by many processes. Here the α-chain of HIF plays the most important role; the synthesis, stabilization, nuclear translocation, dimerization with the β-subunit, DNA binding and co-activator recruitment all need to be correctly executed.

The expression of HIF-1α protein is regulated via several mechanisms but it is generally agreed that the dominant mode of regulation is via combination of protein stabilization and enhanced co-activator recruitment under hypoxia (Kallio et al. 1998).

The HIF-β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively active and virtually insensitive to changes in oxygen availability. The α unit is also constitutively transcribed and translated but in contrast to the β subunit it is also constantly degraded with a half-life as short as five minutes. Under hypoxia the α subunits are rapidly stabilized, transported into nucleus, dimerized with the β subunit and thereby forming an active transcription complex.

Regulation of the α subunit occurs via region referred to as oxygen dependent degradation domain ODDD. In normoxia ODDD is hydroxylated by prolyl 4-hydroxylase enzymes (PHDs) at conserved proline residues (Pro402 and Pro 564) and subsequently becomes recognized by pVHL (figure 5). pVHL is a component of a ubiquitin-protein ligase complex. It functions by recognizing the ODDD domain, tagging it with ubiquitin moieties that earmark HIF-1α to be degraded by the proteasome. If cells are subjected to hypoxia PHD enzymes are virtually inactivate and the recognition by pVHL fails to occur, leading to HIF-1α accumulation. Therefore hydroxylated ODDD functions as the key regulator of HIF-1α protein stability (Cockman et al. 2000; Kamura et al. 2000; Maxwell et al. 1999; Ohh et al. 2000; Tanimoto et al. 2000). Mutations of pVHL disable the HIF-1α recognition process which leads to HIF-1α protein accumulation. This is a typical feature seen in renal clear cell cancer (RCC) (Clifford et al. 2001; Maxwell et al. 1999).

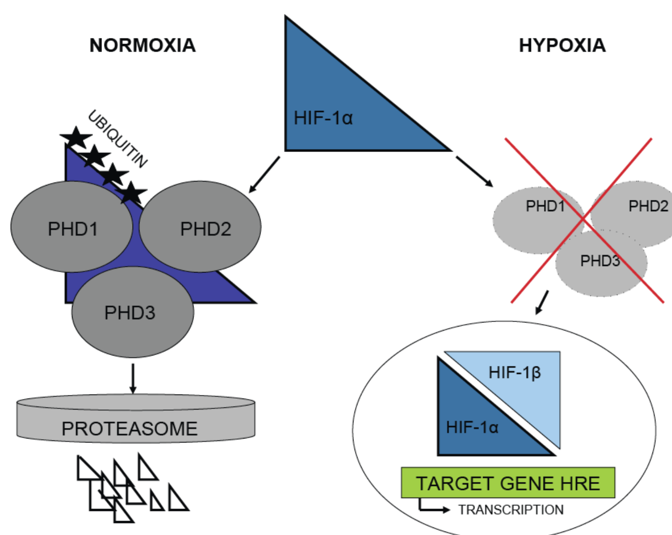

Figure 5. Oxygen dependent HIF regulation

Another hydroxylase, namely asparaginyl hydroxylase factor inhibiting HIF-1α (FIH1) also targets HIF-1α. Similarly to prolyl hydroxylases, FIH is an O_2-dependent enzyme adding another component to oxygen sensing machinery. HIF-1α hydroxylation by FIH disrupts the interaction between HIF-1α and its coactivators p300/CBP thus impairing HIF transcriptional activity (Mahon et al. 2001; Webb et al. 2009)

In HIF-1α there are two transcription activation domains (N-terminal NTAD and C-terminal CTAD) that are connected via an inhibitory domain. These transactivation domains are released from suppression upon exposure to hypoxic environment thus allowing the increased transcription of its target genes. The N-terminal activation domain NTAD located at aa 531 – 575 overlaps with the ODDD (aa 401 – 698) and therefore its regulation is hard to distinguish from that of protein stability. At the C-terminal end the transactivation domain is called CTAD at aa site 786 – 826. CTAD interacts with co-activators such as cAMP-response element-binding protein (CREB) binding protein CBP p300, an interaction which is greatly enhanced by hypoxia. The enzymatic hydroxylation of either of two prolyl residues located within NTAD and CTAD suffices the recognition of normoxic HIF-1α and HIF-2α by pVHL. In HIF-3α there is only a single prolyl residue (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001) .

HIF-β subunit is essentially insensitive to lowered oxygen availability, but the α-subunit's accumulation and activity are rapidly induced in response to reduced O_2–levels. In both subunits there are protein domains that mediate the O_2–dependent regulation.They are also both members of the bHLH–containing PAS domain family of transcription factors. PAS is an additional auxiliary dimerization site named after the first three proteins where this domain was found, namely after the Drosophila proteins period, ARNT (dioxin receptor / aryl hydrocarbon receptor) and single-minded. The basic domain as well as the carboxy-terminus are mandatory for DNA-binding of HIF-1. On the other hand the

HLH domain and the aminoterminus of PAS are required for dimerization of the α and β subunits upon activation.

In both HIF subunits there are two PAS-domains designated PAS-A and PAS-B. Protein-protein interactions are frequently mediated by PAS-domains and this usually occurs as a consequence of ligand or cofactor binding to their hydrophobic cores (Taylor and Zhulin 1999). In the formation of HIF heterodimers one or both of the HIF-α PAS domains have been functionally implicated (Chapman-Smith et al. 2004; Jiang et al. 1996). Their participation has also been implicated in HIF-α nuclear localization, HIF stabilization via heat shock protein 90 (Hsp90) association (Gradin et al. 1996; Isaacs et al. 2004; Kallio et al. 1998; Minet et al. 1999).

bHLH is a structural motif of proteins that characterize a family of transcription factors. It has two α helices that are connected by a flexible loop. One helix is in general smaller than the other and allows dimerization and packing against the other helix via the loop. The larger helix typically contains a amino acid residue that contains the DNA binding E-box CANNTG regions although some bHLH transcription factors bind to different but similar sequences (Murre et al. 1994).

Proteins belonging to this group share many characteristics. Many of the bHLH transcription factors, like HIF and c-Myc, have been associated with cancer due to their effects on growth and metabolism. As many of these factors are heterodimeric, the dimerization of the subunits controls their activity.

Although hypoxia is a central regulator of cancer progression, not all tumours are hypoxic and many O_2-independent HIF-inducing mechanisms have been described. Dysregulation of mTOR induces HIF-1α expression. Loss of function of promyelocytic leukemia nuclear bodies (PML), liver kinase B1 (LKB1), phosphate and tensin homolog (PTEN) or tuberous sclerosis complex 1 or 2 (TSC1/TSC2) tumour suppressors are all able to alter mTOR function (Bernardi et al. 2006; Shackelford et al. 2009; Zhong et al. 2000; Zundel et al. 2000) and thereby affect HIF-1α translation. The inhibition of PHD2 by tricarboxylic acid (TCA) cycle intermediates succinate and fumarate reduces HIF-1α hydroxylation, ubiquitination and proteasomal degradation. Decreased levels of 2-OG following isocitrate dehydrogenase mutation in turn affects HIF-1alpha levels through inhibiting PHD2 (Zhao et al. 2009). So, alterations in mitochondrial metabolism have major implications in HIF-1α non-hypoxic regulation.

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD) –dependent histone deacetylases that have been shown to participate in gene transcription, DNA repair, metabolism and organism lifespan (Haigis and Sinclair 2010). HIF-1α and HIF-2α transactivation can be modulated according to NAD^+ levels; NAD^+ -dependent deacetylase sirtuin 1 (SIRT1) has been described to bind and deacetylate HIF-2α thereby increasing its transcriptional activation. HIF-1α, however, was not modified by SIRT1 (Dioum et al. 2009). Poly(ADP-ribose) polymerase 1 (PARP1), also a NAD^+ -dependent enzyme, was recently described to bind HIF-α and promote its transactivation (Elser et al. 2008). HIF-1α has been, however, linked to Sirt6 (Zhong et al. 2010). From studies with *Sirt6-/-* mice and cells it became evident that Sirt6 influences glucose consumption as knockout cells and mice showed elevated

expression of glycolytic genes, many of which were identified as HIF-1α targets. Supporting, these *Sirt6*$^{-/-}$ cells and mice also displayed increased rates of glucose consumption (Zhong et al. 2010).

Nitric oxide (NO) can also modulate HIF-1α expression levels. Increased expression of nitric oxide synthase isoforms and increased NO levels have been shown to increase HIF-1α protein stability in human oral squamous cell carcinoma. Nuclear colocalization of endothelial nitric oxide synthase, estrogen receptor beta, HIF-1α and HIF-2α in chromatin complexes has been described to occur in prostate cancer where it associated with aggressive disease progression (Nanni et al. 2009; Quintero et al. 2006).

2.4.4 Functions of HIF

The hypoxia-dependent HIF system has the ability to cooperate with the hypoxic conditions present during embryonic development and so HIF activity is an absolute requirement for normal embryonic development. Loss of both HIF-1α alleles or HIF-2α and Arnt in a mouse embryo has been shown to lead to massive developmental defects that are lethal at E10.5. Key morphological defect is the failure to develop placenta (Abbott and Buckalew 2000; Adelman et al. 2000). Also cellular hypoxia and HIF component expression is essential for the development of the heart (Aitola and Pelto-Huikko 2003; Krishnan et al. 2008).

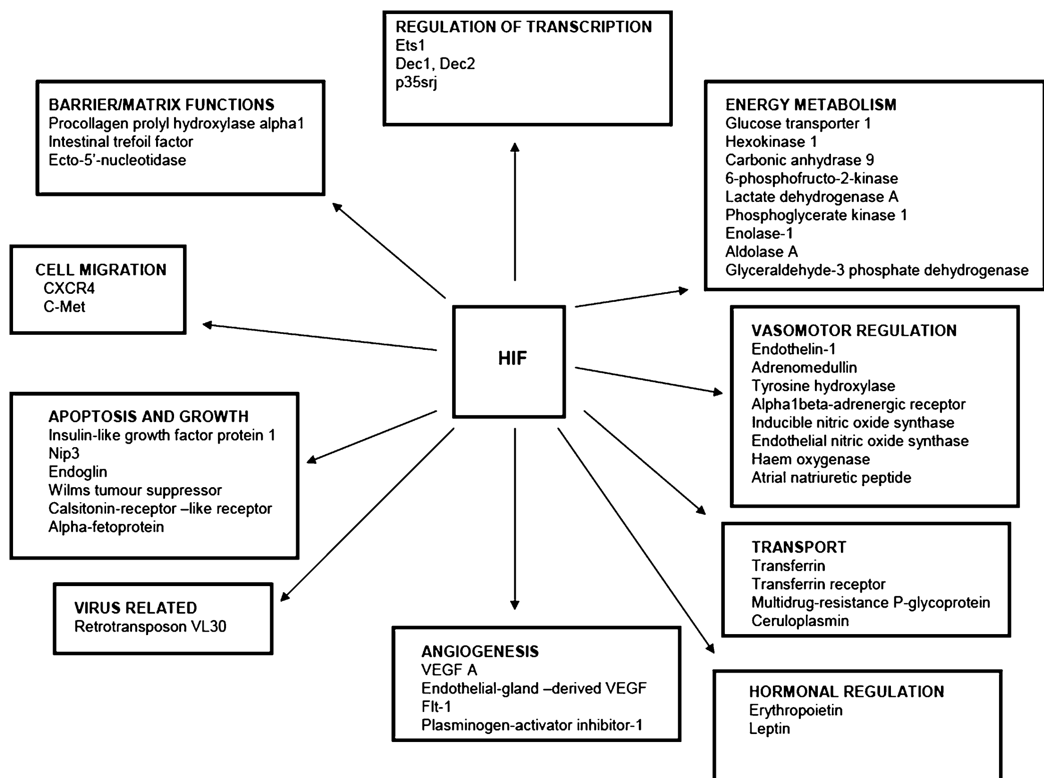

Figure 6. Examples of the transcriptional targets of HIF

Regulation of metabolism can be viewed as the principal, primordial function of HIF-1 (Figure 6). Under hypoxia and in metastatic cancer transition from oxidative to glycolytic metabolism occurs and glucose uptake is increased. This adaptation is primarily mediated by HIF-1. It regulates the expression of main factors in metabolic adaptation: pyruvate dehydrogenase kinase 1 (PDK1), lactate dehydrogenase A (LDHA), as well as apoptosis regulating BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3-like protein (BNIP3L). HIF-1 activates the transcription of *SCL2A1* and *SLC2A3* which encode glucose transporter 1 and 3 (GLUT1 and GLUT3) as well as the transcription of hexokinase encoding genes *HK1* and *HK2*, the first two enzymes in the glycolytic pathway (Iyer et al. 1998). Glucose is then further metabolized to pyruvate via actions of HIF-1 encoded target genes. Pyruvate can be either converted to acetyl coenzyme A by pyruvate dehydrogenase (PDH) or to lactate by LDHA. HIF-1 actively suppresses metabolism through TCA by activating PDK1 which phosphorylates and inactivates PDH. As this inhibits the conversion of pyruvate to acetyl coenzyme A it thus attenuates mitochondrial respiration and reactive oxygen species (ROS) production (Kim et al. 2006). Also the delivery of NADH and FADH2 to the electron transport chain is reduced, a crusial event in the adaptation to hypoxia as has been shown in the HIF-1α null mouse embryo fibroblasts where PDK1 expression is not induced by hypoxia and these cells die of excess ROS production. In concert with HIF-1 *PDK1* transcription is regulated and activated by MYC which provides an amplification of the hypoxic responses (Kim et al. 2007; Kim et al. 2006). BNIP3 and BNIP3L mediate the selective mitochondrial autophagy by competing with Bcl2 thereby freeing Beclin 1 to trigger autophagy (Bellot et al. 2009; Semenza 2008). Both BNIPs are activated by HIF-1. It has been shown that HIF-1 –dependent BNIP3 expression is crucial for cell survival under prolonged hypoxia although initially it was associated with hypoxic cell death (Kim et al. 2006; Zhang et al. 2008).

Recently it was shown that HIF-1α affects the pentose-phosphate pathway (PPP) (Zhao et al. 2010). PPP functions to convert intermediates from glycolysis into a nucleic acid biosynthesis substrate, ribose-5-phosphate (Tong et al. 2009) . In the referred study the authors show that in drug resistant leukemia cells HIF-1α favours the non-oxidative arm of PPP when directing glucose carbon flux indicating that HIF-1α is able to redirect glucose metabolism so that it can be used both as an energy source as well as in RNA and DNA synthesis. This would bear significant benefits for tumour cell growth and survival under hypoxia.

HIF-2α regulates somewhat different set of genes than HIF-1α but even though HIF-2α has a critical role in metabolism (Figure 6). For example, *cytochrome c oxidase* is regulated by both HIF-1α and HIF-2α which makes electron transport chain more efficient (Semenza et al. 1996). In addition, HIF-2α stimulates the expression of SOD2, an antioxidant enzyme functioning in cellular redox homeostasis (Garcia et al. 1995). In renal cancer HIF-2α has been shown to regulate *heme oxygenase 1* (HMOX1) which functions as an antioxidant (Bertout et al. 2008). Supportingly, HIF-2α loss increases ROS production, activates p53 and leads to cell death.

HIF-2α is linked to protecting cells against hypoxic stress. Studies done with *PHD1-/-* mice show that PHD1 inhibition increases tolerance of ischemia in skeletal muscle and

in liver (Aragonés et al. 2008; Schneider et al. 2010) As HIF-2α is negatively regulated by PHD1 it indicates that the ischemia tolerance is predominantly dependent on HIF-2α, possibly by glucose metabolism modulation indirectly via peroxisome proliferator-activated receptor (PPAR) (Aragonés et al. 2008; Huang et al. 2002).

Lipids provide a major source of energy in tissues such as liver and heart. In hypoxic condition lipid storage is enhanced and catabolism through beta-oxidation is inhibited (Boström et al. 2006; Huss et al. 2001) HIF-2α regulates lipid metabolism as has been shown in mice with liver-specific *Vhl*-deletion and that are therefore pseudohypoxic. In these livers genes that are involved in beta-oxidation are reduced and genes needed for lipid storage are enhanced, phenotypes which are known to rely more on HIF-2α than on HIF-1α.

2.5 Von Hippel-Lindau protein (pVHL)

The von Hippel-Lindau syndrome is clinically viewed as an autosomal-dominant hereditary disease predominantly manifested by haemangioblastomas of the central nervous system, phaeochromocytomas and clear-cell renal-cell carcinomas (RCC). On a cellular level, however, VHL disease is autosomal recessive in its pattern of inheritance since it requires the inactivation of both alleles in order to develop but because of the occurrence of the second hit is virtually guaranteed, the clinical perception is justified. The disease was first described in the literature in 1927 (Lindau 1927). The disease is caused by a germ line mutation in the *VHL* tumour suppressor gene located at chromosomal locus 3p25 (Latif et al. 1993). Following the Knudson two-hit model patients who have inherited a mutated *VHL* gene develop highly vascularised benign tumours only after a second inactivation of the remaining *VHL* copy (Kaelin 2002). Although most of the tumours associated with the VHL disease are benign, the clear-cell type kidney cancer is malignant and is the major cause of death among patients with VHL disease (Ohh and Kaelin 2003).

The translation of *VHL* produces two biologically active proteins $pVHL_{19}$ and full length $pVHL_{30,}$ collectively referred to as pVHL. If either of these proteins is re-introduced in VHL-deficient RCC cells, their ability to form tumours in nude mice is suppressed and promotes cell cycle exit. Therefore, pVHL acts as a tumour suppressor (Iliopoulos et al. 1995; Schoenfeld et al. 1998). However, tumours producing exclusively the shorter transcript have been identified and it has been shown that this transcript alone does not function as a tumour suppressor (Gnarra et al. 1994). In addition, VHL mRNA is ubiquitously expressed and it has been shown to be expressed in fetal kidney suggesting it bears a role also in normal renal tubular development and differentiation (Kessler et al. 1995).

The best understood role of pVHL is its function as an E3 ubiquitin ligase, the substrate recognition component in a complex that also includes elongins B and C as well as Cullin 2 and Rbx1 (Ohh et al. 2000; Tanimoto et al. 2000). This complex serves to target proteins to proteasomal degradation. In pVHL there are two domains with different functions: Alpha helical domains bind to elongins B and C, a complex without which

pVHL is highly unstable. Beta sheet domains serve as substrate-docking sites for target proteins. Many substrates are known for this E3 ubiquitin ligase complex including atypical protein kinase C, VHL-interacting deubiquitinylating enzymes and RNA polymerase subunits. pVHL has functions that are important for its tumour suppressor role: pVHL regulates transcription, controls microtubule dynamics, regulates neuronal apoptosis, maintains the primary cilium and possibly even stabilizes p53. However, the best characterized *bona fide* substrates are the α subunits of the HIF-family members 1, 2 and 3. The substrate binding domain of VHL (beta domain) binds to hydroxylated HIF-α and targets it for proteasomal degradation. The hydroxylation of proline 564 in the HIF-α ODDD-site is both necessary and sufficient for VHL binding. As hydroxylation of ODDD occurs only under normoxic conditions, also the HIF-α recognition by pVHL occurs only when oxygen partial pressure is sufficient. The regulation of HIF-α by pVHL/complex E3 is a prerequisite for normal cell function. Also, the interaction between HIF-α and VHL has a critical role in the progression of cancer. VHL deficient tumours display an overproduction of HIF-mediated gene products such as VEGF. Supporting this, VHL-deficient cells show an overabundance of numerous HIF target genes even under normoxic conditions (Iliopoulos et al. 1996).

2.6 2-oxoglutarate and iron(II)-dependent family of dioxygenases

In humans there are approximately 70 members in the 2-oxoglutarate (2OG) and iron (II)-dependent dioxygenase family (Aravind and Koonin 2001; Schofield and Ratcliffe 2004). Reactions catalysed by these enzymes include hydroxylations, desaturations, and ring closures (Hewitson et al. 2005; McDonough et al. 2010). They are involved in various other processes other than oxygen sensing, namely DNA repair, chromatin remodelling, collagen assembly, lipid metabolism, diabetes ad obesity (Duncan et al. 2002; Gerken et al. 2007; Kivirikko and Savolainen 1980; Trewick et al. 2005; Valegård et al. 1998).

The enzymatic activities of these dioxygenases are regulated by many factors. As any enzymes, the 2OG family of enzymes is regulated by the overall level of the enzyme, the availability of the primary substrate and by the availability of co-substrates and co-factors. For 2OG-dependent enzymes the required co-substrates and co-factors are oxygen, iron(II) and 2-OG. Also the removal of other products such as succinate and carbon dioxide is needed for their proper function. For the above mentioned features it is feasible that these enzymes have a crucial role in integrating and adapting many vital functions such as oxygen supply and demand, energy metabolism and iron balance to mention a few.

2.7 Prolyl 4-hydroxylases

Twenty amino acids are used as building blocks of most polypeptides. Great molecular diversity is conferred by chemical modifications of these amino acids. Some modifications are permanent, others reversible. Proteins can be modified by polypeptide chain cleavage or molecule or functional group additions. Additions can be as big as entire

proteins, carbohydrates or lipids or only few atoms like in phosphorylation, acetylation, carboxylation, methylation or sulfation.

Post-translational modifications typically cause profound changes in the structure and function of a protein. Hydroxylation of proline is the most common post-translational modification in mammals as collagen hydroxylation is essential for its triple-helical folding and stability (Holmgren et al. 1998; Kivirikko et al. 1989; Sakakibara et al. 1973). This irreversible reaction yields 4-hydroxyproline and is catalyzed by prolyl-4-hydroxylase enzymes for which two main classes exists: the collagen prolyl 4-hydroxylases and the HIF prolyl 4-hydroxylases. As substrates for these enzymes are diverse, so are the biological outcomes. Prolyl hydroxylation generally stiffens the structure of proteins such as that in collagens (Myllyharju and Kivirikko 2004). Therefore protein conformation and interactions between proteins usually changes and in turn enable further modifications.

Trent and colleagues described in 1983 an egg-laying defect (egl) in the nematode worm *Caenorhabditis elegans. Egl-9* gene was subsequently isolated and characterized to be responsible of this phenotype (Trent et al. 1983). In 1999 Darby and co-workers demonstrated Egl-9 protein to play a role in a paralysis caused by a diffusible toxin produced by certain strains of *Pseudomonas aeruginosa* and that loss-of-function mutations in *Egl-9* gene confer protection against this paralysis (Darby et al. 1999). Later rat SM-20 was described to be the vertebrate homolog of Egl-9 (Moschella et al. 1999) and following its identification SM-20 was shown to be essential in nerve growth factor –dependent survival of neuronal cells (Lipscomb et al. 1999) as well as in the differentiation and growth regulation of rat muscle cells (Moschella et al. 1999; Wax et al. 1994). In year 2000 Dupuy and co-workers published their work describing SCAN domain –containing 2 (SCAND2) and C1orf12 which were shown to be human homologues of rat SM-20 (Dupuy et al. 2000). Following these findings Taylor (Taylor 2001) described a conserved gene family Egl-Nine (EGLN) of which rat SM-20 and *C.elegans* are part of. This family was shown to include three genes of conserved genomic structure of five coding exons and they were termed EGLN1, EGLN2 and EGLN3 (Table 1).

Table 1. PHD nomenclature

PHD1 – EGLN2 – HPH3 – HIF-PH4-1
PHD2 – EGLN1 – HPH2 – HIF-PH4-2
PHD3 – EGLN3 – HPH1 – HIF-PH4-3

These proteins belong to an evolutionarily conserved subfamily of 2-OG dependent dioxygenases. They use oxygen and 2-OG as co-substrates and ferrous iron (Fe2+) and ascorbate as co-factors so that molecular oxygen is split to hydroxylate their substrates and simultaneously oxidize and decarboxylate 2-OG into succinate (Schofield and Zhang 1999). In mammalian cells there are in total four members belonging to the subfamily as a fourth member, a transmembrane prolyl 4-hydroxylase (P4H-TM), has recently been described (Koivunen et al. 2007). An enzyme bearing similarity to mammalian hydroxylases, Skp1, has been characterized in the plant *Dictyostelium* (van der Wel et

al. 2005), and in it has been suggested that enzymes with similar catalytic centers may exist also in prokaryotes (McDonough et al. 2006). PHD2 has been found in the simplest animal, *Trichoplax adhaerens* (Loenarz et al. 2011).

Having a requirement for molecular oxygen as a co-substrate O_2 concentration is inevitably the most ubiquitous factor governing prolyl hydroxylase–catalyzed reactions and the requirement for O_2 is absolute for PHDs. However, strong body of evidence shows that various parameters have an effect on these enzymes (Pan et al. 2007). Other oxygen-independent factors influencing PHDs are iron, ascorbate and many TCA cycle intermediates. Also ROS and NO are able to modify PHD function.

PHDs need ferrous iron in order for these enzymes to be correctly assembled into an active conformation. In a complete reaction Fe^{2+} is transiently oxidized into Fe^{4+}. For the enzyme to be recycled, the Fe^{4+} needs to be restored in Fe^{2+} state. When 2-OG is converted into succinate, and no hydroxylation of the peptide substrate occurs, Fe^{2+} is oxidized into Fe^{3+}. The Fe^{3+} reduction back to Fe^{2+} depends on the presence of ascorbate. This makes intracellular ascorbate and Fe^{2+} levels important regulators of PHD enzymatic activity.

2-OG and succinate are TCA cycle intermediates having an effect on PHD activity. In human cancers succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH) mutations have been identified. Inactivating mutations in them lead to the accumulation of succinate and fumarate. Succinate is a competitive inhibitor of PHD's, a function which is reversed by 2-OG. In TCA cycle succinate is converted into PHD inhibiting fumarate by SDH enzyme. Fumarate-driven inhibition of PHD's and the following HIF-1α activation has been proposed to drive tumourigenesis. In cells lacking the tumour suppressor FH fumarate accumulates and dioxygenases are inhibited by fumarate competetively thus stabilizing the HIF-α subunit. This process has been termed pseudohypoxia and it has been suggested to favour tumour development (Isaacs et al. 2005; O'Flaherty et al. 2010; Pollard and Ratcliffe 2009). IDH1 and IDH2 participate in the oxidative decarboxylation of isocitrate which produces 2-OG and CO_2 while converting NAD^+ into NADH. Mutant IDH's, however, convert 2-OG into 2-hydroxyglutarate thus depleting cellular 2-OG and downstream byproducts succinate, fumarate and malate (Reitman et al. 2011).This produced 2-hydroxyglutarate is an (R)-enantiomer. Recently it has been shown that this (R)-2HG possesses the ability to stimulate PHD activity and therefore to diminish HIF levels (Koivunen et al. 2012).

Also other glucose metabolites pyruvate, oxaloacetate, citrate, isocitrate and malate have been reported to bind 2-OG binding sites of these enzymes thereby inhibiting their activity (Dalgard et al. 2004; Hewitson et al. 2007; Selak et al. 2005).

Mitochondria have also been suggested, albeit still controversially, to participate in oxygen sensing by regulating PHDs. In moderate hypoxia (1,5% O_2) mitochondria have been shown to stimulate the production of ROS. ROS in turn are known inhibitors of PHDs and thus HIF-α degradation (Kaelin 2005; Klimova and Chandel 2008). A number of findings support the role of mitochondria as oxgen sensors and regulators of PHD activity via ROS production (Klimova and Chandel 2008; Waypa et al. 2010). The role may be restricted only to moderate hypoxia as many studies have shown that HIF-α stabilization under anoxia occurs despite absence of mitochondria suggesting

additional factors other than mitochondrial ROS to inhibit PHD activity (Kaelin 2005; Klimova and Chandel 2008) (Table 2). Furthermore, debate is still ongoing whether PHD modulation via ROS release is direct or indirect, or what in the first place triggers mitochondria to release ROS in response to hypoxia. Suggestions have also been made towards the possibility that mitochondria rather than modulation of PHD function by ROS production instead signal to PHDs indirectly through their oxygen consumption (Klimova and Chandel 2008).

Table 2. Factors influencing PHD function

Regulators of PHDs

PHD1	PHD2	PHD3
O_2	O_2	O_2
Siah2	FKBP38	Siah2
glucose metabolites	glucose metabolites	glucose metabolites
HIF-1	AhR	TRiC
	TGF-beta	MORG1
	NO	HIF-1
	HIF-1	

2.7.1 Targets of PHDs

Bearing in mind that the name ”PHD proteins” reflects only their functionality as prolyl 4-hydroxylases and does not commit them to be HIF specific, other substrates and hydroxylase activity independent functions exist.

The RNA polymerase II is a complex responsible for DNA to mRNA translation. RNA polymerase II large subunit Rpb1 contains an LXXLAP motif and has been shown to be hydroxylated on proline 1465 by PHD1 leading to subsequent recognition by pVHL. The exact consequences of Rpb1 hydroxylation and pVHL recognition are thus far unknown but it has been suggested to have a role in transcription elongation altering gene expression in stress situations that lead to DNA damage (Kuznetsova et al. 2003; Mikhaylova et al. 2008). PHD2 has been shown to be able to bind ING4 and OS-9 and recruit them to HIF-1 (Ozer and Bruick 2005).

Activating transcription 4 (ATF-4) is a transcription factor belonging to the ATF/CREB family of basic region leucine zipper transcription factors which is rapidly stabilized in response to hypoxia. ATF-4 has been shown to be proteasomally degraded and this has been shown to be prolyl hydroxylase-dependent (Ameri and Harris 2008; Köditz et al. 2007). The specific hydroxylase for this is most likely to be PHD3 as both PHD1 and PHD2 fail to interact with ATF-4. Also pVHL is unable to interact with ATF-4, and the regulation of the transcription factor has been suggested either to be dependent on SCFbTrCP ubiquitin ligase (Lassot et al. 2001) or not (Wottawa et al. 2010).

Nuclear factor kappa beta (NF-κB) is a transcription factor implicated in tumour development and it has fundamental importance in innate immune responses and inflammation. NF-κB is regulated by a cascade of inhibitory proteins, IκB’s. IKKβ is a

kinase that phosphorylates IκB, an event leading to the ubiquitination and degradation of IκB. Thereby NF-κB is freed from the inhibition and gets activated. IKKβ also contains the conserved LXXLAP motif. PHD3 has been shown to interact with IKKβ and physically blocking its interaction with Hsp90 which is required for IKKβ phosphorylation. Thus, PHD3 inhibits the activation of NF-κB (Xue et al. 2010). Moreover, PHD1 overexpression has been shown to decrease cytokine-stimulated NF-κB activity (Cummins et al. 2006).

Within the G-protein-coupled receptor family the β-adrenergic receptors regulate cardiovascular and pulmonary functions in response to catecholamines. Hypoxia increases the β2-subtype of adrenergic receptors (β2AR), an event mediated by prevention of its prolyl hydroxylation, subsequent recognition by pVHL and proteasomal degradation (Xie et al. 2009). The specific interacting prolyl 4-hydroxylase has been shown to be PHD3 and its depletion in normoxia leads to an increase in β2AR levels.

FIH hydroxylates endogenously many proteins with ankyrin repeat domains (ARDs). The biological outcome of FIH hydroxylating ARD is still unclear, but it has been suggested that ARD-proteins might act as competitive inhibitors of FIH-dependent HIF-1α C-terminal activation domain hydroxylation, the most convincing example being the Notch1 ARD (Coleman et al. 2007).

2.7.2 Structure and general function of PHDs

The active site of the prolyl hydroxylase enzymes contains eight beta strands folded into a beta barrel jelly roll motif. Two histidine residues and a carboxylate group serve as ligands for 2-OG and iron which are positioned by the motif (Elkins et al. 2003; McDonough et al. 2006).

PHDs function in an O_2-concentration–dependent manner rendering them progressively less active with dropping O_2 supply. They have high K_m-values for oxygen (100-250 µM) towards oxygen as compared to the tissue pO_2 levels and are considered as cellular oxygen sensors (Hirsilä et al. 2003; Koivunen et al. 2006) FIH which functions as a dimer has even higher K_m-value of 90 µM for oxygen which capacitates it to remain active even under low oxygen tensions (Koivunen et al. 2004). The addition of a hydroxyl group by prolyl hydroxylases leads to exclusion of a water molecule from pVHL-HIF-α interface thus allowing the formation of two additional hydrogen bonds between these two. This further stabilizes their interaction and enhances the ubiquitylation of HIF-α (Hon et al. 2002).

Prolyl hydroxylases catalyse a forward reaction but not a reverse reaction. Therefore, when the level of a prolyl 4-hydroxylase increases, the net reaction rate also increases. Accordingly, PHD2 is the most abundant of this group of enzymes and it also is the dominantly active one (Berra et al. 2003) whilst when the levels of PHD1 ad PHD3 increase they also contribute to the regulation of HIF level and activity (Appelhoff et al. 2004).

In *Caenorhabditis elegans* and *Drosophila melanogaster* genetic studies have shown a single prolyl 4-hydroxylase, Egl9 and Fatiga respectively, to have a critical function in HIF-α stability (Bruick and McKnight 2001; Epstein et al. 2001; Lavista-Llanos et al.

2002). In mammalian cells PHD1-3 have apparently originated from gene duplication. The existence of three such closely related proteins suggests these proteins have distinct properties in respect to regulating HIF function in higher organisms.

Initially it has been shown that all PHDs have the ability to hydroxylate HIF-α *in vitro*. They all have also been shown to be able to, when overexpressed, suppress HRE-mediated reporter gene activity (Huang et al. 2002; Metzen et al. 2003b). Their abundance in a particular cell type at a particular time reflects a lot to their function like in the case of the transcriptional bias between HIF-1α and HIF-2α target genes. Both HIF-1α and HIF-2α have highly conserved sites of prolyl hydroxylation but the effects of prolyl hydroxylases on these transcription factors are not equal. It has been shown that PHD2 is the main regulator of HIF-1α whereas PHD1 and -3 have more effect on HIF-2α. This has been proposed to be a consequence of differential action of the enzymes on the NTAD and CTAD of HIF-1α and HIF-2α (Appelhoff et al. 2004).

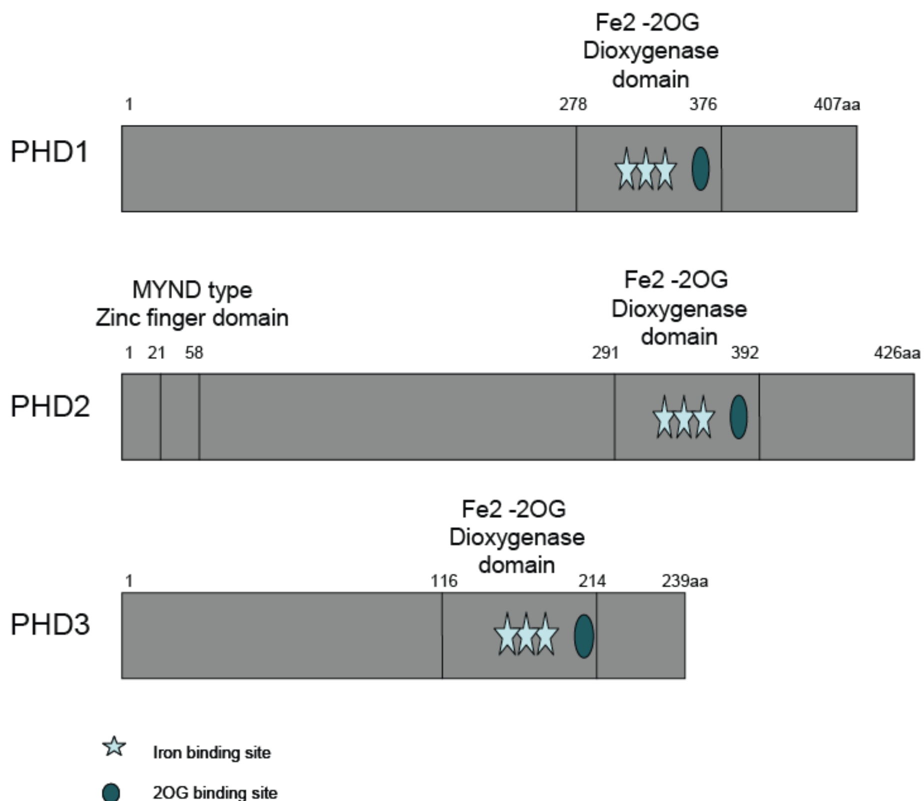

Figure 7. Domain structure of PHDs

In short, isoform-specific patterns of PHD induction by hypoxia and estrogen (Appelhoff et al. 2004) in different cell types confer variability to the relative abundance of the PHDs as well as on their relative contribution to HIF regulation. Differences in their preference toward hydroxylation sites within HIF-α subunit have been shown to exist. Similarly, the selectivity of PHDs between HIF-1α and HIF-2α gives potential to selectively alter HIF activation characteristics.

2.7.3 PHD1

Human PHD1 isoenzyme (also kown as EGLN2 or HPH3) is a 43.6 kDa protein encoded by 407 amino acids with 42 % similarity to other prolyl hydroxylase isoforms (Figure 7) (Taylor 2001). It was described as Falkor in mice (Bruick and McKnight 2001; Epstein et al. 2001) and EIT-6 in estrogen dependent breast cancer cell line ZR75-I (Seth et al. 2002). It belongs to a conserved SM-20 family of growth regulatory immediate-early genes. In normoxic conditions PHD1 mRNA is widely expressed albeit in varying levels amongst different human cancers. In comparison to its homologs PHD1 mRNA levels remain relatively stable in response to hypoxia (Berra et al. 2003; Cioffi et al. 2003; Epstein et al. 2001) or are in some cases significantly reduced (Appelhoff et al. 2004). PHD1 protein levels are lower than would be predicted based on the levels of PHD1 mRNA (Appelhoff et al. 2004). In contrast to PHD2 and -3, PHD1 protein levels are not induced by hypoxia. Within cells PHD1 locates mainly in the nucleus (Metzen et al. 2003b). In tissue level the highest PHD1 protein levels are found in renal tubules, gall bladder epithelium, pancreatic and salivary gland ducts, in myoepithelial and luminal cells of breast ducts, in testicular Leydig and Sertoli cells as well as in chorionic trophoblastic cells. Also adipocytes show some expression of PHD1 (Soilleux et al. 2005).

PHD1 has been observed as a doublet in cell extracts. In immunoblotting experiments a variety of cell lines have revealed a predominant species at predicted molecular weight (PHD1p43). The faster migrating species (PHD1p40) has been shown to be reduced by hypoxic exposure (Appelhoff et al. 2004). The shorter species has been described to be produced by internal initiation at M34 and it has shorter half-life (approximately 50 minutes) compared to the longer species (approximately 100 minutes). PHD1 isoforms are potentially targeted and regulated by ubiquitin ligases Siah1/2, but apparently these particular pathways do not contribute to setting the steady-state–levels of PHD1 (Tian et al. 2006).

PHD1 levels are also responsive to other factors than hypoxia. Oestrogen has been shown to influence the levels of PHD1 (Appelhoff et al. 2004; Erez et al. 2003). Cell confluence seems to have an effect on the levels of PHD1. The levels of both forms are increased in lower cell confluence but the relative proportion of the shorter species is increased (Tian et al. 2006).

Information about the function of PHD1 has long been elusive. It is able to hydroxylate HIF-1 similarly to other PHDs. PHD1 depletion has also been linked to the survival of ischemic skeletal muscles in response to mitochondrial dysfunction and ROS production. *Phd1*$^{-/-}$ skeletal muscle cells have been shown to be hypoxia-tolerant due to metabolic reprogramming that reduces mitochondrial oxidative metabolism and oxygen consumption (Aragonés et al. 2008). In estrogen stimulated normoxic cells PHD1 siRNA-depletion has been shown to have an influence on HIF-1α induction (Appelhoff et al. 2004). PHD1 has been shown to hydroxylate IKK proteins (Cummins et al. 2006).

2.7.4 PHD2

PHD2 is a 46 kDa protein that has been shown to be most active of the three isoforms in hydroxylating HIF-1α both in normoxia and in mild hypoxia (Figure 7) (Appelhoff et

al. 2004; Berra et al. 2003). PHD2 has been shown to be specifically active towards the N-terminal oxygen dependent degradation domain of HIF-1α rather than that of HIF-2α. It is active towards HIF-1 also in hypoxia where it by direct binding to HIF-1 and inhibition of N-terminal transcriptional activity influences HIF-1 without affecting its proteolysis. Structural domain analyzes have shown that PHD2, in addition contains a unique N-terminal MYND–type zinc finger domain (an acronym for Myeloid translocator protein 8, Nerwy and Deaf-1). The catalytic domain locates in the C-terminal region. The MYND domain inhibits PHD2's catalytic activity (Choi et al. 2005b). MYND domain has been suggested to function by anchoring PHD2 to mitochondrial or endoplasmic reticulum (Barth et al. 2009). This feature of PHD2 N-terminus has been suggested to confer to its specificity towards HIF-1α.

Transcript levels of PHD2 are, as are those of PHD3, regulated by HIF-1, and the hypoxic induction of PHD2 attenuates HIF-α levels. The *phd2* gene contains a hypoxia-responsive element in its promoter area which makes it a direct HIF target gene (Aprelikova et al. 2004; Metzen et al. 2005; Pescador et al. 2005).

PHD2 is expressed widely in different tissues (Willam et al. 2006). On mRNA level, PHD2 is particularly abundant in adipose tissue as well as in the heart (Oehme et al. 2002) and is generally quite uniformly expressed. PHD2 protein levels correlate well with that of mRNA (Appelhoff et al. 2004). Strong PHD2 staining has been reported in skin endothelial cells and in the basal proliferative layer of epidermis (Jokilehto et al. 2006). Markedly high stainings have also been reported from tracheal respiratory epithelial cells (Soilleux et al. 2005)

PHD2 is mainly expressed in the cytoplasm of cells (Metzen et al. 2003a). It is responsive to hypoxia as its protein levels are markedly increased in response to lowered oxygen availability. Similarly to PHD3, PHD2 has been suggested to create a negative feedback loop with HIF-1 in order to correctly respond to increased HIF-1 activity in hypoxia. PHD2 is also responsive to aryl hydrocarbon receptor as this can upregulate PHD2 expression in normoxia (Seifert et al. 2008)

Other factors besides hypoxia have been shown to regulate PHD2. Transforming growth factor beta (TGF-β1) is a growth factor that efficiently downregulates PHD2 both on mRNA as well as on protein level through the Smad signalling pathway (McMahon et al. 2006). NO is a potent inhibitor of PHD2 activity in normoxia but under hypoxia NO opposingly induces its activity. This discrepancy may be explained by the facts that NO in normoxia efficiently binds ferrous iron in the catalytically active center, but in hypoxia its ability to inhibit mitochondrial respiration chain provides PHDs with increased cellular oxygen thus increasing their activity (Hagen et al. 2003; Metzen et al. 2003b)

The post-transcriptional activity of PHD2 has been proposed to be regulated by amplified in osteosarcoma 9 (OS9) protein (Baek et al. 2005) but this finding has been challenged since (Brockmeier et al. 2011). ING4 tumour suppressor protein and FKBP38 have also been shown to interact with and regulating the activity of PHD2 (Barth et al. 2007; Ozer and Bruick 2005)

PHD2 has diverse function in respect to cell proliferation. Depending on the cell type, both activating and inhibiting functions have been addressed. A mutation inactivating PHD2 gene causes erythrocytosis indicative of PHD2's critical role in erythropoiesis regulation (Percy et al. 2006). PHD2 suppresses hypoxia-induced endothelial precursor cell proliferation (Takeda and Fong 2007). In normal growth the demand for PHD2 has been reported; in human pulmonary artery smooth muscle cells PHD2 is needed for FGF-2 and platelet-derived growth factor (PDGF)–induced proliferation (Schultz et al. 2009) and genetic inhibition of PHD2 results in ischemic cardioprotection (Hyvärinen et al. 2010; Hölscher et al. 2011).

PHD2 is also known to regulate vessel formation and shape in hypoxic conditions (Mazzone et al. 2009). Recently it has been shown that PHD2 regulates arteriogenesis and artery homeostasis by a mechanism where it controls specific differentiation state in macrophages via NF-κB –pathway (Takeda et al. 2011)

2.7.5 PHD3

2.7.5.1 General

HIF prolyl 4-hydroxylase enzyme PHD3 is a 27 kDa protein otherwise known as EGLN3 or HPH1 (Figure 7). Originally the rat homolog of PHD3 was identified as SM-20 in a differential screen of cultured smooth muscle cells (Wax et al. 1994). Its activation in fibroblasts was shown to be responsive to p53 activation (Moschella et al. 1999; Wax et al. 1994; Wax et al. 1996). In vascular smooth muscle cells SM-20 mRNA levels were shown to increase if cells were stimulated with growth factors and also upon smooth muscle cell differentiation (Moschella et al. 1999; Wax et al. 1994) Also injuries to the blood vessel wall led to SM-20 mRNA upregulation (Wax et al. 1996). In rat embryo fibroblasts the activation on temperature-sensitive p53 also led to an increase in SM-20 expression leading to apoptosis of these cells (Madden et al. 1996). Interestingly, tumour cells lacking p53 and stably expressing SM-20 were able to form less colonies in colony forming assays suggesting that could function downstream of p53 to drive growth arrest or even apoptosis (Madden et al. 1996).

Following SM-20 identification as a proapoptotic protein it was discovered that *SM-20* expression is upregulated in symphatetic neurons after nerve growth factor (NGF) withdrawal and that overexpression of SM-20 is sufficient to induce apoptosis even in the presence of NGF (Lipscomb et al. 1999). This cell death was shown to be caspase-dependent and SM-20 to be a mitochondrial protein. Also it was shown that SM-20 could be detected by immunofluorescence in punctate pattern colocalizing with cytochrome oxidase (Lipscomb et al. 2001).

SM-20 has also been shown to be expressed in addition to neurons in all smooth, skeletal ad cardiac muscle, in the epithelial cells of the endocrine organs and kidney. Also proliferating myoblasts of the dermomyotome and the developing heart tube express SM-20 at E8.5 (Moschella et al. 1999).

2.7.5.2 Expression and localization of PHD3

In normal cells PHD3 mRNA and protein levels are usually quite low or below detection limit. Upon hypoxic exposure PHD3 -levels are strongly upregulated. However, many studies have reported that in many cancer cell types, PHD3 inducibility in response to hypoxia is diminished and so the expression levels are low or absent (Appelhoff et al. 2004; Tuckerman et al. 2004). Decreased PHD3 mRNA levels and a lack of hypoxic upregulation have been reported at least in human prostate and breast carcinoma cell lines (Place et al. 2011). Mechanistically this phenomenon has been linked to aberrant *PHD3* promoter methylation (Hatzimichael et al. 2010; Place et al. 2011).

On cellular level, PHD3 protein localizes evenly throughout the cytoplasm and nucleus (Metzen et al. 2003b). Hypoxia has not been shown to influence the subcellular localization of PHD3.

mRNAs of all PHD isoforms are detectable in heart, liver, kidney, brain, testis and lung. The highest PHD3 mRNA level is found in the myocardium. Hypoxia induces PHD3 mRNA in the liver, testis and heart (Willam et al. 2006). PHD3 also shows peri-ischemic enhancement in response to myocardial infarction, maximal at 7 days after infarction (Willam et al. 2006), implying that PHD3 has, together with PHD2, strong influence on the pathophysiology of conditions where oxygen supply is below normal.

PHD3 upregulation has been linked to increasing age (Rohrbach et al. 2005). Aging is a factor that has been shown to aggravate adaptation to hypoxia by decreasing the expression of hypoxia-inducible genes such as *VEGF* and *Glut1* (Cataldi et al. 2004; Rivard et al. 2000b; Rivard et al. 1999; Xia et al. 1997). Also a decrease in hypoxia-induced HIF-1α stabilization has been described to be age-dependent (Rivard et al. 2000a). This has been correlated with an increase in the expression of PHD3 in mouse heart samples as well as in human heart left ventricles (Rohrbach et al. 2005) suggesting that PHD3 may at least partly be responsible for the age-dependent adversary effects. Interestingly, the elevated expression of PHD3 has been noted to be counteracted by reduced calorie intake in heart, skeletal muscle and liver (Rohrbach et al. 2005).

Expression of PHD3 occurs early during development in the skeletal muscle (Moschella et al. 1999). At E8.5 PHD3 expression is seen in the dermomyotomal cells of the rostral somites from where its expression progresses caudally. Highest levels are seen in the muscle primordial and mature muscles (Moschella et al. 1999), which suggests PHD3's involvement in the regulation of skeletal muscle differentiation. Skeletal muscle formation is mainly governed by the family of myogenic regulatory factors, which include myogenin. Indeed, PHD3 has been shown to be a binding partner for myogenin and increase its levels. Moreover, PHD3 has been shown to antagonize VHL-mediated ubiquitination and degradation of myogenin (Fu and Taubman 2010).

PHD3 has been reported to be overexpressed in pancreatic cancer (Bruegge et al. 2007; Gossage et al. 2010; Metzen and Ratcliffe 2004). In a screen of human pancreatic cancer specimens PHD3 was shown to be upregulated more than 10-fold, most notably so in well-differentiated tumours. Interestingly, metastatic lesions were reported to exhibit higher PHD3 mRNA levels compared to normal pancreatic tissue, but less than in an

average cancer sample supporting the notion that more differentiated tumours express more PHD3 (Su et al. 2010).

Inflammatory stimuli have been noted to induce PHD3 expression (Walmsley et al. 2011). Neutrophils have been shown to express all 3 PHD3 isoforms constitutively at mRNA level; however, only PHD3 was induced in neutrophils in hypoxia on protein level (Walmsley et al. 2011).

2.7.5.3 Regulation of PHD3

PHD3 is most robustly induced by hypoxia on mRNA and protein level in comparison to PHD1 or PHD2 (del Peso et al. 2003; Metzen et al. 2003b). It is also induced by other factors besides hypoxia; in cultured smooth muscle cells and in arterial wall PHD3 is induced by growth factors (Menzies et al. 2004; Wax et al. 1994; Wax et al. 1996). Also during myogenesis PHD3 is strongly upregulated (Lieb et al. 2002). In addition, PHD3 upregulation has been associated with p53-mediated growth arrest and apoptosis in Ras-transformed rat embryo fibroblasts (Madden et al. 1996).

In HIF-deficient cells PHD3 mRNA upregulation by hypoxia has been shown to be hindered. Moreover, in VHL-deficient cells PHD3 was shown to be highly abundant and this could be reverted by restoration of VHL function. These findings suggest that the hypoxic induction of PHD3 is mediated by HIF forming a negative feedback loop to limit the hypoxic HIF response. Similarly to PHD2, increase in PHD3 levels can compensate for reduced oxygen availability (Henze et al. 2010). In glioblastomas this negative feedback loop has been shown to protect tumour cells against hypoxia-induced cell death (Henze et al. 2010). *Phd3* gene has a hypoxia-responsive element in its promoter region through which it is upregulated under hypoxia (Pescador et al. 2005). The enhancer element responsible for hypoxia- and HIF-mediated upregulation of PHD3 is located in the first intron of the gene over 12 kB downstream of transcription initiation site. *In vivo* it binds endogenous HIF-1α in an oxygen-dependent manner. Among hypoxia-related genes it is unusual for a HRE to reside at such distance from the transcription initiation site. For example in the case on PHD2 gene the HRE is located 0.5 kb upstream of the initiation site.

Even though HIF-1α itself drives upregulation of PHD3 in response to hypoxia, HIF-2α seems to be the preferential regulator of PHD3. In response to overexpressing HIF-2α PHD3 is upregulated much more prominently than in response to HIF-1α overexpression while PHD2 levels remain unchanged (Aprelikova et al. 2004).

PHD3 (as well as that of PHD2) mRNA and protein levels are increased in response to TCA intermediates pyruvate and oxaloacetate independently of hypoxia (Dalgard et al. 2004). These metabolites bind to the 2-OG site of PHD3 (and PHD2) and thereby inactivate HIF-1α decay in normoxia (Lu et al. 2005). Also it has been shown that under conditions where PHD3 is inhibited by hypoxia, its function on HIF-1α can be restored by increasing the intracellular concentration of 2-OG. This restoration leads to a decrease in HIF-1α levels, reversal of hypoxic glycolytic changes and PHD3-mediated cell death (Tennant and Gottlieb 2010).

On protein level, the degradation of PHD3 is partly regulated by ubiquitin ligases of the SIAH family (Habelhah et al. 2004; Nakayama et al. 2004). Also it has been suggested that PHD3 is a substrate of cytosolic chaperonin protein TRiC (TCP-1 ring complex) and thus be regulated by it (Masson et al. 2004). TRiC belongs to group II chaperonins which have implications in neurodegenerative diseases where they promote and assist the correct folding of polypeptides (Albanèse et al. 2006).

In severe hypoxia (0-1% O_2) PHDs are nearly inactive but in milder hypoxia (2-5%) they retain at least some of their enzymatic activity. However, in mild hypoxia HIF-1α is stabilized which is explainable by the fact that PHD3 (and to some extent PHD1) are substrates for the RING finger E3 ligase Siah1/2 which targets PHD3 to be proteasomally degraded (Nakayama et al. 2004).The susceptibility of PHD3 to be degraded by Siah1/2 is limited by PHD3's ability to assemble into higher order complexes, the composition of which is controlled by the ambient oxygen tension. PHD3 can homo- or heterodimerize and it has been suggested that these complexes quite likely contain also other proteins besides PHDs that possibly influence the localization and activity of PHD3 (Nakayama et al. 2007b). Further it has been suggested that the reduced activity of PHD3 under hypoxia is partly due to its relocalization into higher order complexes where it can less actively function as a prolyl 4-hydroxylase and that in the lower order complexes PHD3 would be more active and more susceptible to degradation by Siah2 (Nakayama et al. 2007b).

RING domain E3 ubiquitin ligases like SIAH interact with adaptor proteins that attract different binding partners through protein-protein interaction domains sch as WD40-repeats and leucine-rich repeats. One such protein is also MAPK Organizer 1 (Morg1) which has been shown to interact with PHD3 to enhance its activity and function as a scaffold to restrict HIF-1α expression (Hopfer et al. 2006).

Large complexes formed by PHD3 under hypoxia have been shown to contain human PRP19 (pre-mRNA processing factor 19)–protein. The role of hPRP19 in hypoxia is not known, but in normoxic conditions it is known to have a role in splicing, ubiquitination and cell growth (Ohi et al. 2005). The connection between PHD3 and PRP19 is similar to that of PHD3 and Morg1 (Hopfer et al. 2006) as it utilizes the C-terminal WD40 domain of PRP19. The interaction between PHD3 and PRP19 has been reported to increase in hypoxia albeit mechanistically this remains elucive. However, the interaction has been shown to prevent PHD3 –dependent apoptosis induced by hypoxia. Possibly importantly, PHD3 has been shown to facilitate the interaction between N- and C-terminal regions of PRP19, suggesting PHD3 to function as a scaffold protein (Sato et al. 2010).

Alternative splicing of PHD3 has been proposed to bear some importance in its regulation. In addition to full length *PHD3* gene two shorter products are consistently being generated. The smaller product, 438 base pairs in length, has a large deletion in exon 1. The intermediate product of 646 base pairs has a deletion of exon 4 (Cervera et al. 2006; Hirsilä et al. 2003). Both alternative splicing products have been detected in elevated levels in cancer samples compared to normal tissues. The shorter splice variant has no activity towards HIF-1α when overexpressed in hypoxia in contrast to the intermediate variant that preserves the ability to hydroxylate HIF-1α (Cervera et al. 2006).

Another level of PHD3 regulation is provided by methylation. PHD3 promoter has been shown be subject of aberrant CpG methylation and thus basal mRNA expression is silenced. This has been shown to occur in B-cell dyscracias (Hatzimichael et al. 2010) as well as in melanoma and prostate, breast and renal cell carcinoma cell lines (Place et al. 2011). Interestingly, neither HIF-1α protein levels nor hypoxic response are compromised in these PHD3-methylated cell lines suggesting this mode of regulation does not affect the transcriptional response through HIF pathway, but that it is used by malignancies of diverse cell types (Place et al. 2011). It has also been suggested that PHD3 silencing through methylation occurs through the loss of some interaction partners (Place et al. 2011).

MicroRNAs (miRNA) are a family small interfering RNAs that modify transcriptional and posttranscriptional processes (Bartel and Chen 2004). From a genome-wide miR-screen of mechanically stretched neonatal rat cardiomyocytes microRNA-20a was identified to be upregulated in an *in vitro* hypoxia-reperfusion model. It was shown significantly inhibit hypoxia-mediated apoptosis while its knockdown induced apoptosis of these cardiomyocytes. In subsequent studies miR-20a was shown to directly target and downregulate PHD3 (Frank et al. 2012).

2.7.5.4 Function and targets of PHD3

Although the function of PHD3 is less well known than that of PHD2, it has been shown to be the primary regulator of HIF-1 under more severe and prolonged hypoxia, and its role may link to more tissue-specific events (Appelhoff et al. 2004). PHD3 hydroxylates most efficiently the C-terminal ODD-domain of HIF-α and has almost no hydroxylation activity over N-terminal ODDD (Appelhoff et al. 2004). It has been shown to be induced in sympathetic neurons in response to nerve growth factor withdrawal and when overexpressed it is able to provoke apoptosis in pheochromocytoma cells (Lipscomb et al. 1999; Straub et al. 2003b) .

In neurons PHD3 functions downstream of c-Jun and the neuronal apoptosis occurring naturally during development is dependent on PHD3 (Lee et al. 2005). This apoptosis inducing capability is unique among PHDs and it is dependent on its hydroxylase activity as studies have shown the catalytically inactive mutant to be unable to provoke apoptosis. This is not dependent on HIF-α as stable variants of either HIF-1α or HIF-2α do not affect the apoptosis-inducing function of PHD3 (Lee et al. 2005). In the early studies it was noted that PHD3 mRNA-levels increase in primary symphatetic neurons right at the time when these cells undergo apoptosis after NGF withdrawal (Lipscomb et al. 1999). In PHD3-deficient mice the development of symphatetic neurons is dependent on PHD3 as after NGF withdrawal PHD3 –deficient neurons are resistant to apoptosis (Schlisio 2009; Schlisio et al. 2008). These mice have abnormal sympathoadrenal development and in the super cervical ganglia and in the adrenal medulla the number of cells is increased (Bishop et al. 2008). These abnormalities have been linked to the deregulation of β2-AR which is known to be specifically hydroxylated by PHD3 and its loss leads to upregulation of β2-AR.

In pheochromocytomas it has been proposed that the genetic defects in *VHL, NF1, c-RET* and *SDH* all act directly on or upstream of PHD3 to impair apoptosis and thus

give rise to neoplasias since for instance SDH inhibition leads to intracellular succinate accumulation and inhibition of PHD3 function (Lee et al. 2005). Whether similar route is true for all neoplasias of neural crest origin remains to be seen.

Neutrophil lifespan is regulated by apoptosis and their susceptibility to go into apoptosis is in turn regulated via the oxygen sensing pathway and neutrophil survival has been shown to link to PHD3 in hypoxia (Walmsley et al. 2011).

This cell death is markedly delayed in hypoxia. Studies have linked PHD3 expression to this delay as in *Phd3*$^{-/-}$ mice increased levels of neutrophil apoptosis and clearance has been reported. In conjuction with the defect of *Phd3*$^{-/-}$ mice to drive apoptosis, an upregulation of proapoptotic mediator Siva1 and loss of its binding partner Bcl-xl have been reported (Walmsley et al. 2011). These findings support the notion that PHD3 has a role in regulating neutrophil survival in hypoxia.

In the development of neuroblastomas a pathway involving PHD3 has been proposed where the elimination of excess neurons during embryo development is a crusial event. PHD3 has been shown to function on this pathway by regulating kinesin KIF1Bbeta levels (kinesin family member 1B) (Schlisio et al. 2008). Interestingly, KIF1Bbeta is a tumour suppressor gene and the loss-of-function mutations of this kinesin have been linked to formation of some tumours of neural crest origin (Benn et al. 2000; Cheng et al. 1995; Ichimiya et al. 1999; Wang et al. 2006).

PHD3 has been shown to have other hydroxylation targets besides HIF such as ATF-4 (Köditz et al. 2007) and β2AR (Xie et al. 2009). In colorectal cancer cells PHD3 seems to function as a tumour suppressor as it inhibits IKKβ phosphorylation and thereby NF-κB activity (Xue et al. 2010). Recently PHD3 has been shown to have a role in regulating pyruvate kinase M2 and thereby having an effect on glycolytic metabolism (Chen et al. 2011; Luo et al. 2011b). ATF-4 (also known as CREB2, TAXREB67 or C/ATF) is a transcription factor that is translationally induced by anoxia and following endoplasmic reticulum stress mediates the unfolded protein response. Therefore it is critical in mediating cell fate. Studies have shown that the zipper II domain of ATF-4 interacts with PHD3 and that an ODD domain is located adjacent to zipper II domain of ATF-4. Mutations of five prolyl residues within this domain were sufficient to stabilize ATF-4 in normoxia as was downregulation of PHD3 by siRNA showing that PHD3 confers oxygen dependently stability to ATF-4 protein although direct demonstrations of ATF-4 hydroxylation by PHD3 is still lacking (Köditz et al. 2007).

PHD3 targets paired box 2 transcription factor (Pax2) for degradation. Pax2 is known to regulate tissue development and cellular differentiation and its function attenuates towards adulthood (Lang et al. 2007). Deregulated expression has been reported in cancers like breast, prostate, liver, kidney and leukemia (Muratovska et al. 2003). Hypoxic upregulation of Pax2 has been shown in RCC (Luu et al. 2009). PHD3 has been shown to negatively regulate Pax2, but the precise mechanism remains unclear (Yan et al. 2011).

NF-κB is a transcription factor family that has been linked to many biological and pathological processes (Hayden and Ghosh 2004). NF-κB is kept inactive by inhibitor of

κB (IκB) family of inhibitory proteins. If IκB function is inhibited by the activation of IκB kinases (IKK's), NF-κB is released from its restrictors allowing translocation into nucleus where it regulates target gene expression. IKK activation in turn is dependent on phosphorylation regulated by heat shock protein 90 (Hsp90). The activation of NF-κB is seen in many types of cancer, for example in colorectal cancers (Hardwick et al. 2001). PHD3 expression has been reported to be decreased in colorectal cancer and this decrease has been associated with higher tumour grade and metastasis. Studies show that PHD3, independently of its hydroxylase activity, has the ability to inhibit NF-κB signalling by directly inhibiting the interaction between IKKβ and Hsp90 thereby inhibit NF-κB signalling (Xue et al. 2010). For many types of cancers NF-κB signalling is a survival prerequisite and PHD3 upregulation via inhibiting this signalling may be detrimental. NF-κB also inhibits skeletal muscle differentiation and its binding and transcriptional activity has been shown to decrease with differentiation (Guttridge et al. 1999; Lehtinen et al. 1996). It has been shown that PHD3 negatively regulates NF-κB also in this setting (Fu and Taubman 2010).

PHD3 can to some extent compensate for the loss of PHD2 as has been shown with *Phd3*$^{-/-}$ mice that are homozygous for a conditional *Phd* allele. In mice that lack both PHD2 and PHD3 HIF-1α levels were higher than in mice lacking only either alone. Simultaneous loss of *Phd3* and *Phd2* also led to severe hepatic steatosis similar to mice with pVHL loss. Also, the dilated cardiomyopathy and premature mortality seen with *Phd*$^{-/-}$ mice was worsened by concurrent loss of *Phd3*. However, no overproduction of erythropoietin was seen in accordance with PHD3 indicating that the compensation differs depending on the HIF target and tissue (Minamishima et al. 2009). The loss of PHD3 alone does not phenocopy the changes seen with PHD2 loss, consistent with the notion that PHD2 is the primary regulator of HIF under normal conditions. The importance of PHD3 is, however, poised to modulate HIF under conditions like prolonged hypoxia.

PHD3 upregulation has been linked to this apoptosis in embryonic rat heart-derived cell line via its ability to reduce Bax-Bcl-2 complex formation. Stressed cells rely heavily on the interactions of Bcl-2 family of proteins. Bcl-2 is known to promote cell survival whereas the other members of the family promote apoptosis. Therefore an interaction of PHD3 with Bcl-2 would inhibit the anti-apoptotic function of Bcl-2 (Liu et al. 2010). Chronic hypoxia is known to prevent adipogenesis HIF-1α–dependently via PPARgamma inhibition (Yun et al. 2002). However, transient HIF-1α expression has been reported in the initial stages of adipogenesis in normoxia (Imagawa et al. 1999). Therefore it is conceivable that oxygen sensing has a physiological role in these early stages. PHDs have been indicated in adipogenesis with PHD3 expression being apparent in the late stages.

In many studies PHD3 has been shown to inhibit cell growth whereas cell growth is accelerated by its downregulation. PHD3 overexpression has also been linked to morphological changes. Appearance of stress fibres and increase in focal adhesion kinase (FAK) phosphorylation have been reported with catalytic-active PHD3 overexpression independently of HIF-1.These are both markers for focal adhesions that anchor F-actin to transmembrane proteins thus facilitating communication of the cell with its environment. These findings imply PHD3 may have a role in tumour cell invasion and cell migration (Su et al. 2010).

Recent research has identified PHD3 in the regulation of glycolytic activity under hypoxia. Chen et al showed that PHD3 interacts with the glycolytic enzyme pyruvate kinase. They showed that in normoxia downregulation of PHD3 increases the relative amount of PK-M2 which is the more active form of the two PK-M forms, namely PK-M1 and PK-M2 (Chen et al. 2011). Following this Luo et al described that PK-M2 and PHD3 interact in hydroxylase activity –dependent manner, and that this interaction enhances PK-M2 binding to HIF-1α. This in turn facilitates PK-M2 coactivator function. Moreover this study demonstrates PHD3 downregulation to reduce glucose uptake and lactate production, and to increase the O_2 consumption of cancer cells (Luo et al. 2011b). These findings highlight PHD3's role in regulating HIF-1α transactivation and reprogramming of the metabolic profile of cancer cells.

2.7.6 Factor Inhibiting HIF (FIH)

Factor inhibiting HIF (FIH) is an asparaginyl hydroxylase which facilitates the repression of HIF-1 transcriptional activity by binding to VHL. It thereby blocks the association of HIF with its coactivators CBP/p300, an event that inhibits HIF from being transcriptionally active. FIH hydroxylates an asparagine residue at the C-terminal transactivation domain of HIF in an oxygen–dependent manner. From this reaction a hydroxyasparagine residue is created which in turn sterically inhibits the binding of coactivators.

Mice with a null mutation in the *Fih* gene appear to be normal as what comes to classical HIF functions, *i.e* erythropoiesis, angiogenesis and development. FIH null mice do, however, have elevated metabolic rate, hyperventilation tendencies, reduced body weight *etc*, showing that FIH activity has clear implications in metabolism regulation (Zhang et al. 2010).

Recently, another FIH substrate class has been identified. These studies have highlighted the importance of asparaginyl hydroxylation as a fairly common posttranscriptional modification of cellular proteins that contain an ankyrin repeat domain (ARD). Initially IκB proteins were identified as substrates for FIH (Cockman et al. 2006).

2.8 p62

The signal adaptor protein p62 (also known as SQSTM1 and A170, hereafter designated as p62) is a ubiquitously expressed cellular protein conserved in all metazoan but not in fungi or plants. It is often found in cytosolic aggregates and intracellular inclusions together with polyubiquitinated proteins in patients with neurodegenerative diseases, liver disease or myopathies (Zatloukal et al. 2002). p62 mutations have been identified in Paget's disease of bone, a chronic disorder characterized by defects in normal bone remodelling process (Laurin et al. 2002). p62 was originally described as a cytosolic, phosphotyrosine-independent protein functioning as a ligand for the p56lck Src homology 2 domain (Park et al. 1995). Subsequently it was shown that p62 noncovalently interacted with ubiquitinated proteins via its C-terminal domain (Vadlamudi et al. 1996). Later p62 was identified as a partner for the atypical protein kinase C's (aPKCs) interacting

through its PB1-domain (Sanchez et al. 1998). Studies with knock-out, knock-in and transgenic mice have proven it to serve as a platform for various cellular functions such as bone remodelling, obesity, cell survival, inflammation, apoptosis, autophagy and cancer (Figure 8) (Duran et al. 2008; Durán et al. 2004b; Moscat and Diaz-Meco 2009; Moscat et al. 2006; Rodriguez et al. 2006). It has also been suggested that p62 would control the localization of aPKCs to the NF-κB cascades (Moscat et al. 2006).

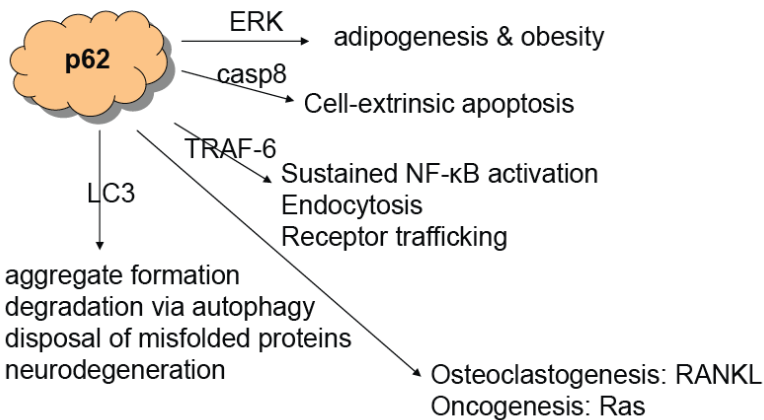

Figure 8. Functions of p62

Early on it was shown that p62 is expressed in a punctate pattern in liver cells exposed to a stressful environment (Stumptner et al. 1999). In liver diseases cytoplasmic inclusions termed Mallory bodies and hyaline bodies are typically seen, and they usually contain p62 (Zatloukal et al. 2002). p62 containing inclusion that colocalize with ubiquitin have been seen in many neurological conditions such as Alzheimer's and Pick's and Parkinson's disease (Kuusisto et al. 2001). Much research has been done on the role of p62 in neurodegenerative diseases and its crucial role in protein aggregation has become apparent (Braak et al. 2011; Kuusisto et al. 2002). In general, p62 is a major player in the control of cellular signalling, protein trafficking and protein catabolism.

2.8.1 Structure of p62

p62 is a 440-amino acid protein (Geetha and Wooten 2002) and it has potential to function as an intersection for signal transduction within cells. Proteins that are involved in signal transducing generally harbour various domains that can combinatorily be used in the assembly of multiprotein complexes (Pawson and Nash 2000). p62 is not only a cytosolic protein even though its cytosolic functions are better described. p62 contains a nuclear localization signal (NLS) and is therefore a subject of active transportation into the nucleus. It has been proposed that phosphorylation of p62 at the Cdk1 sites would control its ability to shuttle to the nucleus and localize to PML bodies. In prostate tumour samples 18% of the samples have been reported to be positive for nuclear p62 at least in a fraction of the cells studied (Pankiv et al. 2010). Nuclear protein quality control has been shown to be of upmost importance as most polyglutamine expansion diseases are connected to the formation of nuclear protein aggregates (Shao and Diamond 2007). It

has been shown that p62 is actively transported back and forth to and from the nucleus and that this transport is modulated by phosphorylation.

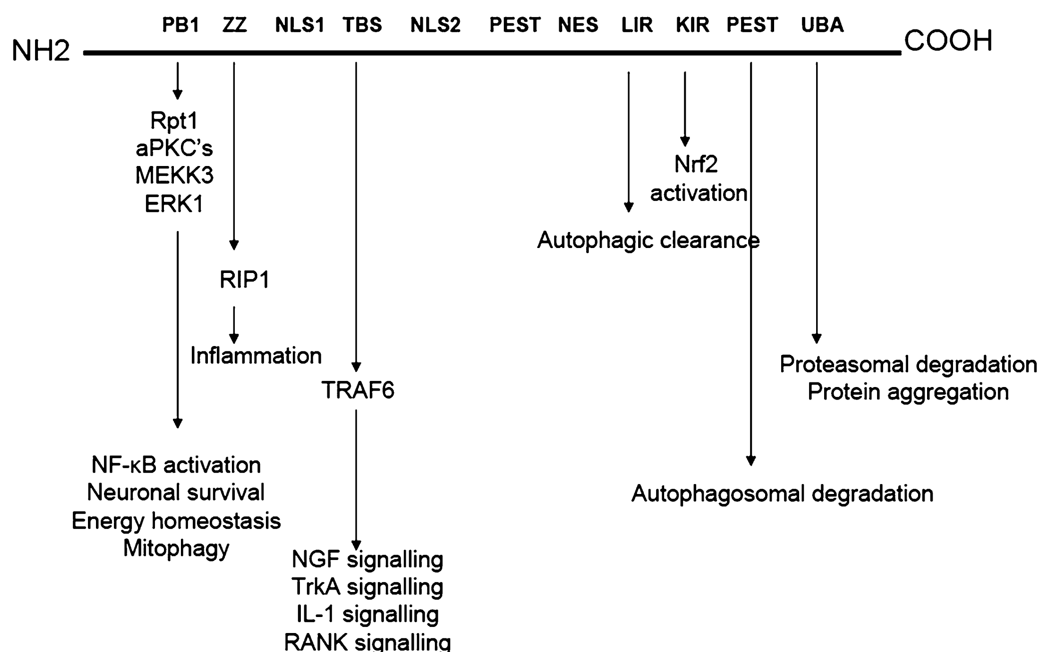

Figure 9. p62 domains and their functions

2.8.1.1 PB1 domain

Phox/Bem1p –domain modules (PB1 domains) are evolutionarily conserved protein-protein interaction modules that are present in many signalling molecules such as polarity protein Par-6, aPKCs, MEK5 and NBR1 (Figure 9; neighbor of BRCA1 gene 1) conferring specificity and fidelity to cellular signalling. These proteins bind each other through their PB1 domains like in the case of p62 and aPKCs where the binding is involved in the activation of the transcription factor NF-κB (Durán et al. 2004b; Sanz et al. 2000). Via this domain p62 binds aPKC, MEKK3, MEK5 and ERK1 and thus is able to function as signal integrator and diversifier of signalling pathways (Moscat et al. 2006).

Also, PB1 domain enables p62 to self-oligomerize, which is an important feature of p62 since it gives rise to p62 bodies. Self-oligomerization is also important for the degradation of p62 by autophagy (Ichimura et al. 2008a; Pankiv et al. 2007). Proteasomal protein Rpt1 interacts with the PB1 domain of p62 triggering the degradation of ubiquitinated p62 cargo by 26S proteasome (Geetha et al. 2008; Seibenhener et al. 2004).

2.8.1.2 ZZ domain

Another domain within p62 is the ZZ-type zinc finger domain located in close proximity to PB1 domain (Figure 9). It binds receptor interacting protein 1 (RIP1) (Bjørkøy et al.

2005) which enables p62 to link aPKC to this signalling complex (Sanz et al. 1999). RIP1 interacts with death receptors such as TNF giving rise to a complex that is able to activate several downstream pathways including NF-κB and p38MAPK (Festjens et al. 2007). The exact role of p62 ZZ-domain still remains elusive.

2.8.1.3 TRAF6-binding domain

TRAF6 is an E3 ubiquitin ligase needed for protein polyubiquitination and p62 harbors a domain that binds TRAF6 (Figure 9). It has been shown that p62 serves as a scaffold for TRAF6 to mediate ubiquitination of growth factor receptors such as TrkA, TrkB and TrkC (Geetha et al. 2005; Moscat et al. 2006) and the activation of NGF-induced NF-κB signalling (Wooten et al. 2005). A naturally occurring isoform of p62, ZIP2, lacks the TRAF6 binding site and it has been shown to promote signalling via Trk receptors in certain settings (Gong et al. 1999).

2.8.1.4 NLS and NES

There are two nuclear localization signal domains (NLS1 and NLS2) and one nuclear export signal domain (NES) in the p62 protein (Figure 9) (Pankiv et al. 2010). These domains enable p62 to shuttle between cytoplasm and nucleus, an activity that is regulated by phosphorylation of a site close to NLS2. Blocking the nuclear export leads to accumulation of ubiquitinated proteins in the nucleus. This is turn promotes the rapid transport of p62 into the nucleus as well (Pankiv et al. 2010).

2.8.1.5 PEST sequence

PEST regions are sequences that often are flanked by regions of positively chaged amino acids. These regions are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (Rechsteiner and Rogers 1996). These regions are often a structural feature of regulatory molecules and serve as signals for rapid degradation by the 26S proteasome. In p62 the PEST region probably serves to facilitate the rapid autophagosomal degradation in response to conditions such as hypoxia (Figure 9) (Pursiheimo et al. 2009).

2.8.1.6 UBA domain

Many proteins such as ubiquilin-1 and NBR1 contain a ubiquitin associated (UBA-type) binding domains (Waters et al. 2009; Wu et al. 2002). In the C-terminus of p62 protein there is a fifty amino acids long three helix bundle UBA domain that preferentially binds to the Lys63 –linked polyubiquitin chains (Figure 9) (Long et al. 2008). The UBA domain of p62 binds polyubiquitinated proteins and shuttles them to proteasomal degradation (Seibenhener et al. 2004). The UBA domain of p62 mediates its accumulation into aggresome-like structures or aggresomes together with ubiquitinated, misfolded proteins which then can be cleared out via autophagy (Bjørkøy et al. 2005; Johansen and Lamark 2011). Although the specificity of the UBA-domain mediated transport is still controversial, it has been demonstrated that at least neuronal proteins such as Tau and TrkA are subject to transport by p62 (Babu et al. 2005; Geetha et al. 2008). It has

been shown that p62 directly binds to an autophagic effector protein LC3 (Pankiv et al. 2007) and subsequently the motif of 11 amino acids in p62 protein were idenfied to be the LC3 –binding region. It was designated as LC3-interaction region (LIR) (Ichimura et al. 2008b).

Mutations in the UBA domain have been shown to link to the development of and predisposing to the Paget's disease of bone (Hocking et al. 2010). A naturally occurring p62 splice variant ZIP3 which lacks the UBA domain is expressed in many tissues such as hippocampus and cortex (Croci et al. 2003). Lack of UBA domain disrupts the proteasomal targeting of proteins as well as inhibits aggregate formation ad autophagic protein clearance.

2.8.1.7 LIR and KIR domains

p62 harbours an LC3 interaction region LIR (Figure 9). LC3 (microtubule-associated protein 1 light chain 3) is a mammalian homolog of yeast Atg8 (autophagy-related protein-8). Atg proteins are ubiquitin-like proteins that are required for the formation of autophagosomal membranes. Based on amino acid similarity two groups of Atg8-homologues exist in mammals, namely the LC3-group and the GABARAP-group (gamma-aminobutyric-acid type A receptor associated protein). The LIR motif mediates binding to both LC3- and GABARAP-subfamilies.

KEAP1 interaction region KIR of p62 mediates the induction of stress responsive cellular defence genes by associating p62 and KEAP1, an association which leads to hyperactivation of NF-E2–related transcription factor 2 (Figure 9) (Komatsu et al. 2010). Nrf2 is an important regulator of the responses elicited by oxidative stress. Interestingly, the induction of p62 gene by oxidative stress is itself mediated by Nrf2. Keap1 (Kelch-repeat domain of Kelch-like ECH-associated protein 1) is a Cullin 3-based ubiquitin ligase adapter that facilitates the ubiquitination and subsequent proteolysis of Nrf2 thereby regulating its activity (Komatsu et al. 2010).

2.8.2 Function of p62

p62 interacts with and serves as a scaffold to wide variety of signalling proteins such as ERK, RIP, TRAF6, caspase 8 and aPKC. It is known to be an inclusion body component, structures which are observed in liver diseases and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). On cellular level p62 is seen in cytosolic aggregates or speckles. These structures are formed when p62 oligomerizes via its PB1 domain, binds ubiquitinated proteins via its UBA-domain and binds the LC3-protein via the LC3 interaction region (LIR) which is needed for the formation of autophagosome (Pankiv et al. 2007). In these speckles p62 functions as signal-organizer and interacts with caspase-8 and TRAF6. During cytokine signalling p62 recruits TRAF6-IRAK complex to the speckles. TRAF6 gets modified by the K63-linked polyubiquitin chain catalyzed by the TRAF6 ubiquitin ligase itself. K63-linked ubiquiting chains often have an effect on functional and spatial protein regulation as opposed to K48-linked polyubiquitin chains which most often provide a signal for proteasome-mediated protein degradation. Interaction of p62 and TRAF6 has been shown

to regulate phosphorylation and ubiquitination of the IKK complex and thereby regulate NF-κB activity. NF-κB activation is seen in many tumourigenic processes (Benhar et al. 2002). p62 has been shown to activate NF-κB. The loss of p62 causes accumulation of Ras-induced ROS production resulting in oxidative stress situation within cells. This in turn activates c-Jun N-terminal kinase (JNK) and apoptosis (Duran et al. 2008).It has also been suggested that p62-TRAF6 –signalling hubs may also modulate receptor trafficking and thereby be involved in signalling regulation (Geetha et al. 2005).

By triggering TRAF6—NF-κB pathway p62 speckles favour cell survival. p62 can also favour apoptosis by promoting aggregation of cullin-3 -modified polyubiquitinated caspase-8. Caspase-8 aggregation leads to full processing of the enzyme and robust stimulation of effector caspases suggesting that p62 could function as a coordination point between extrinsic apoptosis and other signalling pathways (Jin et al. 2009).

p62 also shuttles polyubiquitinated proteins for degradation by autophagy (Komatsu and Ichimura 2010). It has also been shown to be involved in clustering and degradation of depolarized mitochondria and formation of aggresomes (Geisler et al. 2010; Okatsu et al. 2010).

2.8.3 Regulation of p62

p62 expression levels are regulated at transcriptional level (Nakaso et al. 2004). The p62 gene contains seven introns and eight exons. The splice sites comply with the GT/AG – rule with the exception of introns 6 and 7 using unusual GC dinucleotides. The promoter region of p62 is rich in CpG which makes this region more susceptible to damage caused by oxidative stress (Vadlamudi and Shin 1998). The p62 promoter is TATA-less as is the vast majority of human protein coding genes. From the 5'-flanking region binding sites for many transcription factors such as AP-1, SP-1, NF-κB and Ets-1 are found (Vadlamudi and Shin 1998). Also, p62 gene is regulated by Nrf2 with which it forms a positive feedback loop (Jain et al. 2010). As CpG islands are often targets for epigenetic regulation of gene expression it is highly likely that p62 expression will turn out to be under such regulation as well, although this still remains to be proven.

Oncogenic Ras has been shown to transcriptionally regulate p62 levels thereby reducing oncogenic transformation through impaired NF-κB activation (Duran et al. 2008). Ras is an oncogene most frequently mutated in human cancers (Bos 1998).Ras transformants have been shown to target the AP-1 element in the promoter region of p62 by a mechanism involving ERK and PI3K, both downstream targets of Ras.

p62 is also a proteotoxic stress responsive protein. Exposure to oxidants, sodium arsenite, cadmium, ionophores, proteasomal inhibitors or overexpression of polyglutamine-expanded proteins are all able to induce p62 (Nagaoka et al. 2004).

Analysis of p62 levels in the different phases of cell cycle has revealed that p62 gets heavily phosphorylated during early mitosis (Pankiv et al. 2010). This study demonstrates that p62 is phosphorylated at threonine 269 and serine 272 by cdk1 (Pankiv et al. 2010). Cdk1 is a kinase that controls the transit through S/G2 –phase and early mitosis (Malumbres and Barbacid 2009). The expression of nonphosphorylatable p62 in cancer cells has

shown that these cells actually exit mitosis to G1 faster than cells with wild type p62 cells. Also in the nonphosphorylated cells lagging chromosomes are seen indicative of increased genomic instability. Together these findings suggest that p62 phosphorylation restrains cell transformation (Pankiv et al. 2010).

p62 is localized to autophagosomes via its LC3-interaction region (LIR). It is constantly degraded via autophagy and if autophagy is dysfunctional p62 gets markedly accumulated resulting in p62-positive inclusion within cells (Komatsu et al. 2007). p62 is degraded by autophagy through direct interaction with LC3. However, mutations in the PB1-domain resulting in defective oligomerization greatly attenuates the degradation of p62 suggesting that for inclusion body formation both p62 accumulation and oligomerization are needed and that PB1-domain facilitated oligomerization is crucial for p62 degradation via autophagy (Ichimura and Komatsu 2010). Along with its PB1 binding partner NBR1 p62 has been shown to regulate the packing and delivery of aggregated, misfolded and in general polyubiquitinated proteins to clearance machinery in metazoa (Kirkin et al. 2009). This function is extremely important in cell survival regulation as toxic aggregate accumulation has been implicated in various pathological conditions and p62 is a component of these aggregates (Zatloukal et al. 2002).

2.8.4 p62 in disease

Recently p62 has gained a lot of attention as it has been associated with many diseases. Paget's disease of bone (PDB) is a condition where bone destruction and regrowth is abnormal. Studies on genotypically modified p62 null mice have shown that p62 regulates osteoclastogenesis and bone homeostasis through NF-kB activation (Durán et al. 2004a). This disorder is characterized by aberrant osteoclastogenic activity, which has been linked to the mutations of p62 (Laurin et al. 2002). In PDB the UBA-domain of p62 is either truncated or in some other way has lost its function (Layfield et al. 2006).

In Alzheimer's disease protein aggregates form neurofibrillary tangles (NFTs), in steatohepatitis Mallory bodies and in hepatocellular carcinoma intracytoplasmic hyaline bodies and p62 is associated with all of these structures (Kuusisto et al. 2002; Salminen et al. 2012). Accumulation of misfolded and polyubiquitinated proteins increase the expression of p62. This upregulation serves to protect the cells by localizing these misfolded proteins as aggregates in cytoplasmic inclusions (Nakaso et al. 2004; Zatloukal et al. 2002). p62 has been attributed in the formation of NFT's in the brains of Alzheimer's disease patients since its absence hinders the degradation of tau protein, a substrate protein for TRAF6 which binds to the p62 UBA-domain. Tau degradation is an important event since its accumulation leads to formation of insoluble K63-polyubiquitinated aggregates.

p62 has been linked also to obesity and insulin resistance. p62 knockout mice have significantly more body fat than their wild type counterparts and they showed symptoms of being diabetic. It has been suggested that the increased ERK activation and adipogenesis followed by p62 depletion could lead to obesity and leptin and insulin resistance (Rodriguez et al. 2006). Also a connection between p62 and mTOR/Raptor has been showed recently, implicating p62 to function as a connector between

autophagy and mTORC1 activity, maybe to thereby control adipogenesis (Duran et al. 2011).

2.8.5 p62 and cancer

Cancer is a group of diseases where p62 function has been much investigated. In tumourigenesis cell proliferation needs to overcome the difficulties of harsh conditions as well as nutrient and oxygen scarcity. The ability of a cell to activate autophagy, to reprogram metabolism, to control toxic compound production and misfolded protein accumulation are all terms that must be met by the tumour cells. p62 has the ability to modulate all these factors and it is therefore a central regulator of tumourigenesis.

During cell division high levels of protein synthesis and anabolic metabolism is needed. This is regulated by the serine/threonine kinase mTOR (mammalian target of rapamycin) which is a central regulator of cell growth and autophagy. These functions render mTOR to be in the crossroads of integrating nutrient sensing and cell-size control. This pathway has been reported to be aberrantly activated in various types of cancers owing to the activation of upstream oncogenes such as PI3K and AKT as well as the loss of tumour suppressor genes like PTEN and LKB1 (Duran et al. 2011; Kim et al. 2002; Menon and Manning 2008).

mTOR regulates two multiprotein complexes, namely mTORC1 and mTORC2. p62 is known to associate specifically with mTORC1 as p62 interacts with mTORC1 core component Raptor (Duran et al. 2011). mTORC1 orchestrates various signals that aim to coordinate cell growth and proliferation by means of controlling cell growth and inhibiting autophagy.

Upstream of this signalling is the TSC1-TSC2 complex. These proteins inhibit mTORC1 activity under conditions of environmental stress such as for instance hypoxia.The TSC complex negatively regulates Rheb, a Ras-related small G protein, the function of which is essential for mTORC1 activation (Huang et al. 2008). p62 has been proposed to function in the other mTORC1 activation path, namely via amino acid signalling, a task that would place p62 in the heart of nutrient sensing cascade (Duran et al. 2011). This proposal is supported by the finding that in p62-deficient cells mTORC1 activity is decreased and autophagy is upregulated (Duran et al. 2011). This in turn creates a feedforward loop (as p62 itself is a substrate for autophagy; (Bjørkøy et al. 2005) where mTORC1 activation by p62 increases its levels further increasing the activity of mTORC1 (Moscat and Diaz-Meco 2011).

Hypoxia is known to suppress mTORC1. In severely hypoxic cells ATP levels decrease and activate AMP-activated protein kinase (AMPK) (Liu et al. 2006). AMPK phosphorylates TSC2 to inhibit mTORC1 activity. Also, HIF-1α has been shown to upregulate expression of REDD1 which represses mTORC1. Further, BNIP3, a hypoxia-inducible pro-autophagy protein inhibits RHEB (homologue enriched in the brain) which decreases mTORC1 activity (Li et al. 2007). This HIF-1α–regulated inhibition of mTORC1 would seem to be beneficial as it would reduce protein synthesis and increase autophagy during hypoxic stress. Also HIF-2α has a role in regulating mTORC1. HIF-

2α has been shown to stimulate mTORC1 in order to under hypoxia promote cell proliferation. HIF-2α target genes include RB1CC1 (RB1-inducible coiled-coil protein 1) which has been implicated in interacting with TSC1 and disrupting TSC1-TSC2 –complex which would lead to mTORC1 activation (Gan et al. 2005). Similarly, HIF-2α targets TGFβ, PDGFβ and insulin-like growth factor 1 (IGF1) to activate AKT and thus mTORC1 (Roberts et al. 2009).

Many tumourigenic processes use the NF-κB signalling cascade as many NF-κB transcriptional products function as ROS scavengers (Duran et al. 2008). In p62 deficient mice it has been shown that Ras-induced transformation is impaired due to a decrease in NF-κB activation. Decreased NF-κB activation leads to decreased levels if ROS scavengers and thus ROS levels increase and cause more apoptosis (Duran et al. 2008).

These findings have been validated in a lung cancer model with p62 ablation. *p62-/-* mice were shown to be completely resistant to the formation of lung adenocarcinomas and adenomas whereas the wild type counterparts developed them efficiently showing that p62 is required for tumourigenesis (Duran et al. 2008). This has been proposed to function through p62-NF-κB axis as a study shows that during development of pancreatic ductal adenocarcinoma in which Ras activates NF-κB and NF-κB in turn transcriptionally induces p62 (Ling et al. 2012). p62 would then most likely oligomerize with TRAF6, further enhancing NF-κB activation (Duran et al. 2008). Moreover, the lack of p62 completely inhibits Ras-induced nuclear translocation of NF-κB. These studies reveal a p62-dependent pancreatic carcinogenic process where the activation of NF-κB occurs by combined efforts of Ras and p62 activation. Also, it exemplifies a situation where Ras-induced p62 overexpression is utilized to channel a pathway designed for the activation of NF-κB by RANK and IL-1 to increase cancer cell survival (Sanz et al. 2000).

p62 loss has been shown to lead to JNK activation and apoptosis (Duran et al. 2008). This apoptosis has been linked to the increased production of ROS. TRAF6 oligomerizes with p62 and therefore it is plausible that also TRAF6 is relevant in tumour formation TRAF6 mRNA has been reported to be overexpressed and its gene amplified in human lung cancer samples (Starczynowski et al. 2011). Supporting, TRAF6 inactivation has been shown to reduce NF-κB activation and therefore inhibit tumourigenesis. This suggests that p62-TRAF6 is an important complex for Ras-induced tumourigenesis of the lung. Further it suggests that Ras-driven transformation depends on p62-TRAF6 complex which it channels to function in tumour formation instead of its physiological function as bone remodelling regulator. TRAF6 has been shown to be able to confer normal cells with cancer cell-like characteristics when ectopically overexpressed (Starczynowski et al. 2011)

In line, a recent study shows that also p62 overexpression can drive tumourigenesis (Mathew et al. 2009b). In this study it was show that cells deficient in both apoptosis and autophagy are stressed and the accumulation of p62 leads to enhanced tumourigenicity. The precise mechanism is undefined but the authors do report of increased aneuploidy. In this study p62 overexpression was correlated with reduced NF-κB activation in contrast to other works that have shown p62 overexpression to activate NF-κB (Durán et al.

2004a). This discrepancy may be explained by the use of immortalized baby mouse kidney (IBMK) cells with defective apoptosis and autophagy machinery (Mathew et al. 2009a) while the other groups used Ras-transformed cells with intact apoptosis and autophagy machinery. It is feasible that in IBMK cells when deprived of nutrients and oxygen the accumulation of p62 at some level inhibits NF-κB pathway. Also it is possible that other factors are influenced by elevated p62 levels and thus the outcomes are different. Nevertheless it will be interesting to see how p62 functions to balance between being an activator of NF-κB and an inhibitor when metabolically stressed. It has been already demonstrated that p62 functions to activate caspases in cancer (Jin et al. 2009) so potentially p62 serves to balance between proapoptotic and antiapoptotic signalling.

2.9 Eukaryotic intracellular protein clearance

Cells need to control the turnover of their intracellular constituents on a regular basis. Eukaryotic cells have three main routes for this: the ubiquitin-proteasome system (UPS), the lysosomal pathway and the autophagosomal pathway. Multiprotein barrel-shaped complexes known as proteasomes predominantly degrade short-lived proteins of cytosol and nucleus as well as misfolded retrotranslocated ER proteins. To be degraded by the proteasome a protein usually needs to be covalently modified by ubiquitin. Three enzymes perform this ubiquitin moiety addition to lysine residues, namely E1, E2 and E3. E1 is a ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 a ubiquitin ligase. Ubiquitin can itself form a substrate for further rounds of ubiquitination, a cycle which leads to formation of a ubiquitin chain. These chains serve as a recognition motif for proteins to be escorted to the proteasomal degradation (Glickman and Ciechanover 2002).

Turnover of plasma membrane proteins such as receptors and channels is mainly governed by the lysosome pathway. Lysosomal degradation is characterized by vacuolar type H^+-ATPase (v-ATPase) mediated organelle acidification. In this degradation pathway, endocytosed proteins can be either recycled to the plasma membrane or captured into luminal vesicles.

Cytosolic entities such as organelles and protein aggregates which can not be degraded by the proteasomes are most suited for degradation via autophagy. In this process cytosol and organelles are captured by double-membrane preautophagosomal structure which then can fuse with late endosomes or lysosomes (Ciechanover 2005; Klionsky and Emr 2000).

2.9.1 Autophagy

In unstressed situations cells perform homeostatic functions such as cytoplasm and organelle turnover at low basal levels. In situations of sub-lethal stress cells utilize response mechanisms that enable them to adapt to changed environment and if possible, repair the damage. Immediate cytoplasmic alterations take place that are not dependent on transcriptional reprogramming. Specific subcellular structures such as autophagosomes are formed in order to eliminate damaged organelles and to mobilize energy reserves

within the cell (Klionsky 2004). This way, when extracellular nutrients become scarce, cells are capable of recycling intracellular macromolecules and organelles in order to generate macromolecules and thereby survive harsh conditions. This lysosome-mediated degradation process termed autophagy, a term derived from Greek words "auto" (self) and "phago" (to eat). Autophagosomes form through maturation process where they fuse with lysosomes to form an autolysosome. The luminal content of the double-membrane bound autolysosome is then degraded. These autophagosomes then mediate the first step of macroautophagy where long-lived proteins, organelles and/or portions of the cytoplasm are sequestered. Autophagosome formation is regulated by two ubiquitin-like conjugation systems. In the first part an autophagy gene Atg12 is conjugated to Atg5, an event mediated by Atg7 (an E1-like enzyme) and Atg10 (an E2-like enzyme). Following this Atg8 is conjugated to the lipid phosphatidylethanolamine regulated by Atg7 together Atg3 (an E2-like enzyme) (Xie and Klionsky 2007). Macroautophagy or *sensu stricto* autophagy is considered a life supporting phenomenon in cells as it supports cellular homeostasis in uni- and multicellular organisms and protects the cell from intra- and extracellular insults such as oxidative, metabolic and chemotherapeutic stress. Its fundamental role is to provide amino acids in response to starvation in order to maintain new protein synthesis and energy balance. In yeast the *Atg* mutants are highly sensitive to nitrogen deprivation but do not exhibit any phenotype in nutrient-rich conditions. Autophagy is also indispensable for sporulation of *S. cerevisiae*, fruit body formation of slime mold and dauer formation of *C. elegans* (Tsukada and Ohsumi 1993). The scope of autophagy influences a variety of other processes in metazoans, such as clearance of unwanted and misfolded protein aggregates (Renna et al. 2010), developmental cell death (Berry and Baehrecke 2008; Denton et al. 2009) and mitochondrial clearance during erythrocyte maturation (Kundu et al. 2008; Novak et al. 2010; Sandoval et al. 2008). In immunity its role is well recognized and well beyond its actions in removal of surplus organelles. In mice during neonatal starvation autophagy enables survival by preventing energy depletion (Kuma et al. 2004). In turn, autophagy deficiency in brain ($atg5^{-/-}$ or $atg7^{-/-}$) leads to neuronal degeneration, mitochondria damage and the accumulation of polyubiquitin-containing protein aggregates (Komatsu et al. 2006; Komatsu et al. 2007). Targeting autophagy deficiency to liver ($atg7^{-/-}$) results in accumulation of protein aggregates, liver injury and hepatocyte cell death (Komatsu et al. 2005). In addition to adaptive autophagy recent research highlights the importance of basal autophagy that takes place constitutively at low rates irrespective of any stress situation and operates to control the quality of cellular components.

Having a clearly protective function in many pathological conditions such as Crohn's disease (Brest et al. 2010), neurodegeneration (Wong and Cuervo 2010) and pathogenic infections (Deretic 2010) autophagy's role in cancer has remained rather elusive. Autophagy is induced in hypoxic tumour regions where it supports cell survival (Degenhardt et al. 2006).

2.9.1.1 p62 and autophagy

First suggestion linking p62 and autophagy came from studies showing p62 binding to Atg8/LC3 through its LIR-domain (Komatsu et al. 2007). Since then p62 has been shown

to regulate the packing and delivery of misfolded, aggregated and thus polyubiquitinated proteins and organelles to be degraded via autophagy (Pankiv et al. 2007). These studies gave p62 a role in regulating cell survival as the disposal of toxic protein aggregates is crucial for cell survival. Proofs of p62 being a part of these aggregates further supported this role (Zatloukal et al. 2002).

In tumourigenesis controlling the levels of p62 is of great importance. Several studies have shown that p62 accumulation by genetic inactivation of key autophagy molecules like Atg7 leads to formation of liver tumours (Inami et al. 2011; Takamura et al. 2011). This can be reversed by simultaneous ablation of p62 suggesting that p62 overexpression leads to a state of chronic inflammation and cancer in the liver (Komatsu et al. 2007). This may seem contradictory as autophagy is considered to be a cellular survival mechanism. However, many studies have shown that an essential autophagy gene *Beclin-1* is in fact a tumour suppressor which supports the notion that autophagy in essence is a cancer inhibiting process (Yue et al. 2003). Furthermore, well-established tumour suppressors PTEN and TSC proteins promote autophagy (Huang et al. 2008; Inoki et al. 2005) supporting the view that autophagy is tumour suppressive and p62 a major regulator of that process.

Atg7 –deficient mice have impaired autophagy and in the brains and livers from these mice p62-positive aggregates are seen (Komatsu et al. 2007). Simultaneous p62 –ablation leads to total disappearance of these aggregates supporting its role in aggregate formation and as such, it would be a key molecule in the quality control of proteins. However, other studies have also shown that autophagy impairment and accumulation of p62 leads to inhibition of proteasome function (Korolchuk et al. 2009a). It has been suggested that this would reduce the rate of polyubiquitinated protein clearance. In this case the appearance of aggregates of polyubiquitinated proteins would not be due to the structural role of p62 but rather because of proteasome blockage (Korolchuk et al. 2009b). This would be in line with the finding that p62 ablation together with autophagy inhibition leads to the disappearance of the aggregates.

ROS have been attributed in the autophagy-mediated tumour suppression by p62. In livers that are deficient of autophagy enhanced ROS production is seen. Also the activation of Nrf2 is commonly seen in these livers as Nrf2 controls the expression of key ROS scavengers (Taguchi et al. 2011). p62 overexpression has been linked to Nrf2 activation as p62 can chelate Nrf2 inhibitor Keap1 (Copple et al. 2010; Komatsu et al. 2010; Lau et al. 2010). This would provide a mechanistical outline where reduction in autophagy accumulates p62, a substrate for autophagy. Impaired autophagy could cause an accumulation of dysfunctional mitochondria leading to a state of increased oxidative stress. The accumulation of p62 would in turn lead to enhanced expression and activation of Nrf2 to alleviate oxidative stress. However, in autophagy-deficient liver tumours the ablation of p62 does not lead to worsened hepatotoxicity but instead is prevented by it. Also in contrast to above mentioned scenario, genetic inactivation of Nrf2 has been shown to inhibit p62 accumulation in hepatocytes defective in autophagy (Inami et al. 2011; Takamura et al. 2011) further highlighting the complex relation of p62 and cancer.

3. AIMS OF THE STUDY

Hypoxia is a common feature of tumours posing a considerable obstacle in treating cancer. In cancer cells the main sensors of oxygen, and therefore critical regulators of cells response to hypoxia, are the prolyl 4-hydroxylase domain containing enzymes, PHDs. The main goal of this thesis work was to elucidate the role of one PHD isoform, PHD3, in cancer cell survival.

In more detail, the specific goals were

I. to assess the effect of PHD3 on cancer cell survival under hypoxia

II. to assess the role of PHD3 in normoxic cancer cells

III. to identify molecular regulators such as p62 influencing PHD3 activity in cancer cells under hypoxia

and

IV. to characterize the biological significance of the interaction of PHD3 and p62

4. MATERIALS AND METHODS

More detailed information on methods and reagents is available in the original publications (I-IV).

Cell lines:

I, II,III) HeLa (from ATCC, Rockville,MD)
I,III) UT-SCC2 (cell line description: Jokilehto et al 2006; Landsford et al 1999)
II) HaCat keratinocytes, embryonic kidney HEK293, osteosarcoma U2OS, head and neck squamous cell carcinoma UT-SCC9
III) UT-SCC7, UT-SCC7, UT-SCC9

Patient samples:

III) Tumour samples were obtained after informed written consent from patients with oral and oropharyngeal HNSCC (Turku University Hospital). Normal samples were obtained from patients undergoing uvulopalatopharyngoplasty. Patient material has been described in Jokilehto et al 2006.

Cell culture:

I, II, III, IV) Cell were cultured in DMEM + 10%FCS with penicillin-streptomycin and L-glutamine. For UT-SCC2 and UT-SCC9 –cells non-essential amino acid supplement was used.

Hypoxia treatments:

I, II, III, IV) Cells were cultured in 1% oxygen with 5% humidity in Ruskinn $InVivo_2$ hypoxia chamber

Transfections:

The following transfections were used:
I, II, IV) FuGene HD (Roche Applied Biosciences, UK)
I) Effectene (Qiagen, CA)
II, III) Oligofectamine (Invitrogen CA)

Plasmid constructs:

I) PHD3-EGFP construct has been described previously (Metzen et al2003). PHD3R206K-EGFP construct was generated with QuikChange Site-Directed mutagenesis kit (Stratagene, CA).For PHD3-dsRed construct PHD3 open reading frame was generated by PCR and cloned into pDsRed-Monomer-N1 –vector (Clontech, Germany).
II, IV) pDestmyc-p62 and pEGFP-LC3 have been described in detail in Lamark et al 2003 and Bjorkoy et al 2005.

Small interfering RNA's:

II, III, IV) siRNA's against PHD1, PHD2, PHD3, p62, HIF-1α and HIF-2α and non-target siRNA were obtained from MWG Biotechnology, Germany. siRNAs against LC3 were obtained from Santa Cruz Biotechonoly, CA

RNA extraction, cDNA preparation and analysis:

III) RNA extraction was done using RNeasy mini kit (Qiagen) and cDNA with M-MMLV RT (H-) (Promega). RT-PCR analysis was done using Applied Biosystems equipment.

TagMan primers and probes:

III) PHD primers were obtained from Eurogentec

Chemicals:

The following chemicals were used:
I, II) Hydroxylase inhibitor DMOG (Cayman Chemical,MI)
I, II, III, IV) Hoechst 33342 (Sigma-Aldrich)
I, II, III, IV) ECL-kit (Pierce, Germany)
I, II) Mowiol-DABCO (Carpinteria, CA)
I) Propidium iodide (Invitrogen)
II) DFO (Sigma-Aldrich)
II, IV) 3-MA (Sigma-Aldrich)
II, IV) Bafilomycin A1 (LC Laboratories,MA)
II, IV) EHNA (Sigma-Aldrich)
II, IV) MG-132 (Calbiochem, CA)
III) Aphidicolin
IV) hematoxylin

Antibodies:

The following antibodies were used:
I) PHD3 (Bethyl Laboratories, TX)
I, III, IV) PHD2 (Novus Biologicals)
I, III) Caspase-3 (Promega, WI)
I) HSP70 (Stressgen, CA)
I) 20S proteasome (Biomol, Germany)
I) Beta-actin (Sigma Aldrich)
I) Hemaglutin (Roche Diagnostics)
I) Cy3-secondary antibody (Jackson Immunochemicals)
I) Cy5-secondary antibody (Jackson Immunochemicals)
II, IV) guinea pig polyclonal p62 (Progen Biotechnik, Germany)
II) mouse monoclonal P-ERK1/2 – (Santa Cruz Biotechnology, CA)
II) goat polyclonal ERK-2 (Santa Cruz Biotechnology, CA)
II, IV) mouse monoclonal AC-40 (Sigma Aldrich)
II) rabbit polyclonal anti-LC3 (Novus Biologicals, CO)
II) mouse Golgin-97 (Molecular Probes, OR)

II) mouse monoclonal anti-Myc (Sigma Aldrich)
II, III, IV) anti-mouse HRP, anti-rabbit HRP, anti-guinea pig HRP (DAKO, CA)
II) Alexa Fluor-555-labelled anti-guinea pig (Invitrogen)
III) anti-cyclinB1 (Santa Cruz Biotechnology, CA)
III) anti-cyclinD1 (Santa Cruz Biotechnology, CA)
III, IV) HIF-1alpha (BD Transduction Laboratories)
III, IV) PHD3 (Novus Biologicals – Sigma Aldrich)
IV) Myc (Sigma Aldrich)
IV) Bright Vision plus Poly-HRP-anti mouse/rabbit/rat IgG (ImmunoLogic)

Protein detection:

I, II, III, IV) Western blot analysis was performed to detect protein expression

Cell imaging and image processing:

I) Cell images and fluorescence recovery after photobleaching (FRAP) were done using Zeiss LSM510 confocal microscope at Turku Centre for Biotechnology Imaging Core. Quantifications and analysis was done by ImageJ software (NIH). FRAP analysis software was developed in-house (FRAPCalc, Dr. Rolf Sara, Turku, Finland).
II, IV) Cell imaging was done with Zeiss LSM510 confocal microscope and analyzed with Zeiss LSM software
III) Cell imaging was done using Zeiss Lumar V12 stereo microscope and image analysis with ImageJ

Immunohistochemistry:

IV) Heat-mediated antigen retrieval was used and tissue sections were visulized with Olympus BX60 microscope

Cell proliferation analysis:

III) Cell proliferation was done using colorimetric ELISA BrdU assay (Roche Diagnostics)

Proximity ligation assay:

IV) Duolink PLA assay was obtained from Olink Bioscience and done according to manufacturers instructions

Flow cytometry and analysis:

I, III) FACS analysis was done with FACSCalibur (Becton Dickinson, CA). Cell cycle analysis was performed with CellQuest Pro software (Becton Dickinson, CA).

Database studies:

III) Oncomine™ was used for database studies

Statistics:

III) Statistical analysis were done with Spearman correlation and two-tailed Student's t-test

5. RESULTS

5.1 PHD3 forms subcellular bodies in oxygen and hydroxylase activity dependent manner (I)

In most cancer cells PHD3 expression in normoxia is at a very low or undetectable level, but is strongly induced by hypoxia (Appelhoff et al. 2004; Aprelikova et al. 2004; del Peso et al. 2003; Marxsen et al. 2004). Under hypoxic conditions, however, PHD3 loses much of its enzymatic activity (Hirsilä et al. 2003) but is reactivated after reoxygenation. PHD3 expression pattern was studied with immunocytochemistry in both normoxia and hypoxia as well as in reoxygenation by using squamous cell carcinoma (SCC2) cells as a model. Transfecting SCC2 cells with siRNA against PHD3 (previously validated by Berra et al 2003) caused a complete loss of PHD3 signal as compared to control siRNA. In normoxia SCC2 cells expressed hardly any PHD3 protein. After 48-hour hypoxia, however, PHD3 showed clear upregulation that localized both to cytoplasm and nucleus as reported previously (Metzen et al. 2003b). In hypoxic SCC2 cells PHD3 was detected in punctuated structures that after 1 hour reoxygenation were even more pronounced in size and number. In PC12 and HeLa cells aggregates were more pronounced in normoxic conditions as compared to hypoxia. HeLa cells were used to further study the nature of the aggregates. Cells were transfected with PHD3-EGFP and subsequently grown under normoxia. Even though many cells showed dispersed PHD3 expression pattern, a great number of cells expressed PHD3 in subcellular aggregates. These structures were mainly cytoplasmic or perinuclear as judged from costaining with lamin A/C. The expression level of PHD3-EGFP from aggregate-forming and nonforming cells was compared showing that there was no direct correlation between the amount of PHD3 within these cells. This aggregation of PHD3-EGFP was detected in similar manner also in PC12 and SCC2 cells.

The influence of oxygen on the formation of the PHD3 bodies was studied. HeLa cells expressing ectopic PHD3-EGFP were exposed to 1% of oxygen or kept in 21% of oxygen for 8 hours. A strong reduction in the aggregation was detected under hypoxic conditions as compared to normoxia. Not only did hypoxia reduce the number of cells displaying aggregates it also reduced the number and size of the aggregates.

As the effect of hypoxia on the number of aggregates was so clear it was next studied whether the hydroxylase activity of PHD3 was necessary for PHD3 -body formation. HeLa cells were treated with a well characterized hydroxylase inhibitor dimethyloxaloylglycine (DMOG) for 8 hours. This clearly reduced the number of PHD3-bodies within cells. To specifically inhibit the hydroxylase activity of PHD3 but not that of PHD1 and PHD2 a point mutation was introduced to PHD3. In this construct the 2-oxoglutarate coordinating arginine 206 was mutated into lysine (PHD3R206K). Similarly to DMOG, PHD3R206K showed clearly reduced aggregate size and number. This clearly showed that PHD3-body formation is strictly dependent on PHD3 hydroxylase activity.

Similarities to other proteins forming aggresome-like structures like SQSTM1/p62 were observed. To test whether oxygen is a requirement for general protein aggregation cells

were transfected with EGFP-p62 and subsequently treated with either normoxia or 8 hour hypoxia. As previously described p62 forms cytoplasmic aggregates in normoxia. No difference in the aggregation of EGFP-p62 was observed under hypoxia which suggested that oxygen is not a requirement for protein aggregation.

5.2 PHD3 bodies colocalize with proteasomal components (I)

The most common form of proteasomes is referred to as the 26S proteasome. It contains a 20S core unit and 19S regulatory particle and, once active, they degrade damaged and unneeded proteins via proteolysis. Aggresome-like structures are known to contain components of the 26S proteasome as well as various chaperones and ubiquitin. To see whether PHD3 bodies localize in the vicinity of proteasomal components cells were cotransfected with PHD3-dsRed and LMP2-EGFP, a structural component of 20S. A near-complete colocalization between these two proteins was observed one day after transfection. Similarly to LMP2, endogenous components of proteasome (with 20S antibody) were shown to colocalize with ectopic PHD3-EGFP. Also colocalization with chaperone Hsp70 and ubiquitin was detected. No vimentin cage around PHD3 bodies was observed which suggested that the aggresome-like structures formed by PHD3 exhibit different characteristics form those of typical aggresomes despite other similarities.

To study the localization of endogenous PHD3 in respect to proteasomal components SCC2 cells were transfected with egfp-tagged LMP2 and subsequently treated with 48 hour hypoxia. Hypoxia-induced endogenous PHD3 showed a partial colocalization with LMP2. To test whether PHD3 would colocalize with proteasomal components also outside the aggresome-like structures 40 µM DMOG was used to partially disperse PHD3 protein from the aggregates. No colocalization outside the aggregates was detected.

During aggresome formation degradation-destined proteins are transported along microtubules to the perinuclear region, an event which can be inhibited by inhibiting microtubule function. Using 5 µM nocodazole, a known inhibitor of microtubule polymerization, the aggregation of PHD3 was completely inhibited. Supporting the notion that proteasomal function is needed for PHD3 body localization, proteasome inhibitor MG132 caused significant clustering of PHD3 along the microtubuli.

The dynamics of PHD3 bodies was studied using FRAP, a method measuring the recovery of fluorescent signal after photobleaching the object fluorochrome. FRAP of both cytoplasmic and nuclear PHD3 aggregates were investigated. Aggregates that resided in the cytoplasm showed overall more rapid recovery rate which indicated a fast movement of PHD3 into these structures. Cells were imaged live showing cytoplasmic movement and convergence of PHD3 aggregates. In contrast, juxtanuclear aggregates showed slower recovery and in some instances were highly stable. Supporting this, the mobile fraction within perinuclear aggregates varied between 8% – 30% whereas in the cytosolic aggregates the mobile fraction covered 23% – 96% of the protein. By using nocodazole post-aggregation a clear reduction in PHD3 movement was observed, thus

supporting the finding that PHD3 bodies are highly dynamic structures with features of aggresome-like structures.

5.3 PHD3 bodies associated with apoptosis in normoxic cancer cells (I)

Proteins inducing proteasomal aggregation have been characterized to induce caspase 3 –dependent apoptosis and aggresomes have the ability to block the function of proteasomes (Bennett et al. 2005; Kristiansen et al. 2005). Normoxic PHD3 has been shown to induce caspase-dependent neuronal apoptosis after NGF withdrawal (Lipscomb et al. 2001; Straub et al. 2003a). HeLa cells were used to study whether PHD3 induced apoptosis in these settings when force expressed. Judged by nuclear fragmentation and caspase-3 activation apoptotic cell death was observed in normoxia and reoxygenation. This apoptosis-inducing function could be linked to PHD3 hydroxylase activity since the use of both DMOG and mutationally inactivated PHD3 led to loss of apoptotic activity.

As noted before, the emergence of PHD3 bodies and PHD3-induced apoptosis were dependent on oxygen availability as well as on PHD3 hydroxylase activity. To clarify the connection between PHD3 aggregation and PHD3-induced apoptosis flow cytometric evaluation of apoptosis occurence was performed. Maximal apoptosis levels were detected 48 hours after transfection with PHD3-egfp. Similarly, based on nuclear fragmentation analysis, PHD3-EGFP–expressing HeLa cells showed maximal apoptosis 48 hours after transfection. This holds true only in respect to PHD3-aggregating cells since cells with dispersed PHD3 showed no signs of apoptosis.

5.4 SQSTM1/p62 –expression is dependent on oxygen availability (II)

p62 is an adaptor protein involved in basal cellular pathways including signal transduction, transformation and protein degradation. The response of p62 protein levels to hypoxia was studied in order to clarify its possible fate in hypoxic tumour microenvironment. Several cell lines (HeLa, HaCat, HEK293, U2OS and SCC9) were subjected to either normoxic or hypoxic conditions and p62 expression levels were quantified with western blotting. A marked reduction in the protein levels was observed already after 24 hours of hypoxic treatment and the reduction was even more prominent after 48 hours of hypoxia. The downregulation of p62 protein expression was reversed with reoxygenation within 6 hours.

The tendency of p62 to form distinctive cytoplasmic bodies is well characterized (Bjørkøy et al. 2005; Wooten et al. 2006). The effect of hypoxic treatment followed by reoxygenation on these aggregates was studied. Punctate staining seen in normoxic conditions was seen in hypoxia as well albeit in greatly lesser extent. The overall staining intensity of p62 was also highly decreased in hypoxia. Reoxygenation was able to restore the punctate, high expression of p62.

5.5 Oxygen depency of p62 is VHL- and HIF –independent and does not mimick PHD inhibition (II)

A large part of hypoxia responses are mediated by HIF-1. For further elucidation on the role of HIF over p62 oxygen depency carcinoma cells were transfected with HIF-1α and HIF-2α siRNAs. Downregulation of HIF isoforms 1 and 2 had no effect on hypoxic downregulation response of p62.

pVHL is needed for normal regulation of HIF. A renal carcinoma cell line (RCC4) with dysfunctional pVHL and constitutive HIF-1α expression was used to elucidate whether pVHL plays a role in p62 regulation. Along with the lines of HIF-1α siRNA having no effect on p62 expression, wild type RCC4 as well as pVHL–rescued RCC4 cell line (RCC+VHL) efficiently downregulated p62 expression in response to hypoxia. This implied that not only is the hypoxic downregulation of p62 HIF-1α–autonomous it is also pVHL–independent. To see whether downregulation of p62 in normoxia can be achieved by inhibiting PHD activity pan-PHD–inhibitors (DMOG and DFO) as well as siRNAs against individual PHD enzymes were used. As compared to hypoxic downregulation of p62, the inhibition of hydroxylase activity, despite of HIF-1α stabilization, could not alter normoxic p62 levels.

By using real-time PCR p62 mRNA-level responses to hypoxia were studied. No relevant changes in the levels of p62 mRNA were detected in hypoxia while the protein levels were greatly reduced. This indicated that the hypoxia response of p62 protein is not a consequence of mRNA-level regulation.

5.6 Hypoxia enhances autophagy-mediated clearance of p62 (II)

Two parallel pathways to clearing unneeded or damaged proteins exist in cells: the ubiquitin-proteasome pathway (UPS) and the lysosome/autophagy pathway. As p62 protein levels were significantly reduced in response to hypoxia and no effect on mRNA level was seen, the effects of hypoxia on p62 protein translation and protein stability were investigated. Blocking translation with a known protein translation inhibitor cycloheximide had no marked effect on normoxic p62 protein levels. However, in hypoxia, cycloheximide treatment accelerated the disappearance of p62 protein which indicates that hypoxia quickens the posttranslational clearance of p62. Proteasomal inhibitor MG132 was administered to cells after 16 hours hypoxic treatment to see if p62 clearance was mediated by UPS. Even though UPS inhibition stabilized HIF-1α in normoxia as expected, it did not accumulate normoxic p62 nor did it prevent the proteins disappearance in response to hypoxia. This shows p62 is not degraded by UPS in hypoxia.

Autophagy has been shown to degrade p62 (Bjørkøy et al. 2005; Pankiv et al. 2007). Following hypoxic treatment cells were treated with autophagy inhibitors to see whether hypoxic disappearance of p62 would be due to autophagic pathway. No effect on normoxic p62 expression was seen. In hypoxia, however, the degradation of p62 was markedly attenuated. Accordingly, using siRNA against autophagosome membrane protein LC3

the hypoxic downregulation of p62 was inhibited. This showed that autophagy-mediated p62 expression was greatly enhanced in hypoxia.

5.7 p62 has a role in hypoxia-induced ERK phosphorylation (II)

p62 null mice have decreased oxygen consumption and increased Ras/ERK –signaling (Rodriguez et al. 2006). Knowing that hypoxia elevates Ras/ERK signalling (Kwon et al. 2006; Minet et al. 2000) the role of p62 in controlling the Ras/ERK signalling was studied. Analysis of phosphorylated ERK in HeLa cells exposed to hypoxia showed that p62 expression decreased in response to hypoxia whereas the levels of phosphorylated ERK1/2 increased leaving the total levels of ERK remained unchanged. In turn, total p62 depletion in hypoxia with siRNA treatment resulted in enhanced ERK1/2 phosphorylation implying that p62 depletion in hypoxia may allow the increased hypoxic phosphorylation of ERK1/2.

5.8 PHD3 is upregulated in HNSCC tumours (III)

Many cancer cell types have been shown to express PHD3 in response to hypoxia such as human osteosarcoma cell line U2OS (Marxsen et al. 2004) and breat cancer cell line MCF7 (Appelhoff et al. 2004). To study the PHD3 expression in response to hypoxia in human head and neck squamous cell carcinoma (HNSCC) were used. Primary cell lines established from HNSCC patients were obtained and grown in normoxia or hypoxia. In three out of five cell lines PHD3 protein levels were upregulated in response to hypoxia and an induction in PHD3 mRNA was seen in all five cell lines. In order to further elucidate the PHD3 mRNA expression we investigated tumour samples from HNSCC patients. In comparison to anatomically matching healthy controls PHD3 expression was five-fold in the tumour samples. This PHD3 expression associated with hypoxia as judged by a strong correlation between PHD3 and Glut-1, a known hypoxia marker showing that PHD3 transcription is hypoxia-activated in HNSCC.

5.9 PHD3 is needed for HNSCC growth in hypoxia independent of its hydroxylase activity (III)

Strong PHD3 upregulation in HNSCC in response to hypoxia indicates it serves a growth-promoting purpose for these cells. To verify this we studied UT-SCC –cells. Cells were grown in hypoxia and studied for survival under PHD3 depletion using siRNA against PHD3. A marked reduction in the cell number was observed when hypoxic cells were depleted of PHD3 whilst in normoxia no obvious effects with siPHD3 were seen.

As reduction in cell number can be a consequence of either reduced proliferation or increased cell death we looked into the possible effect of PHD3 depletion on cell growth. Indeed, UT-SCC transfected with siPHD3growing in hypoxia showed clear reduction in proliferation. Following this we adressed whether the enzymatic activity of PHD3 is needed for its hypoxic proliferation promoting function. In a rescue experiment we

used a hydroxylase-inactive mutant in comparison to wild type PHD3 to rescue the cells from PHD3 depletion. The proliferation rate of these cells in hypoxia was followed for 48 hours. Both constructs showed similar enhancement of proliferation after PHD3 depletion indicating that PHD3 hydroxylase activity is not a requirement for hypoxic cell survival.

5.10 Hypoxia-induced G1-S block is enhaced by the inhibition of PHD3 (III)

Decreased proliferation rate of PHD3-depleted, hypoxic UT-SCC cells implicated possible deficiencies in the progression of cell cycle. Hypoxia is a known downregulator of cell cycle progression. Interestingly, depletion of PHD3 form hypoxic UT-SCC cells lead to even more pronounced inhibition of G1 to S phase transition.

5.11 Hyperphosphorylation of retinoblastoma protein is reduced in PHD3 depleted hypoxic cells (III)

In order for the cell cycle to proceed retinoblastoma protein (Rb) needs to be hyperphoshorylated (pRb). Motivated by the finding that PHD3 depletion in hypoxia leads to a block in the cell cycle at G1 to S transition we sought to see if the phosphorylation status of Rb would be in any way affected by this depletion. In UT-SCC cells grown under hypoxia we detected that the amount of pRb was clearly diminished in response to PHD3 reduction. As hyperphosphorylation of Rb during cell cycle progression is dependent on a complex formed by cyclin D1 and cyclin dependent kinases 4 and 6, we looked into the levels of cyclin D1 in response to siPHD3 treatment in hypoxia. As expected, a clear decrease in cyclin D1 level was detected with this treatment thereby clarifying the route by which PHD3 potentiates cell cycle progression in hypoxia. Supporting this, the levels of p27, a known cell cycle arrest –promoting kinase inhibitor, was strikingly increased in response to PHD3 depletion in hypoxia.

5.12 PHD3 is expressed in aggresome-like structures in normoxic carcinoma cells where it colocalizes and interacts with p62 (IV)

In our previous studies we showed that PHD3, when forced expressed in normoxia, has a tendency to form aggresome-like structures (I). Therefore we sought out to clarify the biological role of these PHD3 bodies. We used cancer samples from head and neck region as well as those from breast cancer, both of which express PHD3 in rather high levels (II). The samples were then studied for PHD3's expression pattern with immunocytochemistry. In these samples we detected large cytoplasmic aggregetes positive for PHD3. This implicated that PHD3 bodies may bear biological significance in cancer. In comparison, analysis of non-cancerous tissue that has a high PHD3 expression, like placenta, was studied. In non-cancerous tissues we found that not only were the PHD3 aggregates fewer in number but they also we smaller than in cancerous tissue samples.

As similarities between PHD3 bodies and aggresome-like structures were clear we studied the potential colocalization of PHD3 and p62 within these structures. In comparison to PHD2 that showed absolutely no colocalization with p62, PHD3 resided in the same aggregates in practically all cells. This localization was apparent with both exo- and endogenous proteins.

Following the finding that PHD3 and p62 reside in the same structures, the actual physical interaction between the two proteins was looked into. Co-immunoprecipitation studies with Flag-antibody as bait showed specific interaction between p62 and PHD3 in vivo.

To verify the interaction and possibly specify the sites of interaction proximity ligation –based method (PLA) was used. PLA is a highly sensitive method that possesses the ability to detect even the weakest interactions and by amplifying the signal obtained from two proteins residing in close proximity to one another make the signal visible to microscopic analysis. With this method we were able to show in situ interaction between PHD3 and p62.

5.13 Aggregation of PHD3 is induced by p62 (IV)

As shown by our previous work (I) PHD3 forms aggregates in a majority of carcinoma cells when overexpressed. p62 is a known aggregation-inducing protein and a signal adaptor. To see whether p62 is a required for PHD3 aggregation, we studied how the depletion of p62 with siRNA would influence PHD3 aggregate formation. To this end we transfected HeLa cells with PHD3-EGFP followed by siRNA-transfection either with scrambled siRNA or siRNA specifically targeting p62 mRNA. The depletion of p62 markedly attenuated PHD3 aggregate formation and allowed PHD3 to be diffusively expressed throughout the cell showing that p62 is indeed needed for PHD3 protein aggregation. Supporting this, cotransfection experiments with p62 plasmids lacking either UBA-domain (needed for ubiquitin binding) or PB1-domain (needed for p62 self-oligomerization) and egfp-PHD3 showed that only wild type p62 with aggregation capacity is able to drive aggregation of PHD3.

In previous studies (I) we have shown that PHD3 aggregation is dependent on oxygenation status as in hypoxia PHD3 aggregation is lost. We have also shown that p62 protein is downregulated rapidly in response to hypoxia (II). Motivated by this we asked whether forced expression of p62 in hypoxia would be sufficient to drive PHD3 back into aggregates. Therefore we cotransfected carcinoma cells with both PHD3 and p62 and subsequenetly grew them in hypoxia for 24 hours. After transfection p62 expression level comparable to normoxia was reached after 24 hours of hypoxia treatment and aggregation of p62 was similar to that seen in normoxia. Interestingly, p62 expression in hypoxia was able to restore the PHD3 aggregation. This indicates that p62's ability to direct PHD3 expression is independent of oxygenation status.

5.14 PHD3 protein levels are regulated by p62 (IV)

PHD3 levels are usually very low in normoxic cancer cells. p62 in contrast is expressed abundantly across wide range of cancer cells. In keeping with the finding that p62 regulates the localization of PHD3 expression we asked whether p62 also directly influences the protein levels of PHD3 in normoxia and thereby accounts for the low normoxic PHD3 levels. To study this we depleted p62 from normoxic HeLa cells and analyzed both mRNA- and protein levels for PHD3. On mRNA level we saw no differences in PHD3 between control and p62-depleted cells. However, on protein level p62 depletion led to marked, consistent and strong elevation of PHD3 levels. Supporting this hypoxic force expression of p62 led to strong downregulation of PHD3 protein levels and p62 depletion resulted in slowed reduction of PHD3 protein levels in response to reoxygenation. In conclusion the data indicates that p62 is a potent regulator of PHD3 expression.

5.15 The biological outcome of PHD3 regulation by p62 is the restriction in HIF-1α and PHD3 interaction (IV)

The main function of PHD3 is considered to be the hydroxylation of HIF-1α in prolonged hypoxia although the direct *in vivo* interaction has proven to be quite challenging to show. To clarify the biological significance of the regulation of PHD3 by p62 we used PLA to study if p62 expression restricts PHD3 from functioning on its targets, ie HIF-1α. In normoxia PHD3 levels are low, and we detected fairly low amounts of interactions between PHD3 and HIF-1α. However, when p62 was depleted from these cells, the amount of interactions was increased by 3-fold. Along wthe lines of this, hypoxic overexpression of p62 reduced the interactions between PHD3 and HIF-1 significantly. Moreover, the location site of the interaction, which under hypoxia was mainly nuclear, greatly shifted to be mainly cytoplasmic. This led us to conclude that p62 governs the expression of PHD3 in order to inhibit an untimely hydroxylation of PHD3's targets.

6. DISCUSSION

In this thesis the role of PHD3 as a regulator of human carcinoma cells was investigated and its function both in normoxia and in hypoxia was studied. PHD3 elevation was shown to have an opposite effect depending whether the upregulation occurs in normoxia or in hypoxia indicating a more versatile function for PHD3 than previously described

6.1 Cell cycle regulation by PHD3 under hypoxia

Cell cycle progression is slowed down in response to hypoxia in cancer cells as all available energy needs to be directed to basic survival functions. PHD3 levels are elevated in hypoxia in many cell types. During this thesis work I studied the biological function of this upregulation. It was noticed that if carcinoma cells grown in 1% O_2 were depleted of PHD3 the number of cells was greatly decreased in comparison to PHD3-expressing cells. Two possible explanations for the phenomenon were considered; either the cells that are devoid of PHD3 go into apoptosis or that these cells grow slower. The results showed that the rate of apoptosis was not affected but instead a significant decrease in the cell cycle pace was observed. The G1 -phase population of cells showed clear increase and concomitantly S phase population was decreased. This indicated a cell cycle block at G1 to S transition that was demonstrated by several methods. Thereby it was concluded that PHD3 expression is needed to drive cell cycle under hypoxia. As an increase in the levels of p27 was observed following PHD3 depletion in hypoxia the cell cycle inhibitors are feasible targets for PHD3 and their regulation might potentially explain the inhibition of the cell cycle by PHD3 depletion. However, more work needs to be done in order to reveal the mechanism as to how PHD3 influences the cell cycle progression.

All three PHD isoforms have been shown to be able to hydroxylate HIF *in vitro*. Initially it has been thought that HIF-1α is the sole target for all the PHDs. However, it has become clear that they serve also other purposes albeit the understanding of these other functions is less clear. The numerous cell cycle regulators and their inhibitors provide an interesting group of novel PHD3 target molecules that may operate outside the HIF pathway.

6.2 PHD3 aggregation

Much of the research on PHDs has been focusing on the conditions of low oxygen *i.e* hypoxia which is a common feature of solid tumours and therefore holds its ground to be an area of intense investigation. It is becoming evident that many normal cellular functions in normoxia and in non-HIF pathways are dependent on PHDs. Several studies have shown that PHD3 protein is expressed in cancer cells both in the nucleus as well as in the cytoplasm (Metzen et al. 2003b; Willam et al. 2006) whereas PHD1 and PHD2 are more selectively expressed either in the nucleus or in the cytoplasm, respectively. The enzymatic activity of these hydroxylases is greatly diminished in hypoxia yet PHD2 and

PHD3 are balancing their lessened activity in hypoxia by upregulating their expression. Moreover, in many tumours PHD3 levels are elevated even though they are seemingly within normoxic range (Boddy et al. 2005; Jokilehto et al. 2006).

PHD3 levels in normoxia are kept low by proteasomal degradation thus allowing PHD2 to be the primary regulator of HIF-1 in normoxia. In normoxia PHD3 is targeted to proteasomal degradation by Siah ubiquitin ligases (Nakayama et al. 2004). To investigate the function of PHD3 in normoxic carcinoma cells forced expression studies were used. HeLa cells were transiently transfected with either PHD3 or enzymatically inactive mutant PHD3. We found that not only is PHD3 expressed diffusely throughout the cell it is also expressed in juxtanuclear aggregates or aggresome-like structures that contain components of the proteasomal system. The aggregation of PHD3 occurred only in normoxia which implicated that the aggregation was dependent on oxygen. Furthermore, the aggregation was dependent on the enzymatic activity of PHD3 as the enzymatically inactive PHD3 mutant did not form aggresome-like structures. This indicated that mere enhanced production is not the reason for aggregation as hypoxia itself greatly increases PHD3 protein levels but the protein needs to accumulate above certain threshold as an active enzyme in order to form these structures. In verification that the aggregation was not merely a consequence of overexpression endogenous PHD3 expression was studied in these carcinoma cell lines and was seen in aggresome-like structures albeit they were smaller than those seen with forced expression This may also implicate that PHD3 aggregation is dependent on the presence of another protein that may be absent in hypoxic conditions.

It could be speculated that when cancer cells are faced with a situation where an untimely elevation in the PHD3 protein levels occur, the activity of the enzyme is controlled not only via degradation but also by harnessing the expressed protein into aggregates so that it cannot target its substrates. Were this assumption true the aggresome-like structures would be cytoprotective in nature. However, the formation of the aggregates coincided with apoptosis in a subset of carcinoma cells. It is known that aggresome-like structures can be cell survival enhancing structures in some cases and cell death promoting structures in others. In the subset of carcinoma cells that were prone to apoptosis with PHD3 aggregation was driven into aggregates, also the proteasomal components localized to the same sructures. However, in another carcinoma cell strain where PHD3 did not induce aggresome-linked apoptosis, also the proteasomal components failed to localize into them. This would suggest that the tendency of PHD3 to promote apoptosis may in fact be regulated by other factors such as those involved in proteasome assembly and that the proapoptotic function of PHD3 would need other components. Also, an open question is how does an aggregation of one protein species within a cell influence the function of adjacent proteins.

Aggregation of PHD3 in normoxia heavily relies on its hydroxylase activity as with point mutated construct no speckle-like expression of PHD3 was detected. It could be proposed that the aggregation of PHD3 is a consequence or byproduct of hydroxylation of some PHD3 target. Of the known PHD3 non-HIF targets ATF-4 could be the suitable candidate as ATF-4 hydroxylation and stability has been shown to be regulated by PHD3 (Köditz et al. 2007). ATF-4 is an integral part of the unfolded protein response

and thus would be a *bona fide* partner of PHD3 aggregation. However, ATF-4 is a nuclear protein and PHD3 aggresome-like structures are mainly seen in the cytoplasm so at least direct interdependence between PHD3 aggregation and ATF-4 hydroxylation seems unlikely.

Another interesting possibility is the chaperonin TriC, the substrate of which PHD3 is (Masson et al. 2004). TRiC can alter the aggregation state of huntingtin (htt) polyglutamine (polyQ) –proteins in mammalian cells and its inhibition leads to enhanced aggregate formation and toxicity (Kitamura et al. 2006). It may be that disturbancies in the function of TRiC account also for the aggregation of PHD3.

By definition, proteins aggregate when they either accumulate in excess amounts or when they are misfolded. Protein aggregation is also known to be an integral part of cell survival under stressed conditions and thus is under strict control. Only few proteins are known to drive proteasomal components into aggresome-like structures. p62 is a known inducer of aggregation. Investigation of PHD3 bodies showed great resemblance to those formed by p62 and colocalization and immunoprecipitation studies confirmed their existence in same structures. These findings suggested that PHD3 might bear potential to drive proteins into aggresome-like structures. Also it indicated that PHD3's activity may be regulated by associating and dissociating it to and from some form of complexes. Later this was shown to be true by studies showing that PHD3 forms complexes with itself as well as with other hydroxylases (Nakayama et al. 2007a).

6.3 p62 regulation by oxygen availability

p62 is a highly versatile signal adaptor protein and its can easily be spotted in cytosolic aggregates within cancer cells. These speckles can be signal organizing centers formed by PB1-domain driven p62 oligomers and p62-aPKC complexes as well as polyubiquitinated proteins (Moscat et al. 2009). In these speckles p62 has been shown to interact with caspase 8 and TRAF6.

Several studies have shown p62 to be widely expressed across various cancer types and it is a key component of cellular transformation. For example, p62 has been shown to regulate crusial events like autophagy, nutrient sensing and oxidative detoxification of proteins. p62 has been shown to inhibit ERK activation and therefore may function as cellular oxygen consumption regulator (Rodriguez et al. 2006). Our studies show that p62 is highly regulated by oxygen availability in a wide range of cancer cell types as its protein levels are rapidly decreased when cells are moved to hypoxia (II). Upon reoxygenation the levels are accordingly restored. No effects were seen on mRNA level suggesting the regulation to be post-translational. We showed that p62 is degraded in hypoxia via autophagy. This finding is supported by the literature as hypoxia is a known inducer of autophagy. Importantly, HIF did not seem to induce downregulation of p62 protein like that seen in response to hypoxia but when cells were exposed to prolonged DMOG treatment a clear downregulation in p62 protein levels was seen. It is conceivable that p62 dowregulation in hypoxia occurs via two separate pathways allowing more precise control over p62 protein expression. In the acute phase of hypoxia

autophagic degradation is HIF-independent which may be due to the fact that the need to downregulate p62 is more urgent than the need for HIF-mediated transcriptional responses.

Some investigations have suggested p62 to be upregulated under adverse tumor conditions such as nutrient deficiency. Therefore at first sight our results on the downregulation of p62 by hypoxia may seem contradictonary. It remains to be investigated how the expression of p62 differs in diverse cancers spatialy and temporarly and whether hypoxia and other tumor microenvironment conditions have an opposite effect on p62 expression.

6.4 The connection between PHD3 and p62

Both p62 and PHD3 show speckle-like expression pattern in various cancer cell lines as well as in clinical samples. As bodies formed by PHD3 resembled those formed by p62 we sought to see whether they were co-expressed in these speckles. Indeed a clear colocalization between the two proteins was seen in normoxic carcinoma cells. Moreover, p62 was shown to interact with PHD3 and this interaction was dependent on the presence of the PB1-domain of p62.

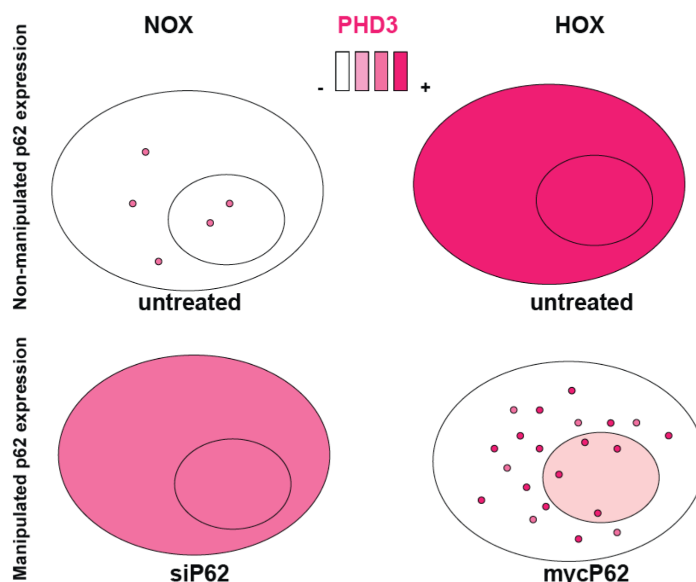

Figure 10. Effect of p62 protein amount on PHD3 expression level and location (marked with pink colour). In untreated normoxic cells (upper left corner) PHD3 is expressed in small amount (depicted as pink dots). In hypoxia PHD3 expression increases and is seen both in cytoplasm as well as in the nucleus (upper right corner). In normoxia, where p62 is in many cancers expressed abundantly, depletion of p62 with siRNA leads to enhanced PHD3 expression as compared to the normal normoxic situation (lower left corner). Moreover, in normoxic p62-depleted situation, PHD3 gets expressed evenly throughout the cytoplasm and nucleus. In turn in hypoxia where p62 levels rapidly fall, p62 forced expression drives PHD3 back into aggregates (lower right corner).

In further support of p62's regulatory role over PHD3 the expression pattern of p62 was shown to negatively parallel with the expression of PHD3 indicating that PHD3 upregulation depends on the downregulation of p62. Under hypoxia force expression of p62 was able to drive PHD3 back into aggregates suggesting that p62 expression is sufficient to control the localization of PHD3 (Figure 10). This finding gives rise to the idea that simultaneous presence of p62 and PHD3 as a diffuse protein is an unwanted situation in a cell. It was then showed that the localization of PHD3 into aggresome-like structures requires p62 function. This gives a plausible explanation as to why p62 needs to be downregulated in hypoxia; as PHD3 is considered to be the primary regulator of HIF in prolonged hypoxia PHD3 levels need to be elevated (Figure 10). In normoxic situation PHD2 has been shown to target HIF for degradation so in normoxia the highly abundant p62 harnesses PHD3 expression into cytosolic aggregates (Figure 11). In keeping with this, p62 turned out to be a major regulator of PHD3 protein levels in normoxia as p62 depletion from normoxic cancer cells greatly increased PHD3 expression. In corroboration with the previous results that had shown PHD3 to bear apoptotic potential when overexpressed in normoxia, the p62–depleted normoxic carcinoma cells faced extensive apoptosis within 48 hours after depletion. This would support the theory that p62 functions as a regulator of PHD3 proapoptotic activity.

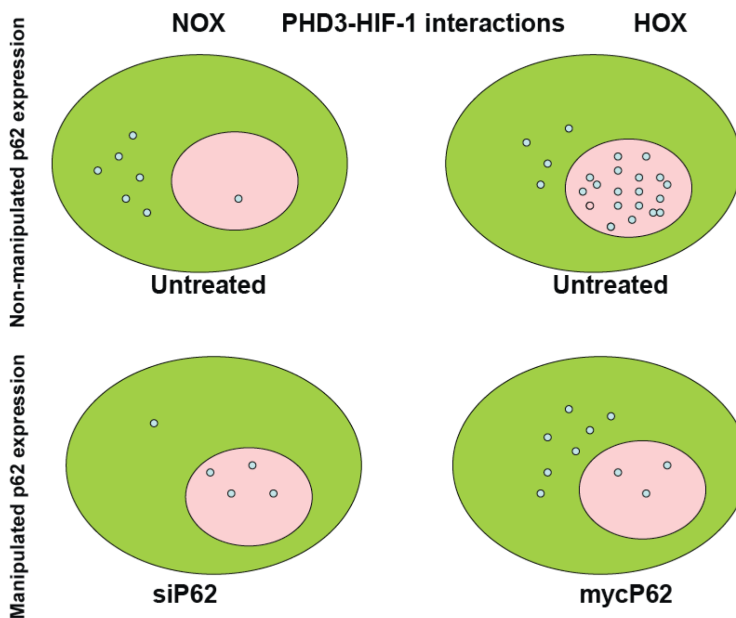

Figure 11. PHD3-HIF–interaction site fluctuation (PHD3-HIF-1 interactions are marked with small dots) in response to p62 expression. Interactions were detected with proximity ligation assay. In untreated normoxic cells, where PHD3 expression is low, some interactions could be detected (upper left corner) In hypoxia most of the interactions were detected from nucleus, as expected (upper right corner). In normoxia p62 depletion led to a decrease in the overall interaction amount, but noticeably relatively more interactions were detected in the nucleus. In hypoxia p62 overexpression led to a decrease in the nuclear interactions

My work establishes a connection between PHD3 and p62 and as such connects the oxygen sensing pathway and the multitude of cellular functions relayed by p62. As the interaction between the two brings together such crucial pathways, previously established interaction partners for both PHD3 and p62 may gain better understanding.

p62 has traditionally been viewed as a cytosolic protein, but as it contains two nuclear localization signals and one nuclear exit signal, it does posses the ability to shuttle back and forth. In normoxic conditions p62 is abundantly expressed and in many cancers its expression is upregulated and it has been shown that the protein level of p62 positively correlates with aggressive progression of prostate and breast tumours (Kitamura et al. 2006). Correspondingly, PHD3 levels are kept low in normoxia but when moved to hypoxia, p62 is rapidly degraded, possibly for enabling PHD3 upregulation. The apoptosis inducing capacity of some PHD3 aggregates may well connect to the presence of p62 in these aggregates.

mTOR is a serine/threonine protein kinase which centrally influences cell growth, metabolism and survival. mTOR activity has been reported to be inhibited under hypoxia in a HIF-1α-dependent mechanism (Brugarolas and Kaelin 2004; Liu et al. 2006). p62 in turn is an intergral part of the mTORC1 as p62 is required for mTORC1 translocation to the lysosome (Duran et al. 2011). PHD3 interaction partner ATF-4 upregulates autophagy genes following proteasomal inhibition (Korolchuk et al. 2009a). ATF-4 has also been shown to regulate REDD1 expression, a negative regulator of mTOR. REDD1 is essential for mTOR complex 1 regulation under hypoxia (Vadysirisack and Ellisen 2012). mTOR hyperactivity in a variety of human cancers has been reported in several studies (Guertin and Sabatini 2007; Karbowniczek et al. 2008; Molinolo et al. 2007; Robb et al. 2007). TSC, composed of TSC1 and TSC2 is a critical negative regulator of mTOR (Huang et al. 2008) and suppressed by IKKβ (Lee et al. 2007).

I propose that in normoxic situations one of the biological outcomes of the PHD3-p62 interaction is that PHD3 protein levels low are thereby kept low. Thereby IKKβ would remain functional and able to suppres TSC1 (Lee et al. 2007) thus allowing mTOR to remain hyperactive (Figure 12). mTOR hyperactivity is common in the multistep process of oncogenesis and thus observed in many cancers, and it has been suggested to contribute to the development of the Warburg effect. Interestingly, mTOR induces PKM2 under normoxia (Sun et al. 2011) and PKM2 was in a recent paper demonstrated to be an interaction partner of PHD3 (Luo et al. 2011a). In line, in hypoxia the decline in p62 levels confers to the decreased mTOR signalling. The dualistics role of PHD3 is emphasized in hypoxia where its upregulation is needed in order to restrict prolonged HIF-1 activation. These findings provide another level of regulation as to how cancer cells adjust and respond to oxygen-deprived environment.

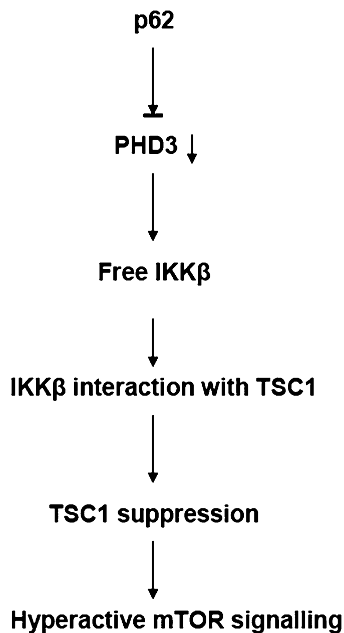

Figure 12. PHD3-p62 balance influences basic mechanisms in normoxic cancer cell growth. Under normoxia p62 keeps PHD3 expression low. As PHD3 has been reported to physically block IKKβ activation, low PHD3 amounts enable IKKβ to be active. IKKβ has been shown to interact with tuberous sclerosis complex 1 (TSC1), which in turn suppresses TSC1 and allows hyperactive mTOR signalling.

It still remains open as to how PHD3 induces apoptosis in certain normoxic settings. One possible explatation may come from the fact that PHD3 blocks the interaction between IKKβ and Hsp90 and thus prevents IKKβ phosphorylation (Xue et al. 2010). IKKβ phosphorylation is a prerequisite for NF-κB activation. Active NF-κB in turn is needed for Ras-transformed cancer cell survival (Duran et al. 2008). Therefore it may be that the apoptosis-inducing function of PHD3 links to the inhibition of IKKβ and NF-κB (Figure 12). This is supported by the link between p62 and NF-kB: p62 is needed for the activity of NF-κB as in Ras-transformed cells p62 depletion causes cell death (Duran et al. 2008). In short the proposal here is that p62 interacts with PHD3 in normoxia to keep its levels low and enabling NF-κB activity.

6.5 Implications of PHD3 in neurodegenerative disorders

PHD3 is expressed in aggresome-like structures and to great extent colocalizes and negatively correlates with the expression of p62. PHD3 is known to induce apoptosis in stressed neuronal cells. p62 can shuttle between cytoplasm and nucleus at high rate, and in the nucleus it can be found in PML bodies (Pankiv et al. 2010). It has been shown that cytosolic aggregation of p62 also serves to regulate its transportation between compartments (Pankiv et al. 2010). p62 connects to a wide variety of neuronal disorders such as Parkinson's and Alzheimer's disease (PD, AD, respectively) as well as ALS where p62 is often associated with intracellular inclusions. PD and AD are characterized by hypoxia, and hypoxia is believed to worsen the disease progression (Ogunshola

and Antoniou 2009). Our unpublished preliminary data shows that PHD3-containing aggregates are present in the brain tissue sections from patients with frontotemporal dementia (FTD) and ALS. It would be of interest to further study the potential function of PHD3-p62 aggregates in neurodegenerative diseases.

6.6 PHD3 in cancer

The role of PHD3 in cancer still remains a mystery. Its levels are kept very low in normoxia but rapidly upregulated in response to hypoxia to be again downregulated upon reoxygenation. This pattern suggests that PHD3 would be only of use in hypoxic conditions. It is known to be expressed in many cancers but thus far it has been studied more extensively only in neuronal settings. One link to cancer is the report showing that the loss of PHD3 is associated with the development of pheochromocytomas as the loss of PHD3 leads to failure to execute apoptosis (Lee et al. 2005).

The results shown in this thesis suggest that PHD3 needs to be carefully regulated so that its unnecessary expression in normoxia could be avoided. Our unpublished data supports this notion: In normoxia PHD3 seems to be safely stored within cells as a ready translated protein. This has been suggested by experiments where normoxic PHD3 has been depleted with siRNA with no effect on the protein levels of PHD3 allthough at the same time mRNA levels are reduced. This suggests that siRNA is targeting the newly transcribed PHD3 mRNA but there is a pool of PHD3 protein ready to be used when needed and stored in the cells possibly in these aggregates. This stored protein could then be rapidly released when needed. We have preliminary data indicating PHD3 needs to upregulated in response to other situations besides hypoxia. In our experiments PHD3 upregulation can be seen in normoxic cells grown to near confluence. This upregulation can not entirely be the result of local hypoxia created by dense cell culture as HIF– levels do not preceed but follow the PHD3 upregulation. This normoxic storing and release upon stress like confluence could be seen as comparative to that happening in response to hypoxia, but in hypoxia the PHD3 protein levels are dramatically much more elevated that it needs new protein synthesis as well, and the mere release from a storage aggregates would not be sufficient.

7. SUMMARY

In normoxia PHD3 levels are kept low and the protein that exists in the cells is stored into aggresome-like structures. This aggregation heavily relies on the expression of the adaptor protein p62. The storing of PHD3 is necessary for cell survival as we and others have shown that PHD3's untimely and uncalled expression is detrimental for the cells. To support this, the thesis shows that depleting p62 in normoxia leads to elevated PHD3 protein levels. Normoxic release from these aggregates would allow PHD3 to target its hydroxylation substrates such as HIF-1α and possibly others that are yet unidentified. To support this it is shown that the amount of interactions between PHD3 and HIF-1α as well as the sites of these interactions are carefully fine tuned by the levels of p62 and thereby PHD3. In the well-balanced cellular system PHD3 needs to be restricted in its expression.

This work clarifies the roles of oxygen sensors in cancer cell biology, in particular the PHD3 isoform and emphasizes its bipartite role as an oxygen sensor and HIF-1 regulator but also a critical mediator in other signalling pathways that are regulated by p62.

Although much remains to be elucidated, this thesis work describes a novel interaction which potentially gives new directions to the search for therapeutically important convergence points in cancer cell growth.

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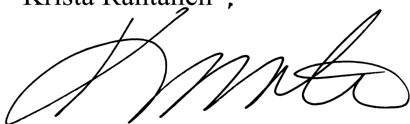

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