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## University of Turku

Faculty of Medicine
Institute of Biomedicine
Department of Medical Biochemistry and Genetics
Turku Doctoral Programme of Molecular Medicine
Turku Centre for Biotechnology

## Supervised by

Professor Panu Jaakkola, MD, PhD
Department of Oncology
Faculty of Medicine
University of Helsinki
Turku Centre for Biotechnology
Finland

## Reviewed by

Professor Thomas Kietzmann, MD, PhD
Faculty of Biochemistry and
Molecular Medicine
University of Oulu
Finland

Professor Cecilia Sahlgren, PhD
Faculty of Science and Engineering
Åbo Akademi University
Finland

## Opponent

Professor Johanna Myllyharju, PhD
Faculty of Biochemistry and Molecular Medicine
University of Oulu
Finland




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# ABSTRACT

**Petra Miikkulainen**

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University of Turku, Institute of Biomedicine, Department of Medical Biochemistry and Genetics, Turku Doctoral Programme of Molecular Medicine, Turku Centre for Biotechnology, Turku, Finland
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The family of HIF prolyl hydroxylase enzymes (PHDs) is known for their function of post-translationally regulating hypoxia-inducible transcription factors (HIFs) in oxygen-dependent manner. HIFs are the key transcription factors mediating the cellular responses to low oxygen concentration, i.e. hypoxia, by activating target genes that in turn drive the cellular adaptation. Among the PHDs, PHD3 shows particularly high expression in clear cell renal cell carcinoma (ccRCC) tumors and derived cell lines. However, the role of PHD3 expression in ccRCC has remained elusive.

In this thesis work, PHD3 is shown to have widespread functions in ccRCC. PHD3 regulates glucose metabolism and lactate production, as well as number of translational machinery components and activation of mTOR downstream signaling. PHD3 has previously been linked to cell cycle progression and my work extended the knowledge on PHD3 enhancing cell cycle progression, proliferation and 3D colony formation in ccRCC cells. PHD3 was also found to regulate cell migration and several proteins related to cytoskeleton and attachment. Moreover, the work revealed a novel mechanism for HIF-2α post-transcriptional regulation as PHD3 was found to maintain HIF-2α mRNA level in ccRCC cells. This new relation of PHD3 and HIF2A was further examined in clinical ccRCC tumor samples, in which a positive correlation of HIF2A and PHD3 expression was demonstrated. The results indicate a substantial role for PHD3 in promoting ccRCC growth and other processes linked to malignant progression.

KEYWORDS: HIF prolyl hydroxylase PHD3, kidney cancer, ccRCC, metabolism, cell cycle, hypoxia-inducible factor HIF-2α

# TIIVISTELMÄ

**Petra Miikkulainen**

**SOLUN HAPENTUNNISTAJA PHD3:N UUDET TOIMINNOT KIRKASSOLUISESSA MUNUAISSYÖVÄSSÄ**

Turun yliopisto, Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Molekyylilääketieteen tohtoriohjelma, Turun Biotekniikan keskus, Turku, Suomi

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Prolyylihydroxylaasientsyymit (PHD:t) toimivat solujen hapentunnistajina säädellen hypoksiasta indusoituvien transkriptiofaktorien (HIF:n) proteiinin pysyvyyttä hapesta riippuvaisesti. HIF:n tehtävä soluissa on välittää geeninluennantason mukautumista vähähappiseen ympäristöön, i.e. hypoksiaan, aktivoimalla kohdegeenejään. HIF:n kohdegeenit ovat usein solujen aineenvaihduntaan ja energiatasapainoon sekä muun muassa verisuonenmuodostukseen liittyviä geenejä, joiden proteiinituotteet toimivat solutason mukautumisessa hypoksiaan. PHD-entsyymiperheen jäsenistä PHD3:n on osoitettu olevan korkeasti ilmentynyt erityisesti kirkassoluisissa munuaissyövissä ja munuaissyöpäsolulinjoissa. Korkean PHD3 ilmentyvyyden merkitystä kirkassoluisessa munuaissyövässä ei kuitenkaan tunneta vielä tarkasti.

Väitöskirjatutkimuksessani osoitan PHD3:n osallistuvan useiden tärkeiden toimintojen säätelyyn munuaissyöpäsoluissa. Tulosteni perusteella PHD3 säätelee solujen sokerimetaboliaa ja laktaatin muodostumista, sekä osallistuu solujen proteiinintuotantokoneiston ylläpitoon ja mTOR-viestinnän aktivaatioon. Lisäksi tutkimukseni tulokset laajentavat tietämystä PHD3:n roolista solusyklin säätelyssä ja solukasvussa sekä osoittavat PHD3:n säätelevän solujen liikkumista. PHD3:n osoitettiin toimivan myös uudenlaisena HIF-2α:n mRNA:n pysyvyyttä säätelevänä tekijänä, ja vastoin aiempaa tietämystä PHD3:n osoitettiin toimivan HIF-2α:n ilmentymistä edistävänä tekijänä. Tätä uudenlaista säätelyä tuki myös kliinisestä munuaissyöpäaineistosta tehty löydös PHD3:n ja HIF-2α:n ilmentymistasojen korrelaatiosta. Tulokseni osoittavat että PHD3:lla on useita rooleja munuaissyöpäsoluissa, jotka liittyvät syöpäsolujen kasvuun ja pahanlaatuisuuteen.

AVAINSANAT: Prolyylihydroxylaasi PHD3, munuaissyöpä, kirkassoluinen munuaissyöpä, metabolia, solusykli, hypoksiasta indusoituva transkriptiotekijä HIF-2α

# TABLE OF CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| 2D, 3D | Two dimensional, three dimensional |
| 2-OG | 2-Oxoglutarate |
| ACC2 | Acetyl coenzyme A carboxylase β |
| Acetyl-CoA | Acetyl coenzyme A |
| ARNT | Aryl hydrocarbon receptor nuclear translocator protein |
| AFT-4 | Activating transcription factor 4 |
| ATP | Adenosine triphosphate |
| β(2)AR | Beta2-adrenergic receptor |
| bHLH | Basic helix-loop-helix |
| C/N-TAD | C-terminal/N-terminal transactivation domain |
| CAIX | Carbonic anhydrase IX |
| ccRCC | Clear cell renal cell carcinoma |
| CDK | Cyclin dependent kinase |
| CHX | Cycloheximide |
| CKI | Cyclin dependent kinase inhibitor |
| DMOG | Dimethyloxaloylglycine |
| E2F1 | E2 transcription factor 1 |
| ECM | Extra cellular matrix |
| EGFR | Epidermal growth factor receptor |
| EGLN | Egg-laying nine homolog |
| eIF4G | Eukaryotic translation initiation factor 4 G |
| ENO1 | Alpha enolase |
| FIH | Factor inhibiting HIF |
| G6PD | Glucose 6-phosphate dehydrogenase |
| GAPDH | Glyceraldehyde phosphate dehydrogenase |
| GFPT1 | Glutamine - fructose-6-phosphate transaminase 1 |
| GLUT1 | Glucose transporter 1 |
| GO | Gene ontology |
| HIF | Hypoxia inducible factor |
| HK1 | Hexokinase 1 |
| hKIS | Human kinase interacting stathmin |
| hnRNP | Heterogeneous nuclear ribonucleoproteins |
| HNSCC | Head and neck squamous cell carcinoma |
| HPH | HIF prolyl hydroxylase |
| HRE | Hypoxia-responsible element |
| IDH1/2 | Isocitrate dehydrogenase 1/2 |
| JNK2 | c-Jun N-terminal protein kinase 2 |
| KIF1Bβ | Kinesin family member 1B |

| | |
|---|---|
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LDHA/B | Lactate dehydrogenase isoform A/B |
| MCT1 | Monocarboxylate transporter 1 |
| MDH2 | Malate dehydrogenase |
| MMP | Matrix metalloproteinases |
| mRNA | Messenger RNA |
| mTOR | Mechanistic target of rapamycin |
| mTORC1/2 | Mechanistic target of rapamycin complex 1/2 |
| NADH | Nicotinamide adenine dinucleotide |
| NF-ϰB | Nuclear factor- kappaB |
| OCR | Oxygen consumption rate |
| ODDD | Oxygen dependent destruction domain |
| PARP-1 | Poly (ADP-ribose) polymerase-1 |
| PAS | PER-ARNT-SIM, period circadian protein - aryl hydrocarbon receptor nuclear translocator protein - single-minded protein |
| PDGF | Platelet derived growth factor |
| PDH | Pyruvate dehydrogenase |
| PDK1 | Pyruvate dehydrogenase kinase 1 |
| PFKP | Phosphofructokinase, platelet |
| PGAM | Phosphoglycerate mutase |
| PHD | Prolyl hydroxylase domain protein |
| PI3K | Phosphoinositide 3-kinase |
| PKM2 | Pyruvate kinase M2 |
| pVHL/*VHL* | von Hippel-Lindau protein, von Hippel-Lindau gene |
| Rb | Retinoblastoma protein |
| RBP | RNA binding protein |
| RCC | Renal cell carcinoma |
| ROS | Reactive oxygen species |
| RPL | Ribosomal protein large subunit |
| RRM | RNA recognition motif |
| RT-qPCR | Reverse transcription quantitative PCR |
| shRNA | Small hairpin RNA |
| siRNA | Small interfering RNA |
| Skp2 | S-phase kinase-associated protein 2 |
| TALDO | Transaldolase |
| TCA | Tricarboxylic acid |
| TGF-α | Transforming growth factor alpha |
| TOP mRNA | 5' terminal oligopyrimidine motif containing messenger RNA |
| TPI1 | Triosephosphate isomerase |
| tRNA | Transfer-RNA |
| VEGF | Vascular endothelial growth factor |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III). In addition, unpublished data is presented in this thesis. The original articles have been reproduced with the permission of the copyright holders.

I Miikkulainen P, Högel H, Rantanen K, Suomi T, Kouvonen P, Elo LL and Jaakkola PM. HIF prolyl hydroxylase PHD3 regulates translational machinery and glucose metabolism in clear cell renal cell carcinoma. *Cancer & Metabolism* 2017, 5:5.

II Högel H, Miikkulainen P, Bino L and Jaakkola PM. Hypoxia inducible prolyl hydroxylase PHD3 maintains carcinoma cell growth by decreasing the stability of p27. *Molecular Cancer* 2015, 14:143.

III Miikkulainen P, Högel H, Seyednasrollah F, Rantanen K, Elo LL and Jaakkola PM. HIF prolyl hydroxylase PHD3 maintains high HIF2A mRNA level in clear cell renal cell carcinoma. *Manuscript.*

# 1 INTRODUCTION

Cancer is a major public health issue and among the leading causes of death worldwide. Cancer affects millions of people – estimations of 14 million new cancer cases and 8 million cancer-related deaths every year worldwide (Cancer Research UK). The incidence of cancer is rising due to the increase in lifespan and changes in life style and nutrition. However, the cancer mortality has simultaneously decreased due to early diagnosis and better treatment options, such as targeted therapies. The most common types of cancer include lung, female breast, bowel and male prostate cancer, which account up to 40 % of all new cancer diagnoses each year. Kidney cancer is among the ten most common cancer types and it affects men twice as often as women (Cancer Research UK; Hsieh *et al.*, 2017).

Renal cell carcinoma (RCC) is the most common type of kidney cancer, constituting a heterogeneous group of tumors originating from kidney tubular epithelial cells. RCCs are often detected in advanced, metastatic state and thus RCCs are associated with high mortality (Hsieh *et al.*, 2017). Also, metastatic RCCs are often to some extent resistant to the used treatments such as radiation and chemotherapy. In addition, a high level of intratumoral heterogeneity leads to varied response to treatment (Bielecka *et al.*, 2014; Hsieh *et al.*, 2017). Thereby, understanding the basic biological mechanisms of RCC growth and progression could benefit the development of treatment options.

Clear cell renal cell carcinoma (ccRCC) is the major form of RCC characterized by frequently occurring mutations in the von Hippel-Lindau tumor suppressor gene (*VHL*) (Hsieh *et al.*, 2017). Due to the inactivating mutation, the *VHL* gene product (pVHL) fails in its function in promoting the degradation of the hypoxia-inducible factor α-subunits (HIF-α). The hypoxia-inducible factors (HIFs) are key transcription factors responding to low oxygen concentration, i.e. hypoxia. HIFs function by activating transcription of the target genes that mediate the cellular adaptation to hypoxia. However, in ccRCC, constant activation of the HIF signaling pathway leads to highly vascularized and metabolically active tumors. High expression of HIF-α subunits, and more specifically HIF-2α, is indispensable for ccRCC development and growth (Schodel *et al.*, 2016).

HIF prolyl hydroxylases (PHDs) are oxygen sensing enzymes that regulate HIF-α protein stability in the presence of oxygen. Among the three PHDs, PHD3 is often upregulated in cancer and interestingly, several hydroxylase-independent functions have been demonstrated for PHD3. In cancer cells, PHD3 has been shown to be involved in the regulation of survival mechanisms and metabolism (Jaakkola and Rantanen, 2013). Among various types of cancer, PHD3 is most abundantly expressed in ccRCC (Högel *et al.*, 2011). This thesis investigates the role of PHD3 in ccRCC cells, with focus on proteome level changes in response to PHD3 expression level and determining functions of PHD3 in various essential cellular processes.

# 2 REVIEW OF THE LITERATURE

## 2.1 Malignant transformation

The most prominent feature distinguishing normal and transformed cells is the escape from growth control and the acquired ability to divide in an uncontrolled manner. In general, malignant transformation starts with genetic mutations. Although mutations are common and can in most cases be repaired, mutations escaping the repair mechanisms can be transferred to the next generation of cells during cell division and accumulate. In particular, mutations leading to the activation of an oncogene or silencing of a tumor suppressor gene appear to have severe consequences and cause major effects on cellular behavior associated with permanent cell growth. As a consequence, this leads to formation of a continuously growing cellular mass, a tumor. In general, benign and malignant tumors can be distinguished. While benign tumors usually do not spread, malignant tumors have the ability to invade the surrounding tissue and to enter the blood or lymph system. As a result, they infiltrate other organs and form metastases, which then undergo again malignant progression usually at the expense of the healthy individual. Hence, a wide range of different types of malignant tumors that share common features are referred to as cancer disease.

Decades of research have revealed that formation of malignant tumors from the initial transformed cell population is a multistep process, in which cells acquire features that enable unlimited growth and the ability to invade the surrounding tissues. Several hallmarks of cancer have been recognized that contribute to malignant transformation (Figure 1). These hallmarks include sustaining proliferative signaling that enables cells to divide without extracellular growth stimulus, enabling replicative immortality by maintaining telomerase activity, evading signaling leading to growth suppression and avoiding programmed cell death. Malignant cells are also able to induce angiogenesis to supply nutrients and oxygen to fill the needs of the growing tumor mass. The major inductor of angiogenesis is the lowered oxygen level inside the tumor, which drives the expression of pro-angiogenic factors responsible

for vascularization when the tumor mass exceeds the limits of oxygen diffusion into tissue. Cancer cells also activate the invasion to the surrounding tissues and formation of metastasis in distant tissues (Hanahan and Weinberg, 2011; Fouad and Aanei, 2017).

In addition to sustaining growth and promoting invasion of the tumor mass, other important features distinguishing cancer cells from normal cells have been characterized (Figure 1). These include promotion of genomic instability, tumor inflammation, evasion from immune surveillance and destruction, as well as reprogramming the cellular energy metabolism. Changes in tumor metabolism have been recognized early on. However it has become clear in more recent years that cancer cells promote certain metabolic pathways in order to favor the production of biosynthetic intermediates and other important building blocks that are needed for continuous cell proliferation (Hanahan and Weinberg, 2011; Fouad and Aanei, 2017).

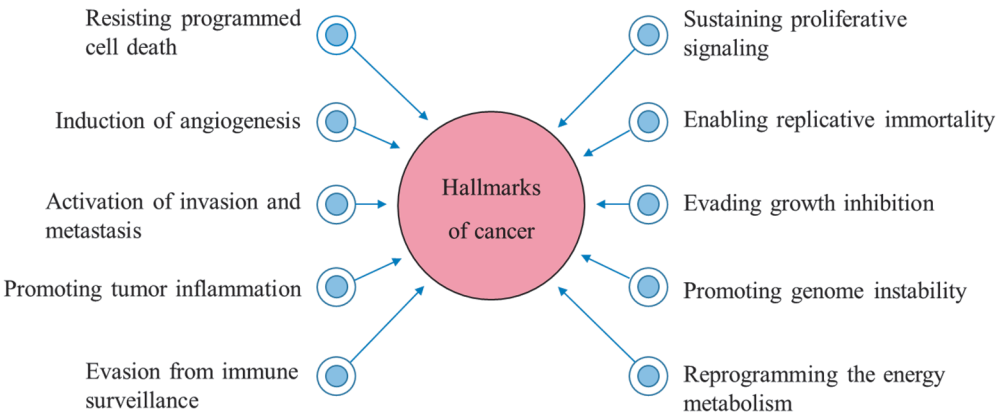


**Figure 1**. Hallmarks of cancer.

In addition to acquired features in cellular signaling and behavior, also the tumor microenvironment can drive cancer progression. The microenvironment can cause selective pressure towards a more aggressive cancer cell phenotype. Major environmental factors affecting cancer progression include inadequate oxygen availability, the composition of the extracellular matrix and tissue pH (Hanahan and

Weinberg, 2011; Muz *et al.*, 2015). Cancer cells are also able to activate other cell types such as fibroblasts, endothelial cells and macrophages to support the tumor progression by releasing growth factors and chemotactic cytokines, and by remodeling and depositing extracellular matrix (ECM) components (Quail and Joyce, 2013).

What once thought to be a disease caused by genetic alterations has shown to be an extremely complex cascade of events ranging from mutations in the DNA to the systemic features such as evading the immune surveillance and to the environmental selection. Ultimately, the most striking aspect is that the cascade cannot be prevented.

## 2.2 Renal cell carcinoma

Renal cell carcinomas (RCCs) are cancers of the kidney, which most commonly originate from epithelial cells of the proximal convoluted tubules in nephrons. Proximal tubules transport the primary urine in the nephrons and thus are a part of the ductal system of the kidney. RCCs are the most common type of kidney cancers (over 90%) and the third most common type of urinary tract cancers, affecting men more often than women (Ljungberg *et al.*, 2011; Ferlay *et al.*, 2015).

RCCs can be categorized into three major subtypes based on the histopathological classifications: clear cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC (chRCC). ccRCC is the most common subtype counting up to 80% of all cases. Besides the major three subtypes, also cases of unclassified RCC with no clear histopathological features occur. Each of the major subtypes can also be associated with a distinct hereditary syndrome caused by certain germline mutations in the genome. While ccRCC is most often associated with von Hippel-Lindau disease that is caused by loss-of-function mutation in the *VHL* gene, pRCC cases are associated with mutations in the proto-oncogene *MET* or in the fumarate hydratase (*FH*) gene. The chRCC and other rare types of kidney cancers are linked with the Birt-Hogg-Dubé syndrome, which are caused by mutations in the tumor suppressor gene folliculin (*FLCN*) (Verine *et al.*, 2010; Shuch *et al.*, 2015; Hsieh *et al.*, 2017).

However, the hereditary syndromes only count up to less than 5% of all RCCs, whereas the majority of RCC cases have gained a somatic mutation in both alleles of a particular gene. In addition to the well-known genetic mutations causing the hereditary syndromes, also several other genes have been implicated in sporadic RCC cases including for example *PBRMI*, *SETD2*, *PAB1*, and *PTEN* (Hsieh *et al.*, 2017).

### 2.2.1 Clear cell renal cell carcinoma

The ccRCC is the major subtype of renal cell carcinomas. The nomenclature clear cell in ccRCC arises from the fact that the lipid- and glycogen-rich cancer cells appear as "clear" or "empty" on histological hematoxylin and eosin staining. It is well-known that ccRCCs display a highly glycolytic metabolism and highly vascularized phenotype. Also, genes related to metabolism and angiogenesis are often overexpressed in ccRCC (Hsieh *et al.*, 2017). The metabolic shift towards high glycolytic activity and fatty acid synthesis also has a clinical significance in ccRCC, as worse patient outcome correlates with high expression levels of genes involved with fatty acid synthesis, glycolysis and pentose phosphate pathway (Cancer Genome Atlas Research Network, 2013).

Majority of clinical ccRCC cases are characterized with loss-of-function of the VHL tumor suppressor protein (pVHL). This can be due to a genetic mutation, chromosomal aberrations affecting the *VHL* locus or epigenetic modifications in the *VHL* gene (Gnarra *et al.*, 1994; Herman *et al.*, 1994; Cancer Genome Atlas Research Network, 2013). The best-known molecular function of pVHL is in the proteasomal degradation pathway. The well-characterized targets of pVHL are the α-subunits of the hypoxia-inducible transcription factors (HIFs). Thus, inactivation of pVHL leads to accumulation of HIF-α, which exist as two major isoforms, HIF-1α and HIF-2α. The role of pVHL in ccRCC formation and progression has been widely studied and it is generally accepted that the *VHL* mutations are among the earliest oncogenic events in ccRCC formation (Iliopoulos *et al.*, 1995; Lubensky *et al.*, 1996; Li and Kaelin, 2011; Gossage *et al.*, 2015; Schodel *et al.*, 2016).

Moreover, the role of accumulated HIF-α isoforms has been studied in depth and it is generally accepted that high HIF-1α and especially HIF-2α contribute to the tumor initiation and progression by activating their target genes (Schodel *et al.*, 2016). The HIF pathway target genes are involved in adaptation to low oxygen concentration,

such as glycolytic pathway, angiogenesis and cell survival (described in detail in section 2.4) but the same target genes have tumor promoting properties when constantly expressed. Thus, with inactive pVHL, the overexpression of the metabolic enzymes and factors inducing vascularization is a direct consequence of HIF-α accumulation (Wiesener *et al.*, 2001; Turner *et al.*, 2002; Hu *et al.*, 2003; Schodel *et al.*, 2016).

However, loss of *VHL* alone is insufficient to induce ccRCC formation as reported by several studies or as evidenced by a long latency in ccRCC development in individuals with germline mutations in *VHL* (Kaelin, 2007; Kapitsinou and Haase, 2008). Additional genetic events are required for developing ccRCC tumors. Large-scale genomic studies of clinical ccRCC samples have revealed several novel gene mutations including *PBRM1, SETD2, BAP1, KDM5C, KMD6A* and *MTOR* (Dalgliesh *et al.*, 2010; Pena-Llopis *et al.*, 2012; Cancer Genome Atlas Research Network, 2013; Sato *et al.*, 2013). Of these genes, *PBRM1* has been shown to be mutated in approximately 30% - 40% of cases, *BAP1* in 6% - 15% of cases, and other genes have a mutation frequency of less than 10% of cases (Varela *et al.*, 2011; Pena-Llopis *et al.*, 2012; Hakimi *et al.*, 2013). *PBRM1*, *SETD2, BAP1, KDM5C* and *KMD6A* encode chromatin-regulating and histone-regulating proteins (Dalgliesh *et al.*, 2010; Varela *et al.*, 2011; Pena-Llopis *et al.*, 2012). The gene product of *MTOR*, mechanistic target of rapamycin (mTOR) is a protein complex that functions in sensing a range of intra- and extracellular stimuli in order to regulate energy homeostasis and cell growth (Saxton and Sabatini, 2017). Identification of the new gene mutations are opening interesting avenues in understanding molecular level of ccRCC pathology.

In addition to pVHL inactivation and other mutations, the HIF-α isoform profile may play an important role in the pathogenesis of ccRCC. In the normal kidney proximal tubular cells, only HIF-1α is expressed (Rosenberger *et al.*, 2002; Schietke *et al.*, 2012). Interestingly, however, the immunohistochemical analysis of early pre-cancerous lesions in kidneys of patients with VHL disease shows increased expression of both HIF-1α and HIF-2α accompanied by abundance of HIF target gene expression. These findings suggested that the increased HIF-2α expression in kidney tubular cells with pVHL inactivation is an early event and likely contributing to the cancer progression (Mandriota *et al.*, 2002; Rosenberger *et al.*, 2002; Raval *et al.*, 2005; Schietke *et al.*, 2012).

## 2.3 Oxygen sensing system

Molecular oxygen ($O_2$) is a prerequisite of aerobic life. $O_2$ accounts to approximately 20.9% of the total atmospheric volume, which corresponds to an $O_2$ partial pressure ($pO_2$) of 159 mmHg (Wenger *et al.*, 2015). In mammals, respired $O_2$ is carried in the blood by erythrocytes in which $O_2$ is bound to hemoglobin. Via capillaries, the blood stream distributes $O_2$ to all organs and almost all tissues throughout the body. At the cellular level, 80% of $O_2$ is used as a terminal electron acceptor in the mitochondrial electron transport chain of the oxidative phosphorylation. The oxidative phosphorylation is a process of efficient energy production in cells converting energy from nutrients to adenosine triphosphate (ATP), which is the key energy source for cellular events. About 20% of $O_2$ in a cell is used for $O_2$–dependent enzyme reactions such as protein hydroxylation. Thus, mammalian cells require oxygen for maintaining energy homeostasis and cellular functions. Under anaerobic conditions, cells are able to produce energy only for relatively short periods via substrate phosphorylation, thus, allowing some flexibility at the fluctuating oxygen concentrations, such as in heavy stress (Semenza, 2012).

To assure survival in different environments, cells have developed mechanisms to adapt to changes in oxygen availability. On the molecular level, adaptation to lowered $O_2$ level depends on the activation of hypoxia-inducible factors (HIFs). The HIF transcription factors activate a transcription of various target genes that mediate the adaptation to low oxygen concentration. This activation process is also called as hypoxic response, which includes modulation of erythropoiesis, angiogenesis, cellular metabolism and proliferation, among other functions (Semenza, 2012; Bishop and Ratcliffe, 2014; Ivan and Kaelin, 2017).

The ability of the cells and the whole organism to sense oxygen in their environment and to alter their behavior in response to changes in the oxygen concentration is tightly regulated at the cellular level. In the presence of oxygen, the HIFs are being modified by hydroxylation and rapidly degraded. When the oxygen concentration decreases, the oxygen-dependent enzymes responsible for hydroxylation are inactivated allowing transcriptional activity of HIFs that provides adaptive responses. By this sophisticated pathway, mammalian cells have gained a survival advantage for adapting to the environmental changes (Semenza, 2012; Bishop and Ratcliffe, 2014; Ivan and Kaelin, 2017).

### 2.3.1 Hypoxia in physiology and in cancer

In the human body the oxygen concentration progressively reduces after entering the lung alveoli until it reaches all tissues via the blood circulation. The normal oxygen partial pressure varies greatly between tissues, ranging from 60-50 mmHg in highly vascularized tissues to around 5-10 mmHg in bone and cartilage with the least vasculature (Carreau *et al.*, 2011). Thus, within the normal physiological range the oxygen availability can vary detrimentally and different cell types better tolerate lower oxygen levels than others. A condition where cells or tissues are lacking adequate levels of oxygen to maintain the ATP production necessary for survival is called hypoxia. In hypoxic conditions, cells adapt their signaling to meet the needs of their environment by regulation of angiogenesis, metabolism, proliferation, differentiation and death – leading to a transcriptional program, which is called the hypoxic response. Non-malignant cells often respond to severe hypoxia by a restricting cell growth and eventually by cell death (Carreau *et al.*, 2011; Wilson and Hay, 2011).

Hypoxia is also a frequently occurring factor in several pathological conditions, such as in stroke, inflammation and in solid tumors. In tumors, hypoxic regions are formed since the diffusion of oxygen is limited due to the weak blood supply from the structurally and functionally abnormal tumor vasculature, as well as due to the high proliferation rate of cancer cells (Carreau *et al.*, 2011; Wilson and Hay, 2011). In contrast to non-malignant cells, cancer cells can adapt to continuous deprivation of oxygen, survive and proliferate. Furthermore, hypoxia drives the transcriptional program towards glycolytic metabolism, induces angiogenesis, activates epithelial-mesenchymal transition and cell invasion, promotes immune evasion and stem cell maintenance, which are among the well-characterized hallmarks of cancer (Figure 1) (Hanahan and Weinberg, 2011; Wilson and Hay, 2011; Muz *et al.*, 2015). Thus, limited oxygen supply within the tumor can serve as a selective pressure towards a more aggressive phenotype. In clinical aspects, tumor hypoxia is often associated with increased malignancy and resistance to radiation and chemotherapy, thus resulting in poor prognosis. Moreover, successful radiation therapy needs $O_2$ as the oxygen radicals are responsible for the secondary effects of the radiation treatment. In addition, hypoxia can cause cell cycle arrest or quiescence in some cell types that also contribute to the poor response to treatment (Wilson and Hay, 2011; Muz *et al.*, 2015).

## 2.4 Hypoxia-inducible factors

The primary regulators of the hypoxic response in mammals are hypoxia-inducible factors (HIFs). HIFs are heterodimeric transcription factors consisting of an α- and β- subunit (also known as the aryl hydrocarbon nuclear translocator, ARNT) (Semenza and Wang, 1992; Wang *et al.*, 1995). HIF-α has three known paralogs in mammals and most of the vertebrates, HIF-1α, HIF-2α and HIF-3α. The genes encoding HIF-α isoforms are *HIF1A*, *HIF2A* (also known as *EPAS1*, endothelial PAS domain-containing protein 1) and *HIF3A,* respectively (Ema *et al.*, 1997; Gu *et al.*, 1998; Iyer *et al.*, 1998). HIF-β is a common subunit of heterodimeric transcription factors that also exists in several paralogs. HIF-β is constitutively expressed both under normal and lowered oxygen tensions, whereas the HIF-α subunits are highly regulated by the oxygen levels (Semenza, 2012).

HIF-1α was the first discovered HIF-α paralog along with an identification of a hypoxia response element (HRE) in the erythropoietin (EPO) gene. EPO is a highly hypoxia-inducible hormone that stimulates erythrocyte proliferation (Goldberg *et al.*, 1988; Semenza *et al.*, 1991). Since its discovery, HIF-1α has been recognized as a central transcription factor in the oxygen sensing mechanism in mammals, responsible for the induction of genes mediating adaptation from normal physiological oxygen levels to hypoxia (Semenza, 2012). A few years later, HIF-2α was described having a similar structure and function as a hypoxia-inducible transcription factor (Tian *et al.*, 1997; Wiesener *et al.*, 1998).

HIF-1α and HIF-2α are considered as the major isoforms responsible for adaptation to low oxygen levels and they share similarities in their structure and function. HIF-3α differs in structure, and its functions are less studied. Both HIF-1α and HIF-2α consist of PER-ARNT-SIM (PAS) domains that are required for dimerization, basic helix-loop-helix domain (bHLH) for DNA binding, oxygen dependent degradation domains (ODDD) and transactivation domains NTAD and CTAD (Jiang *et al.*, 1996; Jiang *et al.*, 1997).

HIF-3α has shown to be expressed in multiple splice variants (Makino *et al.*, 2002; Maynard *et al.*, 2003; Pasanen *et al.*, 2010). The full-length HIF-3α transcriptionally activates genes in response to hypoxia, however, HIF-3α has only one transactivation

domain. Some of the splice variants have been reported to function as inhibitors of HIF-1α and HIF-2α or competing HIF-1/2α binding with HIF-β (Duan, 2016).

The HIF-1α protein levels are rapidly induced under hypoxia. Thus, HIF-1α is generally thought to induce the acute response to low oxygen availability. HIF-2α, on the other hand, often shows a delayed upregulation in response to hypoxia when compared to HIF-1α. Moreover, HIF-2α has been demonstrated to remain upregulated over a longer period of time. Hence, it has been suggested that HIF-2α has a role in adaptive responses to chronic hypoxia (Ivan and Kaelin, 2017).

Recent advantages in technology, including next-generation sequencing have enabled comprehensive studies of the transcriptional responses to hypoxia. The HIF responsive transcriptional program includes a vast group of genes mediating changes in erythropoiesis, angiogenesis, metabolic reprograming, cell cycle regulation, and cell survival among others (Semenza, 2012) (Figure 2). Furthermore, some of the HIF target genes are regulated across different tissues or cell types in response to hypoxia, whereas the others are highly tissue specific.

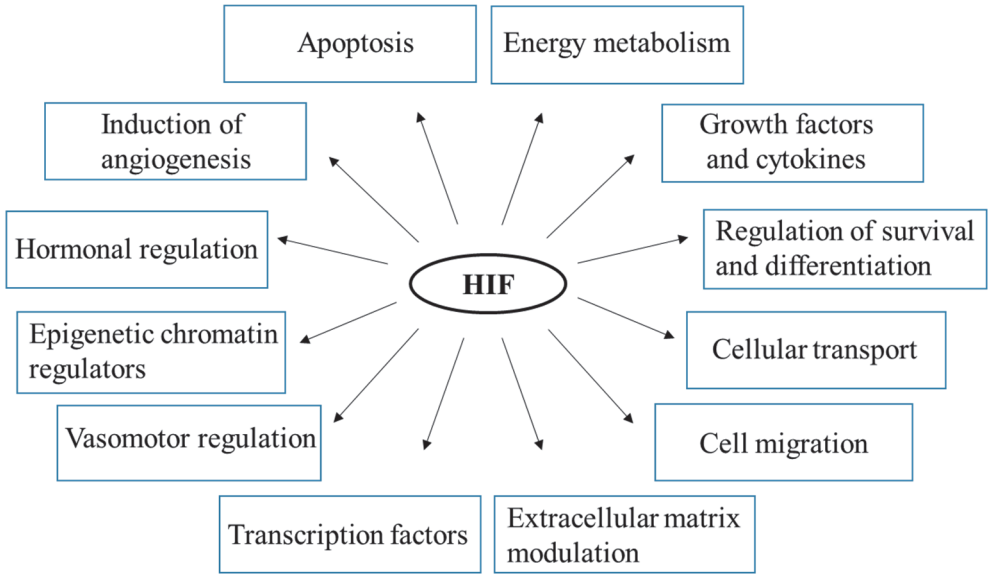


**Figure 2.** Cellular processes regulated by HIF signaling (modified from (Schofield and Ratcliffe, 2004; Bishop and Ratcliffe, 2014)).

Despite the similarities in structure and function, HIF-1α and HIF-2α have overlapping but to at least some extent distinct set of target genes. In general, HIF-1α is considered as a regulator of fundamental biological processes, such as metabolism and vascularization. HIF-1α has been shown to regulate several glycolytic enzymes including phosphofructokinase (PFK), pyruvate kinase (PKM), lactate dehydrogenase (LDH) as well as glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX), and vascular endothelial growth factor (VEGF). On the other hand, HIF-2α is thought to provide a mechanism of fine tuning the responses under various conditions, and is linked to the induction of other growth-factors, such as transforming growth factor alpha (TGF-α) and platelet derived growth factor (PDGF), as well as cell cycle regulation (cyclin D1) and matrix modifying factors (MMPs) (Semenza, 2012; Schodel *et al.*, 2016; Ivan and Kaelin, 2017). Moreover, the expression of HIF-2α and HIF-3α has been proposed to be more tissue specific, which may indicate more specialized roles in different tissues and developmental stages.

Importantly, the hypoxia-inducible gene products also play a role in various pathologies, including tumorigenesis and cancer progression (Semenza, 2012; Duan, 2016; Schodel *et al.*, 2016). Several of the HIF target genes belong to the essential biological processes that are disturbed in cancer, thus contributing to the hallmarks of cancer (Hanahan and Weinberg, 2011). Moreover, the HIF signaling pathway shows crosstalk with several other signaling pathways, such as mTOR, p53, NF-κB and Notch to promote cancer progression (Sahlgren *et al.*, 2008; Cavadas *et al.*, 2013; Obacz *et al.*, 2013; D'Ignazio *et al.*, 2016).

The major regulation of HIF-α occurs on protein level. In addition to the major oxygen-dependent mechanism (described in detail in section 2.4.1), HIFs are regulated at the protein level by several cytokines and growth factors. However, the transcriptional and post-transcriptional regulation of *HIF1A* and *HIF2A* is still not well characterized (Ke and Costa, 2006).

### 2.4.1 Oxygen-dependent regulation of HIF-α

HIF-α is post-translationally hydroxylated in an oxygen-dependent manner, which directly affects the HIF-α protein stability. Proline residues that are targeted by the hydroxylation can be found in all HIF-α subunits, however, HIF-3α only has one of

the target proline residues due to a lack of C-terminal activation domain, while HIF-1α and HIF-2α bear two proline hydroxylation sites (Semenza, 2012; Duan, 2016). The oxygen-regulated stability of HIF-α protein provides a direct mechanism for transducing changes in cellular oxygen availability to changes in gene expression. This mechanism enables the cells to sense oxygen levels and consequently to undergo adaptive changes that either lead to enhancing oxygen delivery or to promoting cell survival in a hypoxic environment. The transcription and synthesis of HIF-α protein are constitutive regardless of the oxygen availability, but under normal oxygen availability, normoxia, HIF-α protein is rapidly degraded (Wang *et al.*, 1995; Kallio *et al.*, 1997).

Figure 3 illustrates the oxygen-dependent regulation of the HIF-α protein stability under normal oxygen conditions and under hypoxia. Under normoxia, a family of HIF prolyl hydroxylase enzymes (PHDs) hydroxylates the HIF-α. Hydroxylation is a transient and reversible post-translational modification that marks the target protein for further processing. In the case of the HIF-α, hydroxylation serves as a mark for recognition by ubiquitin-proteasome machinery, which is a principal mechanism of protein decay in cells. Hydroxylation of two proline residues ($Pro^{402}$ and $Pro^{564}$ in human HIF-1α; $Pro^{405}$ and $Pro^{531}$ in human HIF-2α) within the oxygen dependent destruction domain (ODDD) promotes the interaction of HIF-α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Epstein *et al.*, 2001; Jaakkola *et al.*, 2001; Ivan *et al.*, 2001; Masson *et al.*, 2001; Hirsila *et al.*, 2003; Pereira *et al.*, 2003). The pVHL complex mediates ubiquitylation of HIF-α and thus marks it for proteasomal degradation (Maxwell *et al.*, 1999; Cockman *et al.*, 2000).

Under hypoxia, PHDs are inactive due to the requirement of molecular oxygen as a co-substrate in the hydroxylation (Maxwell *et al.*, 1999). Thus, HIF-α remains stable and it is translocated into the nucleus where it dimerizes with HIF-β. The complex binds to the hypoxia response element (HRE) in the regulatory region of a target gene, recruits transcriptional co-activators such as CREB binding protein (CBP/p300), steroid receptor coactivator (SRC-1) and transcription intermediary factor 2 (TIF-2), and activates the transcription (Semenza *et al.*, 1991; Semenza and Wang, 1992; Wang and Semenza, 1995; Arany *et al.*, 1996; Carrero *et al.*, 2000).

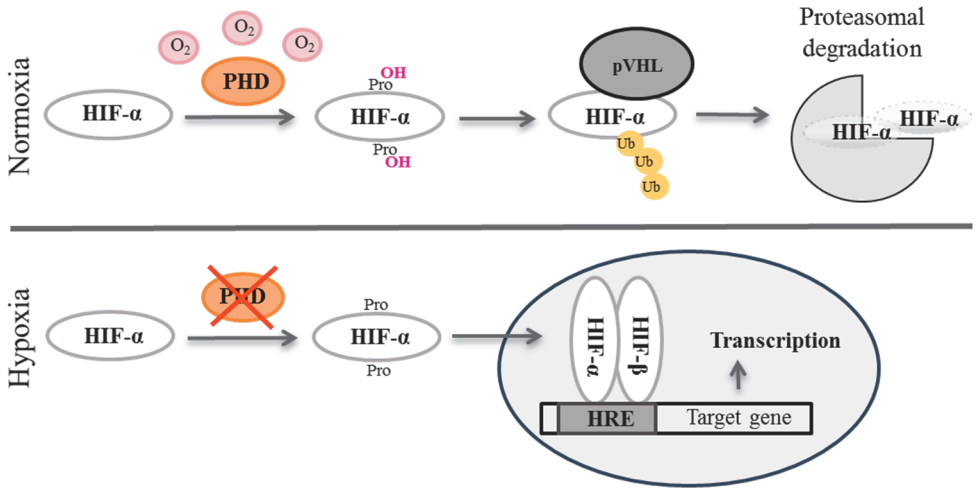


**Figure 3.** Schematic representation of oxygen-dependent regulation of HIF-α. Under sufficient oxygen availability, PHD enzymes hydroxylate HIF-α, which promotes the recognition by pVHL. pVHL mediates the ubiquitylation and marks HIF-α for proteasomal degradation. Under hypoxia, oxygen-dependent PHD enzymes are inactive leading to stabilization of HIF-α. HIF-α enters the nucleus and dimerizes with HIF-β. The complex binds to hypoxia response element (HRE) of target genes to active their transcription.

PHDs are hydroxylase enzymes that require molecular oxygen ($O_2$) as well as ferrous iron ($Fe^{2+}$) and ascorbate for their enzymatic activity (Schofield and Zhang, 1999; Jaakkola *et al.*, 2001). Characteristics of PHDs are described in more detail in section 2.5. In addition to hydroxylation by PHDs, Factor inhibiting HIF-1 (FIH1) regulates HIF-α through oxygen-dependent asparaginyl hydroxylation. Asparaginyl hydroxylation prevents HIF-α interaction with the transcription coactivator CBP/p300 leading to disruption in transcription activation (Hewitson *et al.*, 2002; Lando *et al.*, 2002).

### 2.4.2 Transcriptional and post-transcriptional regulation of HIF-α

The studies involved in transcriptional regulation of HIF-α have emerged in recent years. However, transcriptional regulation of HIF-α is still relatively unknown. In

several cell lines studied, *HIF1A* and *HIF2A* transcription was not induced by hypoxia, highlighting the importance of protein level regulation as the major mechanism of HIF-α hypoxic induction (Hamidian *et al.*, 2015).

Nuclear factor kappa B (NF-κB) has been shown to regulate the transcription of *HIF1A* (Nizet and Johnson, 2009). Also, loss of c-Jun N-terminal protein kinase 2 (JNK2) results in decrease in both *HIF1A* and *HIF2A* expression (Sala *et al.*, 2017). For *HIF2A* expression, direct regulation by transcription factor E2F1 have been shown in several cell lines, including *VHL* mutated 786-O ccRCC cell line. Also, the deubiquitylase Cezanne was linked to the regulation of *HIF2A* by demonstrating that Cezanne regulates the stability of E2F1 (Moniz *et al.*, 2015). Moreover, Poly (ADP-ribose) polymerase-1 (PARP-1) has been suggested to directly enhance *HIF2A* transcription when studied in *Parp* knockout mouse embryonic fibroblasts (MEFs) (Gonzalez-Flores *et al.*, 2014). In neuroblastoma cells, *HIF2A* expression is regulated by estrogen-related receptor alpha (ERRα) (Hamidian *et al.*, 2015) and by mTORC2 complex (Mohlin *et al.*, 2015). In keeping with this, HIF-2α protein levels have been reported to be regulated by the mTORC2 (Nayak *et al.*, 2013).

Post-transcriptional regulation is the control of gene expression at mRNA level. Post-transcriptional regulation of mRNAs include different steps of mRNA processing and maturation such as alternative splicing, regulation of nuclear export and stability and degradation of mRNAs. There are numerous factors involved in the complex regulation of mRNA processing including RNA binding proteins (RBPs) and non-coding RNAs (Halbeisen *et al.*, 2008).

After transcription, regulation of mRNA stability mainly occurs through RNA binding proteins (RBP), which bind to the RNA recognition motif (RRM) in the transcript. Binding of different RBPs results in different fates of the mRNA, whether it is rapidly decayed, sequestrated into storage or stabilized and translated. RNA binding proteins and/or non-coding RNAs recruit other factors involved in mRNA decay and subsequently the mRNA molecule is degraded by specific enzyme complexes. Regulation of mRNA stability provides yet another level in the complex regulation of protein abundancy within the cell (Wu and Brewer, 2012). Control of HIF-1α mRNA stability has been linked to RNA-binding proteins HuR and PTB. HuR and PTB have been shown to directly bind to *HIF1A* transcript and to regulate the translation of *HIF1A*. Moreover, it has been proposed that post-transcriptional

regulation of hypoxia-inducible mRNAs is an important regulatory step under hypoxic condition (Schepens *et al.*, 2005; Galban *et al.*, 2008)

### 2.4.3 Functions of HIF-2α in ccRCC

The role of the HIF-1α and HIF-2α in cancer progression has been studied in depth. Overexpression of both HIF-1α and HIF-2α leads to increased tumor growth and is associated with poor clinical outcome in many cancer types (Semenza, 2010; Keith *et al.*, 2011). In ccRCC, HIF-1α and HIF-2α are highly expressed at the protein level due to the frequently occurring inactivation of pVHL (Maxwell *et al.*, 1999; Krieg *et al.*, 2000; Turner *et al.*, 2002).

One intriguing characteristic of ccRCC is that generally all the tumors are expressing HIF-2α protein, but only a subset of tumors are also expressing HIF-1α. Based on their HIF-α expression profile, ccRCC tumors and derived cell lines can be divided into two different groups; those expressing both HIF-1α and HIF-2α and those expressing high HIF-2α but no HIF-1α (Gordan *et al.*, 2008). Similarly, ccRCC-derived cell lines that are commonly used for molecular level studies, such as 786-O and A498 are expressing high levels of HIF-2α and lacking functional HIF-1α (Maxwell *et al.*, 1999; Shen *et al.*, 2011). Moreover, genomic analyses have revealed that renal cell carcinomas commonly have deletions in chromosome 14 in the region that harbors the *HIF1A* gene and the loss of HIF-1α expression is associated with poor patient prognosis (Monzon *et al.*, 2011; Shen *et al.*, 2011; Cancer Genome Atlas Research Network, 2013).

Clinical ccRCC tumors with solely HIF-2α expression were associated with increased expression of commonly used proliferation marker and with a larger tumor volume (Gordan *et al.*, 2008). In addition, HIF-2α overexpressing xenograft tumors appear large and fast growing, while HIF-1α overexpression leads to reduced tumor size (Maranchie *et al.*, 2002; Raval *et al.*, 2005; Biswas *et al.*, 2010; Shen *et al.*, 2011). In line with these results, short hairpin RNA (shRNA) mediated silencing of HIF-2α in ccRCC cells resulted in smaller *in vivo* tumors (Kondo *et al.*, 2003; Zimmer *et al.*, 2004). Conversely, HIF-1α knockdown increased ccRCC cell proliferation both *in vitro* and *in vivo* (Shen *et al.*, 2011). The pre-clinical findings have led to the conclusion that high HIF-2α expression results in more tumorigenic phenotype in ccRCC.

Furthermore, recent clinical phase 1 trial demonstrated clinical benefit for the inhibition of HIF-2α in patients with advanced ccRCC (Courtney *et al.*, 2017).

Similarly to high HIF-2α protein expression, ccRCC shows high HIF-2α mRNA expression (Turner *et al.*, 2002; Sandlund *et al.*, 2009). However, despite the association of HIF-2α and tumor growth, the mRNA expression of HIF-2α was not found to correlate with ccRCC patient survival (Sandlund *et al.*, 2009).

The well-studied transcription factor and oncogene c-Myc is often amplified in the ccRCC genome, and overexpression of c-Myc protein leads to enhanced cell proliferation and growth via regulation of genes involved in cell cycle and tumor metabolism (Klatte *et al.*, 2012; Cancer Genome Atlas Research Network, 2013). HIF-2α has been shown to enhance c-Myc activity and subsequently to increase the expression of c-Myc target genes, such as cell cycle related proteins cyclin D2 and E2F1. Simultaneously, c-Myc can also modulate the cell cycle regulation by repressing multiple genes, for example cell cycle inhibitors p21 and p27. It has been proposed that the high proliferation rate and increased tumor growth in HIF-2α expressing ccRCCs is mediated by c-Myc and its target genes (Gordan *et al.*, 2007a; Gordan *et al.*, 2008). In contrast, HIF-1α acts as an inhibitor of c-Myc activity and thus HIF-1α expression results in decreased proliferation in ccRCC.

## 2.5 HIF prolyl hydroxylases

The HIF prolyl hydroxylases belong to a well conserved superfamily of 2-oxoglutarate (2-OG)-dependent dioxygenases. These enzymes are widespread throughout the kingdom of aerobic living organisms and various members are found in plants, microorganisms, and mammals. Experiments in the nematode *C. elegans* were the first identifying the HIF prolyl hydroxylases as members of the Egl-Nine (EGLN) gene family, which have functions in growth, differentiation and apoptosis in the worm´s muscle and nerve cells. Later mammalian orthologues from rodents and humans were described (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Taylor, 2001; Ivan *et al.*, 2002). They were found to encode three prolyl hydroxylase domain protein isoforms which were then called PHD1, PHD2 and PHD3. The three PHDs are sometimes also referred to as HIF prolyl 4-hydroxylases 1-3 (HIF-P4H-1, HIF-

P4H-2 and HIF-P4H-3) or EGLN2, EGLN1 and EGLN3, respectively. In addition to the three soluble PHD isoforms, also a fourth member has been described, abbreviated as P4H-TM. P4H-TM acts in hydroxylating HIF-α *in vitro*, but unlike other PHDs, it possesses a transmembrane domain (TM) and associate with the endoplasmic reticulum (Oehme *et al.*, 2002; Koivunen *et al.*, 2007b). Further, another member of the 2-OG-dependent dioxygenase family has been identified which acts as HIF asparaginyl hydroxylase and was called Factor Inhibiting HIF (FIH1) (Mahon *et al.*, 2001; Lando *et al.*, 2002; Hewitson *et al.*, 2002).

Human PHD1-3 have a relative sequence similarity of 42-59% (Taylor, 2001) and they share many similar features but differ greatly in their function. All PHDs have been shown to function as oxygen sensors *in vitro* as they hydroxylate HIF-α subunits in the presence of oxygen and the essential cofactors. It is well established that PHD2 is the main regulator of HIF-1α in normoxia whereas PHD3 is thought to be the main regulator of HIF-2α and partly retaining hydroxylase activity under prolonged hypoxia (Berra *et al.*, 2003; Aprelikova *et al.*, 2004). PHD3 and PHD1 have also been assumed to function in a more tissue specific manner (Appelhoff *et al.*, 2004). The tissue specificity and differential affinity towards HIF hydroxylation have been suggested to serve more variability in the regulation of hypoxia response across the tissues (Epstein *et al.*, 2001; Hirsila *et al.*, 2003; Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004).

Of the three isoforms, PHD2 and PHD3 are hypoxia-inducible whereas PHD1 expression levels are stable in many cell lines despite the oxygen levels. PHD3 shows the most robust hypoxic induction in several cell lines studied (Appelhoff *et al.*, 2004). PHD2 and PHD3 are known HIF target genes, which cause the upregulation under hypoxia and moreover, creates a negative feedback loop for HIF regulation under hypoxia and re-oxygenation. The upregulation of PHDs under hypoxia has also been proposed to compensate their reduced activity (Epstein *et al.*, 2001; Berra *et al.*, 2003; del Peso *et al.*, 2003; D'Angelo *et al.*, 2003; Aprelikova *et al.*, 2004).

For their enzymatic activity, all PHDs require molecular oxygen ($O_2$) and 2-OG for co-substrates as well as ferrous iron ($Fe^{2+}$) and ascorbate as co-factors. $O_2$ is cleaved in the hydroxylation process; one atom is transferred to the target proline residue and the other one reacts with 2-OG to form succinate and $CO_2$ (Schofield and Zhang, 1999; Loenarz and Schofield, 2011). In this reaction $Fe^{2+}$ is oxidized into $Fe^{3+}$. $Fe^{3+}$ needs to be reduced to $Fe^{2+}$ to maintain the full activity of PHDs.

Ascorbate is not directly used in the hydroxylation reaction, but it has been reported in several studies, that ascorbate is required for full enzymatic activity of PHDs. It has been suggested that the reduction of $Fe^{3+}$ to $Fe^{2+}$ is dependent on ascorbate (Jaakkola *et al.*, 2001; Hirsila *et al.*, 2003; Masson and Ratcliffe, 2003; Flashman *et al.*, 2010a). The $K_m$ values of PHD enzymes for $O_2$ are relatively high, which indicates that the enzymes are highly sensitive for changes in cellular $O_2$ level (Hirsila *et al.*, 2003; Koivunen *et al.*, 2006; Stiehl *et al.*, 2006; Flashman *et al.*, 2010b). Furthermore, it has been shown that all the PHDs hydroxylate the conserved residue of $Pro^{564}$ in HIF-1α, but only PHD1 and PHD2 have been shown to hydroxylate $Pro^{402}$, which is considered to evolve later (Berra *et al.*, 2003; Hirsila *et al.*, 2003; Chowdhury *et al.*, 2016).

The expression of PHDs varies between the different tissues. PHD2 is expressed widely and uniformly within different tissues, highlighting the role of PHD2 as a major regulator of the hypoxic response in normal physiology. PHD1 is expressed in low levels in most tissues, but an especially high expression has been reported in testis and placenta and moderate expression levels in brain, liver, heart and adipose tissue. Moreover, PHD3 is widely expressed in different tissues, with most abundant expression in heart and skeletal muscle, epithelial cells, and placenta (Lieb *et al.*, 2002; Oehme *et al.*, 2002; Cioffi *et al.*, 2003). Furthermore, alternative splicing has been described for PHDs and the differentially spliced variants may exhibit functions distinct for the full length proteins in normal physiology and in pathological conditions (Hirsila *et al.*, 2003; Cervera *et al.*, 2006; Tian *et al.*, 2006).

The roles of the different PHD isoforms in physiology and in development are also well demonstrated by the knockout (KO) mouse models. PHD2 KO mice are not viable until birth and the embryos die between 12.5 to 14.5 embryonic days due to severe cardiac defects and insufficient vascularization of the placenta (Takeda *et al.*, 2006; Minamishima *et al.*, 2008). PHD1 KO mice are viable and no severe developmental defects appear, but the mice are showing differential regulation of glucose metabolism in skeletal muscles and in liver, a shift from oxidative metabolism to anaerobic ATP production. The metabolic shift was shown to impair muscle performance under normal conditions, but also to provide protection against acute ischemia in skeletal muscles (Aragones *et al.*, 2008). A similar protective effect of PHD1 loss in case of acute ischemia has also been demonstrated in liver (Schneider *et al.*, 2010). In addition, several other phenotypic effects have been reported for PHD1 KO mice, including impaired insulin sensitivity and glucose

tolerance, increased hepatic steatosis, as well as deregulated proliferation in several tissues (Tambuwala *et al.*, 2010; Mollenhauer *et al.*, 2012; Thomas *et al.*, 2016).

PHD3 KO mice are also viable. However, PHD3 KO mice have an impaired development of the sympathetic nervous system, seen as an elevated blood pressure and other increased sympathetic activity. The report suggested that the effect on sympathetic nervous systems is due to the role of PHD3 in controlling apoptosis of the sympathetic neurons in developing individual. Interestingly, the cardiovascular system and placenta of the PHD3 knockout mice were reported to appear normal, although the PHD3 is usually expressed in high levels in these tissues (Takeda *et al.*, 2006; Bishop *et al.*, 2008). Moreover, PHD3 KO has also been shown to protect heart tissue from ischemia/reperfusion injury, a similar protective effect that is also seen in organs of PHD1 KO mice. The effect has been thought to be mediated by inhibition of cardiomyocyte apoptosis when PHD3 is silenced (Xie *et al.*, 2015).

### 2.5.1 Expression and regulation of PHD3

Initially, PHD3 was identified as a rat homolog SM-20 in smooth muscle cells and found to be induced by growth factor stimuli (Wax et al. 1994). Since its discovery, PHD3 has been widely studied in many experimental systems, and it is considered to be the primary regulator of HIF-2α and partly maintaining the enzymatic activity under moderate to severe hypoxia (Stiehl *et al.*, 2006; Ginouves *et al.*, 2008). Under normoxia, PHD3 mRNA and protein levels are low but under hypoxia the levels are strongly upregulated (Appelhoff *et al.*, 2004). PHD3 is expressed in both nucleus and cytoplasm, and the hypoxic exposure has no effect on subcellular localization (Metzen *et al.*, 2003).

Immunohistochemical analyses have shown that PHD3 protein is widely expressed across different tissues, especially in epithelia (Soilleux *et al.*, 2005). Also, PHD3 is strongly expressed in cardiac, skeletal and in vascular smooth muscle (Moschella *et al.*, 1999; Lieb *et al.*, 2002; Soilleux *et al.*, 2005). In skeletal muscle, the expression of PHD3 occurs in the early developmental stage and thus PHD3 has been suggested a role in the differentiation of skeletal muscle (Moschella *et al.*, 1999; Fu *et al.*, 2007). PHD3 also functions in neuronal cells, where apoptosis mediated by PHD3 is needed during a normal development of sympathetic neurons.

PHD3 is transcriptionally regulated by HIFs, and HIF-2α is suggested to be the primary regulator of PHD3 (Aprelikova *et al.*, 2004; Pescador *et al.*, 2005). It has been under debate, why the HIFs are inducing the expression of PHDs, which in turn limit the activity of HIFs. In general, it has been thought that the upregulation compensates the reduced oxygen availability and provides rapid responses to HIF signaling when re-oxygenation occurs.

Besides HIFs, PHD3 is also regulated by other factors. Under hypoxia, PHD3 protein is proteasomally degraded by the ubiquitin E3 ligases Siah1a/2 (Nakayama *et al.*, 2004). It has also been suggested that re-localization into protein complexes regulates PHD3 activity (Nakayama *et al.*, 2007). Moreover, methylation and alternative splicing of *PHD3* gene, as well as micro-RNAs provide another level of regulation for PHD3. In various human cancer cell lines and in some B-cell lymphoma subtypes and plasma cell dyscrasias, *PHD3* promoter methylation causes total mRNA expression silencing (Huang *et al.*, 2010; Hatzimichael *et al.*, 2010; Place *et al.*, 2011). Also, alternative splicing of *PHD3* gene has been described (Hirsilä *et al.* 2003; Cervera *et al.* 2006). Micro-RNAs (miRNAs) are small interfering RNAs regulating transcription and post-transcriptional processes, and miR-20a has been demonstrated to directly target PHD3 mRNA (Frank *et al.*, 2012).

In addition to protein stability and genome level regulation, glycolytic pathway and tricarboxylic acid (TCA) cycle intermediates pyruvate and oxaloacetate has been shown to regulate the expression of PHDs, including PHD3. Furthermore, pyruvate and oxaloacetate are also able to bind to the 2-oxoglutarate site of PHD2 and PHD3, thus inactivating their ability to hydroxylate HIF-α in the presence of oxygen (Dalgard *et al.*, 2004; Lu *et al.*, 2005). In addition, TCA cycle intermediates fumarate succinate act as inhibitors of PHD activity *in vitro* (Selak *et al.*, 2005; Koivunen *et al.*, 2007a; Hewitson *et al.*, 2007).

### 2.5.2 Functions of PHD3

PHD3 has been demonstrated to act as a major negative regulator of HIF-2α protein stability by post-translational hydroxylation. Moreover, PHD3 partly retains its enzymatic activity under hypoxia, and thus PHD3 is characterized as major regulator of hypoxic response under prolonged hypoxia (Aprelikova *et al.*, 2004; Stiehl *et al.*, 2006; Ginouves *et al.*, 2008). Besides HIF-α, many other hydroxylation targets have been demonstrated for PHD3. The known hydroxylation targets of PHD3 and their key functions are listed in the table 1.

PHD3 hydroxylates activating transcription factor 4 (ATF-4) (Koditz *et al.*, 2007) that is involved in the unfolded protein response, and beta2-adrenergic receptor (β(2)AR) that mediates several stress responses (Xie *et al.*, 2009). PHD3 has also been shown to hydroxylate HCLK2, which is a key element in the DNA damage response (Xie *et al.*, 2012). Furthermore, PHD3 regulates pyruvate kinase M2 (PKM2). PHD3 has been shown to hydroxylate PKM2 and thus to enhance the coactivator function of PKM2 on HIF-1α transactivation, which results in increase of HIF target genes GLUT1, LDHA and PDK1 (Luo *et al.*, 2011). Another study has reported that PHD3 directly binds to PKM2 and affects the PKM2 tetramer formation (Chen *et al.*, 2011). More recently, PHD3 was also linked to regulation of fatty acid metabolism, by hydroxylating acetyl coenzyme A carboxylase 2 (ACC2), and thus mediating the repression of fatty acid oxidation in response to nutrient abundance (German *et al.*, 2016).

**Table 1.** Hydroxylation targets of PHD3.

| Target protein | Key function | References |
|---|---|---|
| HIF-α | Cellular adaptation to low oxygen concentration | (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Masson *et al.*, 2001; Huang *et al.*, 2002; Hirsila *et al.*, 2003; Appelhoff *et al.*, 2004; Stiehl *et al.*, 2006) |
| ACC2 | Fatty acid oxidation | (German *et al.*, 2016) |
| Actin (non-muscle) | Cytoskeleton structure and cell movement | (Luo *et al.*, 2014) |
| ATF-4 | Unfolded protein response | (Koditz *et al.*, 2007) |
| β(2)AR | Stress response | (Xie *et al.*, 2009) |
| HCLK2 | DNA damage response | (Xie *et al.*, 2012) |
| Pax2 | Cell proliferation and differentiation | (Yan *et al.*, 2011) |
| PKM2 | Glucose metabolism | (Luo *et al.*, 2011) |
| Sprouty2 | Regulation of receptor tyrosine kinase signaling | (Anderson *et al.*, 2011) |
| NF-κB signaling | Stress response and survival | (Fu and Taubman, 2010) |

In addition to these hydroxylation targets, several other functions and interactors have been characterized for PHD3 independently on the enzymatic activity. These targets represent proteins in various important cellular processes, such as cell survival via regulation of the cell cycle, apoptosis and NF-κB signaling, cell motility, and immune cell functions (Place and Domann, 2013). Among the aforementioned processes, several reports have shown that PHD3 functions as negative regulator of NF-κB signaling via inhibitors of IkappaBs (IKKBs); this appears to be partly dependent on the hydroxylase activity and partly on other mechanisms (Fu and Taubman, 2010; Xue *et al.*, 2010; Fujita *et al.*, 2012; Fu and Taubman, 2013). NF-κB has a key role in the immune and stress response, as well as in inflammation. NF-κB signaling is considered to be an essential survival advantage of cancer cells. In the regulation of apoptosis, PHD3 has various targets, including Bcl-2, hPRP19 and KIF1Bβ, and moreover, PHD3 directly mediates apoptotic cell death in neuronal cells and in cancer cell lines (Jaakkola and Rantanen, 2013; Place and Domann, 2013). On the contrary, PHD3 has been shown to enhance cell cycle progression under hypoxic conditions in cancer cells and hence to serve as a survival promoting factor under a reduced oxygen level (Högel *et al.*, 2011).

PHD3 has also been shown to regulate glucose metabolism. Two independent studies show that PHD3 enhances glucose uptake and lactate production, as well as decreases $O_2$ consumption via regulation of PKM2 in cancer cells, including ccRCC cells, and thus shifting the metabolism towards increased glycolysis (Chen *et al.*, 2011; Luo *et al.*, 2011). The report by Luo *et al.* demonstrates that the regulation of glycolysis takes place via HIF-1α that has been generally considered as a master regulator of glycolytic enzyme gene expression in ccRCC cells. In addition, PHD3 has been reported to directly interact with the pyruvate dehydrogenase (PDH) complex protein PDH-E1β, and to regulate the stability of the PDH complex. Depletion of PHD3 reduced the activity of the PDH complex and thus PHD3 was suggested to play a role in regulation of pyruvate to acetyl coenzyme A conversion (Kikuchi *et al.*, 2014). Another study demonstrates that PHD3 has a role in insulin sensitivity in liver via regulation of HIF-2α and insulin receptor substrate-2 (Irs2) (Taniguchi *et al.*, 2013). To summarize based on the above mentioned findings, PHD3 has many targets and at least partly specific roles in certain tissues and conditions.

### 2.5.3 PHD3 in cancer

PHD3 is overexpressed in various types of cancer, including head and neck squamous cell carcinomas (HNSCC), breast cancer, pancreatic cancers and especially in ccRCC (Sato *et al.*, 2008; Högel *et al.*, 2011; Tanaka *et al.*, 2011; Place and Domann, 2013). On the other hand, decreased levels of PHD3 in comparison to adjacent healthy tissue have been reported for some cancer types (Xue *et al.*, 2010; Rawluszko *et al.*, 2013; Ma *et al.*, 2017).

The overexpression of PHD3 in non-small cell lung cancer (NSCLC) and in pancreatic cancer is an unfavorable prognostic factor (Couvelard *et al.*, 2008; Gossage *et al.*, 2010; Andersen *et al.*, 2011) whereas in gastric cancers and in breast cancers the upregulation of PHD3 has been reported as favorable prognosticator (Peurala *et al.*, 2012; Su *et al.*, 2012). In gliomas, the PHD3 expression decreases along with tumor stage, with simultaneous increase in cell proliferation. Mechanistically, loss of PHD3 in gliomas has been shown to increase epidermal growth factor receptor (EGFR) activity and thus to regulate cell survival and proliferation. The report suggested that loss of PHD3 in gliomas would lead to a growth advantage under unfavorable conditions (Garvalov *et al.*, 2014; Henze *et al.*, 2014). Based on these results, the role of PHD3 in cancers is somewhat controversial and both the expression level and the function seem to be tissue specific. The expression level of the originating tissue may play a major role in PHD3 expression level in cancer cells. However, it cannot be concluded that the expression levels of PHD3 results in uniform outcome across different cancer types, but it can serve as a predictive marker in certain types of cancer. Also, the varying functions of PHD3 could be at least partially explained by signaling crosstalk with pathways activated in specific cancer types.

Several reports show that PHD3 is overexpressed in ccRCC (Sato *et al.*, 2008; Högel *et al.*, 2011; Tanaka *et al.*, 2011; Fu *et al.*, 2015). PHD3 has also been suggested as a marker for poor prognosis in ccRCC patients (Garcia-Donas *et al.*, 2013). The overexpression of PHD3 has been thought to account for high HIF-α expression in *VHL* mutated ccRCC cells. However, some indications of specific functions of PHD3 in ccRCC cells have been reported. As described in section 2.5.2, PHD3 has been demonstrated to regulate glucose metabolism in ccRCC cells via the glycolytic enzyme PKM2 and its function to be a transactivator of HIF-α (Luo *et al.*, 2011).

## 2.6 Tumor metabolism

Changes in metabolism are universally detected across solid tumors and cancer cell lines. Thus, the metabolic reprogramming has been considered as an important hallmark of cancer (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016). By reprogramming the activity of diverse metabolic pathways, cancer cells improve their survival under fluctuating nutrient availability. The major nutrient supporting the survival and the massive biosynthesis is glucose (Pavlova and Thompson, 2016).

The classic example of an altered metabolic pathway in cancer cells is known as aerobic glycolysis or "The Warburg effect". Glycolysis is a pathway used for energy production under low oxygen availability or under a stress condition, such as heavy muscle work. As early as in the 1920s, Otto Warburg made an observation that cancer cells exhibit constitutive glucose uptake and high consumption, in concordance with high lactate production, in the presence of oxygen (Warburg *et al.*, 1927; Warburg, 1956). Later, the effect has been found universally in all solid tumors and different cancer cells types and it represents one of the most prominent differences in the metabolism between cancer cells and non-malignant cells (Koppenol *et al.*, 2011).

According to Warburg's original hypothesis, cancer cells use the aerobic glycolysis due to impaired mitochondrial function. However, it has become clear that cancer cells have functional mitochondria, but the glycolytic pathway is upregulated by the activation of oncogenes (Pavlova and Thompson, 2016). Furthermore, the high glycolytic activity is favored as it yields important intermediates to other biosynthetic pathways, such as pentose phosphate pathway (PPP), hexosamine pathway, serine/glycine biosynthesis and pathways for phospholipid production. Enabling the activity of the biosynthetic pathways is an essential aspect of tumor metabolism, as the cancer cells are in constant need of the macromolecules required for tumor growth. In keeping with this, the rate-limiting enzymes that mediate the reactions from the glycolytic pathway to the branching biosynthetic pathways are frequently upregulated in tumors (DeBerardinis and Chandel, 2016; Pavlova and Thompson, 2016).

In glycolysis, glucose is sequentially converted into pyruvate in enzyme-catalyzed reactions in the cytoplasm (Figure 4). Each molecule of glucose yields two molecules

of pyruvate and two molecules of ATP, the energy converted in a form useful for cellular events. In addition, the pathway yields two molecules of NADH coenzyme that can be used in cellular redox reactions as a reducing agent. The rate limiting enzymes of glycolysis are considered to be hexokinase, phosphofructokinase, and pyruvate kinase. After glycolysis, pyruvate can be further converted into lactate and secreted out from the cells or directed into the tricarboxylic acid (TCA) cycle after oxidative decarboxylation of pyruvate into acetyl coenzyme A (acetyl-CoA) (Nelson and Cox, 2000).

In the TCA cycle, acetyl-CoA is condensed with oxalacetate to form citrate. A following series of reactions consuming water oxidize citrate and regenerate oxaloacetate. As a consequence of these reactions, carbon dioxide and reduced equivalents in the form of NADH are formed. In mammalian cells the reactions take place in the mitochondrial matrix (Figure 4). The generated NADH molecules are then directed into oxidative phosphorylation in the presence of oxygen. In short, the electrons from NADH are then transferred to $O_2$ that serves as a final electron acceptor in the oxidative phosphorylation in the inner mitochondrial membrane. The remaining protons are then the proper driving force for the ATP synthase (Nelson and Cox, 2000).

Several upstream regulators have been shown to contribute to the metabolic shift in cancer cells. As an example, the transcription factors HIF-1α and c-Myc have been reported to upregulate the expression of facilitated glucose transporter 1 (GLUT1) pyruvate dehydrokinase (PDK), hexokinase 1 (HK1), and lactate dehydrogenase A (LDHA), among others enzymes of the glycolytic pathway. LDHA is the enzyme responsible for conversion of pyruvate to lactate. Also, c-Myc has been demonstrated to induce monocarboxylate transporter (MCT1) levels, which facilitates the transport of lactate into the extracellular space (Gordan *et al.*, 2007b; Wahlstrom and Henriksson, 2015; Semenza, 2013). In addition, PI3K/Akt signaling has shown to function as a key regulator of glucose uptake and the first steps of the glycolytic pathway (Pavlova and Thompson, 2016).

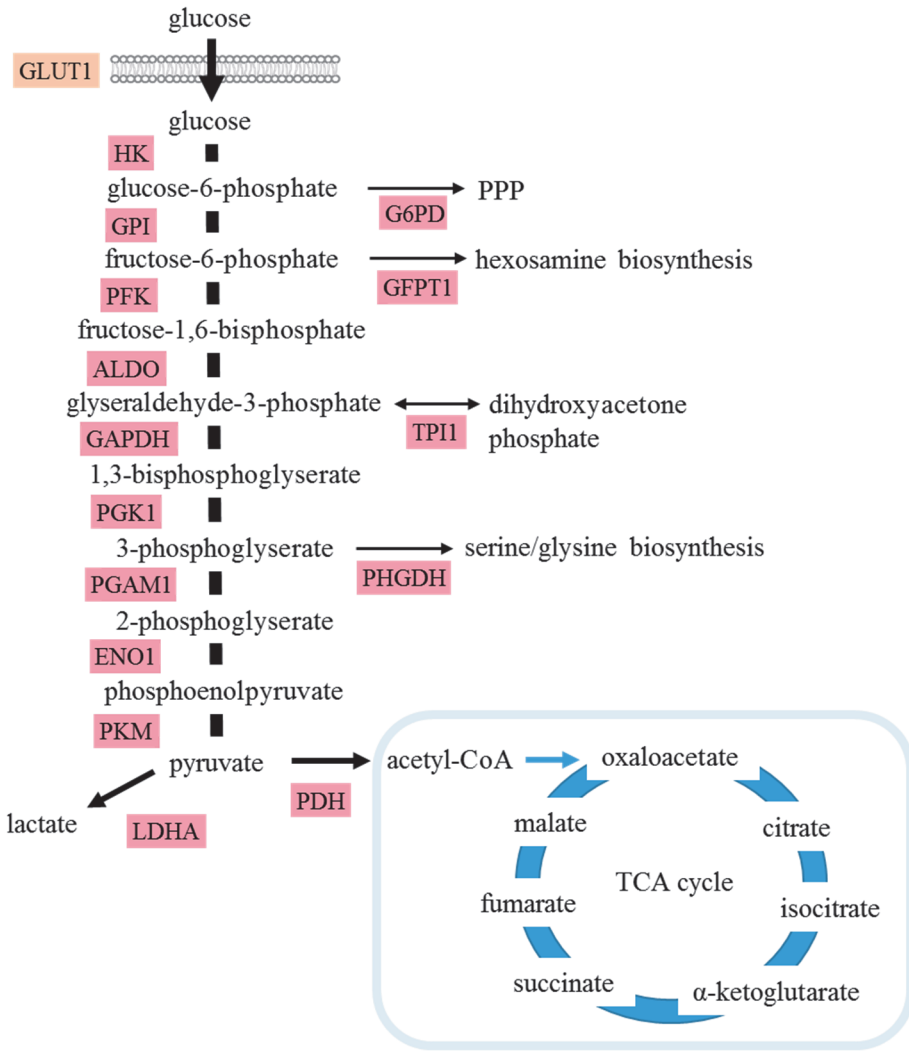


**Figure 4.** Representative illustration of glycolysis and glycolytic enzymes, and the tricarboxylic acid (TCA) cycle. Red-colored blocks represent enzymes involved in glycolysis and in branching pathways, orange-colored block represents glucose transporter protein. Abbreviations: GLUT1 glucose transporter 1, HK hexokinase, GPI glucose-6-phosphate isomerase, G6PD glucose-6-phosphate dehydrogenase, PPP pentose phosphate pathway, PFK phosphofructokinase, GFPT1 glutamine--fructose-6-phosphate transaminase 1, ALDO aldolase, GAPDH glyceraldehyde-3-phosphate dehydrogenase, TPI1 triosephosphate isomerase 1, PGK1 phosphoglycerate kinase, PGAM1 phosphoglycerate mutase 1, PHGDH phosphoglycerate dehydrogenase , ENO1 alpha-enolase, PKM pyruvate kinase isoform M, LDHA lactate dehydrokinase A, PDH pyruvate dehydrokinase.

Another advantage of using glycolysis is that high oxidative phosphorylation activity causes accumulation of reactive oxygen species (ROS). Overproduction of ROS may have damaging effect on cells (Sullivan and Chandel, 2014). Furthermore, ATP and NADH produced by the TCA cycle have shown to act as major negative regulators of glycolysis. Hence, by converting excess pyruvate to lactate, cancer cells prevent accumulation of cytosolic NADH and ATP, thereby promoting glycolytic activity (Pavlova and Thompson, 2016).

Lactate is excreted from the cells and accumulation of extracellular lactate also participates in promoting tumor progression. Extracellular lactate has been reported to promote immune evasion via several types of immune cells and to induce angiogenesis via stabilization of HIF-1α signaling. Lactate has also been shown to induce matrix metalloproteinase (MMP) proteolytic activity that enhances the extracellular matrix (ECM) degradation and thus cancer cell invasion to the surroundings (Romero-Garcia *et al.*, 2016).

To summarize, in order to keep up with the proliferation rate cancer cells increase the import of nutrients and favor the glycolytic pathway in energy production. Thus, cells are able to produce large amounts of biosynthetic pathway intermediates. By exploiting the flexibility of the metabolism, cancer cells are able to survive and maintain their excess proliferation in environments with changing nutrient- and oxygen availability.

### 2.6.1 Metabolic reprogramming in ccRCC

RCCs in general are considered to be a metabolic disease, as the gene mutations commonly occurring in RCCs are also important regulators of metabolic pathways (Linehan and Ricketts, 2013). ccRCC cells are packed with lipid droplets and glycogen, which clearly implicate excess activity of energy metabolism and storage. ccRCC tumors and cell lines display high glycolytic activity, fatty acid synthesis and other characteristic metabolic features. Moreover, a metabolic shift towards glycolysis and fatty acid synthesis also has a clinical significance in ccRCC progression (Cancer Genome Atlas Research Network, 2013).

Metabolic reprogramming in ccRCC is commonly related to the inactivation of pVHL that results in accumulation of transcription factor HIFs (Wettersten *et al.*,

2017). HIF signaling has an essential role in cellular adaptation to low oxygen levels by modulating metabolism. Genes involved in glucose uptake, glycolysis and lipid synthesis are well-known target genes of the HIF signaling pathway, and thus upregulation of several metabolic factors (such as GLUT1, PKM2, PDK1, LDHA, PGAM, HK1) in ccRCC tumors and cell lines is a direct consequence of HIF activation (Semenza, 2007; Schodel *et al.*, 2016; Wettersten *et al.*, 2017). In addition, metabolomics analyses on ccRCC tissues show increased glycolytic metabolites extending the understanding of the metabolic shift into functional level (Hakimi *et al.*, 2016; Wettersten *et al.*, 2015).

On the contrary, TCA cycle enzymes and intermediates, such as fumarate, malate, α-ketoglutarate (also known as 2-oxoglutarate, 2-OG), fumarate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH) are often downregulated in ccRCC (Catchpole *et al.*, 2011; Minton *et al.*, 2015; Wettersten *et al.*, 2015; Hakimi *et al.*, 2016). Furthermore, the metabolism of several amino acids, including glutamine, tryptophan and arginine are also modulated in ccRCC to serve the needs of multiple metabolic pathways, including protein and nucleotide synthesis and glutathione synthesis, among others (Wettersten *et al.*, 2017). Lipids are used in energy production but also for membrane synthesis in order to support proliferation. Increased expression of fatty acid synthesis related enzymes, such as fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1) are found upregulated in ccRCC tumors, and on the contrary, decreased levels of enzymes involved in β-oxidation that catabolizes the fatty acids (Wettersten *et al.*, 2015).

## 2.7 Cell cycle – phases and control

In multicellular organisms, tight regulation of cell proliferation and cell death must be maintained in order to keep the cell number and tissue homeostasis constant. The cell cycle is a tightly regulated process that results in the duplication and transmission of genetic information from one cell generation to the next. Disruption of the regulation leads to an imbalance in homeostasis and uncontrolled cell growth.

The eukaryotic cell cycle is divided into four phases: M (mitosis), $G_1$ (the growth period between mitosis and the initiation of DNA replication), S (synthesis, the

phase of DNA replication), and $G_2$ (the second growth phase between the completion of DNA replication and mitosis) (Figure 5). In the G1 phase, extracellular growth factors, i.e. mitogens, are responsible for activating signaling cascades leading to active protein translation and anabolic metabolism, which eventually lead to duplication of cellular components and the cell size growth. The critical point in the late $G_1$ phase, where progression through the cell cycle becomes independent of mitogenic stimuli, is called the restriction point (R). The progression through the restriction point is one of the major points of regulation in the cell cycle. During the S phase, chromosomes are replicated and the newly synthetized DNA is quality controlled. At the $G_2$ phase, cells are growing and preparing for the division. The second important cell cycle checkpoint in the G2 phase ensures DNA quality before entering the M phase, as damaged or incomplete DNA synthesis stops the cell cycle progression. During the M phase, chromosomes condense, microtubules form a bipolar mitotic spindle, and chromosomes align to the center of the cell. The sister chromatids are separated on to opposite ends to the spindle. The finalizing step is the division of the cytoplasm, cytokinesis that forms two daughter cells (Lodish *et al.*, 2000; Israels and Israels, 2001).

However, not all cells in a multicellular organism are constantly undergoing cycling. Cells can "exit" the cell cycle by entering a phase called G0, resting phase or quiescence. Cells can re-enter the cell cycle after G0, but also the re-entry is highly regulated, thus providing additional control of cell proliferation (Lodish *et al.*, 2000).

The control of cell cycle progression is based on checkpoints and transitions that ensure that the cell is ready to proceed to the next phase. The eukaryotic cell cycle is regulated by heterodimeric protein kinase complexes. The complexes are formed by two subunits, the regulatory kinase subunits called cyclins and catalytic subunits called cyclin-dependent kinases (CDKs) (Lodish *et al.*, 2000). Normal cells are dependent on external stimuli such as growth factors or mitogens to pass through the early G1 phase. The external stimuli cause a cascade of intracellular phosphorylation events that eventually activates the cyclin/CDK complexes. Each CDK subunit can bind to different cyclins forming different heterodimers. Cyclin/CDK complexes have distinct functions in each phase of the cell cycle and the complexes phosphorylate specific target proteins to drive the cell cycle forward (Lodish *et al.*, 2000; Israels and Israels, 2001).

The periodicity of the cyclin/CDK complexes is achieved by a well-orchestrated synthesis and degradation of cyclins, which assures the accurate transitions between the different cell cycle phases. In G1 phase cyclin D is highly expressed in several homologues, which associate with CDK4 and CDK6 to form activated complexes (Figure 5). The G1 phase cyclin/CDK complexes phosphorylate the retinoblastoma (Rb) protein, which is the master regulator of G1 progression through the restriction point (R) (Donjerkovic and Scott, 2000; Foster *et al.*, 2010). Once hyper-phosphorylated, Rb dissociates from the transcription factor E2F, allowing transcription of genes required to proceed through R and to G1/S transition (Suryadinata *et al.*, 2010). Rb remains hyper-phosphorylated throughout the rest of the cycle and is dephosphorylated once the mitosis is completed (Donjerkovic and Scott, 2000).

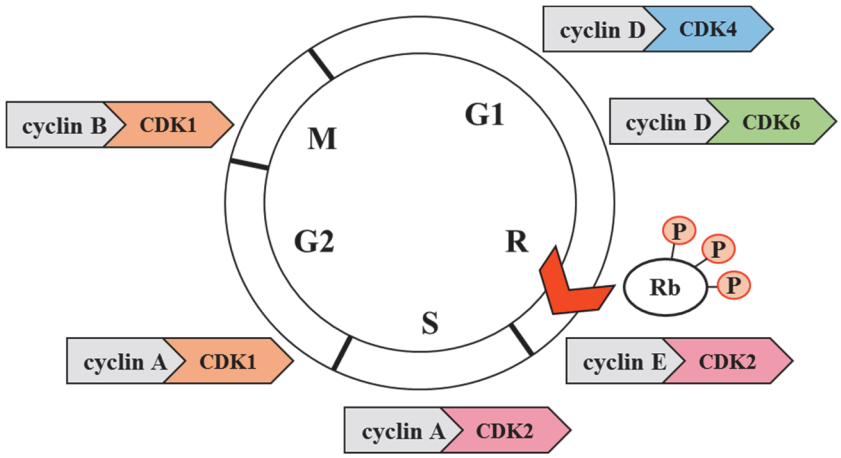


**Figure 5.** Cell cycle phases controlled by cyclin/CDK complexes. G1 – growth 1 phase, R – restriction point, S – synthesis (DNA replication), G2 – growth 2 phase, M – mitosis, Rb – retinoblastoma protein, P – phosphate group.

In late G1, subsequent from E2F release, cyclin E expression is upregulated. The cyclin E/CDK2 complex is required for the G1/S transition and rapidly degraded once the S phase is initiated (Figure 4) (Hwang and Clurman, 2005). Cyclin A is upregulated at the G1/S boundary and S-phase initiates upon the formation of cyclin A/CDK2 complex. Mitotic cyclin B/CDK1 complex upregulated during G2 phase drives the cell cycle through M phase (Israels and Israels, 2001).

In addition to cyclins and CDKs, two families of CDK inhibitors (CKIs) are involved in cell cycle regulation by inhibiting the catalytic activity of CDKs. The INK4 (inhibitors of cyclin dependent kinase 4) family members include $p15^{INK4b}$, $p16^{INK4a}$ $p18^{INK4c}$ and $p19^{ARF}$ that are all inhibiting the activity of CDK4 and CDK6 and their activity is restricted to early G1 phase. The Cip/Kip family includes p21, p27 and p57 functioning at multiple phases of the cell cycle by targeting all CDKs and also by directly binding to cyclins (Sherr and Roberts, 1999; Malumbres, 2014).

### 2.7.1 The cell cycle inhibitor p27

The Cip/Kip family members are often found deregulated in cancers and have been suggested to function as tumor suppressors due to the growth inhibitory effects. p21 and p27 share similarities in their structure, but the proteins are differentially regulated and display different functions in cell cycle regulation. p21 has been shown to mediate cell cycle arrest in response to DNA damage, whereas p27 is upregulated in response to growth-stimulating (or -inhibiting) signals and other external stimulus, such as adhesion and contact inhibition. p57 differs greatly from other family members and its role in the cell cycle regulation has remained elusive (Donjerkovic and Scott, 2000; Coqueret, 2003; Borriello *et al.*, 2011).

p27 (encoded by gene *CDKN1B*) functions in several phases of the cell cycle by regulating the activity of CDK2. Here, the focus is on the function of p27 in G1/S transition by regulating the activity of cyclin E/CDK2 complex. Moreover, p27 also interacts with cyclin D/CDK complexes and it has been suggested to be required for complex formation. Mechanistically, p27 inhibits cyclin E/CDK2 complex until cyclin D expression increases in G1 phase. Abundancy of cyclin D/CDK complexes result in sequestration of p27 by cyclin D/CDK4/6 that leads to activation of cyclin E/CDK2 (Foster *et al.*, 2010). On the other hand, growth-stimulating signals in G1 phase can lead to downregulation of p27 expression and subsequently to the activation of cyclin E/CDK2. Cyclin E/CDK2 phosphorylates Rb that induces the cell cycle progression into S phase. Thereby, increased expression of p27 caused by growth inhibitory signals such as contact inhibition can arrest the cell cycle at G1 (Coqueret, 2003; Foster *et al.*, 2010).

While in the nucleus, p27 functions in cell cycle regulation. However, when localized in the cytoplasm, p27 has been shown to promote cytoskeleton re-organization and

cell migration via GTPases Rac and RhoA (McAllister *et al.*, 2003; Wu *et al.*, 2006). Hence, p27 has been suggested to play a bipartite role in cancer by acting as both tumor suppressor and as a possible oncogene enhancing the metastatic capability. Nonetheless, modulation of the p27 cell cycle inhibitory function is often observed in cancer cells. The inactivation of p27 can occur by several mechanisms, including enhanced degradation, sequestration by cyclin/CDK complexes and post-translational modifications causing nuclear export and cytoplasmic retention of p27 (Bloom and Pagano, 2003).

p27 is mainly regulated on the protein level by the ubiquitin-proteasome pathway. p27 degradation is initiated in the nucleus by phosphorylation on Thr187 by Cyclin E/CDK2 complex and subsequently targeted for ubiquitination and proteasomal degradation (Morisaki *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). The degradation of p27 protein serves as a key event for the cells to enter the S phase. There are also other known phosphorylation sites involved in post-translational level regulation of p27. Ser10 phosphorylation of p27 serves as a marker for nuclear export and has been reported as a major stabilizer of the p27 protein. (Kotake *et al.*, 2005; Besson *et al.*, 2006). Phosphorylation of Thr157 and Thr198 are considered as signals for cytosolic trapping, resulting in low levels of p27 in the nucleus and thus prevention of the cell cycle arrest (Reed, 2002; Fujita *et al.*, 2003). The phosphorylation of p27 is achieved by several kinases responsible for phosphorylation of distinct amino acid residues, including Akt, ERK, human kinase interacting stathmin (hKIS) and Mirk/Dyrk1B (Borriello *et al.*, 2011).

In addition to the proteasomal degradation and subcellular localization, p27 is regulated by several other mechanisms, including via negative regulation by c-Myc. An inverse correlation in the expression of c-Myc and p27 has been shown in several experimental setups. However, the regulation of p27 has been suggested to be highly dependent on cell type (Donjerkovic and Scott, 2000).

PHD3 has previously been linked with cell cycle regulation, as PHD3 silencing leads to upregulation of p27, but not p21 or p16 in human carcinoma cell lines under low oxygen availability. The upregulation of p27 was shown to be accompanied by reduced RB hyper-phosphorylation, decreased levels of cyclin D1 and Cyclin B1 and cell cycle arrest in G1. PHD3 was suggested to increase cancer cell survival during hypoxia by enhancing the cell cycle progression via p27 (Högel *et al.*, 2011).

# 3 AIMS OF THE STUDY

Several studies have demonstrated high expression of PHD3 in clear cell renal cell carcinoma (ccRCC) and derived cell lines independent of the oxygenation status. Moreover, previous work has suggested several functions and individual targets for PHD3 across different cell types, but the role of high PHD3 expression in ccRCC cells have remained elusive.

Previous work from our group has determined the functions of PHD3 in cell cycle and survival mechanisms of carcinoma cells under hypoxia. To have a wider view of the functions of PHD3, the focus of this thesis work was to study large-scale changes in protein expression in response to PHD3 expression. To this end, proteomics tools and label-free quantification of the proteome level data were selected to gain an overall insight of the PHD3 responsive proteins. Moreover, cellular processes found to respond to PHD3 by the proteome-level study were further investigated using molecular biology methods to study the biological functions of PHD3 in ccRCC cells.

To understand the role of high PHD3 expression in ccRCC cell, the specific aims of this thesis were:

1. Study the proteome-level changes in response to PHD3 silencing in ccRCC

2. Study the biological function of PHD3 in ccRCC

3. Study the mechanism of PHD3 in controlling the cell cycle.

# 4 MATERIALS AND METHODS

## 4.1 Cell culture (I-III)

Cell lines used in the study are listed in Table 2. 786-O were cultured in RPMI-1640 medium (Lonza) and RCC4, RCC4+*VHL*, T47D, HeLa, UT-SCC-34 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich). Both media were supplied with 10% fetal bovine serum (FBS, Biowest), L-glutamine (Lonza) and penicillin/streptomycin (Lonza). Cells were cultured in a humidified atmosphere in 37°C containing 5% of $CO_2$. Cells were regularly tested for mycoplasma contamination when growing, and all cell lines were proven negative in each test. For experiments, cells were plated onto well plates with suitable confluency for each experiment. For hypoxic experiments the cells were cultured in 1% $O_2$ in a hypoxic workstation (Invivo$_2$, Ruskinn Technology) with oxygen replaced with 99.5% pure $N_2$ (AGA, Finland).

**Table 2.** Cell lines used in the study.

| Cell line | Description | Used in |
|---|---|---|
| 786-O | Human kidney ductal adenocarcinoma with mutated *VHL* gene | I-III |
| RCC4 | Human kidney cancer with mutated *VHL* gene | I, III |
| RCC4+*VHL* | Human kidney cancer with stable transfection of *VHL* gene | III |
| T47D | Human breast cancer | III |
| HeLa | Human cervical cancer | II |
| UT-SCC-34 | Human primary larynx/head and neck squamous cell carcinoma | III |

### 4.1.1 Small interfering RNA transfection

For small interfering RNA (siRNA) transfections, two stranded oligonucleotides were used at final concentration of 10 nM for 48 or 72 hours as stated in the original publications. Reverse transfections were performed simultaneously as the cells were plated on well plates using Lipofectamine® RNAiMAX (Invitrogen) according to manufacturer's protocol. For adenoviral small hairpin RNA (shRNA) delivery, cells were transduced with either control (shScr) or with PHD3-targeting shRNA (shPHD3) (Vector BioLabs) for 24 hours. Table 3 lists the oligonucleotide sequences of the used siRNAs and shRNAs.

**Table 3.** siRNA and shRNA oligonucleotide sequences used in transient transfections.

| Target | Sequence | Used in |
|---|---|---|
| Scr, non-targeting | 5'-CCUACAUCCCGAUCGAUGAUG(dTdT)-3' | I-III |
| siHIF1A | 5'-AACUAACUGGACACAGUGUGU(dTdT)-3' | III |
| siHIF2A | 5'-GCGACAGCUGGAGUAUGAAUU(dTdT)-3' | III |
| siPHD1 | 5'-ACAUUGCUGCAUGGUAGAA(dTdT)-3' | III |
| siPHD2 | 5'-GACGAAAGCCAUGGUUGCUUG (dTdT)-3' | III |
| siPHD3#1 | 5'-GUCUAAGGCAAUGGUGGCUUG (dTdT)-3' | I-III |
| siPHD3#2 | 5'-AGGAGAGGUCUAAGGCAAUG (dTdT)-3' | I, III |
| sip27 | 5'-AAGCACACUUGUAGGAUAA (dTdT)-3' | II |
| shScr, non-targeting | 5'GACACGCGACTTGTACCACTTCAAGAGAGT GGTACAAGTCGCGTGTCTTTTTTACGCGT-3' | II, III |
| shPHD3 | 5'CCGGCACCTGCATCTACTATCTGAACTCG AGTTCAGATAGTAGATGCAGGTGTTTTT-3' | II, III |

### 4.1.2 Plasmid-DNA transfection

For plasmid transfections, cells were plated and allowed to attach for 24 hours. 0.5 μg of plasmid DNA per well was used to overexpress HIF2A protein in cells. Empty pcDNA3 vector was used as a control. Transfections were performed with Fugene HD (manufacturer) according to manufacturer's protocol.

## 4.2 Protein and mRNA expression level analysis (I-III)

### 4.2.1 Western blot analysis

For protein expression analysis, cells were harvested in SDS-Triton lysis buffer with protease inhibitors (1 mM $Na_3VO_4$, 1 mM PMSF and 10 mM NaF). Protein concentration was measured using Bio-Rad DC Protein assay followed by addition of 2-mercaptoethanol containing SDS loading buffer and sample denaturation by heating at 95°C for 5 minutes prior to loading. Equal amounts of protein were loaded onto and separated on SDS-PAGE in a mini-gel chamber (Bio-Rad) and transferred to a PVDF membrane (Millipore). Membranes were incubated in the primary antibody at 4°C overnight and in the secondary antibody one hour in the room temperature. Primary and secondary antibodies used in the study are presented in Table 4. Protein detection was performed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific). β-actin, α-tubulin or glyseraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control. Western blot band intensities were quantified using Image J (NIH). Intensities were normalized against the housekeeping protein stated in the original publications.

**Table 4.** Primary and secondary antibodies used in the study.

| Antibody | Manufacturer, catalog number | Used in |
|---|---|---|
| **α-tubulin** | Santa Cruz Biotechnology, sc-23948 | I-II |
| **β-actin** | Sigma-Aldrich, Ac-74 | I-III |
| **Alpha-actinin-4** | Enzo Life Sciences, ALX-210-356-C050 | I |
| **Anti-mouse-HRP** | DAKO, P0447 | I-III |
| **Anti-rabbit-HRP** | DAKO, P0399 | I-III |
| **CD70** | R&D Systems, MAB2738 | I |
| **Fibronectin** | Sigma-Aldrich, F3648 | I |
| **Flag** | Sigma-Aldrich, F3165 | II |
| **GAPDH** | Hytest, 5G4-6C5 | I |
| **GLUT1** | Abcam, ab14683 | I |
| **HIF-1α** | BD Transduction Laboratories, 610959 | II, III |
| **HIF-2α** | Novus Biologicals, NB100-122 | II, III |
| **Integrin β1** | BD Transduction Laboratories, 610468 | I |
| **LDHA** | Cell Signaling Technologies, #3582 | I, III |

| | | |
|---|---|---|
| **MDH2** | Abcam, ab181873 | I |
| **p16** | Santa Cruz Biotechnology, sc-468 | II |
| **p21** | Santa Cruz Biotechnology, sc-397 | II |
| **p27** | Santa Cruz Biotechnology, sc-528 | II |
| **p-p27 (S10)** | Santa Cruz Biotechnology, sc-12939-R | II |
| **p-p27 (T157)** | R&D Systems, AF1555 | II |
| **p-p27 (T187)** | Santa Cruz Biotechnology, sc-16324 | II |
| **p-p27 (T198)** | R&D Systems, AF3994 | II |
| **PHD1** | Novus Biologicals, NB100-310 | II-III |
| **PHD2** | Novus Biologicals, NB100-137 | II-III |
| **PHD3** | Novus Biologicals, NB100-139 | I-III |
| **S6** | Cell Signaling Technologies, #2217 | I |
| **p-S6 (S235/236)** | Cell Signaling Technologies, #2211 | I |
| **p-S6 (S240/244)** | Cell Signaling Technologies, #3564 | I |
| **p70 S6K** | Cell Signaling Technologies, #2708 | I |
| **p-p70 S6K (T389)** | Cell Signaling Technologies, #9234 | I |
| **Skp2** | Santa Cruz Biotechnology, sc-7164 | II |
| **STAT1** | Cell Signaling Technologies, #9176 | I |

### 4.2.2 Immunocytochemistry and imaging

For immunocytochemistry, cells were grown on 13mm coverslips, fixed with fresh 4% paraformaldehyde and permeabilizied with 0.1% TritonX-100. Coverslips were incubated with primary and secondary antibodies as indicated in the original publications (I, III). Cell nuclei were stained with the nuclear stain Hoechst 33342 (Invitrogen). Imaging was performed using LSM780 (Carl Zeiss) confocal microscope with C-Apochromat 40x/1.20 W Korr M27 objective at the Cell Imaging Core at the Turku Centre for Biotechnology.

### 4.2.3 Reverse transcription quantitative PCR

For Reverse transcription quantitative PCR (RT-qPCR), cells were lysed and total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to

the manufacture's protocol. RNA concentration was measured and equal amount of RNA was used for complementary DNA (cDNA) synthesis with M-MuLV RNase H-reverse transcriptase (Promega) and random hexamer primers (Promega) according to the manufacturer's protocol. Quantitative PCR reactions were performed with TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Life Technologies) and run with QuantStudio 12K Flex (Thermo Fisher Scientific) at the Finnish Functional Genomics Centre (Turku Centre for Biotechnology). TaqMan primers (Oligomer) and probes (Roche, Universal ProbeLibrary) used are listed in Table 5. Data analysis was performed with QuantStudio 12K Flex Software (Thermo Fisher Scientific) by using ΔΔCq method for quantification differences in gene expression. The expression of the genes of interest was normalized against the expression of a housekeeping gene (glyceraldehyde phosphate dehydrogenase, *GAPDH*).

**Table 5.** TaqMan primers and Universal ProbeLibrary (UPL) probes used in RT-qPCR.

| Gene | Forward primer<br>Reverse primer | UPL probe | Used in |
|---|---|---|---|
| **BAX** | CATGTTTTCTGACGGCAACTT<br>CCAGATCACGCCATTTCAC | #57 | III |
| **GLUT1** | GGTTGTGCCATACTCATGACC<br>CAGATAGGACATCCAGGGTAGC | #67 | II-III |
| **HIF1A** | TTTTTCAAGCAGTAGGAATTGGA<br>GTGATGTAGTAGCTGCATGATCG | #66 | III |
| **HIF2A** | CATCTACAACCCTCGCAACC<br>CAGATAGGACATCCAGGGTAGC | #45 | III |
| **p27** | TTTGACTTGCATGAAGAGAAGC<br>AGCTGTCTCTGAAAGGGACATT | #60 | II-III |
| **p21** | TCACTGTCTTGTACCCTTGTGC<br>GGCGTTTGGAGTGGTAGAAA | #32 | III |
| **PHD2** | CGACCTGATACGCCACTGT<br>GTTCCATTGCCCGGATAAC | #44 | III |
| **PHD3** | ATCGACAGGCTGGTCCTCTA<br>GATAGCAAGCCACCATTGC | #61 | I, III |
| **LDHA** | CATCCTGGGCTATTGGACTC<br>GACATCATCCTTTATTCCGTAAAGA | #53 | III |

## 4.3 Discovery proteomics (I)

### 4.3.1 Peptide extraction

For peptide extraction, proteins were separated on Criterion XT 12% Bis-Tris gel (BioRad) and silver stained. Lanes were cut into 1mm x 1mm pieces and peptides were purified and in-gel trypsinized as follows. The gel pieces were first dehydrated with 100% acetonitrile (ACN) and reduced with 20mM dithiothreitol (DTT) in 100mM $NH_4HCO_3$ for 30 min in +56°C. Next, a second round of dehydration was performed with 100% ACN followed by an alkylation with 55mM iodoacetamide (IAA) in 100mM $NH_4HCO_3$ for 20 min in RT, dark. Third, the gel pieces were dehydrated with 100% ACN and vacuum dried, followed by incubation in solution of trypsin-40mM $NH_4HCO_3$-10% ACN on ice for 20 min. Finally, the gel pieces were fully covered with 40mM $NH_4HCO_3$-10% ACN, and the in-gel trypsinization was performed 18 hours in +37°C. After in-gel trypsinization, the peptides were extracted in two steps; first using 100% ACN for 15 min in +37°C, and secondly using 50% ACN in 5% CHOOH for 15 min in +37°C. The collected supernatants were mixed together, vacuum dried and stored at -20°C until analysis.

### 4.3.2 LC-MS/MS data acquisition and processing

For liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, tryptic peptides were dissolved in 0.2% formic acid (CHOOH) and 200-ng samples were submitted for LC-MS/MS system. Each sample was analysed in three biological replicates using a QExactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The QExactive was coupled to an EASY-nLC 1000 nano flow LC instrument (Thermo Fisher Scientific). Sample loading, solvent delivery, and scan functions were controlled by Xcalibur software (version 2.1.0 SP1.1160, Thermo Fisher Scientific). The compositions of the trap column and the analytical column are described in the original publication. A 45 min gradient from 95% solvent A (98% $H_2O$, 2% ACN, and 0.2% HCOOH) to 90% solvent B (95% ACN, 5% $H_2O$, and 0.2% HCOOH) with 0.3 µL/min flow rate was used for peptide elution.

Details of the MS/MS data acquisition can be found in the original publication. The database search for the raw spectrum files was performed in Proteome Discoverer (version 1.3.0.339, Thermo Fisher Scientific) by using Mascot (Matrix Science, London, U.K.). The search was performed for tryptic peptides, one missed cleavage site was allowed, against UniProtKB/Swiss-Prot human database. Search parameters are described in detailed manner in the original publications.

Progenesis QI for proteomics (Nonlinear Dynamics) was used for peptide quantification. The analysis area of the gradient was set to 0−45 min. Automatic peak picking was used in default sensitivity mode. Peptide abundances were exported from the software and median normalized. Differential expression between the sample groups was determined using the R-package PECA (Suomi *et al.*, 2015). A paired t-test was performed using modified settings. PECA ranks the peptides of each protein by their p-value and direction of change and uses the median p-value of the ranked list as a score. Protein-level significance is then determined from the beta distribution using the score and the total number of peptides per each protein. Finally, the Benjamini-Hochberg procedure was used to calculate the false discovery rate (FDR).

### 4.3.3 Pathway and functional group analysis

Gene Ontology (GO) terms for genes coding for the significantly differentially expressed proteins were analyzed for enrichment of biological process using DAVID (Database for Annotation, Visualization and Integrated Discovery) 6.7. The analyzed protein coding genes were compared against a whole organism (Homo sapiens) background and with the highest classification stringency. Enrichment score was used to rank the annotation groups and only groups with enrichment score above 1.3 (p-value < 0.05) were considered. Also, Cytoscape (version 3.3.0) with the Biological Networks GO plug-in (BINGO, version 3.0.3) was used to identify enriched GO biological processes. The analyzed protein coding genes were compared against a reference set of complete Homo sapiens GO annotations, with p-value adjusted to 0.05 as threshold for significance after correcting for multiple testing by Benjamini and Hochberg FDR correction.

Known interactions between selected proteins were retrieved from the STRING interaction database. Only high confidence (> 0.7) interactions derived from experimental data or curated databases were considered.

## 4.4 Cell cycle analysis, proliferation and 3D cell growth (I-II)

For studying cell cycle by using flow cytometry, siRNA treated cells were incubated 24 hours to reach 50-60% confluence, followed by 24 hours in normoxic (21% $O_2$) or in hypoxic (1% $O_2$) condition. Cells were washed with phosphate buffered saline (PBS), harvested using trypsin-EDTA and fixed with 70% ethanol over night at +4°C. After fixing, the cells were stained with propidium iodide that incorporates to DNA stoichiometrically. Cell cycle analysis was performed using flow cytometer (BD FACSCalibur, BD Biosciences) and BD CellQuest™ Pro software. The proportion of the cells considered as apoptotic was measured determining the fraction of cells in sub-G1 from the flow cytometry data.

To follow cell proliferation on two dimensional well plate surface, siRNA treated cells (1 x $10^4$ cells) were plated on 96-well plates, four wells for each siRNA. Cells were allowed to attach for 24 hours, and the well plates were placed into Incucyte® Live-Cell Analysis System (Essen BioScience). The wells were scanned for confluency every 12 hours in total of 96 hours, and the proliferation rate of the siRNA treated cells vs the control cells was determined accordingly.

For determining three-dimensional growth on Matrigel® matrix, transfected cells were embedded in Matrigel® (Corning) on 96 well plates, 1 x $10^4$ cells per well as triplicate wells for each siRNA treatment. Cell culture medium was applied on top of the Matrigel® and changed every two days. 786-O cells were grown for 7 days and RCC4 cells for 10 days after which the whole wells were imaged with a phase contrast microscope with x2 objective. Average colony size was determined using ImageJ (NIH) software.

## 4.5 Functional analysis on metabolic function (I)

Cell culture medium pH was measured from transfected cells cultured 24 hours in normoxic condition followed by 24 hours of normoxic or hypoxic condition. For normalizing the pH to the cell count, the culture media was removed from the wells and cells were stained for nuclear stain Hoechst 33342. Cell count was determined from images acquired with EVOS Digital Inverted microscope (Thermo Fisher Scientific) and hydronium ion [$H_3O^+$] concentration was normalized to cell count and converted to pH.

For measuring secreted lactate concentration from the cell culture media, transfected cells were cultured 24 hours in normoxic (21% $O_2$) condition followed by 24 hours of normoxic or hypoxic (1% $O_2$) condition. The extracellular lactate concentration was measured from the sample of growth media by using L-Lactate colorimetric assay (Abcam). After sampling, the cell culture media was removed from the wells and the cells were stained for nuclear stain Hoechst 33342. Cell count was determined from images acquired with EVOS Digital Inverted microscope (Thermo Fisher Scientific) and lactate concentration was normalized to cell count.

Oxygen consumption rate (OCR) and basal glycolysis function were measured with Seahorse Analyzer (Agilent Technologies). In OCR measurement, mitochondrial activity of the living cells is measured in real-time by using agents that specifically modulate the components of the electron transport chain in mitochondria. The agents include oligomycin, FCCP, and rotenone/antimycin A, which are serially channeled to the cells, and several status of mitochondrial are measured in real-time. For measurements, transfected cells were seeded (1 x $10^4$ cells) on the 8-well Seahorse XFp Miniplate (Agilent Technologies) in triplicates and allowed to attach for 24 hours. Prior to the analysis, cells were incubated for 1 hour in XF Base medium (Agilent Technologies) supplemented with glucose, glutamine and pyruvate in a non-CO2 incubator at 37°C. Seahorse XF Cell Mito Stress Test Kit was used for determining the OCR and basal glycolysis function by using the manufacturer's protocol.

## 4.6 2D migration assay (unpublished data)

Scratch wound assay is a method to assess 2D migration of cells. siRNA silenced cells were grown until fully confluent monolayer on 12 well plate in normoxia. 6 hours before scratch wounding, proliferation inhibitor aphidicolin (1μg/ml) was applied to the culture medium. Confluent layer of cells was scratch wounded with a pipet tip and the wound was imaged with EVOS Digital Inverted microscope (Thermo Fisher Scientific) at four different spots (0h time-point). Wounds were re-imaged at time-point of 12h (786-0) or 24h (RCC4) and wound closure was calculated from images by measuring the width of the wound by using Image J (NIH).

## 4.7 Statistical analysis (I-III)

Quantified data were reported as means together with their respective standard errors (SEM) unless otherwise stated in the original publications. Two-tailed paired t-test was used to assess the statistical significance of differences between the treated and the corresponding control samples. Nominal p-values were reported in the original publications.

# 5 RESULTS

## 5.1 Discovery proteomics revealed widespread response to PHD3 silencing in ccRCC cells (I)

We studied the effect of PHD3 depletion in 786-O cell line on the proteome level with a discovery proteomics approach in order to gain insight into the functions of PHD3 expression in ccRCC. LC-MS/MS analysis and label-free quantification of the data was used for proteome level analysis of PHD3 depleted cells exposed to ambient oxygen level (21% $O_2$) or hypoxia (1% $O_2$). Moreover, the cell line was selected as it only expresses HIF-2α and not HIF-1α, a characteristics that has been linked to more aggressive ccRCC phenotype (Kondo *et al.*, 2003; Raval *et al.*, 2005; Biswas *et al.*, 2010; Keith *et al.*, 2011).

For LC-MS/MS analysis, 786-O cells were transfected with siRNA targeting PHD3, which had been selected among two distinct sequences and proven effective in PHD3 knockdown both at mRNA and protein level (I, Fig. 1A-B). Followed by PHD3 knockdown, cells were exposed to hypoxia (1% $O_2$) or kept under normoxia. Cells were lysed and peptides were extracted using in-gel trypsinization, followed by injection into mass spectrometer system. Analysis was performed in three biological replicates. Following LC-MS/MS data acquisition, database search was used to identify proteins in each sample and label-free quantification of the data was performed. Differential expression between control and PHD3 silenced samples was tested using peptide-level expression change averaging (PECA) method (Suomi *et al.*, 2015).

The statistical analysis revealed 91 significantly differentially expressed proteins, 63 proteins under hypoxic condition and 28 proteins under normoxia (I, Table 1-2). Interestingly, a wider difference between the most upregulated and downregulated proteins ($log_2$ fold change from 5.3 to -8.0) was seen in hypoxic samples in comparison to the normoxic samples ($log_2$ fold change from 1.5 to -1.6). This could suggest that the effect of PHD3 is more widespread and pronounced under restricted oxygen availability. A set of proteins specify was successfully validated by

immunoblotting in two ccRCC cell lines by using two distinct siPHD3 sequences in 786-O cells (I, Fig. 1D). Protein validation experiments verified the results of LC-MS/MS analysis and also indicated that the effects seen in proteome level analysis are not dependent on the siRNA sequence used.

The protein-coding genes were analyzed with gene ontology (GO) tool the Database for Annotation, Visualization and Integrated Discovery (DAVID) in order to study the biological processes in which the differentially expressed proteins are related to. GO annotations were performed for proteins that were found downregulated or upregulated as a response to PHD3 knockdown. The top annotations (enrichment score above 1.3) for the upregulated proteins included nicotinamide metabolic processes (glucose metabolism), ribosomal subunits, mRNA processing, regulation of apoptosis and regulation of protein modification, protein transport and actin binding. GO annotations for the downregulated proteins showed translation, glycolysis, cellular ATPase activity and sarcomere contraction (I, Fig. 2A). Collectively, the result indicates that PHD3 has far more functions in ccRCC cells than currently known, and that the functions of PHD3 are linked to major cellular events, such as cellular glucose metabolism, protein translation, apoptosis, and protein modification and transport. Figure 6 shows a graphical summary of the biological processes affected by PHD3 in ccRCC cells.

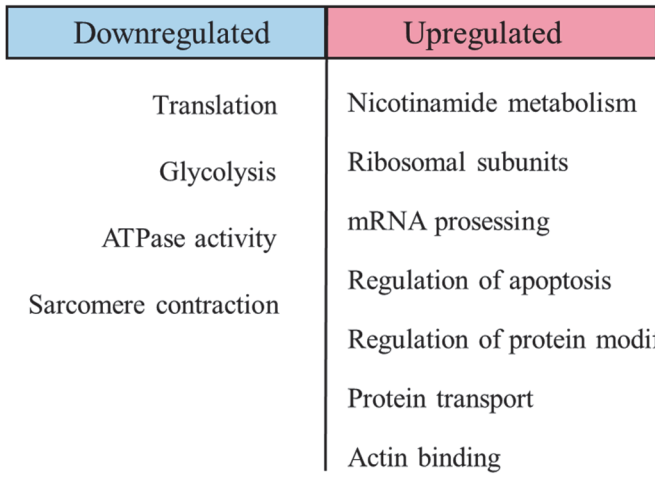


**Figure 6.** A graphical representation summarizing the biological processes upregulated and downregulated in response to PHD3 silencing in ccRCC cells.

### 5.1.1 PHD3 enhances glucose metabolism and lactate production (I)

ccRCC tumors and derived cell lines show high glycolytic activity (Semenza, 2007; Semenza, 2013; Cancer Genome Atlas Research Network, 2013; Hakimi *et al.*, 2016). Interestingly, the proteomic screening of 786-O ccRCC cells revealed that glucose metabolism was one of the biological processes regulated by PHD3. The key enzymes of glycolytic pathway PFKP, TPI1, ENO1, PGAM1 and LDHA, as well as glucose transporter GLUT1 were all found to be significantly downregulated by PHD3 silencing under hypoxic condition (I, Fig. 3A). When further scrutinizing the proteins of the glycolytic pathway, a clear pattern of glycolytic pathway suppression was seen in response to PHD3 silencing in 786-O cells (I, Fig. 3B). In contrast to downregulated glycolytic enzymes, NAD(P)H producing enzymes G6PD, TALDO1, IDH1 and MDH2 were upregulated. G6PD and TALDO1 links glycolysis to pentose phosphate pathway and IDH1 and MDH2 are tricarboxylic cycle (TCA-cycle) enzymes.

Lactate dehydrogenase (LDH) enzymes were also found affected by PHD3 silencing. LDHA was downregulated in response to PHD3 silencing, whereas LDHB was upregulated. LDHA was among the proteins validated with immunoblotting by using two distinct PHD3 targeting siRNA sequences (I, Fig. 1D). LDH enzymes convert pyruvate into lactate in a bi-directional manner, LDHA in pyruvate to lactate conversion and LDHB in lactate to pyruvate conversion (Doherty and Cleveland, 2013). Consequently, both downregulated LDHA and upregulated LDHB are likely to result in decreased pyruvate to lactate conversion and thus decreased extracellular acidification.

To further study the effect of PHD3 on glucose metabolism in ccRCC cells, lactate concentration and extracellular acidification was measured in the cell culture medium after incubation under ambient oxygen level or under hypoxia. In line with the changes on LDHA and LDHB protein expression, extracellular lactate concentration was significantly reduced in 786-O cells with PHD3 knockdown, and the effect was more pronounced in hypoxia. Similar reduction in lactate concentration was seen in RCC4 cells (I, Fig. 3C) which has also been previously demonstrated (Luo *et al.*, 2011). In addition, the pH of the cell culture media was significantly higher with PHD3 knockdown, directly indicating a lower level of acidification of the cell culture medium (I, Fig. 3D).

Furthermore, the oxygen consumption rate (OCR) and glycolysis function of 786-O cells was measured using Seahorse XFp Analyzer (Agilent Technologies). PHD3 silencing increased both basal OCR and maximal OCR measured after stimulating the cells with mitochondrial membrane uncoupling agent FCCP (carbonyl cyanide-4 trifluoromethoxy phenylhydrazone) (I, Fig. 3E). Also, basal glycolysis determined by Seahorse Analyzer was decreased in PHD3-depleted cells compared to control. (I, Fig. 3F). To summarize, downregulation of the glycolytic enzymes in PHD3-silenced ccRCC cells was associated with suppression of glycolysis and extracellular lactate levels and in a metabolic shift of the cells towards oxidative metabolism. Figure 7 shows a graphic summary of the results.

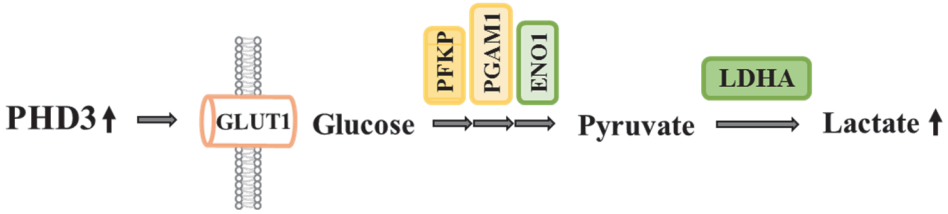


**Figure 7.** A graphic summary of PHD3 regulating high glycolytic activity and lactate production in ccRCC cells. PHD3 promotes the protein expression of glucose transporter protein GLUT1 and several glycolytic enzymes, such as PFKP, PGAM1, ENO1 and LDHA, which results increased glycolytic activity and extracellular lactate levels. Abbreviations: PHD3 – HIF prolyl hydroxylase 3, GLUT1 – glucose transporter 1, PFKP – phosphofructokinase, PGAM1 - phosphoglycerate mutase 1, ENO1 – alpha-enolase, LDHA – lactate dehydrogenase A.

### 5.1.2 PHD3 regulates mTOR downstream signaling and translational machinery (I)

Proteins related to translation and ribosomal subunits were affected by PHD3 depletion based on the proteomic screen (I, Fig. 4A). A number of 60S large subunit ribosomal proteins (RPLs) were downregulated. In addition, asparagine--tRNA ligase (NARS), eukaryotic translation initiation factor 4 gamma 1 (eIF4G1), nascent polypeptide-associated complex subunit (NACA) and RNA binding protein FUS were found to be significantly downregulated (I, Fig. 4A). When investigating the

proteome level data with less stringent statistical cut-off ($p$ 0.05) than used in the original publication, even more ribosomal subunits were found downregulated (Table 6). In addition, several aminoacyl-transfer RNA (tRNA) ligases such as lysine--tRNA ligase (KARS), methionine--tRNA ligase (MARS) and phenylalanine--tRNA ligase (FARSB) were found downregulated. Aminoacyl-tRNA ligases function in transferring the amino acids to the elongating polypeptide chain.

**Table 6.** Downregulated proteins in groups of translation and aminoacyl-tRNA ligase activity based on DAVID gene ontology tool.

| Biological processes - Downregulated | Proteins included in the group |
|---|---|
| Translation | RPL10, RPL13, RPL13A, RPL14, RPL19, RPL27, RPLP0, RPL4, RPL6, RPS10, RPS18, RPS24, RPS7, NACA, NARS |
| Aminoacyl-tRNA ligase activity | KARS, MARS, NARS, FARSB |

The proteome level data suggested that PHD3 may regulate the ribosomal subunits and translation machinery in ccRCC cells, as the annotated proteins included several ribosomal subunits and other proteins of the protein synthesis machinery. In addition to the protein production, a related group of proteins that responded to PHD3 knockdown was mRNA processing. Heterogeneous nuclear ribonucleoproteins (hnRNPs) D, H, L and G, that are essential post-transcriptional regulators, were found upregulated in PHD3 silenced cells (I, Fig. 4A). hnRNPs function mainly in mRNA processing, such as alternative splicing, mRNA stability and decay.

Ribosomal proteins and other components of the translation machinery belong to the group of highly regulated TOP (5' Terminal Oligo Pyrimidine motif) mRNAs. According to literature, TOP mRNAs are directly regulated by the phosphorylation of p70 S6 kinase (p70 S6K) and its target S6 ribosomal protein, which are well-known downstream effectors of mechanistic target of rapamycin (mTOR) (Ruvinsky and Meyuhas, 2006). Interestingly, phosphorylation of both p70 S6K (T389) and S6 ribosomal protein (S235/236 and S240/244) were downregulated upon PHD3 silencing (Fig. 5A-B). Also total p70 S6K protein levels were slightly downregulated as a result of PHD3 knockdown, suggesting that both the expression level and activation of p70 S6K might be regulated by PHD3. These results led to the

conclusion that PHD3 may be involved in the regulation of mTOR downstream signaling and subsequently in the regulation of translational machinery components (Figure 8). However, the detailed mechanism behind the regulation remains to be investigated.


**Figure 8.** A schematic presentation of PHD3 in regulation of mTOR downstream signaling and maintaining translational machinery components. PHD3 was shown to affect the activity of mTOR downstream signaling activation, but the mechanism and target of PHD3 remains to be investigated. Enhanced mTOR signaling activity by PHD3 could result in promotion of protein expression of several translational machinery components, such as ribosomal protein subunits. Abbreviations: PHD3 – HIF prolyl hydroxylase 3, mTOR – mechanistic target of rapamycin, p70 S6K – p70 S6 kinase, S6 – S6 ribosomal protein, P – phosphate group.

## 5.2 PHD3 promotes cell cycle progression via cell cycle inhibitor p27 (I, II)

Previous studies have shown that PHD3 silencing leads to a cell cycle arrest accompanied with reduced phosphorylation of retinoblastoma protein RB and decrease in cyclin D1 protein expression in human cervical cancer cell line HeLa and in primary human head and neck squamous cell carcinoma (HNSCC) cells. Both reduced phosphorylation of RB and reduced expression of cyclin D1 are indicators of cell cycle arrest at the G1/S boundary (Högel *et al.*, 2011). Motivated by these previous results, ccRCC cells were studied for cell cycle regulation by PHD3. Indeed, cell cycle analysis showed that PHD3 silencing leads to G1 arrest in 786-O cells (I, Fig. S3A and II, Fig. 1A-B) as well as in RCC4 cells (I, Fig. S3A). Interestingly, in ccRCC cells, the G1 arrest was found even more prominent than in HeLa and HNSCC cells.

In HeLa cells, the cell cycle block was accompanied with an elevated expression of the cyclin-dependent kinase inhibitor p27. Similarly, a major upregulation in p27 protein expression was detected in 786-O cells. In keeping with this, simultaneous depletion of both PHD3 and p27 restored the cell cycle and cell growth, indicating that PHD3-mediated cell cycle block is dependent on p27 protein expression (II, Fig. 2A-D). The increase in p27 protein expression was found to be specifically regulated by PHD3, as neither PHD1 nor PHD2 silencing had an effect on p27 protein levels (II, Fig. 1C). Also, the role of hydroxylase activity was studied by using panhydroxylase inhibitor dimethyloxalylglycine (DMOG). No effect on p27 protein expression was detected after 6 hours or 24 hours with DMOG treatment suggesting that the upregulation of p27 is not dependent on the hydroxylase activity of PHD3 (II, Fig. S2B).

To further study the level of regulation, mRNA expression of p27 was studied in PHD3 depleted cells. In hypoxic HeLa cells, no difference on p27 mRNA level between control and PHD3 knockdown cells was detected (II, Fig. 3A). However, in 786-O cells, PHD3 silencing leads to a moderate upregulation of p27 also in transcription level (data not shown). In hypoxic HeLa cells, the p27 mRNA expression level was also confirmed as HIF-independent. Double knockdown with siPHD3 and either HIF-1α or HIF-2α targeted siRNA had no effect on p27 mRNA (II, Fig. 3B). Furthermore, neither HIF-1α nor HIF-2α silencing could rescue the upregulation of p27 protein expression by siPHD3 (Fig. 3C and D) that suggests p27 regulation in HIF-independent manner. In line with this, as the cell cycle arrest was observed in 786-O cells that lacks the functional HIF-1α protein (II, Fig. 1A), the results suggest that the PHD3-mediated p27 upregulation is not HIF-dependent.

### 5.2.1 Cell cycle regulation is mediated by p27 protein stability (II)

To study the dynamics of p27 protein regulation, cell synchronization was used to study p27 protein expression in different phases of cell cycle. siRNA-treated cells were synchronized to G0 by serum starvation or to S phase with aphidicolin. After the release from G0 or S, p27 degraded steadily in control cells, whereas in PHD3-silenced cells, the p27 protein appeared more stable (II, Fig. 4A and Fig. S3). In 786-O cells, the difference in p27 expression after release from the serum starvation (G0 arrested cells) was even more prominent (II, Fig. 4A and B).

To further investigate the regulation of p27, cycloheximide (CHX) chase was used to study the dynamics of protein decay. HeLa cells were synchronized either at G0 or S phase and p27 protein stability was followed for six hours after inhibition of the protein synthesis. As expected, the p27 protein level rapidly declined after CHX treatment indicating a fast turnover of p27 protein. In PHD3 silenced cells, however, the p27 protein levels remained up to several hours indicating a more stable p27 protein (II, Fig. 4C-D). At six hours after CHX treatment, the p27 expression was still markedly elevated in PHD3 silenced cells when compared to control cells arrested at G0 (II, Fig. 4C-D). In line with this data, p27 protein stability after S phase arrest was markedly increased upon PHD3 depletion (II, Fig. S3C-D). The data demonstrated that PHD3 silencing leads to more stable p27 protein and that the effect is independent of the cell cycle phase.

p27 is known to be regulated by phosphorylation. Thus, four phosphorylation sites of p27 were studied with PHD3 knockdown at different cell cycle phases. Phosphorylation of threonine residues 187 (T187), 198 (T198) and 157 (T157) were not significantly affected by PHD3 knockdown (II, Fig. 5A-E). Similarly, no changes were detected in the expression level of p27 regulating ubiquitin ligase Skp2 nor in p27 ubiquitylation (II, Fig. S4). However, the phosphorylation of serine 10 (S10) was markedly increased by PHD3 depletion both in HeLa and 786-O cells and both in G0 and G1 phases (II, Fig. 5C-E, Fig. 6A). S10 phosphorylation site is responsible for regulation of p27 stability, which is in line with the protein decay studies.

To further investigate the role of PHD3 on S10 phosphorylation of p27, 786-O cells were synchronized to reside on G0 and S10 phosphorylated p27 expression was monitored up to 8 h after cell cycle release. Similarly to the total p27 protein level, degradation of the S10 phosphorylated p27 was impaired in PHD3 silenced cells (II, Fig. 6B). Finally, the decay of the S10-deficient mutant p27 (p27S10A) protein was compared to the decay of wild type p27 (p27wt) in PHD3-silenced cells treated with CHX. The results showed that PHD3 silencing did not induce p27 when S10 phosphorylation site is removed. PHD3 depletion attenuated the decay of p27wt but not that of p27S10A (II, Fig. 6D). The results confirmed that PHD3 silencing leads to an increase in p27 stability by affecting the phosphorylation of S10 (Figure 9).

To summarize, the results indicated that high PHD3 expression in carcinoma cells results in decrease in the protein stability of the cell cycle inhibitor p27. The stability

of p27 protein is regulated via phosphorylation of S10, and thus PHD3 enhances the cell cycle progression in ccRCC cells and other carcinoma cell types.

## 5.3 PHD3 enhances proliferation and colony growth in ccRCC (I)

In addition to mechanisms of cell cycle progression, further validations were performed on cell proliferation. In order to study the physiological relevance of PHD3 expression in ccRCC cells, we assessed two dimensional (2D) proliferation of ccRCC cells and colony growth in three dimensional (3D) matrix in cells exposed to PHD3 knockdown. 2D cell proliferation was studied using IncuCyte, which determines the proliferation rate of monolayer cells from images acquired in a certain time intervals. PHD3 depletion reduced the proliferation in both ccRCC cell lines studied and even more prominent reduction was seen in RCC4 cells (I, Fig. S3B). Moreover, apoptosis determined as the proportion of sub-G1 cells in cell cycle analysis was not significantly changed in siPHD3-treated cells as compared to control cells (I, Fig. S3C), which suggests that cells exposed to PHD3 knockdown are not driven to controlled cell death. Furthermore, using Matrigel® as a 3D matrix in colony growth assay, PHD3 silencing resulted in significant reduction in colony size in both ccRCC cell lines used (I, Fig. 5C). These findings suggest a crucial role for high PHD3 expression in enhancing ccRCC proliferation and colony formation. Figure 9 summarizes the findings demonstrating the effect of PHD3 in promoting cell cycle progression and thus proliferation.

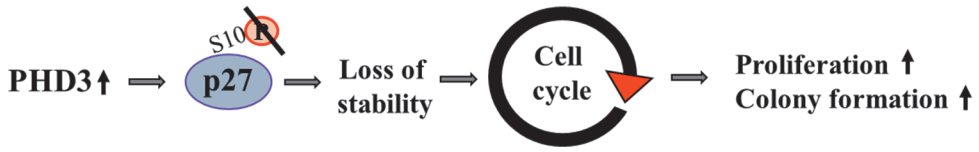


**Figure 9.** A graphic summary of PHD3 regulating proliferation and cell cycle progression via the cell cycle inhibitor p27 protein. PHD3 affects p27 protein phosphorylation at serine 10 (S10) and thus leading to unstable p27 protein. Reduction in p27 protein levels promotes cell cycle progression at G1/S boundary and results in increased proliferation and colony formation in 3D matrix. Abbreviations: PHD3 – HIF prolyl hydroxylase 3, S10 – serine 10, P – phosphate group.

## 5.4 PHD3 silencing attenuates cell migration in ccRCC (unpublished)

To further study the role of PHD3 in cancer progression, 2D cell migration was studied by using a scratch wound assay. In the scratch wound assay, confluent layer of cells is scratched with a pipette tip, and cell migration is determined as a wound closure. Cells were exposed to siRNA-mediated PHD3 knockdown, grown to full confluency and scratched. Aphidicolin (1 μg/ml) was used as a proliferation inhibitor to rule out any differences in cell proliferation during the assay. In 786-O cells, PHD3 knockdown significantly reduced cell migration measured at the 12 hour time-point, whereas PHD2 silencing had no effect (Figure 10). PHD1 silencing also affected cell migration, but PHD1 knockdown also induced significant change in the cellular morphology (Figure 10). PHD1 knockdown had been shown to lead to cell cycle arrest in mitosis (M phase) (Moser *et al.*, 2013) and thus, it is not clear if the observed effect is due to reduced cell migration upon PHD1 silencing or it the cells are facing programmed cell death due to severe cell cycle arrest. A similar effect of PHD3 silencing on cell migration was seen in a preliminary experiment with RCC4 cells (Figure 11).

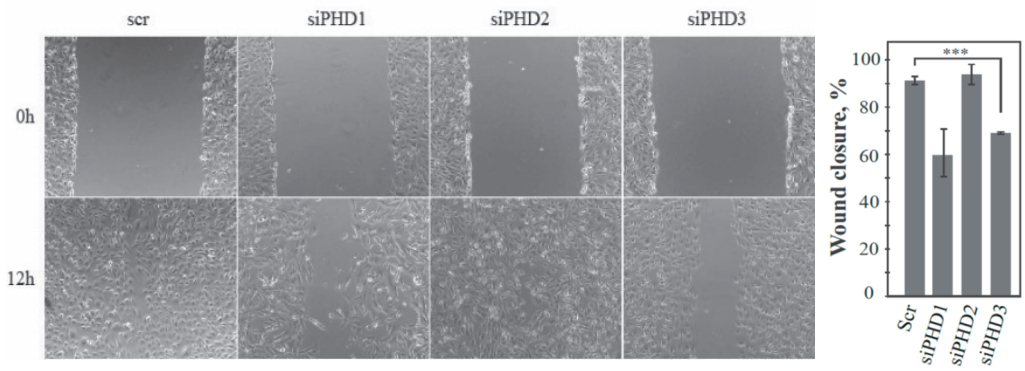


**Figure 10**. 786-O cell migration with silencing of PHD1, PHD2 or PHD3. Representative images were acquired with an EVOS Digital inverted microscope and quantification was performed with Image J. Quantification of four individual experiments, *** marks for $p<0.001$ (Paired, two-tailed t-test).

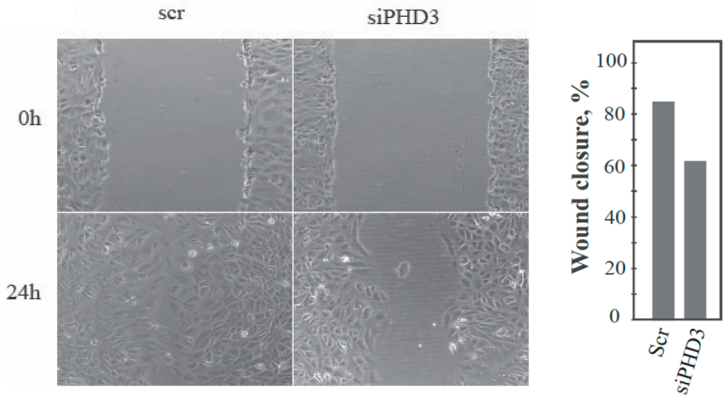


**Figure 11**. RCC4 cell migration with PHD3 silencing. Representative images were acquired with an EVOS Digital inverted microscope and quantification was performed with Image J. Quantification of a preliminary experiment.

The presented data suggest that PHD3 regulates cell migration. Also, according to our proteome level studies several cytoskeletal proteins, and proteins involves in attachment to ECM and cell migration were affected by PHD3 silencing (I, Table 1

and 2). An especially interesting protein considering our observation on migration was integrin β1. Integrin β1 is one of the most commonly expressed integrin family member that mediates cellular attachment to the surroundings via binding to the actin cytoskeleton inside the cell and to the ECM components outside the cell (Hamidi *et al.*, 2016). PHD3 silencing resulted in downregulation of integrin β1 in LC-MS/MS data but the result was also validated with western blot in both 786-O and RCC4 cells (I, Fig. 1D). In immunoblots integrin β1 occurs as a double band, the lower one representing nascent form and the upper band mature, full length form. In RCC4 cells downregulation was seen in both nascent and mature forms while in 786-O cells the mature form of integrin β1 was decreased. It may be that reduced 2D cell migration and the downregulation of integrin β1 in PHD3-depleted ccRCC cells are linked together, but the direct mechanism remains to be studied.

## 5.5 PHD3 maintains HIF-2α mRNA expression in ccRCC cells (III)

When using ccRCC cell lines, we observed a consistent downregulation of HIF-2α protein levels both under normoxia and under hypoxia with PHD3 silencing. The downregulation was evident in both 786-O and RCC4 cells, (III, Fig. 1C-E). This was an interesting finding, as the previous studies show that PHD3 depletion leads to an upregulation of HIF-2α protein expression in some cell lines. Hence, it was tested whether other cell lines respond in a similar fashion to PHD3 depletion as ccRCC cells. Human primary head and neck squamous cell carcinoma cells (UT-SCC-34) showed a massive increase in HIF-2α protein expression upon silencing by siPHD3 (III, Fig. 1F). Further, breast cancer cells (T47D) showed a moderate upregulation of HIF-2α protein upon PHD3 depletion (III, Fig. 1F). The HIF-2α downregulation seemed to occur in ccRCC cells as a response to PHD3 silencing but not in all cell lines. HIF-1α protein level was not affected by PHD3 silencing (III, Fig. 1C).

To further study HIF-2α regulation, HIF-2α protein stability was investigated. Cycloheximide (CHX) was used to study the post-translational decay of HIF-2α. No change in HIF-2α protein stability was observed upon PHD3 knockdown as

compared to the control (III, Fig. 2C). The result suggests that the regulation of HIF-2α expression does not occur at a post-translational level. To scrutinize the effect of PHD3 on HIF-2α, we next studied the *HIF2A* mRNA expression. Indeed, significant downregulation of *HIF2A* mRNA expression was observed with PHD3 depletion in both 786-O and RCC4 cells (III, Fig. 3A). PHD2 depletion had no effect on the expression of *HIF2A* (III, Fig. 3A). Moreover, the expression of *HIF1A* was not changed upon PHD3 depletion (III, Fig. 3B).

To continue investigating the *HIF2A* transcriptional regulation, we used inhibitors of transcription (Actinomycin D) and protein synthesis (CHX) to determine the mRNA stability of *HIF2A*. Interestingly, PHD3 depletion together with the transcriptional inhibitor Actinomycin D resulted in an even further decrease of *HIF2A* (III, Fig. 4A) suggesting that mRNA stability could be affected by PHD3 silencing. Also a significant increase in *HIF2A* expression was detected in PHD3 depleted cells after CHX treatment (III, Fig. 4A). CHX is known to stabilize labile mRNAs (Ohh and Takei, 1995; Newton *et al.*, 1997). To study the dynamics of the *HIF2A* decay, we used Actinomycin D chase until four hours. Rapid decrease in expression of *HIF2A* was observed in siPHD3 treated cells, whereas in the controls, *HIF2A* expression was only slightly decreased (III, Fig. 4B). In support of these data, *HIF2A* mRNA decay is enhanced upon PHD3 silencing. The results are demonstrating a novel regulation of *HIF2A* mRNA level by PHD3.

### 5.5.1 The effect on HIF-2α is specific for PHD3 and independent on hydroxylase activity (III)

In order to study the specificity of PHD3 on HIF-2α expression, silencing of other PHD family members, PHD1 and PHD2 was performed in 786-O cells. No decrease in HIF-2α protein expression was detected with PHD1 or PHD2 depletion (III, Fig. 2A). Also, PHD2 depletion had no effect on *HIF2A* mRNA expression in 786-O and RCC4 cells (III, Fig. 3A). Furthermore, PHD2 silencing had no effect on *HIF2A* mRNA decay (III, Fig. 4B). These results indicate that the downregulation of HIF-2α protein and mRNA expression is specific to PHD3.

We also investigated if the hydroxylase activity of PHDs is responsible for the downregulation of HIF-2α by using a panhydroxylase inhibitor DMOG. No effect on HIF-2α protein expression was detected after exposing the cells to DMOG

neither under normoxic nor hypoxic conditions in 786-O and RCC4 cells (III, Fig. 2B). However, in RCC4 cells stable transfected with VHL (RCC4+*VHL*) we observed an expected increase in both HIF-2α and HIF-1α protein levels in response to DMOG treatment, which serves as a technical control for the experiment. The result suggests that the hydroxylase activity of PHD3 is not directly responsible for HIF-2α downregulation.

### 5.5.2 PHD3 enhances *GLUT1* and *LDHA* expression via HIF-2α (III)

We had identified several enzymes of the glycolytic pathway and glucose transporter 1 (GLUT1) to be downregulated by PHD3 silencing on protein level by using discovery proteomics screening and label-free quantification method. As it is well established that the glycolytic enzymes and GLUT1 are regulated by HIF-α in ccRCC cells, we were interested to know if the downregulation of the glycolytic pathway enzymes is occurring in protein level or if there is a decrease also in mRNA expression. Indeed, when studying GLUT1 and LDHA expression, a significant reduction in expression levels of both genes was seen in PHD3 depleted 786-O and RCC4 cells (III, Fig. 1A). As both cell lines express HIF-2α and only RCC4 expresses functional HIF-1α, we studied if HIF-2α is mediating the effect of PHD3 on *GLUT1* and *LDHA* expression. By using a transient plasmid transfection to overexpress *HIF2A*, both *GLUT1* and *LDHA* expression levels were restored in comparison to the empty vector (III, Fig. 3C). In line with the expression level results, also LDHA protein expression was restored upon forced expression of *HIF2A* on PHD3 depleted cells (III, Fig. 3E). The result indicates that the transcription factor HIF-2α mediates the effect of PHD3 on *GLUT1* and *LDHA* expression (Figure 12).

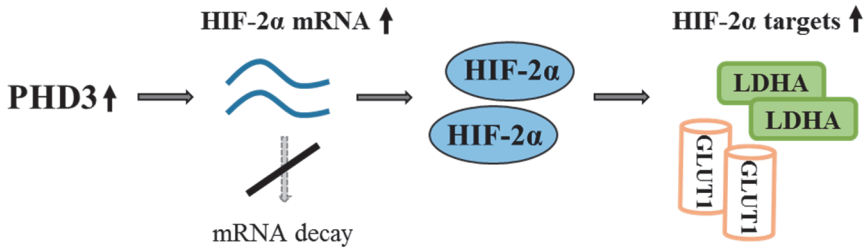


**Figure 12.** A graphic summary showing regulation of HIF-2α mRNA stability and HIF-2α target gene expression by PHD3 in ccRCC cells. High PHD3 maintains HIF-2α mRNA that leads to high HIF-2α protein and target gene GLUT1 and LDHA expression. The detailed mechanism of HIF-2α mRNA stability regulation remains to be investigated. Abbreviations: PHD3 – HIF prolyl hydroxylase 3, HIF-2α – hypoxia-inducible transcription factor 2α, GLUT1 – glucose transporter 1, LDHA – lactate dehydrogenase A.

## 5.6 *PHD3* and *HIF2A* expression correlates in ccRCC tumor samples (III)

The expression levels of HIFs and PHDs were studied in a clinical ccRCC data set by TCGA (Cancer Genome Atlas Research Network, 2013). From the total of 442 tumor samples, TCGA provided normal adjacent tissue samples for 71 patients. Comparative analysis of the tumor samples and their corresponding healthy tissues revealed a significant downregulation of *HIF1A* and upregulation of *HIF2A*, *PHD2* and *PHD3* in ccRCC tumors (III, Fig. 5A). In addition, the correlation between HIFs and PHD1-3 genes was studied in the whole data set (442 samples). *HIF2A* and *PHD3* presented the highest correlation among the studied genes (Pearson correlation 0.196, $p$ value < 0.001) (III, Fig. 5D). Considering the high level of heterogeneity in ccRCC, the data set was narrowed to patients with poor prognosis in order to investigate the correlation in a more homogeneous group of patients. The correlation was re-analyzed by using 40 patients with poor prognosis (survival < 12 months), and even stronger correlation between *HIF2A* and *PHD3* expression levels (Pearson correlation 0.526, $p$ value < 0.001) was observed (III, Fig. 5E), whereas the expression of other PHD family members did not significantly correlate with *HIF1A* or *HIF2A* expression (III, Fig. S3B).

# 6 DISCUSSION

## 6.1 Identification of novel roles for PHD3 in ccRCC

PHD3 is highly expressed in ccRCC tumors and cell lines, but the functions of PHD3 in ccRCC cells are not well known. Proteomics tools are powerful and relatively sensitive methods to study proteome level changes and the approach yields vast amount of data. I used discovery proteomics approach to investigate proteome level changes in response to PHD3 silencing in ccRCC cells. A label-free quantification method and statistical analysis was applied in order to determine differential expression of proteins in PHD3 silenced samples as compared to control samples. Interestingly, 91 proteins were found statistically differentially expressed in PHD3 silenced samples (I, Table 1 and 2).

Gene ontology (GO) analysis was applied to the statistically differentially expressed proteins (I, Table 1 and 2), which revealed several biological processes that were responsive to PHD3 knockdown, such as glucose metabolism, protein translation, mRNA processing, apoptosis, protein modification, intracellular transport and actin binding (I, Fig. 2). Interestingly, the proteins annotated in the aforementioned groups are mostly previously unknown targets of PHD3 while some of the biological processes are previously linked to PHD3. As an example, glucose metabolism has been shown to be affected by PHD3 knockdown (Luo *et al.*, 2011) and also apoptosis and PHD3 have been previously linked by several reports (Jaakkola and Rantanen, 2013; Place and Domann, 2013). Moreover, protein transport was identified as one of the PHD3-responsive processes and PHD3 has been previously linked in regulation of kinesin family member 1B (KIF1Bbeta), a motor protein that is involved in cellular organelle and vesicle transport (Schlisio *et al.*, 2008). Interestingly, also KIF1B has been shown to be involved in apoptosis and especially in clearance of excess neurons during development (Schlisio *et al.*, 2008). Hence, via KIF1Bbeta PHD3 is linked to both regulation of protein transport and apoptosis.

Furthermore, PHD3 depletion resulted in differential expression of actin binding proteins which are known to maintain both structure and function of actin

cytoskeleton and thus are key factors in cell migration. PHD3 has been previously shown to regulate cytoskeleton and cellular morphology via direct hydroxylation of actin and via regulation of focal adhesion kinase (FAK) (Su *et al.*, 2010; Luo *et al.*, 2014). The studies also demonstrated that PHD3 participates in the regulation of cell migration.

To summarize, the results of the proteomic screen show an intriguingly widespread effect of PHD3 in ccRCC cells, as it revealed a number of proteins responsive to PHD3 and also gave hints of the biological processes in which PHD3 expression is needed. However, it is possible that the identified proteins are not direct targets of PHD3, but secondary effects responsive to upstream or downstream effectors. Moreover, the study would have gained more impact if the initial screen would have been conducted with several ccRCC cells lines. Importantly, several proteins and biological endpoints revealed by the proteome level study were validated in second ccRCC cell line (I, Fig. 1, Fig. 3, Fig. 5). The results are indicating that the functions of PHD3 are not restricted to the single ccRCC cell line used in the screening. Nonetheless, the screen provides a starting point for further investigations extending to other ccRCC cell lines and beyond. In one aspect, it would also be interesting to study these proteins and functions in ccRCC cells with wild type *VHL* to further explore the role of PHD3 in more general in ccRCC.

The results of the screen provide a valuable first observation but requires further verification and complementary studies, as the protein expression level data is not fully related to the functional level of the cellular events. The functionality and signal transduction is largely achieved by several post-translational modifications, such as phosphorylation, ubiquitylation, acetylation, SUMOylation and hydroxylation. Thus, the protein expression level results describe a single level of regulation but many other levels remain unrevealed. By the selected approach, we were not able to assess changes in activation or inactivation of certain pathways, and thus complementary screens focusing on different post-translational modifications upon PHD3 silencing would be needed to further understand the role of PHD3 in certain pathways.

Also, the method used in the current study is not applicable for detecting very low abundant proteins in complex samples. To gain more detailed insights into certain proteins of interest, targeted quantitative methods could be employed for detecting low abundance proteins with more precise means of quantification. Future studies should be pinpointed to the processes and pathways revealed by the current

screening and to be extended to numerous proteins that remained undetected or did not match the criteria in terms of statistical analysis. A major interest for future studies would be to investigate the detailed role of PHD3 in regulating protein translation and mRNA processing, as neither of these processes have been previously linked to PHD3 and the group of proteins identified in the current study might contain novel interesting PHD3 target proteins.

## 6.2 PHD3 as a regulator of glucose metabolism in ccRCC

Glycogen and lipid storages are abundant in ccRCC tumors, which implies altered glucose and fatty acid metabolism (Gebhard *et al.*, 1987; Schodel *et al.*, 2016; Hsieh *et al.*, 2017). Furthermore, increased expression of glycolytic enzymes in ccRCC cells and other factors related to metabolic activity, such as glucose transporters, have been demonstrated by several reports (Cancer Genome Atlas Research Network, 2013; Semenza, 2013; Minton *et al.*, 2015; Soltysova *et al.*, 2015; Hakimi *et al.*, 2016). It is generally accepted that ccRCC cells shift their metabolism towards aerobic glycolysis.

In my studies, several glycolytic enzymes were found significantly downregulated in response to siRNA-mediated PHD3 silencing. On the other hand, enzymes involved with branching pathways, such as G6PD and TALDO1 for pentose phosphate pathway and GFPT1 in the hexosamine pathway were found upregulated (I, Fig. 3A-B). Similarly, also tricarboxylic acid cycle enzymes MDH2 and IDH1 were upregulated in response to PHD3 knockdown (I, Fig. 3A-B). The proteome level results were complemented with functional analysis on metabolism. In line with the suppression of glycolytic enzymes, lactate production and extracellular acidification was found decreased and oxygen consumption rate increased (I, Fig. 3C-F). The results imply that high PHD3 expression in ccRCC cells maintains high glycolytic activity and increased lactate production, while resulting in a decrease in oxidative phosphorylation in mitochondria.

LDHA has a key role in lactate production, as the lactate dehydrogenase enzymes functions in converting pyruvate into lactate bi-directional manner (Doherty and Cleveland, 2013). Based on proteome level results lactate dehydrogenase isoform B,

LDHB, was found upregulated in response to PHD3 silencing. This is interesting, as the different isoforms are responding differently. However, both results are in line with the reduced lactate concentration upon PHD3 silencing, since LDHA has converts pyruvate to lactate whereas LDHB from lactate to pyruvate. It can be suggested that PHD3 participates in regulation of cellular pyruvate and lactate levels by having opposite effect on the crucial enzyme isoforms.

LDHA has shown to be overexpressed in cancers and it has been suggested to have a crucial role in cancer cell proliferation, invasion, and metastasis (Doherty and Cleveland, 2013). Moreover, tumor acidification by increased production and secretion of lactate is an emerging area of interest in cancer biology. Tumor acidification has been shown to promote tumor inflammation and to activate vascularization, and increased lactate levels to correlate with metastasis and poor patient outcome (Vaupel, 2010; Doherty and Cleveland, 2013). Thus, the results imply that high PHD3 expression seen in many cancers might have a key role in microenvironmental pH.

The presented results are in line with the previously reported effects for PHD3 knockdown in human cancer cells. In the report by Luo *et al.* it is shown that hydroxylation of pyruvate kinase isoform M2 (PKM2) by PHD3 enhances the binding of PKM2 and HIF-1α. The binding promotes the transcriptional activation of HIF-1α and induction of several genes encoding proteins of the glycolytic pathway. They also show that shRNA-mediated silencing of PHD3 leads to downregulation of LDHA, GLUT1 and pyruvate dehydrogenase kinase 1 (PDK1) at both mRNA and protein level in RCC4 cells (Luo *et al.*, 2011). Moreover, similar downregulation in lactate concentration and upregulation on oxygen consumption rate (OCR) was reported. However, whereas Luo *et al.* suggested the effects of PHD3 to be mediated through HIF-1α, my results show HIF-1-independent functions for PHD3 in glucose metabolism. In our study, we used 786-O ccRCC cells that do not express functional HIF-1α protein together with RCC4 cells, and thus it seems that HIF-1α is unlikely the sole downstream mediator of PHD3 depletion.

Stimulated by our results and the data by others, we studied if PHD3 silencing in 786-O and RCC4 cells leads to downregulation of glycolytic enzymes in mRNA expression level. Indeed, I found that GLUT1 and LDHA mRNA levels were downregulated in response to PHD3 silencing in ccRCC cells (III, Fig. 1A-B). Furthermore, as I had also found a novel negative regulation of HIF-2α by PHD3

in ccRCC cells, I wanted to investigate if decreased HIF-2α levels upon PHD3 silencing mediate the effect on glycolysis related genes. Interestingly, the expression of *GLUT1* and *LDHA* were restored when overexpressing HIF-2α in PHD3 depleted cells (III, Fig. 3C-D). This suggested that HIF-2α mediates the downregulation of at least certain proteins involved in glycolysis pathway. Also, the result was in line with the literature, as HIF-2α have been shown to regulate *GLUT1* and *LDHA* (Maxwell *et al.*, 1999; Hu *et al.*, 2003; Raval *et al.*, 2005; Cui *et al.*, 2016). However, in the early literature considering the unique targets of HIF-1α and HIF-2α, HIF-1α has been characterized as a master regulator of glycolytic enzymes in ccRCC (Hu *et al.*, 2003). It remains to be studied if HIF-2α mediates the downregulation of other glycolytic enzymes upon PHD3 knockdown and if forced expression of HIF-2α could restore the extracellular lactate levels. This could provide a direct link between PHD3 and enhanced glycolytic activity in ccRCC cells.

In addition to PKM2, PHD3 has been reported to interact with the pyruvate dehydrogenase (PDH) complex and to regulate the PDH complex stability in breast cancer cells and in mouse embryonic fibroblasts (MEFs). PDH is an enzyme that catalyzes the conversion of pyruvate to acetyl-co-A, which is an intermediate between glycolysis and TCA-cycle, but also an intermediate for several other biosynthetic pathways. PHD3 depletion was shown to reduce the activity of PDH complex, which can be deduced to result in reduced conversion of pyruvate into acetyl-co-A (Kikuchi *et al.*, 2014). Interestingly, we did not detect any protein expression level changes in PKM2 and PDH complex proteins (PDH-E1α, E1β and E2), while numerous glycolytic proteins were found to response to PHD3 silencing. However, both reports by Luo *et al.* and Kikuchi *et al.* show that PHD3 knockdown has no effect on protein expression, but the activation of PKM2 and in stability of PDH complex, respectively.

On the strength of the several studies investigating the role of PHD3 in glucose metabolism, it can be concluded that in overall the results are supporting each another and they are not conflicting in regards to our data on PHD3 silencing. However, several details remain to be investigated, as the current data shows decreased conversion of pyruvate to lactate, but also the conversion of pyruvate to acetyl-co-A is likely to be impaired. Hence, it would be interesting to study the levels of pyruvate and other glycolytic intermediates as well as the fate of these intermediates in cancer cells upon PHD3 silencing.

Based on my results and data presented by others, I'm proposing in this thesis that PHD3 is a key factor regulating glucose metabolism and lactate production in ccRCC cells. While several mediators of PHD3 have been previously reported, there might also be yet unknown factors linking PHD3 to the regulation of glucose metabolism. Moreover, as several targets independently of HIFs have been reported for PHD3, it is possible that the metabolic effect demonstrated for PHD3 occurs via an upstream effector responsible for overall regulation of cellular energy metabolism.

## 6.3 PHD3 in the control of cell cycle and proliferation

The cell cycle is a precisely regulated process to maintain tissue homeostasis and to prevent uncontrolled growth. The regulation is achieved by timely induction and degradation of cyclin/CDK complexes but also by regulation of CDK inhibitors, such as p27 (Israels and Israels, 2001). Among the major differences between cancer cells and non-malignant cells is the ability to proliferate continuously and to escape from the normal cell cycle control. The escape can be succeeded by overproduction of factors enhancing cell cycle progression and, on the other hand, by suppression of cell cycle inhibitors.

In my thesis work, I have demonstrated that PHD3 silencing leads to cell cycle block and upregulation of cell cycle inhibitor p27 (I, Fig. S3A and II, Fig. 1), decrease in 2D proliferation (I, Fig. S3B) and in 3D colony formation in ccRCC cells (I, Fig. 5C). Furthermore, p27 phosphorylation at S10 was found to be increased in PHD3 depleted cells (II, Fig. 5) and S10 phosphorylation has been previously reported as the major stabilizing phosphorylation of p27 (Ishida *et al.*, 2000; Rodier *et al.*, 2001; Kotake *et al.*, 2005). Thus, high PHD3 expression in ccRCC cells leads to decreased p27 phosphorylation at S10 that subsequently results in loss of p27 protein stability. Together, my results show that high basal PHD3 expression in ccRCC cells enhances cell cycle progression, proliferation and colony formation in 3D matrix, which is achieved at least partly via regulation of p27 protein stability. However, it is possible that also other mechanisms for promoting cell cycle can occur.

S10 phosphorylation of p27 is known to be regulated by several kinases, including human kinase interacting stathmin (hKIS), Akt and ERK2 (Ishida *et al.*, 2000; Boehm

*et al.*, 2002; Fujita *et al.*, 2002). These are potential factors upstream of p27 that could be modulated by PHD3. Also, it is possible that the phosphorylation status of p27 is modified by phosphatase activity, instead of kinase activity. Nonetheless, further studies are needed to determine the upstream factor responsible for modulating p27 phosphorylation at S10.

Transition through the cell cycle is an essential process that has been suggested to be coupled to several other important events, such as metabolism and nutrient availability, translation machinery and attachment to extracellular matrix, among others (Lee and Finkel, 2013; Moreno-Layseca and Streuli, 2014; Polymenis and Aramayo, 2015). As my results indicate that PHD3 modulates several cellular processes in ccRCC cells, including the cell cycle, the translation machinery, glucose metabolism and cell migration, it would be interesting to study if all the processes are linked together via common upstream effector regulated by PHD3. PHD3 is likely not essential factor for all the above-mentioned processes, as we would expect to see more dramatic effect upon silencing a factor ultimately necessary for example in cell cycle progression. However, I'm suggesting that PHD3 is an important modulator of these processes via another factor that is likely to be a central hub in the regulation of cellular behavior and also a promoter of tumorigenesis.

Interestingly, my results also show a reduction in mTOR downstream signaling in PHD3-silenced ccRCC cells. The reduction was seen in phosphorylation of p70 S6K and S6 ribosomal protein, which are direct downstream targets of mTOR complex 1 (I, Fig. 5A-B). The major role of the mTOR pathway is to sense a range of intracellular and extracellular stimuli in order to regulate energy homeostasis and to balance cell growth. The pathway has been shown to regulate several major cellular processes, such as protein translation and ribosome biogenesis, glucose uptake and glycolysis, lipid synthesis, as well as cell growth and cell cycle (Saxton and Sabatini, 2017). Importantly, hyper-activation of mTOR signaling can be found across several cancer types leading to increased growth and enhanced tumor metabolism. The results presented in this thesis suggest that high PHD3 expression in ccRCC cells could maintain mTOR activity and subsequently enhance the mTOR downstream processes. Furthermore, a link between PHDs and mTOR has also been previously suggested (Duran *et al.*, 2013). mTOR exist in two structurally and functionally different complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) (Saxton and Sabatini, 2017) and Duran *et al.* have proposed that PHD enzymatic activity is needed for the activation of mTORC1 by cellular amino acid levels.

It is possible that by modulating the mTOR pathway, PHD3 could have a role in regulation of several cellular processes, as seen based on my study. I'm hypothesizing that the effects of PHD3 are mediated by a central hub responsible for regulation of several events, rather than PHD3 regulating several individual target genes. Moreover, Akt that regulates p27 phosphorylation is also an upstream effector of mTOR, thus making the Akt/mTOR pathway an intriguing candidate for PHD3 target. Within the limits of this thesis, I was unable to determine the specific effect of PHD3 on mTOR signaling, but it would be of great interest to investigate the mechanism in more detail, as mTOR inhibitors are also used in the clinic for treatment of ccRCC (Hsieh *et al.*, 2017).

## 6.4 PHD3 and cell migration

Cell migration is one of the fundamental biological processes indispensable for development and function of an individual. Cell migration is also considered as a crucial step in tumor invasion into the surrounding environment and in the formation of distant metastasis. In the complex multi-step process of migration, actin cytoskeleton dynamics, actin binding proteins and attachment to the surrounding extracellular matrix are essential factors (Yamaguchi and Condeelis, 2007).

My unpublished data shows that PHD3 silencing leads to impaired 2D cell migration in 786-O and RCC4 ccRCC cells (Fig. 10 and Fig. 11). PHD2 knockdown did not have a major effect on cell migration. However, silencing of PHD1 led to major morphological changes in cells and it is known to inhibit mitotic progression by disruption of the mitotic spindle (Moser *et al.*, 2013). Thus, it remained elusive, if the cell migration was hindered in PHD1-depleted cells or if cells were facing programmed cell death due to the cell cycle arrest and major changes in cytoskeletal components. This could have been studied fairly easily by determining cell cycle and population of apoptotic cells exposed to PHD1 knockdown.

Moreover, the discovery proteomics screening revealed number of actin binding proteins (vinculin, fibronectin, tropomyosin, alpha-actinin4 and caldesmon), tubulin subunits and integrin β1 protein expression levels to respond to PHD3 silencing (I,

Fig. 2). Integrin β1 protein expression was further validated in second ccRCC cell line, and the results are convincing that PHD3 silencing decreases the amount of mature integrin β1 (I, Fig. 1D). Together, my results clearly indicate that PHD3 has a role in the regulation of cell migration, attachment and cytoskeleton components in ccRCC cells.

Integrins are crucial links between the actin cytoskeleton and the surrounding extracellular matrix mediating the attachment of the cell to other cells and to ECM components (Hamidi *et al.*, 2016). It remains an interesting question how PHD3 regulates the expression of integrin β1 and if this regulation lies behind the reduced cell migration seen in PHD3-silenced cells. As the reduction was found in the mature form of integrin β1 in 786-O and both nascent and mature form in RCC4 cells, the downregulation could be due to either reduced translation when the nascent levels are affected, by increased degradation of integrin β1 or defect in post-translational modification resulting in disappearance of the mature form. Although the mechanism remains to be investigated, it is possible that the mature integrin β1 is not present on the plasma membrane and does not mediate the attachment of the surroundings to the actin cytoskeleton, thus causing an impaired migration of PHD3-silenced ccRCC cells. Hence, it would be interesting to study the localization of integrin β1 in PHD3-silenced cells by using time-lapse imaging of migrating cells. It is also possible that some of the other actin binding proteins that were found to respond to PHD3 depletion in the screen, or yet unidentified factors, are mediating the effect of PHD3 silencing on cell migration.

PHD3 has also been previously linked to 2D cell migration. On the contrary to my unpublished results on reduced cell migration in ccRCC upon PHD3 depletion, in HeLa cells, PHD3 knockdown has been shown to increase cell migration and actin polymerization. In the same study, it was also shown that non-muscle actin is hydroxylated by PHD3 (Luo *et al.*, 2014). The increase in cell migration was also achieved by inhibition of hydroxylase activity by DMOG, which clearly indicates hydroxylase-dependency. Also in human pancreatic cancer cells and in colon cancer cells PHD3 loss leads to increased cell migration (Place *et al.*, 2013; Radhakrishnan *et al.*, 2016).

Together, these results confirm that there is a mechanism by which PHD3 regulates cell migration and actin cytoskeleton dynamics, and a similar mechanism could occur in ccRCC cells. However, the cell migration is an enormously complex process with

numerous signaling routes and activation cascades. Thus, it is likely that cell-type specific effects occur due to signaling pathway crosstalk. The cell lines used in the studies by other groups have different characteristics than ccRCC cells. As an example, human colon cancer cells have lowered PHD3 expression in comparison to normal colon epithelial cells, whereas ccRCC cells have high basal expression of PHD3. In addition to differential PHD3 expression, also activation and suppression of certain signaling routes might be completely different, thus leading to great differences in phenotype and cellular behavior when it comes to depleting PHD3. Based on the presented data, I'm hypothesizing that PHD3 could regulate or modify a certain factor that can act as a switch in cell migration. Depending on the conditions, knockdown of PHD3 would lead to either enhanced or impaired cell migration. Also, it remains to be studied whether PHD3 silencing affects cell migration and invasion in 3D models.

## 6.5 Novel regulation of HIF-2α by PHD3

Traditionally, the HIF prolyl hydroxylase enzymes (PHDs) has been characterized as key regulators of HIF-α isoforms by post-translational hydroxylation reaction in an oxygen-dependent manner. Hydroxylation is a transient modification that subsequently leads to ubiquitination and proteasomal degradation of HIF-α. This mechanism provides a basis for cellular oxygen sensing and adaptation to lowered oxygen levels, as the regulation of HIF-α stabilization or destabilization can be achieved within minutes in response to available molecular oxygen in the cells (Ivan and Kaelin, 2017). Different PHD isoenzymes have generally been thought to bring flexibility to the system, by their differential affinity towards oxygen as well as towards different HIF-α isoforms. Several reports have shown PHD3 acting as a major negative regulator of HIF-2α in different cell types, and that knockdown of either expression or activity of PHD3 results in major upregulation HIF-2α (Berra *et al.*, 2003; Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004).

In my studies, PHD3 was found to maintain *HIF2A* mRNA expression in ccRCC cells, which represents a novel mechanism of *HIF2A* regulation. PHD3 silencing, but not inhibition of hydroxylase activity, resulted in decrease in HIF-2α protein and mRNA expression (III, Fig. 1C-D, Fig. 2 B and Fig. 3A). When studied further, HIF-

2α mRNA stability was found decreased in PHD3-depleted cells (III, Fig. 4). The results suggest that PHD3 is involved in post-transcriptional regulation of *HIF2A* in ccRCC cells. Furthermore, previously described HIF-2α target genes *GLUT1* and *LDHA* were found decreased upon PHD3 knockdown and forced expression of HIF-2α restored the expression on mRNA and on protein level (III, Fig. 4). Thereby, high PHD3 expression often found in ccRCC leads to enhanced *HIF2A* stability and promotion of HIF-2α target genes. Based on my findings, I'm proposing that high PHD3 expression forms a positive feedback loop in HIF-2α regulation in ccRCC cells, instead of the previously described model of PHD3 limiting HIF-2α expression.

In addition, the clinical relevance of this finding was studied by analyzing the expression levels of HIF and PHD isoforms in ccRCC tumor samples. Positive correlation of *PHD3* and *HIF2A* expression in clinical ccRCC samples was demonstrated and the correlation was found even more pronounced when the data was narrowed to patients with poor prognosis (III, Fig. 5). This further verifies the results from *in vitro* studies on the novel regulation of HIF-2α by PHD3 in ccRCC.

Recently, several mechanisms have been described for HIF-α degradation independently of prolyl hydroxylation and proteasomal degradation (Demidenko *et al.*, 2005; Kong *et al.*, 2007; Bento *et al.*, 2010; Hubbi *et al.*, 2013; Bremm *et al.*, 2014; Liu *et al.*, 2015). Based on these studies, it can be concluded that the regulation of the HIF signaling pathway is far more complex than previously known, involving also lysosomal and autophagosomal degradation in addition to proteasomal decay. Also, studies regarding the HIF-2α transcriptional regulation have emerged in recent years. Transcription factor E2F1 has been shown to directly regulate *HIF2A* expression in several cell lines, including 786-O ccRCC cell line. Moreover, deubiquitylase Cezanne was linked to the regulation of *HIF2A* by the regulation of E2F1 stability by Cezanne (Moniz *et al.*, 2015). In other cell lines, Also Poly (ADP-ribose) polymerase-1 (PARP-1), c-Jun N-terminal protein kinase 2 (JNK2) and mechanistic target of rapamycin complex 2 (mTORC2) has been shown to regulate *HIF2A* expression (Gonzalez-Flores *et al.*, 2014; Mohlin *et al.*, 2015; Sala *et al.*, 2017). These findings clearly indicate that the transcriptional regulation of HIF-2α is complex and sensitive to many different stimuli and that also tissue specific regulation is likely to occur.

Previous literature has described a mechanism of post-transcriptional regulation for *HIF1A* but not for *HIF2A*. RNA binding proteins HuR and PTB have been demonstrated to directly bind to *HIF1A* transcript and to regulate the translation of *HIF1A*. Moreover, post-transcriptional control of hypoxia-inducible mRNAs has been suggested as an important regulatory step under hypoxic condition (Schepens *et al.*, 2005; Galban *et al.*, 2008). It would be interesting to study if motifs for known RNA-binding proteins (RBPs) occur in *HIF2A* sequence. It would be fairly easy to study the possible motifs *in silico*, by using a software that predicts the binding of certain RBPs to the *HIF2A* sequence, following by detailed molecular biology analyses of suitable candidate RBPs. In this thesis work, I also demonstrated that PHD3 knockdown in ccRCC cells leads to upregulation of several mRNA processing factors, including hnRNPs D, H, L and G (I, Fig. 2A and Fig. 4). hnRNPs function in regulation of different steps of mRNA maturation and mRNA stability. It is possible that the regulation of HIF-2α mRNA stability is mediated by some of these factors or some other known RNA-binding proteins.

High HIF-2α expression increases cancer cell proliferation and tumor growth in *in vivo* models (Biswas *et al.*, 2010; Kondo *et al.*, 2003; Maranchie *et al.*, 2002; Raval *et al.*, 2005; Shen *et al.*, 2011). In line with the results from *in vivo* tumors models, a recent clinical phase 1 trial demonstrated clinical benefit for the inhibition of HIF-2α in patients with advanced ccRCC (Courtney *et al.*, 2017). Based on these findings, it is clear that HIF-2α has a key role in ccRCC tumorigenesis and progression. Within the limits of this thesis I was unable to study if HIF-2α mediates the reduced 2D cell proliferation and 3D colony formation seen upon PHD3 knockdown, but based on the established role in ccRCC growth, HIF-2α would be an interesting candidate to link the reduced ccRCC cell growth and PHD3 silencing.

In conclusion, my results show a novel regulation of *HIF2A* expression by PHD3 both *in vitro* and *in vivo* in clinical tumor samples. The results suggest that PHD3 may contribute to ccRCC growth and progression by regulation of HIF-2α and its tumor promoting target genes. Further studies are needed to determine the specific mechanism by which PHD3 regulates HIF-2α mRNA stability and if the regulation occurs also in other cell types than ccRCC cells.

# 7 SUMMARY AND CONCLUSIONS

In this thesis I studied the role of HIF prolyl hydroxylase enzyme PHD3 in ccRCC cells. The ccRCC tumors and derived cell lines with inactivated pVHL express high levels of PHD3 but we lack knowledge of the functions of PHD3 in ccRCC other than oxygen-dependent regulation of hypoxia-inducible factors HIFs. PHD3 has previously been described to play a role in the regulation of cell survival, apoptosis and metabolism. The findings of my thesis work reveal several functions for PHD3 in ccRCC cells, highlighting the role of PHD3 not only in cancer cell survival but also in mediating tumor progression.

PHD3 was demonstrated to enhance cell cycle progression and proliferation, as well as cell migration in ccRCC cells. PHD3 was also shown to regulate glucose metabolism and lactate production, and it was shown to enhance the mTOR downstream signaling and to maintain translational machinery components (Figure 13). These all represent features of malignancy in cancer, and my study proposes that PHD3 is involved in essential processes linked to cancer progression. Furthermore, PHD3 was found to maintain the HIF-2α mRNA stability and expression of HIF-2α target genes, which oppose the previously demonstrated negative regulation of HIF-2α by PHD3. Also, HIF-2α has been proposed as an important factor promoting ccRCC tumor growth and aggressive phenotype. This further underlines the multi-functionality of high PHD3 expression in ccRCC cells.

The findings of this thesis work indicate functional diversity of PHD3 in ccRCC. The results also validated some of the previously shown functions for PHD3. However, the study provided additional insights into previously suggested mechanisms. Thus, both new functions and extension to the previously described roles for PHD3 were achieved. I'm hypothesizing that the shown functions are at least to some extent linked together via common effector that serves as a target for PHD3. An important future perspective would be to determine the mechanism that explains how PHD3 regulates the wide range of cellular processes in ccRCC cells and beyond.

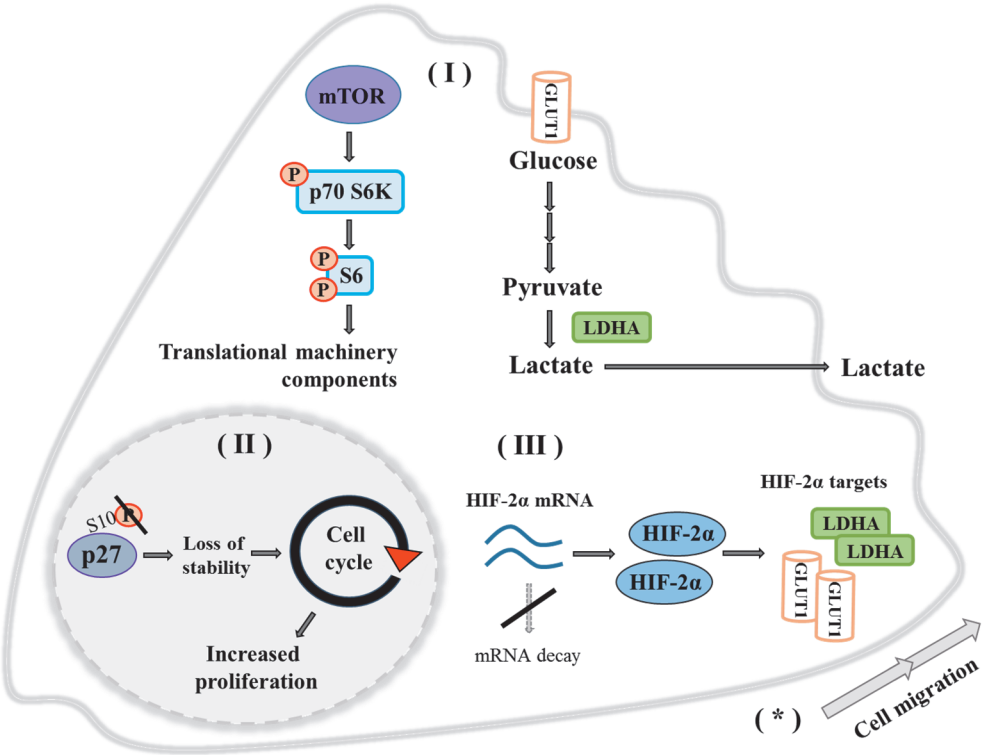


**Figure 13**. A graphical summary of the functions of PHD3 in ccRCC cells. In my thesis work, PHD3 was shown to enhance the mTOR downstream signaling and to maintain translational machinery components, as well as to enhance glycolytic activity and lactate production (I). PHD3 was demonstrated to promote cell cycle progression and cell proliferation via regulating the stability of cell cycle inhibitor p27 (II). PHD3 was also demonstrated in novel post-transcriptional regulation of HIF-2α and in promoting HIF-2α target genes GLUT1 and LDHA (III). In addition, my unpublished (*) results show that PHD3 regulates cell migration in 2D model. Abbreviations: mTOR – mechanistic target of rapamycin, p70 S6K – p70 S6 kinase, S6 – S6 ribosomal protein, P – phosphate group, GLUT1 – glucose transporter 1, LDHA – lactate dehydrogenase A, HIF-2α – hypoxia-inducible transcription factor 2α.

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Petra Miikkulainen

Petra Miikkulainen

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