Turun yliopisto
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# LIFE WITHOUT PHD? – PHD OXYGEN SENSOR PROTEINS IN HYPOXIC CARCINOMA CELL SURVIVAL

Heidi Högel

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***To my family and my dear friends***

*"Genius begins great works,*
*labor alone finishes them."*
*- Joseph Joubert*

# ABSTRACT

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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 (TuDMM), Turku Centre for Biotechnology

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The microenvironment within the tumor plays a central role in cellular signaling. Rapidly proliferating cancer cells need building blocks for structures as well as nutrients and oxygen for energy production. In normal tissue, the vasculature effectively transports oxygen, nutrient and waste products, and maintains physiological pH. Within a tumor however, the vasculature is rarely sufficient for the needs of tumor cells. This causes the tumor to suffer from lack of oxygen (hypoxia) and nutrients as well as acidification, as the glycolytic end product lactate is accumulated.

Cancer cells harbor mutations enabling survival in the rough microenvironment. One of the best characterized mutations is the inactivation of the von Hippel-Lindau protein (pVHL) in clear cell renal cell carcinoma (ccRCC). Inactivation causes constitutive activation of hypoxia-inducible factor HIF which is an important survival factor regulating glycolysis, neovascularization and apoptosis. HIFs are normally regulated by HIF prolyl hydroxylases (PHDs), which in the presence of oxygen target HIF α-subunit to ubiquitination by pVHL and degradation by proteasomes.

In my thesis work, I studied the role of PHDs in the survival of carcinoma cells in hypoxia. My work revealed an essential role of PHD1 and PHD3 in cell cycle regulation through two cyclin-dependent kinase inhibitors (CKIs) p21 and p27. Depletion of PHD1 or PHD3 caused a cell cycle arrest and subjected the carcinoma cells to stress and impaired the survival.

**Keywords:** hypoxia, prolyl hydroxylases, cell cycle, autophagy, cancer

# TIIVISTELMÄ

Heidi Högel

## PHD HAPPISENSORIPROTEIINIT SYÖPÄSOLUJEN SELVIYTYMISTEKIJÄNÄ HYPOKSIASSA

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Turun Molekyylilääketieteen tohtorikoulutusohjelma (TuDMM), Turun Biotekniikan Keskus

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Syöpäkasvaimen mikroympäristöllä on keskeinen rooli solujen välisessä viestinnässä. Syöpäsolujen nopea jakaantuminen vaatii onnistuakseen rakennusmateriaalia uusien solujen rakenteiden tuottamiseen kuten myös ravinteita ja happea energiantuottoon. Normaalissa kudoksessa verisuonisto toimii tehokkaana hapen, ravinteiden ja kuona-aineiden kuljetusverkostona, ja ylläpitää kudosten fysiologista pH:ta. Syöpäkasvaimissa verisuonisto on usein riittämätön niin määrältään kuin laadultaan, jotta se toimisi normaalisti. Tämä aiheuttaa kasvaimeen vähähappisia alueita (hypoksia) ja rajoittaa solujen ravinteiden saantia, jotka edelleen edesauttavat kasvaimen happamoitumista.

Syöpäsoluihin kertyy niiden selviytymistä edesauttavia mutaatioita, jotka mahdollistavat niiden hengissäpysymisen kasvaimen lähes vihamielisissä olosuhteissa. Tällainen on mm. von Hippel-Lindau proteiinin (pVHL) inaktivoituminen munuaissyövässä. Inaktivoitumisen seurauksena hypoksia-indusoitu tekijä HIF on konstitutiivisesti aktiivinen, mikä helpottaa solujen sopeutumista mikroympäristöön energia-aineenvaihdunnan, verisuonten uudelleenmuodostumisen ja solukuoleman säätelyn muuttuessa. HIF:n aktiivisuutta säätelevät normaalisti HIF prolyylihydroksylaasit (PHDt), jotka hydroksyloivat hapen läsnäollessa HIF:n α-alayksikön ja ohjaavat sen hajotukseen pVHL:n ja proteasomien kautta.

Työssäni tutkin PHD entsyymien roolia solujen selviytymisessä ja hypoksia-välitteisessä soluviestinnässä. Tutkimuksissa selvisi, että PHD1 ja PHD3 säätelevät solusyklin eri vaiheita solusykli-inhibiittorien p21 ja p27 kautta etenkin hypoksiassa. Niiden ilmentymisen estäminen aiheutti syöpäsolujen solusyklin pysähtymisen ja heikensi niiden elinmahdollisuuksia.

**Avainsanat:** hypoksia, prolyylihydroksylaasit, solusykli, autofagi, syöpä

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# ABBREVIATIONS

| | |
|---|---|
| 2-OG | 2-oxoglutarate/α-ketoglutarate |
| AKT | v-akt murine thymoma viral oncogene homolog |
| AMPK | AMP-activated protein kinase |
| APC/C | anaphase-promoting complex/cyclosome |
| ARNT | aryl hydrocarbon receptor nuclear translocator protein |
| ATF-4 | activating transcription factor 4 |
| ATG | autophagy-related gene |
| ATM | ataxia-telangiectasia mutated |
| ATR | ataxia-telangiectasia related |
| β-2-AR | beta-2 adrenergic receptor |
| BCL2 | B-cell lymphoma 2 |
| BCR-ABL | breakpoint cluster region protein/abelson murine leukemia viral oncogene homolog 1 -complex |
| CaM | calmodulin |
| CAMKII | calcium/calmodulin-dependent protein kinase II |
| ccRCC | clear cell renal cell carcinoma |
| CDC | cell division cycle |
| CDH1 | cadherin 1 |
| CDK | cyclin-dependent kinase |
| CIP/KIP | CDK interacting protein/kinase inhibitory protein |
| CHX | cycloheximide |
| CKI | cyclin-dependent kinase inhibitor |
| CMA | chaperone-mediated autophagy |
| DDR | DNA damage response |
| DNA-PK | DNA-dependent protein kinase catalytic subunit |
| DSB | double strand break |
| E2F | group of transcription factors |
| ECM | extracellular matrix |
| EPAS | endothelial PAS domain protein |
| EPO | erythropoietin |
| ERK | extracellular signal-regulated kinase |

| | |
|---|---|
| FH | fumarate hydratase |
| GLUT | glucose transporter |
| hCLK2 | human homolog of the *C. elegans* biological clock protein CLK-2 |
| HIF | hypoxia-inducible factor |
| hKIS | human kinase interacting stathmin |
| HNSCC | head and neck squamous cell carcinoma |
| hPRP19 | human pre-mRNA processing factor 19 |
| IDH | isocitrate dehydrogenase |
| IKK | inhibitor of nuclear factor kappa-B kinase |
| INK4 | inhibitors of cdk4 |
| IPAS | inhibitory PAS domain |
| IPNS | isopenicillin N synthase |
| JNK | c-Jun N-terminal kinase |
| KIF1Bβ | kinesin family member 1B subunit β |
| KPC | Kip1 ubiquitination-promoting complex protein |
| LC3 | microtubule-associated protein 1A/1B-light chain 3 |
| LYN | Lck/Yes novel tyrosine kinase |
| MAPK | mitogen-activated protein kinase |
| MEK | mitogen-activated protein kinase kinase |
| MIRK/DYRK1B | minibrain-related kinase/dual-specificity tyrosine-regulated kinase |
| MORG | mitogen-activated protein kinase organizer |
| MPF | mitosis promoting factor |
| mTOR | mammalian target for rapamycin |
| MYC | myelocytomatosis oncogene |
| MYND | myeloid, Nervy, and DEAF-1 domain |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NEMO | NFκB essential modulator |
| ODDD | oxygen-dependent degradation domain |
| ORF | open reading frame |
| OS-9 | osteosarcoma amplified 9 |
| OXPHOS | oxidative phosphorylation |
| PAX | paired box gene |
| PCNA | proliferating cell nuclear antigen |
| PDGF | platelet-derived growth factor |

| | |
|---|---|
| PDH | pyruvate dehydrogenase |
| PERK | protein kinase RNA-like endoplasmic reticulum kinase |
| PFKFB3 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 |
| PHD | HIF prolyl hydroxylase |
| PI3K | phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PIKK | phosphatidylinositol 3-kinase-related kinase |
| PK-M2 | pyruvate kinase muscle isozyme 2 |
| PP2A | protein phosphatase 2 |
| pRB | retinoblastoma protein |
| PTEN | phosphatase and tensin homolog |
| pVHL | von Hippel-Lindau protein |
| RAF | family of serine/threonine-specific protein kinases |
| RAS | protein family of small GTPases |
| RCC | renal cell carcinoma |
| RHO | Ras homolog gene family |
| RING | really interesting new gene |
| RNAi | RNA interference |
| ROS | reactive oxygen species |
| RPB1 | DNA-directed RNA polymerase II subunit |
| RSK1 | ribosomal s6 kinase 1 |
| SCF | Skp, Cullin, F-box containing complex |
| SDH | succinate dehydrogenase |
| SIAH | Siah E3 ubiquitin protein ligase 2 |
| SKP2 | S-phase kinase-associated protein 2 |
| SMT | somatic mutation theory |
| SRC | family of non-receptor tyrosine kinases |
| SSB | single strand break |
| TAK1 | transforming growth factor β activated kinase 1 |
| TCA cycle | tricarboxylic acid cycle |
| TGF | transforming growth factor |
| TME | tumor microenvironment |
| VEGF | vascular endothelial growth factor |
| WAF1 | wild-type p53-activated fragment |

# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III.

**I** Högel H*, Rantanen K*, Jokilehto T, Grenman R, Jaakkola PM. Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. PLoS ONE 6(11): e27112 (2011)

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

**III** Högel H, Miikkulainen P, Jaakkola PM. Prolyl hydroxylase PHD1 attenuates autophagic flux through cyclin-dependent kinase inhibitor p21 in renal cell cancer. *Manuscript*

* equal contribution



In addition, the thesis also presents some unpublished data.

# 1 INTRODUCTION

Cancer is a group of diseases affecting the life of tens of millions of people worldwide. In 2012 alone, 14.1 million new cancer cases and 8.2 million cancer deaths were reported (Ferlay et al., 2013). The incidence of cancer is rising as the lifespan increases. In 2012, 32.6 million people were living with cancer compared to the estimate of 28.8 million people in 2008 (Bray et al., 2013). Although there is a rising trend in the incidence of cancer demonstrated by 5-year prevalence, the mortality has simultaneously decreased (Ferlay et al., 2013). This is due to earlier diagnosis and more efficient as well as better targeted therapies.

The cancer research has been concentrated on understanding the mechanisms of tumor origin and spreading of the cancer. In the recent years, it has become evident that the relationship between the tumor and the surrounding microenvironment is bidirectional, and the tumor is not just about cells proliferating but a special kind of tissue consisting of multiple different components (Hanahan and Weinberg, 2011). This observation has changed also the point of view in the development of cancer therapies; targeting the factors interacting with tumor cells instead of concentrating on the actual cancer cells only could give a more universal approach to cancer therapy.

One common feature among most solid tumors within their microenvironment is the lack of sufficient oxygen, i.e. hypoxia. Most solid tumors are hypoxic because rapid proliferation and tumor growth outgrow functional vasculature. Moreover, the vascularization is abnormal, causing an unbalance between oxygen supply and need. Here, hypoxia serves as a powerful selective pressure towards more aggressive and therapy-resistant cancer type (reviewed in (Hockel and Vaupel, 2001; Vaupel et al., 2001)). It is evident that identifying and understanding the hypoxic signaling mechanisms is critical in order to understand the biology of cancer. In this thesis, I have studied the control of proliferation in hypoxic carcinoma cells and, in particular, the role of an ancient cellular oxygen-sensor protein family, HIF prolyl hydroxylases (PHDs), in the regulation of cell survival in hypoxia.

# 2 REVIEW OF LITERATURE

## 2.1 Biology of cancer

In addition to uncontrolled growth, many things need to go wrong in the process of malignant transformation, i.e. carcinogenesis, before cancer develops. In the center of carcinogenesis is the genomic instability and high mutational rate causing damage to the genome. The random mutations give rise to the oncogenes which drive carcinogenic process by activating proto-oncogenes and inactivating tumor suppressor genes. The function of tumor suppressors is to serve as a brake in carcinogenesis. What is critical at all stages of carcinogenesis is cell proliferation. Damage to the DNA is turned into mutation as it is inherited at the replication process. This enables the clonal expansion of cells (reviewed in (Bertram, 2000)). Figure 1 represents a simplified schematic model illustrating the evolutional nature of carcinogenesis.

The model called somatic mutation theory (SMT) of carcinogenesis follows basic aspects of evolution through selection, fixation and random drift (Michor et al., 2004). However, things are rarely as simple as they seem. Instead of concentrating on the cell only, the cancer should be considered as a physical-biochemical whole where the cellular and microenvironmental factors meet and communicate. Even though cancer is considered as a genetic disease, there are environmental and non-genetic factors involved at many stages of carcinogenesis that makes the idea of a linear process far too simple (Bizzarri and Cucina, 2014). The last decades in the field of cancer research have been revolutionary as the focus of research has moved to understanding the system as a whole, where tumor comprises of multiple cell types including not only the cancer cells but also the extracellular matrix, cells of the immune system and other immune responses as well as the mechanical and chemical forces at the surroundings creating an enormous signaling network.

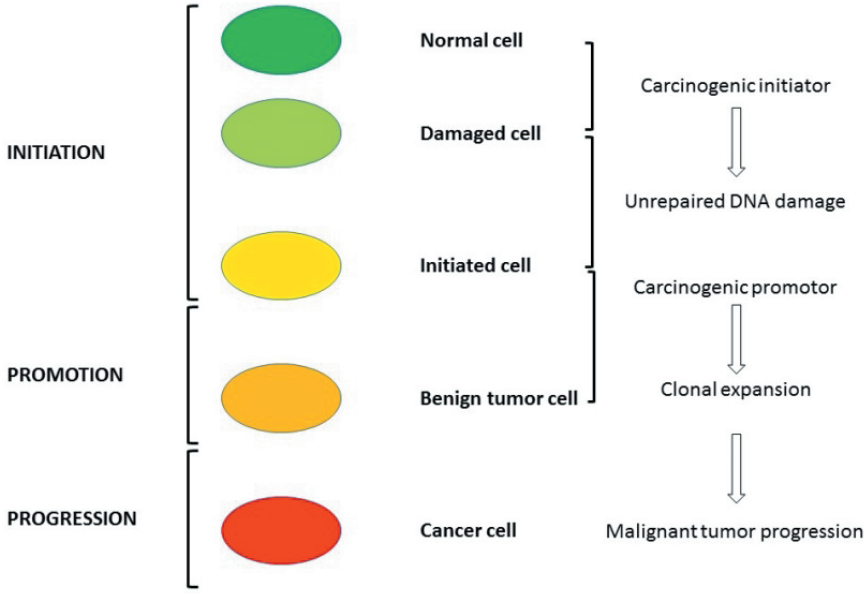


**Figure 1.** Simplified classic multistep model of chemical carcinogenesis. Adapted from (Barrett and Wiseman, 1987).

## 2.2 Hypoxia – a major tumor microenvironmental factor

Tumor microenvironment (TME) comprises of cells, signaling molecules and the extracellular matrix (ECM) between cancer cells, as well as physical factors such as pH and oxygen pressure and mechanical forces. It has been shown that microenvironment has a role in regulating many of the functions deregulated in cancer including cell cycle, cellular architecture, differentiation and apoptosis (Chen et al., 1997; Huang et al., 1998; Paszek et al., 2005; Rana et al., 1994). In line with this, microenvironmental factors act as effective selective pressure when determining the fitness of the cell during carcinogenesis (reviewed in (Gatenby and Gillies, 2008)).

One of the major microenvironmental factors affecting carcinogenesis and further cancer progression is the lack of oxygen, hypoxia. Hypoxia is a state where the partial oxygen pressure ($pO_2$) in the tissue is insufficient to fulfill the cells' needs. The need is dependent on the tissue and cell type and it can even differ within the organ. For example, within kidney medulla 10 to 20 mmHg $pO_2$ is required compared to about

50 mmHg in the cortex (Brezis and Rosen, 1995). In brain the oxygen pressure drops with the brain depth (Dings et al., 1998). Some tissues, e.g. cartilage and bone marrow, can be permanently considered hypoxic as the oxygen pressure is constantly very low. Sensitivity to hypoxia-induced cell death is also cell type specific. For example, the skeletal muscle cells are much more tolerant to the lack of oxygen than the neural cells of the brain (reviewed in (Boutilier, 2001)). Hypoxia is characteristic for most solid tumors and it is caused by rapid tumor growth and inefficient and incomplete growth of new blood vessels that hinders the oxygen and nutrient supply of the cells (reviewed in (Dewhirst et al., 2008; Hanahan and Weinberg, 2011)).

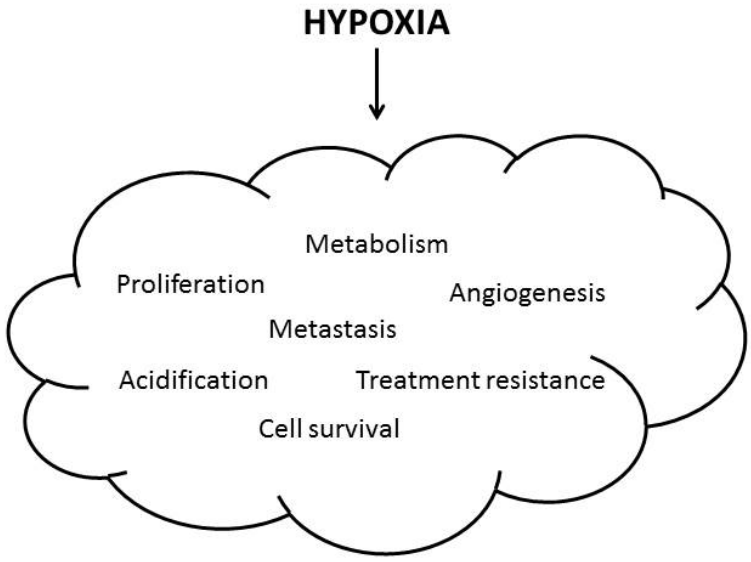


**Figure 2.** Effects of hypoxia in cancer.

Many of the crucial effects on cancer progression and promotion of cancer cell survival, such as angiogenesis, an increase in metastatic potential, continuous cell cycle progression and immortalization, are driven by hypoxia (figure 2) (reviewed in (Bertout et al., 2008; Hanahan and Weinberg, 2011)). In addition to the effect on cancer cell fitness, hypoxia affects the cancer therapy outcome both directly and indirectly. The formation of free radicals is critical for efficient radio- and chemotherapy response that need adequate oxygen supply. Additionally, the sensitivity to therapy is dependent on cell cycle phase, and hypoxia-induced cell cycle arrest protects the cells from cell death (reviewed in (Shah and Schwartz, 2001;

Pawlik and Keyomarsi, 2004)). The studies made already in the 1950s showed the significance of proper oxygenation of the tumor to improve the outcome of radiation therapy (Churchill-Davidson et al., 1957). However, this was later noticed to be more complex as the clinical outcome on usage of hyperbaric oxygen treatment and chemical radiosensitizers were disappointing (reviewed in (Brown, 1983; Henk, 1981)). Nonetheless, later on researchers have realized the value of hypoxia in targeted therapies, for example in imaging and in planning of hypoxia-activated toxins (Brown, 2010; Peitzsch et al., 2014).

Hypoxia activates some crucial cellular survival pathways such as signaling through mTOR and PERK (reviewed in (Bertout et al., 2008)). However, many of the processes activated in hypoxia are regulated by a family of hypoxia-inducible factors (HIFs) and especially by HIF-1 isoform, which mediates the best described mechanisms of hypoxic responses.

## 2.3 HIF-1 mediated hypoxic response

HIFs are heterodimeric transcription factors consisting of α- and β/ARNT-subunits. Both of the subunits are constitutively expressed in the cell but while the β-subunit is stable also in normal oxygen pressure (normoxia), the α-subunit is constantly degraded in normoxia (figure 3) (Wang et al., 1995). This oxygen-dependent regulation of HIF activity is mediated by hydroxylation of two proline residues within the oxygen-dependent degradation domain (ODDD) of the HIF α-subunit (see chapter 2.5) (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). Hydroxyprolines serve as binding motifs for an E3 ubiquitin ligase termed von Hippel-Lindau protein (pVHL) complex, which polyubiquitinates the α-subunit and marks it for degradation through 26S proteasomes. Lack of oxygen causes the hydroxylation reaction to be hindered, the α-subunit is stabilized and transported to nucleus where it forms a dimer with the β/ARNT-subunit and regulates the expression of multiple genes. These genes are involved in glucose transport and glycolysis, angiogenesis, erythropoiesis, cell survival and apoptosis (Ke and Costa, 2006). Among the most extensively studied genes are *EPO* coding for erythropoietin regulating erythropoiesis (Semenza et al., 1991), *VEGF* coding for vascular endothelial growth factor which induces angiogenesis (Levy et al., 1995), glucose transporter coding genes *GLUT1-3* and several growth factor genes such as transforming growth factor *TGF-β* and platelet-derived growth factor *PDGF*.

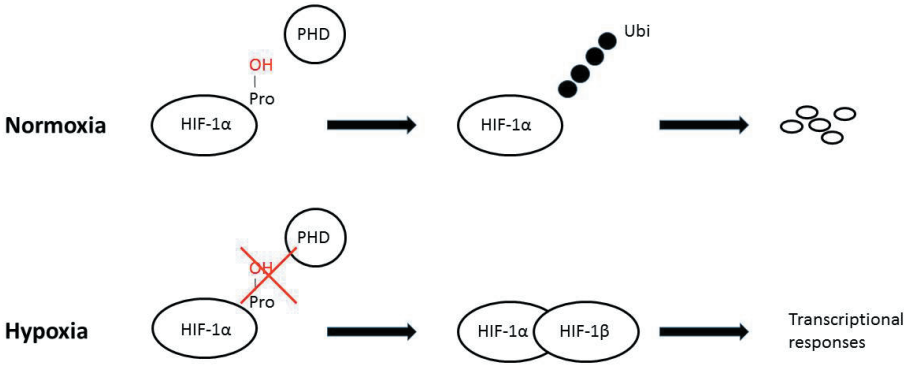


**Figure 3.** Oxygen-dependent regulation of hypoxia-inducible factor HIF-1α by prolyl hydroxylation.

There are three isoforms of HIFα: HIF-1α, HIF-2α and HIF-3α. HIF-1α and 2α have a 48% sequence homology (Ema et al., 1997; Tian et al., 1997) and have similar and partly overlapping functions whereas HIF-3α, also known as inhibitory PAS domain (IPAS), lacks the C-terminal activation domain and has only one of the conserved proline residues. The role of HIF-1 in the regulation of hypoxic response is overbearing. Its activity affects almost all of the features characterized as typical cancerous changes: adaptations on cell metabolism, changes in angiogenesis, pH regulation, increased cell survival and escaping apoptotic signals (reviewed in (Semenza, 2003)). This observation has made HIF-1 and its regulation a tempting target for cancer therapy (Bertout et al., 2008).

## 2.4 HIF-2 – a new player on the field

Much of the hypoxic research has concentrated on the signaling orchestrated by HIF-1. This originates from the finding that HIF-1 dominates the transcriptional regulation in response to hypoxia (Sowter et al., 2003). Even though the studies conducted on renal cell carcinoma indicated independent role for HIF-2 (Raval et al., 2005), only recently the knowledge of the signaling through HIF-2 has been emerging. Specific notice has been taken of the opposing roles of HIF-1 and HIF-2 in the regulation of tumor growth (Gordan et al., 2007a; Imamura et al., 2009; Raval et al., 2005). The pronounced role of HIF-2 instead of HIF-1 in cancer and especially in the case of pVHL inactivation might be due to the VHL-independent regulation of HIF-1, which seems to be specific for HIF-1 and does not affect HIF-2 activity (Demidenko et al., 2005; Isaacs et al., 2002; Kong et al., 2007; Kong et al., 2006). This leads to situation where the activity of HIF-1 is still restricted, but HIF-2 is not.

The effects of HIF-2 activity are not restricted to direct transcriptional regulation but HIF-2 has been shown to, e.g., interact closely with c-Myc (Gordan et al., 2007b; Perez-Escuredo et al., 2016). Expression of HIF-2 enhances the c-Myc mediated gene expression by increasing the promoter binding of c-Myc (Gordan et al., 2007b). Since HIF-1 has also been shown to affect c-Myc target gene activity (Koshiji et al., 2004), it has been suggested that the opposite effects of HIF-1 and HIF-2 on cell cycle progression and tumor growth would be mediated by c-Myc.

## 2.5 HIF prolyl hydroxylases

HIF prolyl hydroxylases (PHDs) belong to a family of 2-oxoglutarate (2-OG)-dependent dioxygenase enzymes. The enzymatic activity of the dioxygenase superfamily is based on molecular oxygen ($O_2$) and 2-OG used in hydroxylation of the substrate. In addition, ferrous iron ($Fe^{2+}$) and ascorbate are needed as cofactors. Although the enzymatic reaction is possible until certain point also in the absence of ascorbate, it is essential for serving as an alternative oxygen acceptor in the uncoupled reaction cycle and reactivating the enzyme by reducing the enzyme bound ferric iron ($Fe^{3+}$) (De Jong et al., 1982; De Jong and Kemp, 1984; Majamaa et al., 1986; Myllylä et al., 1978; Myllylä et al., 1984) . The name dioxygenase refers to the enzymatic reaction where the 2-OG is oxidatively decarboxylated and the substrate is hydroxylated (reviewed in (de Carolis and de Luca, 1994; Kivirikko et al., 1989)).

$$\text{2-oxoglutarate} + O_2 + \text{substrate} \longrightarrow \text{succinate} + CO_2 + \text{hydroxylated substrate}$$

In general, 2-OG-dependent dioxygenases are important mediators of a wide range of processes in organisms from plants and bacteria to worms and mammals. In humans, the 2-OG-dependent dioxygenases are involved, for example, in collagen synthesis, in carnitine biosynthesis and in DNA repair (reviewed in (Schofield and Ratcliffe, 2004)).

The hydroxylation reaction is one of the most common posttranslational modifications in the cell (Khoury et al., 2011). In contrast to many other modifications, such as phosphorylation or acetylation, hydroxylation is an irreversible reaction (Gorres and Raines, 2010). The substrates and targets of 2-OG-dependent dioxygenases are diverse including lysine and proline residues of collagen and aspartic acid or asparagine residues of several epidermal growth factors (de Carolis and de Luca, 1994). The first discovered 2-OG-dependent dioxygenase was

collagen prolyl 4-hydroxylase characterized in 1967 (Hutton et al., 1967). Conversion of proline residues of collagen to hydroxyprolines is essential for the formation of stable collagen triple helices. Collagen prolyl 4-hydroxylases (P4Hs) hydroxylate collagens and more than 15 collagen-like domain containing proteins (reviewed in (Myllyharju, 2003)).

Since 1967, also other prolyl hydroxylases have been characterized. One of these subfamilies consists of three HIF prolyl hydroxylases, named PHD1, PHD2 and PHD3 respectively. PHDs were first discovered in worms (*C. elegans*) and later in mammals including humans (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Taylor, 2001). PHDs have also been found in organisms not expressing HIF that indicates HIF-independent and more ancient role for PHDs. Additionally, even though all three PHDs have been shown to be able to hydroxylate HIF and target it for degradation *in vitro*, PHD2 isoform is most efficient in regulating the activity of HIF-1 (Appelhoff et al., 2004; Berra et al., 2003). Recently, multiple other targets especially for PHD3 have been suggested.

The PHDs show a tissue-specific expression. The gene expression analyses have revealed that PHD1 has the highest expression in testis, PHD2 in heart, brain, liver, adipose tissue and placenta, and PHD3 in heart, brain, liver, placenta, kidney, adipose tissue and skeletal muscle (Cioffi et al., 2003; Lieb et al., 2002; Oehme et al., 2002). In addition, PHD2 and PHD3 are expressed as splice variants (Hirsilä et al., 2003). However, the direct effect on HIF system by the tissue-specificity is yet to be determined.

### 2.5.1 Structure and function of PHDs

The x-ray crystallographic analyses have revealed the structure of 2-OG-dependent dioxygenases the first one being isopenicillin N synthase (IPNS) characterized by Valegård et al in 1998 (Valegård et al., 1998). There is a conserved jelly roll structural domain consisting of eight β-strands at the catalytic site of the enzymes. In addition, the metal binding motif is nearly identical in all 2-OG-dependent dioxygenases using ferrous iron as cofactor (reviewed in (Hausinger, 2004)). The binding of 2-OG at the active site changes the coordination of the metal ion which makes it more reactive towards oxygen. The enzymatic hydroxylation reaction proceeds as the substrate then binds on the catalytic site and oxygen is able to bind in close proximity (reviewed in (Costas et al., 2004; Schofield and Zhang, 1999)).

PHD2 was the first and to date the only HIF prolyl hydroxylase to be crystallized. The crystallization revealed a conserved catalytic domain at the C-terminus and a MYND zinc finger domain at the N-terminus. According to sequence similarities, all three human PHDs share the same active site topology. Thus, the substrate specificity of different PHD isoforms is likely to be determined by other structural domains of the enzymes (McDonough et al., 2006). The PHDs share the same structural components enabling the catalytic properties of enzymatic hydroxylation but the structure of the three PHDs is not consistent. In humans, PHD1 and PHD2 both comprise over 400 amino acids whereas PHD3 consist of only 239 amino acids and has therefore much lower molecular weight (Hirsilä et al., 2003).

### 2.5.2 Regulation of PHDs

The requirements for catalytic activity of prolyl hydroxylases are molecular oxygen ($O_2$), 2-OG and ferrous iron ($Fe^{2+}$) (Epstein et al., 2001; Goldberg et al., 1988; Hirsilä et al., 2003; Hirsilä et al., 2005; Schofield and Ratcliffe, 2004). The role of ascorbate in hydroxylation reaction might be limiting at least in the case of PHDs but its catalytic requirement is not that conserved (Knowles et al., 2003).

In addition to the regulation of the catalytic activity of the PHDs, also the abundance of the PHDs has been proposed to fine tune the oxygen sensing in the cell (Schofield and Ratcliffe, 2005). PHD2 and especially PHD3 are highly induced in hypoxia whereas PHD1 levels remain stable (Appelhoff et al., 2004). Both PHD2 and PHD3 are HIF-1 target genes creating a negative feedback loop and fine tuning the activity of HIF-1 in hypoxia and especially in reoxygenation (Aprelikova et al., 2004; Berra et al., 2001; D'Angelo et al., 2003; del Peso et al., 2003; Epstein et al., 2001). Moreover, in hypoxia PHD1 and PHD3 expression is also regulated by ubiquitin-proteasome pathway by the RING finger E3 ligase Siah2 (Nakayama et al., 2004; Nakayama et al., 2007).

PHDs are all regulated at transcriptional level but only *PHD1* and *PHD3* are regulated both at expressional level and by splicing. There are 17 splicing variants found for *PHD1* and 10 for *PHD3*, in addition to full length transcript (Ensembl). Even though transcribed, not every transcript is coded into a protein. In the case of *PHD1* 14 out of 18 transcripts have an open reading frame (ORF) for 51-407 amino acids. For *PHD3* there are four transcripts out of 11 with ORF ranging from 79 to 239 amino acids of length. Recently the splice variants of *PHD1* have been linked to

smoking behavior and the disease risk of smokers (Bloom et al., 2013). From the splice variants of *PHD3* two have been published to retain the hydroxylase activity and one of them seems to be cancer-specific (Cervera et al., 2006).

#### 2.5.2.1 PHDs in metabolism

What is interesting about the requirements for PHD enzyme activity is that they bound together the sensing of oxygen and the TCA cycle which is important in production of both energy and biosynthesis intermediates (figure 4). The enzymatic activity of the PHDs is dependent on intermediates of the TCA cycle. In contrast to the cofactor function of 2-OG, succinate and fumarate can act as inhibitors of enzymatic activity of PHDs (Isaacs et al., 2005; Koivunen et al., 2007; Selak et al., 2005). TCA cycle is fed by glycolysis and glutaminolysis both of which are targets for alterations in cancer development (figure 4) (reviewed in (Chen and Russo, 2012; DeBerardinis et al., 2008)). In addition, recent studies have revealed the role of reduced form of 2-OG, *(R)*-2HG, as oncometabolite (Chowdhury et al., 2011; Koivunen et al., 2012; Losman et al., 2013; Xu et al., 2011).

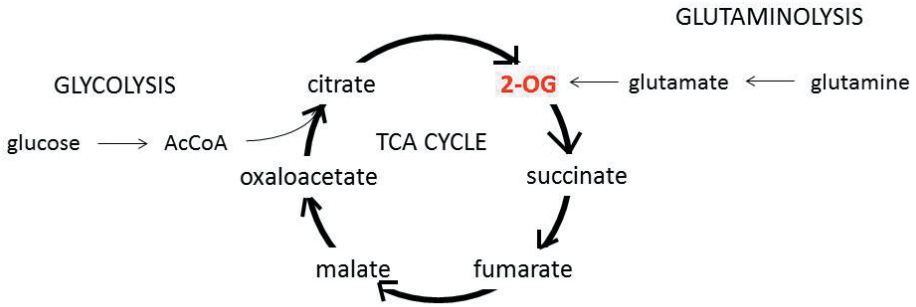


**Figure 4.** 2-OG in the crossroad of metabolic pathways. Whereas TCA cycle intermediate 2-OG acts as a cofactor of PHD enzymes, the intermediates succinate and fumarate are known to inhibit enzymatic functions of PHDs.

The mechanism of pseudohypoxia, where HIF-1 is activated despite normal oxygen conditions, has been found to be dependent on PHD inhibition by these intermediates (Pollard et al., 2005; Selak et al., 2005). Mutations of metabolic genes encoding *FH* (fumarate hydratase) and the subunits of *SDH* (succinate dehydrogenase) as well as *IDH1* and *2* (isocitrate dehydrogenase) resulting in changes in their expression have been discovered in several cancer types including

renal cell carcinoma, paragangliomas, gastric and colorectal cancer, melanoma and glioma (reviewed in (Gottlieb and Tomlinson, 2005; Oermann et al., 2012)). Loss of function of these metabolic enzymes has been thought to cause mitochondrial dysfunction and thus to affect carcinogenesis. However, recently and especially after discovering the role of IDH enzymes in cancer progression also a role of 2-OG-dependent dioxygenases in carcinogenesis has emerged (O'Flaherty et al., 2010; Oermann et al., 2012).

Reprogramming of the metabolic routes is characteristic for cancer (Hanahan and Weinberg, 2011). In relation to 2-OG and in addition to the metabolic enzymes of TCA cycle described earlier, the tendency of some cancer cells to become addicted to glutamine needs to be mentioned (reviewed in (Daye and Wellen, 2012)). Together with glucose, glutamine serves as a main source of energy, carbon and nitrogen for the proliferating cancer cells (DeBerardinis et al., 2007). In glutaminolysis, glutamine is metabolized into glutamate and then into 2-OG which can be fed into TCA cycle (figure 4). The role of glutaminolysis in 2-OG production is especially emphasized during HIF activation or hypoxia (reviewed in (Anastasiou and Cantley, 2012; Daye and Wellen, 2012)).

### 2.5.3 PHD1

PHD1 is probably the least studied of the three PHDs. *In vivo* experiments on mice have shown that PHD1 has a role in energy metabolism of muscles and liver. Mice lacking PHD1 (*Phd1*$^{-/-}$) are more tolerant of ischemia as loss of PHD1 switches the basal cellular metabolism towards glycolytic pathway (Aragones et al., 2008; Schneider et al., 2010). Preferring glycolysis instead of TCA cycle reduces oxygen consumption and ROS production thus protecting the cells against oxidative stress.

In estrogen-dependent breast cancer, estrogen has been shown to induce PHD1 expression (Appelhoff et al., 2004; Seth et al., 2002). The induction affects the cancer progression via recently revealed mechanism. First PHD1 was shown to regulate the abundance of cyclin D1 which is needed for proper cell cycle progression (Zhang et al., 2009). Recently PHD1 was shown to regulate the stability of centrosome component Cep192 (Moser et al., 2013). Together these observations suggest that PHD1 affects cell cycle in G2 phase by regulating the centrosome duplication and maturation which in turn allows the cell cycle progression through cyclin D1 induction (Zeng et al., 2010).

In addition to HIF signaling, hypoxia activates NF-κB signaling associated especially with inflammatory responses (Chen et al., 1999; Cummins and Taylor, 2005). PHD1 has been suggested to regulate the activation of NF-κB by hydroxylating IκB kinase (IKK) β (Cummins et al., 2006). It has also been shown to induce apoptosis in inflamed colon in colitis and inflammatory bowel disease (IBD) (Tambuwala et al., 2010). Although the role of NF-κB in these conditions has been shown (Atreya et al., 2008; Schreiber et al., 1998), the mechanism by which PHD1 mediates the apoptosis is not clear.

PHD1 has also been shown to regulate the ubiquitination and binding of pVHL to the large subunit of RNA polymerase II, Rpb1, in renal cell carcinoma cells (RCCs) (Kuznetsova et al., 2003; Mikhaylova et al., 2008). PHD1 hydroxylates Rpb1 and marks it for ubiquitination. In contrast to hydroxylation targets described above, PHD1 has been shown to target transcription factor ATF-4 involved in stress responses by hydroxylation-independent manner (Hiwatashi et al., 2011).

### 2.5.4 PHD2

Among the three human PHD isoforms PHD2 is most abundant in normoxia (Appelhoff et al., 2004). This is in line with the observations of PHD2 being the most important regulator of HIF-1 activity in normoxia (Berra et al., 2003). The importance of PHD2 among the three isoforms of HIF prolyl hydroxylases has been shown also *in vivo*. From the mice lacking single PHD isoform homozygous *Phd1*$^{-/-}$ and *Phd3*$^{-/-}$ were viable whereas *Phd2*$^{-/-}$ was embryonic lethal. Intact PHD2 is essential for proper placenta and heart development as the mice lacking both alleles of *Phd2* have massive structural defects in heart and in placenta and die before E14.5. This is considered to be independent on HIF hydroxylation as no increase in HIF-1α nor HIF-2α is observed in the hearts of *Phd2*$^{-/-}$ mice (Takeda et al., 2006). Also, the heterozygous deletion of *Phd2* (*Phd2*$^{+/-}$) and the somatic inactivation of *Phd2* from adult mice causes cardiovascular defects leading to premature death (Bishop et al., 2013; Minamishima et al., 2008; Takeda et al., 2008; Takeda et al., 2007). At least a part of the effects has been suggested to be independent on activity of PHD2 enzyme. However, the studies conducted on different conditional knockout models indicate that at least partially the effects of *Phd2* knockout are HIF-mediated (Eckle et al., 2008; Holscher et al., 2011; Hyvarinen et al., 2010; Kerkelä et al., 2013; Takeda et al., 2008).

Mutations in PHD2 have been linked to erythrocytosis in humans (Albiero et al., 2012; Ladroue et al., 2008; Ladroue et al., 2012; Percy et al., 2007; Percy et al., 2006). Further studies have revealed increased serum erythropoietin (Epo) levels of *Phd2* knockout mice but not on *Phd1* or *Phd3* knockout mice. Interestingly, the expression of *Epo* was increased in the kidneys but not in the livers of the *Phd2* knockout mice although the level of HIF-1α and HIF-2α was increased in both organs (Minamishima et al., 2008). This was observed also by Takeda et al. although they failed to detect any HIF-2α in the organs (Takeda et al., 2008).

The expression of PHD2 and its role in carcinogenesis has been studied in several different cancer types including breast cancer (Bordoli et al., 2011; Fox et al., 2011; Peurala et al., 2012), pancreatic cancer (Su et al., 2012), head and neck squamous cell carcinoma (Jokilehto et al., 2006), prostate cancer (Boddy et al., 2005) and glioma (Henze et al., 2010). In head and neck squamous cell carcinoma (HNSCC), the nuclear PHD2 localization has been shown to be associated with tumor aggressiveness and with radiation response (Jokilehto et al., 2006; Luukkaa et al., 2009) whereas in breast cancer high PHD2 expression was associated with increased survival (Peurala et al., 2012). In general, the role of PHD2 in cancer is conflicting. It has been suggested that the contradictory results are at least partly due to the contribution of the stromal PHD2 and especially the involvement of endothelial cells. The studies conducted on *Phd2*$^{+/-}$ mice showed that tumors implanted into these mice were less invasive, had improved oxygenation and were less glycolytic (Mazzone et al., 2009).

In addition to cancer, the role of hypoxia and HIF signaling has been intensively studied in relation to obesity and its consequences as oxygen availability has been shown to affect body weight, adipogenesis and fatty acid synthesis (Quintero et al., 2010; Shukla et al., 2005; Yun et al., 2002). As PHD2 is considered the main regulator of HIF-1α, and as the hypomorphic mice expressing reduced level of *Phd2* show reduced body weight (Hyvarinen et al., 2010), the role of PHD2 has been studied. Recent studies have shown that PHD2 is involved in the regulation of adipose tissue and metabolism. The hypomorphic mice have less adipose tissue and the adipocytes are smaller, and their glucose tolerance and insulin sensitivity is improved (Rahtu-Korpela et al., 2014).

The role of PHD2 in macrophage polarization and maturation has been recently under intensive investigation (Hamm et al., 2013; Ikeda et al., 2013; Takeda et al.,

2011). Conditional loss of PHD2 in hematopoietic cells has revealed that PHD2 is essential for the renewal of progenitor cells in the bone marrow (Singh et al., 2013).

### 2.5.5 PHD3

PHD3 differs from other PHD isoforms in many ways, and its functions have been intensively studied only lately. It is strongly induced by hypoxia and its expression is most abundant among the three PHDs in hypoxia where it is considered to retain part of its activity (Stiehl et al., 2006). In contrast to PHD2, which regulates the normoxic activity of HIF-1, PHD3 has been shown to affect the activity of HIF-1 in hypoxia and in reoxygenation, and to have higher affinity towards HIF-2α than towards HIF-1α (Appelhoff et al., 2004; Cioffi et al., 2003; Luo et al., 2011a). PHD3 has been shown to prefer the P564 residue of HIF-1α over P402, and it has also other hydroxylation targets besides HIF (table 1) (reviewed in (Jaakkola and Rantanen, 2013)).

**Table 1.** PHD3 hydroxylation and non-hydroxylation targets and functions.

| **Hydroxylation targets** | ***References*** |
|---|---|
| HIF-1α | (Appelhoff et al., 2004; Bruick and McKnight, 2001; Epstein et al., 2001; Stiehl et al., 2006) |
| HIF-2α | (Appelhoff et al., 2004; Hirsilä et al., 2003; Taniguchi et al., 2013) |
| PK-M2 | (Luo et al., 2011a) |
| Sprouty2 | (Anderson et al., 2011) |
| Pax2 | (Yan et al., 2011) |
| β(2)AR | (Xie et al., 2009) |
| Non-muscle actin (β, γ) | (Luo et al., 2014) |
| hCLK2 | (Xie et al., 2012) |
| NF-κB signaling | (Fu and Taubman, 2010) |
| **Other targets** | ***Reference*** |
| PK-M2 | (Chen et al., 2011) |
| NF-κB signaling | (Fu, 2016; Fu and Taubman, 2013; Fujita et al., 2012; Xue et al., 2010) |
| ATF-4 | (Köditz et al., 2007) |
| Myogenin | (Fu et al., 2007) |
| Bcl-2 | (Liu et al., 2010) |
| hPRP19 | (Sato et al., 2010) |
| OS-9 | (Baek et al., 2005) |
| Morg1 | (Hopfer et al., 2006) |
| KIF1Bβ | (Schlisio et al., 2008) |
| PDH | (Kikuchi et al., 2014) |

In addition to hydroxylation, PHD3 appears to have hydroxylation-independent functions (table 1). Some of them involve direct interaction between the target and PHD3. The PHD3 targeted proteins represent a variety of signaling pathways, many of which play a significant role in cancer cell survival. For example, PK-M2 has a central role in metabolic reprogramming as it functions as a switch between glycolysis and oxidative phosphorylation (reviewed in (Wong et al., 2014)). PHD3 has been shown to affect PK-M2 by two distinctive mechanisms: first, it regulates the assembly of PK-M2 into a multimer, and second it serves as a co-activator in PK-M2-derived HIF-1 target transcription (Chen et al., 2011; Luo et al., 2011a). NF-κB signaling is another interesting PHD3-targeted pathway involved in carcinogenesis and also in hypoxia (Karin et al., 2002; Koong et al., 1994; Michiels et al., 2002). Recently, HIF signaling was shown to interact with NF-κB signaling pathway (Bruning et al., 2012). In addition to HIF-mediated connection, PHD3 has been linked in several studies to the regulation of NF-κB signaling directly (table 1). It regulates the inhibitors of IκBs (IKKs) which then bind and inactivate NF-κB in the cell (Fu and Taubman, 2010, 2013; Xue et al., 2010) but it also affects NF-κB signaling indirectly (Fujita et al., 2012).

Interestingly, PHD3 binds targets that have been shown to mediate apoptosis and cell death (Bcl2, hPRP19; see table 1). Some of the targets are involved in apoptosis and cell death processes but no direct binding of PHD3 has been shown (KIF1Bβ, see table 1). Also PHD3 itself has shown to be apoptotic and mediate apoptotic cell death in neuronal and also in carcinoma cells (Lee et al., 2005; Lipscomb et al., 1999; Lipscomb et al., 2001; Rantanen et al., 2008). In breast cancer, PHD3 expression has been shown to associate with low grade and low proliferation rate (Peurala et al., 2012).

## 2.6 Controlling cell cycle

The number of cells in a multicellular organism is quite stable even though many cell types are capable of post-mitotic proliferation. This is regulated by balance between restraining uncontrolled cell division and by inducing cell death. The initiation of cell proliferation and DNA replication processes are carefully regulated. At the restriction sites and during the S phase (DNA synthesis/replication) of the cell cycle, genetic damages are identified and cell cycle is halted for repair. If the damage is

beyond repair, the cell can be directed to exit the cell cycle into senescence or programmed cell death.

In addition to creating a whole new organism, cell cycle and division enable many other functions. Reproduction, renewal, repair and blood cell production are examples of continuous use of division potential. Breakage of the balance between proliferation, differentiation or entrance into quiescence, senescence or apoptosis disturbs the sophisticated regulatory machinery and drives the cells into chaos eventually leading to development of cancer.

### 2.6.1 Phases of the cell cycle

Cell cycle describes the life of a cell from the division to the next division. The actual cell division during mitosis (M) comprises the shortest time of the cycle, only about 10% of the total cell cycle time. 90% of the time cells are in a phase called interphase describing the gap between divisions. The interphase is further split into three different phases, two of which are gaps or growth phases (named G1 and G2) and one is synthetic phase (S). During the first gap or the growth phase (G1), the cells are growing and preparing for entering mitosis. This phase is dependent on mitogenic factors until the restriction point. The chromosomes are prepared for the replication and cellular components are duplicated. The G1 is the phase where the ultimate decision to enter cell cycle or quiescence (G0) is made. In synthesis phase S, cells are duplicating the centrosomes for the formation of the mitotic spindle and replicating the DNA, which is then introduced into two daughter cells in mitotic cell division. In G2 between the synthesis phase and mitosis, the cells are growing and passing the final restriction point to enter mitotic cell division (figure 5) (reviewed in (Campbell and Reece, 2002)).

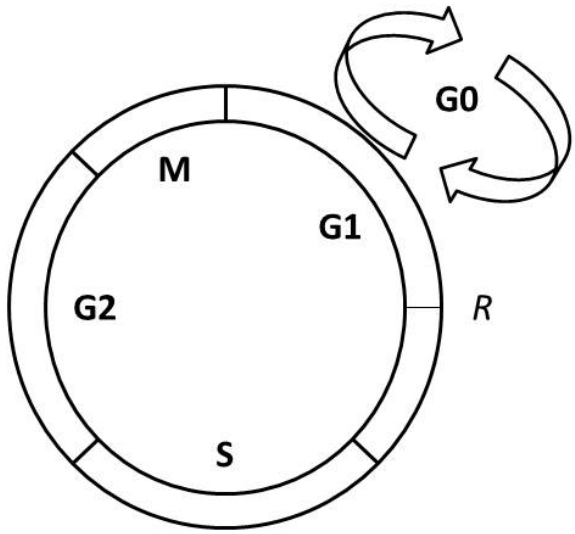


**Figure 5.** Schematic picture of mammalian cell cycle. G0 – quiescence, G1 – growth/gap 1, R – restriction point, S – synthesis, G2 – growth/gap 2, M – mitosis.

### 2.6.2 Cell cycle machinery

The cell cycle is sophisticated and highly organized machinery and its phases are strictly regulated. Signaling pathways regulating cell cycle need to communicate with the pathways and machineries controlling and sensing the environmental factors outside the cell and also the metabolic status, redox status and possible damages inside the cell. The mitosis is the most critical: DNA needs to be replicated only once and this has to be made correctly with no errors as DNA damage serves as initiation for diseases like cancer (Kastan and Bartek, 2004). Restriction points and checkpoints at the different phases of the cell cycle serve as quality control. Cells can respond to damages and malfunctions by repair, by halting the cell cycle or by initiating programmed cell death.

The cell cycle control is based on checkpoints which make sure that the earlier phase of the cell cycle is completed correctly (Hartwell and Weinert, 1989). The major point of regulation of the cell cycle is the G1 phase (Pardee, 1974). The other phases of the cycle are much less dependent of the outer regulatory machinery than the events taking place during G1 and G0. The restriction point R has been mapped about 3-4

hours after M phase has been completed (Zetterberg and Larsson, 1985). After this point, the cells are independent of mitogenic signals and proceed into synthesis phase without outer stimulus (Campisi et al., 1982). The passage through the restriction point is regulated by regulatory protein which was first characterized as labile growth-factor sensitive protein "R" (Rossow et al., 1979). The best candidate for "R" protein thus far has been cyclin D which is induced and regulated in G1 in response to growth factors (Sherr and Roberts, 1999). Together with its catalytic partners cdk4 and cdk6 (table 2), it phosphorylates and initiates the inactivation of retinoblastoma protein pRb that is the key event in G1 progression and surpassing restriction point control (figure 6) (reviewed in (Giacinti and Giordano, 2006; Weinberg, 1995)).

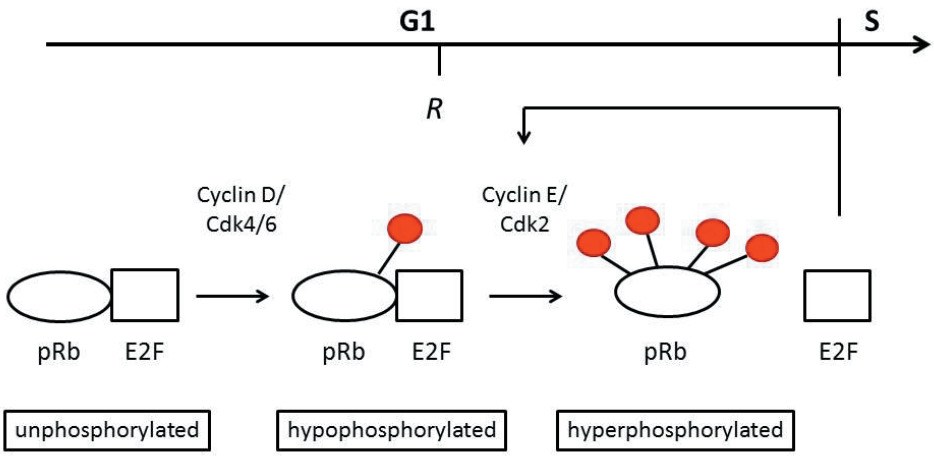


**Figure 6.** pRb phosphorylation in G1 phase. Cyclin D is expressed and assembled with its catalytic partner cdk4 or cdk6 in response to mitotic signals, and it phosphorylates pRb during early and mid-G1. In late G1 cyclin E/Cdk2 complex completes the hyperphosphorylation of pRb. E2F and cyclin E/Cdk2 transaction enable the S phase entry.

However, it has been proposed that there is also another restriction point in late G1 that is dependent on nutrient availability and growth of cell size. Therefore it has been suggested that this later checkpoint would have more in common with the START checkpoint characterized in yeast in 1974 (Foster et al., 2010; Hartwell et al., 1974). The yeast as well as the mammalian nutrient-dependent checkpoints are regulated by TOR (mTOR for mammalian TOR) (reviewed in (Fingar and Blenis, 2004; Schmelzle and Hall, 2000)). As a part of nutrient sensing signaling pathway, mTOR mediates cellular adaptation to the nutrient availability through

transcriptional and translational mechanisms (reviewed in (Rohde et al., 2001; Schmelzle and Hall, 2000)). The importance of energy and biomolecule supply in cell division has been in the spotlight for decades. Surprisingly however, the precise nutrient-dependent regulation of cell cycle has been obscure and the link between metabolic pathways and cell cycle machinery has just begun to be revealed. Recently it was found that APC/C-Cdh1 E3 ubiquitin ligase responsible for the timed degradation of the cell cycle regulatory factors mediates also the stability of glycolytic activator PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3) and glutaminase 1, a key enzyme of glutaminolysis, thus linking the bioenergetics directly to cell cycle control (Almeida et al., 2010; Bolanos et al., 2010; Colombo et al., 2010; Moncada et al., 2012). An even more fundamental phenomenon might be the recent observation made on mitochondria. The research of Lippincott-Schwartz and her group indicates that polarization of mitochondria during G1 is essential for the G1/S transition (Mitra et al., 2009). Indications of the role of mitochondria on cell cycle have also been published earlier as cyclin D and p53 both affect mitochondrial functions (Matoba et al., 2006; Wang et al., 2006).

Even though most of the regulatory machines work at the G1 phase there are multiple other checkpoints along the way. In addition to G1, DNA damage control takes place also during the S phase at the intra-S-phase checkpoint and during G2 phase (reviewed in (Bartek and Lukas, 2001; Karnani and Dutta, 2011; O'Connell et al., 2000; Stark and Taylor, 2006)). DNA damage response (DDR) transduces the signals between factors recognizing the damages and the factors acting on cell cycle regulation. The PIKKs (phosphoinositide 3-kinase related kinases) ATM, ATR and DNA-PK respond to DNA damage such as impaired base pairing, base loss, single-strand breaks (SSBs) and to the most toxic lesion, the double stranded breaks (DSBs) in cooperation (Bartek and Lukas, 2007; Jackson and Bartek, 2009). The multiple points of control for DNA damage assure the integrity of the DNA before entering mitosis. During the mitosis, there is still one checkpoint, spindle-attachment checkpoint, which prevents the anaphase to proceed in case the chromosomes are not correctly attached at the metaphase plate (reviewed in (Elledge, 1996; Wells, 1996)).

#### 2.6.2.1 Cyclins and cdks

The main engine of the cell cycle machinery are the cyclin-dependent kinases (cdks) and their binding partners the cyclins (reviewed in (Yasutis and Kozminski, 2013)). Cdks are Ser/Thr kinases which are ubiquitously expressed but their activity is

regulated by phosphorylation, dephosphorylation, intracellular localization and binding to cyclins. The holoenzymes consisting of catalytic cdks and regulatory cyclins form a network orchestrating the progression through different phases of the cycle (table 2) (reviewed in (Massagué, 2004)). The regulatory function of cyclins is dependent on their expression which is strictly restricted to certain phases of the cell cycle (figure 7).

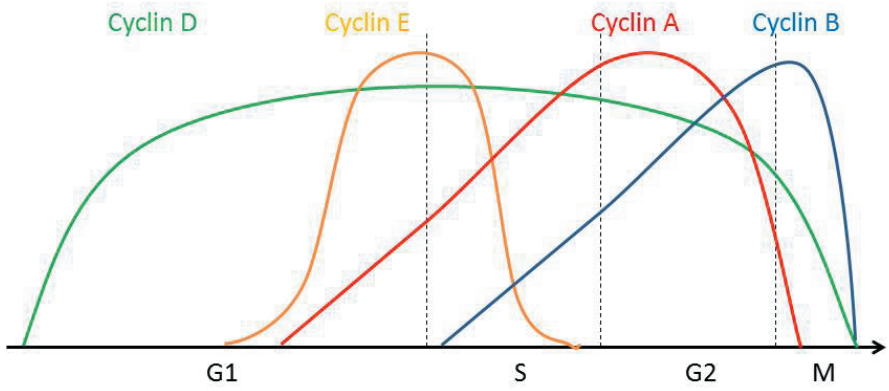


**Figure 7.** Expression of cyclins during cell cycle progression.

The key event in decision to enter the cell division is passing the restriction point in G1 regulated by phosphorylation of pRb (figure 6). pRb represses transcription of many genes needed for cell cycle progression through forming a complex with E2F transcription factor (reviewed in (Lipinski and Jacks, 1999)). During G1, cdk4 and cdk6 form active complexes with D- and E-type cyclins (G1 cyclins) which phosphorylate and thereby inactivate pRb and release pRb binding partner E2F (figure 6) (Harbour et al., 1999; Watanabe et al., 1999). Cyclin E and a S cyclin called cyclin A are among the targets of E2F taking part in regulating next steps of the cell cycle thus creating a positive feedback loop (Duronio et al., 1996; Ohtani et al., 1995). Expression of cyclin E and its interaction with cdk2 allows the completion of pRb phosphorylation (hyperphosphorylated pRb) and G1/S transition (figure 6). Once in S phase, cyclin E is degraded and its activity is lost (figure 7) (reviewed in (Hwang and Clurman, 2005)).

**Table 2.** Cyclins and cdks involved in cell cycle control.

| Cyclin | cdk | Cell cycle phase |
|---|---|---|
| cyclin D1, D2 and D3 | cdk4, cdk6 | G1 |
| cyclin E1 and E2 | cdk2 | G1, G1/S |
| cyclin A1 and A2 | cdk2 | S |
| | cdk1, cdk2 | G2/M |
| cyclin B1 | cdk1 (cdc2) | G2/M |

The free cdk 2 released from the complex during cyclin E degradation forms a new complex with cyclin A resulting in the phosphorylation of E2F thus allowing the completion of the S phase (Kitagawa et al., 1995; Krek et al., 1995; Xu et al., 1994). Cyclin A is essential for two distinct points of cell cycle. In complex with cdk2, it enables the S phase completion through E2F phosphorylation but complex formed with cdk1 in late S phase is involved in G2 progression and mitosis (Pagano et al., 1995; Rosenblatt et al., 1992). The involvement of cyclin A in S phase is more evident than its role in mitosis, and the exact role of cyclin A/cdk complexes in G2 phase has not been well defined even though it seems to be required for G2/M transition (Furuno et al., 1999; Pagano et al., 1992; Strausfeld et al., 1996). Recently, however, it was shown that the pool of cyclin A complexes in G2 regulates the timing of mitotic entry (De Boer et al., 2008).

Degradation of cyclin A is followed by expression and activation of mitotic cyclins, B-type cyclins (figure 7). Cyclin B and especially its proper localization is essential for progression through mitosis (Gallant and Nigg, 1992; Li et al., 1997). Together with its kinase partner cdk1 cyclin B forms the core of maturation/mitosis-promoting factor MPF needed for G2/M transition (Doree and Hunt, 2002; Labbe et al., 1989). The activity of MPF remains until cyclin B is degraded at metaphase/anaphase transition during mitosis (figure 7) (Labbe et al., 1989; Sherwood et al., 1993).

#### 2.6.2.2 Cyclin-dependent kinase inhibitors CKIs

There are two groups of cyclin-dependent kinase inhibitors (CKIs): the INK4 family comprises of four members, $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$, and the Cip/Kip family of three members, $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Roy and Banerjee, 2014). They form the next level of regulation by inhibiting the functions of cdks. The members of the INK4 family (inhibitors of cdk4) inhibit the activity of

cdk4 and cdk6 whereas the Cip/Kip family members have a broader spectrum of targets restraining the activities of cdks 2, 4 and 6, and the ability to bind also cyclins (reviewed in (Sherr and Roberts, 1999)).

Even though named as inhibitors, all cyclin D/cdk complexes have been found to be bound on Cip/Kip family members and still to remain active (reviewed in (Sherr and Roberts, 1999)). It has been suggested that CKIs p21, p27 and p57 would even be necessary for efficient cyclin D/cdk complex formation (Cheng et al., 1999; LaBaer et al., 1997). This has led into conclusion that complexes containing cdk2 are the main inhibitory targets of Cip/Kip proteins and binding to cyclin D/cdk complexes regulates the inhibitory actions of Cip/Kip family members on cdk2 (Roussel, 1999). What seems to be critical in whether functioning in complex assembly of cyclin D or as an inhibitor of cdk is the expression level: lower levels favor the complex assembly whereas higher levels are inhibitory (reviewed in (Pateras et al., 2009)).

Cip/Kip family members share a sequence homology in the amino terminus and have overlapping binding targets, although there are also other functions which separate the three from each other. p21, also known as Waf1 or Cip1, is a target of p53 and is activated in DNA damage response (el-Deiry et al., 1993). Thus it mediates the cell cycle arrest in G1 in response to DNA damage. p21 affects DNA replication also directly by binding PCNA (proliferating-cell nuclear antigen) and inhibiting DNA polymerase δ (Waga et al., 1994). In addition to its function as an inhibitor of cdk2 activity, p21 has been shown to regulate E2F transcriptional activity by direct binding (Afshari et al., 1996). Furthermore, p21 is an important mediator of the permanent cell cycle arrest, cellular senescence (reviewed in (Kong et al., 2011)).

The p27 is an essential part of the cell cycle functioning as a kind of brake. Mainly, it operates in quiescence and in early G1 and its expression is induced in contact inhibition. Later in G1, a mitogenic stimulus causes reduction of p27 expression and release of cdk2, or rebinding of p27 on cyclin D complexes (Blain et al., 2003). Creating a feedback loop, cdk2 mediates the phosphorylation of T187 on p27 targeting it for proteasomal degradation (Montagnoli et al., 1999). Regulation and functions of p27 are discussed more detailed later on (chapter 2.6.3).

The third and less studied member of the Cip/Kip family is p57. It has been proposed that the main function of p57 would be somewhere else than cell cycle

regulation. Indeed, in mice p57 expression but not p21 or p27 is essential for embryogenesis (Yan et al., 1997; Zhang et al., 1997). The p57 has also been shown to regulate actin polymerization through direct binding to Rho downstream effector LIM-kinase 1 (Yokoo et al., 2003).

The INK4 family members p15, p16, p18 and p19 share 40% sequence homology and have restricted target repertoire. The binding of cdk4 and cdk6 only constrains the inhibitory function on D-type cyclins (see table 2 for binding partners). Mechanistically, INK4 CKIs compete for binding to cdk4 and cdk6 and inhibit cyclin D from binding (Cánepa et al., 2007). The cell cycle inhibitory functions of INK4 CKIs, however, are dependent on Cip/Kip CKIs. As an INK4 family member binds its target cdk4 or cdk6, cyclin D is released and degraded. The loss of cyclin D causes release and thus activation of p27 from cyclin D/cdk complexes, and inhibition of cdk2 by free p27 (reviewed in (Cánepa et al., 2007)).

### 2.6.3 Cyclin-dependent kinase inhibitor p27

The Cip/Kip family members are often deregulated in cancer and several lines of evidence support the idea of their role as tumor suppressors. This applies especially for p27, which is considered as the prime mediator of cell cycle arrest in G1. However, it is never mutated in cancer but instead its expression and localization is changed (reviewed in (Besson et al., 2008)). It has been suggested that p27 could serve as both tumor suppressor and oncogene depending on its localization in the cell as its cytoplasmic localization has been shown to induce migration (Besson et al., 2004b; Sun et al., 2001; Wang et al., 2011).

The expression of p27 is mainly post-translationally regulated by the ubiquitin-proteasome system (Pagano et al., 1995) through at least two distinct pathways that are dependent on the phase of cell cycle and the localization of p27 (Carrano et al., 1999; Hara et al., 2005; Hara et al., 2001; Kamura et al., 2004; Nourse et al., 1994; Reynisdóttir et al., 1995; Sheaff et al., 1997). One of the ubiquitination reactions is initiated by phosphorylation of threonine 187 (T187) by cyclin E/cdk2 complex in proliferating cells (Montagnoli et al., 1999; Nguyen et al., 1999; Sheaff et al., 1997; Vlach et al., 1997). The p27 phosphorylated on T187 is targeted by SCF-Skp2 ubiquitin ligase complex and directed for degradation which allows S phase entry (Carrano et al., 1999; Ganoth et al., 2001; Sutterluty et al., 1999; Tsvetkov et al.,

1999). In addition, the requirement for cyclin A/cdk2 complexes for T187 phosphorylated p27 ubiquitination has been demonstrated (Zhu et al., 2004). In resting quiescent cells, however, phosphorylation of serine 10 (S10) markedly increases p27 stability (Ishida et al., 2000; Rodier et al., 2001). At cell cycle re-entry, S10 phosphorylation serves as an export signal from nucleus to cytoplasm (Besson et al., 2006; Ishida et al., 2002) where the degradation is directed by KPC ubiquitin ligase complex (Kamura et al., 2004; Kotoshiba et al., 2005). The third phosphorylation involved in p27 regulation at G0 and G1 is the phosphorylation of threonine 198 (T198) which stabilizes p27 in G0 by protecting it from degradation but which is needed in early and mid-G1 for its association with cyclin D1/cdk4 complexes and its degradation (Kossatz et al., 2006). The fourth of the main phosphorylation sites is threonine 157 (T157) preventing nuclear re-entry of p27 (Shin et al., 2005).

There are multiple kinases taking part in the regulation of p27 at different phases of the cell cycle. Phosphorylation of S10 has been shown to be mediated by Mirk/Dyrk1B kinase in G0 (Deng et al., 2004) and by hKIS at G0/G1 (figure 8) (Boehm et al., 2002). Efficient phosphorylation of T187 has been shown to be due to phosphorylation of tyrosine residues by Src-family kinase Lyn and Bcr-Abl complex (Grimmler et al., 2007). Phosphorylation at T198 and at T157 in G1 were found to occur in response to mitogenic stimuli through the activity of PI3K/PTEN/Akt and Ras/Raf/ERK pathways (figure 8) (Fujita et al., 2002; Fujita et al., 2003; Larrea et al., 2009; Liang et al., 2007; Liang et al., 2002; Motti et al., 2004; Shin et al., 2002). T157 and T198 are directly phosphorylated by Akt (Liang et al., 2002; Shin et al., 2002), and T198 has also been shown to be a target of RSK1 which is an effector of both PI3K/PTEN/Akt and Ras/Raf/ERK pathways (Fujita et al., 2003; Larrea et al., 2009). Akt and ERK2 have been reported to phosphorylate p27 also at S10 at least *in vitro* (Fujita et al., 2002; Ishida et al., 2000).

In addition to phosphorylation, the activity of p27 is regulated by its abundance at different compartments of the cell. During quiescence in G0 p27 is trapped into nucleus where the phosphorylation of S10 by Mirk/Dyrk1B stabilizes it (Deng et al., 2004). In response to mitogenic stimuli, Mirk/Dyrk1B expression is decreased and expression of hKIS increases (Boehm et al., 2002; Deng et al., 2004). hKIS further phosphorylates S10 and causes p27 to translocate into cytoplasm where KPC1 and KPC1 ubiquitin ligases target it for degradation (Kamura et al., 2004) (figure 8).

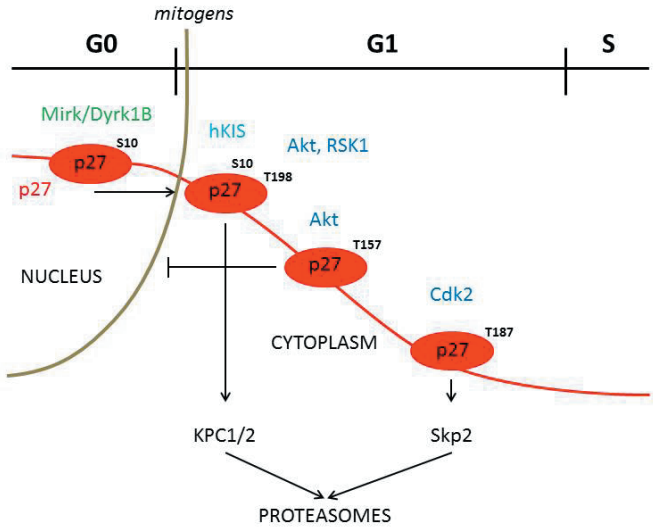


**Figure 8.** Kinases phosphorylating p27, and the subcellular localization of p27 in G0 and in G1. In quiescent cells, phosphorylation of S10 by Mirk/Dyrk1B stabilizes and traps p27 into nucleus. Serum stimulation leads to decreased expression of Mirk/Dyrk1B and increased expression of hKIS which again phosphorylates S10. Together S10 and mitogens serve as export signal from the nucleus to cytoplasm.

### 2.6.3.1 Diverse roles of p27

It has become evident that CKIs have also other functions besides their role as cyclin-dependent kinase inhibitors in cell cycle regulation. All of the Cip/Kip CKIs are important regulators of development as well as transcription, apoptosis and migration (reviewed in (Besson et al., 2008; Sherr and Roberts, 1999)). The important role of p27 in carcinogenesis has been revealed as the loss of p27 has been observed in a variety of malignancies and furthermore this has been shown to be associated with tumor aggressiveness (Blain et al., 2003; Bloom and Pagano, 2003; Catzavelos et al., 1997; Loda et al., 1997; Philipp-Staheli et al., 2001; Porter et al., 1997; Slingerland and Pagano, 2000). Moreover, the dual role of p27 as tumor suppressor and oncogene has become more and more evident (Besson et al., 2004a; Besson et al., 2008; Kossatz and Malek, 2007). Whereas p27 serves as a cell cycle inhibitor when localized into nucleus, the cytoplasmic mislocalization drives migration and metastasis (reviewed in (Besson et al., 2004a). The fact that cytoskeleton needs to be reorganized during the cell cycle for division to occur makes the cell cycle machinery potential mediator of migration defects.

## 2.7 Cell cycle and cancer

As the cell's potential to divide is essential for malignant cancer to develop, the players of the cell cycle control are very often somehow deregulated in cancer. It is not surprising that cancer cells typically harbor mutations on genes related to cell cycle control enabling autonomous growth. These changes on the genome allow the cells to acquire survival mechanisms overcoming normal growth restrictions and ability to bypass proliferation suppressing signals, escape apoptosis and maintain continuous replication potential (reviewed in (Hanahan and Weinberg, 2011)). Loss of restriction point control enables accumulation of further mutations and chromosomal abnormalities (Bertram, 2000).

The most important cell cycle phase relative to tumorigenesis and cancer progression is the G1 phase, where most of the regulation restricting the cell cycle progression is taking place. Many of the oncogenes and tumor suppressor genes are somehow linked to G1 phase (table 3) (reviewed in (Ho and Dowdy, 2002). The tumor suppressors pRb, p53 and PP2A often deregulated in tumors as well as the activated Ras necessary for transformation are among the G1 regulators (Gille and Downward, 1999; Hahn et al., 1999; Sherr and McCormick, 2002; Westermarck and Hahn, 2008). The involvement of cell growth regulators Myc and PTEN in tumorigenesis has also been shown (Boehm et al., 2005). Both of these factors are part of highly complex Akt/AMPK/mTOR signaling network taking part in many actions essential for cell survival, growth and proliferation (reviewed in (Massagué, 2004)).

**Table 3.** Examples of oncogenes and tumor suppressor genes related to cell cycle restriction point control.

| Oncogene/tumor suppressor gene | Cell cycle phase affected |
|---|---|
| pRb | G1 |
| p53 | S |
| Ras | G1, G2 |
| PP2A | G2, M |
| Myc | G1, G2 |
| PTEN | G1 |
| p27, p21 | G0, G1 |

In addition, most human cancers bear mutations in cell cycle regulators directly involved in G1 phase (Sherr, 2000). Malfunctions as overexpression of cyclins and cdks as well as reduction in expression of CKIs, have been reported in many cancers (Malumbres and Barbacid, 2001; Shapiro, 2006). It has been suggested that deregulation of cdks would be observed in 80-90% of tumors (Diaz-Moralli et al., 2013). Unlike CKIs of the INK4 family p14, p15 and p16 that are often silenced through deletions or other mutations, the Cip/Kip CKIs p21 and p27 are rarely mutated but their expression is deregulated or the localization is altered (reviewed in (Besson et al., 2004a)).

Since restriction of cell proliferation is a key point in battle against cancer, many of the inhibitors under intensive research are related to cell cycle control. Over the past decade, many small molecule inhibitors of cdks have shown some encouraging results (reviewed in (Collins and Garrett, 2005; de Carcer et al., 2007)). Moreover, recent findings linking the regulation of bioenergetics directly to the regulatory elements of cell cycle has revealed a novel group of promising targets for cancer therapy.

### 2.7.1 Hypoxic cell cycle control

The override of growth restriction is characteristic for cancer and it is the divider between normal and cancer cell. As hypoxia is a common feature of solid tumors, it is a major microenvironmental factor driving these mechanisms (reviewed in (Allen and Louise Jones, 2011; Hanahan and Weinberg, 2011; Noman et al., 2011; Vaupel, 2008; Zhou et al., 2006). One of the main challenges in sustaining proliferation in hypoxic environment is to exceed restriction points of the cell cycle.

#### 2.7.1.1 CKIs in hypoxia

On many cell types although not all, hypoxia causes a cell cycle arrest in G1/S transition which is mediated by reversible hypophosphorylation of pRb, decrease in activity of G1/S-specific cyclin/cdk complexes and increase of CKIs p27, p21 and p16 (Gardner et al., 2001; Green et al., 2001; Krtolica et al., 1998, 1999; Krtolica and Ludlow, 1996; Ludlow et al., 1993; Pettersen and Lindmo, 1983; Spiro et al., 1984; Zygmunt et al., 2002). Up to this point the picture is quite clear but when it comes to defining the roles of the different CKIs or the exact mechanism of the cell cycle

arrest in hypoxia it becomes more blurry. Although hypoxia increases p21 expression, there are publications showing that the hypoxic cell cycle arrest actually does not require p21 (Amellem et al., 1998; Gardner et al., 2001; Green et al., 2001). In addition, there is no mutual understanding about the requirement of two other CKIs upregulated in hypoxia, p16 and p27, in the hypoxic cell cycle arrest (Gardner et al., 2001; Green et al., 2001). It has been suggested that the hypoxia mediated cell cycle arrest would be coordinated by multiple CKIs instead of upregulation of a single pathway regulating G1/S transition (Box and Demetrick, 2004).

The expression of p27, although unregulated in hypoxia, seems to be independent on HIF-1 though the results have been distinct (Carmeliet et al., 1998; Gardner et al., 2001; Goda et al., 2003; Horree et al., 2008; Koshiji et al., 2004). Moreover, it has been suggested that p27 could mediate oxygen-dependent cell cycle arrest even independently on pRb (Graff et al., 2005). This is in line with observations of pRb-independence of immediate oxygen-dependent cell cycle arrest in late G1 whereas active pRb would be needed for the arrest earlier in G1 and for the maintenance of the arrested state in prolonged hypoxia (Amellem et al., 1998; Amellem et al., 1996). These studies also suggest an important role for pRb in cell cycle re-entry during reoxygenation. The idea of two distinct oxygen-dependent restriction points during G1, one functioning as immediate response blocking G1/S transition and DNA synthesis, and another one responding to prolonged hypoxia through the activation of pRb could explain the conflicting but also somewhat overlapping results.

Thus the requirements for hypoxia-driven cell cycle block are controversial. The HIF-1 and HIF-related hypoxia sensing seem to play a critical role, even though the role of HIF-1 seems to be dependent on cell type especially in the case of transformed cancer cells compared to nontransformed cells but also among different cancer cell types (Box and Demetrick, 2004; Eliasson et al., 2010; Goda et al., 2003; Högel et al., 2011; Semenza, 2011). It has been proposed that also the microenvironmental factors such as nutrient availability and redox status would affect the role of HIF in cell cycle arrest. In hematopoietic cells hypoxic cell cycle arrest seems to be mediated by both HIF-dependent and HIF-independent mechanisms (Eliasson et al., 2010). At least in some cases, the forced expression of HIF-1 or HIF-2 is alone sufficient to cause cell cycle arrest in G1 (Hackenbeck et al., 2009).

The cell cycle arrest dependent on HIF-1 activity is mediated by induction of p21 (Carmeliet et al., 1998; Goda et al., 2003; Koshiji et al., 2004). The mechanism however seems to be independent from dimerization of HIF α-subunit and ARNT. The expression of p21 is downregulated in normal oxygen pressure by c-Myc whereas in hypoxia HIF-1α has been shown to bind the promoter of p21 gene *CDKN1A* and replace c-Myc mediated regulation of p21 expression (Koshiji et al., 2004). The role of HIF-2 however, is distinct from HIF-1 as it has been previously shown to enhance the activity of c-Myc thereby resisting the effects of HIF-1 (see chapter 2.4) (Gordan et al., 2007b). The dilemma of opposing roles of the two HIFs is to be determined but the stoichiometry between the players of c-Myc signaling and the two HIFs might be essential (Gordan et al., 2007a).

#### 2.7.1.2 Other hypoxic cell cycle regulators

Besides c-Myc and HIF, hypoxia activates also other major signaling pathways affecting cell proliferation. The mechanism of HIF-independent hypoxic activation of the transcription factor NF-κB through IKK and the upstream kinase TAK1 has been recently published, although the increased activity of NF-κB in hypoxia has been revealed much earlier (Bandarra et al., 2014; Culver et al., 2010; Koong et al., 1994; Melvin et al., 2011). In its inactive state, NF-κB is sequestered in the cytoplasm and bound to its inhibitor IκB. The activation of NF-κB requires phosphorylation of IκB by IKK complex comprising major subunits IKKα, IKKβ and IKKγ/NEMO (reviewed in (Liu et al., 2012)). Upstream from IKKs, there are multiple signaling pathways. For the catalytic activity of IKKs, phosphorylation needs to occur at IKKα and IKKβ, although the exact mechanism for IKK complex activation has not been revealed (Mercurio et al., 1997). One of the upstream effectors is TAK1 which functions also on both MAPK and JNK signaling pathways (Ninomiya-Tsuji et al., 1999; Wang et al., 2001). In the case of NF-κB signaling TAK1 and also IKK complex seem to be activated through $Ca^{2+}$ release and CaMKII pathway (Culver et al., 2010).

The release of calcium and increase in intracellular $Ca^{2+}$ is one of the early events after hypoxic exposure in almost all cell types (Toescu, 2004). Even though serving as universal second messenger among variety of species from unicellular organisms to mammals, the essential targets of $Ca^{2+}$ regulating cell proliferation have remained elusive. The complexity of $Ca^{2+}$-dependent signaling can be accounted for by its nature as signal transducer of variety of hormones, growth factors and cytokines as

well by its direct binding to numerous target proteins (reviewed in (Kahl and Means, 2003)). The many signals transmitted by $Ca^{2+}$ make the unique responses of the cell possible but also makes it difficult to solve the $Ca^{2+}$-dependent signaling as a whole. The need of extracellular $Ca^{2+}$ is not universal since transformed cells are able to proliferate even in $Ca^{2+}$ free media. However, the role of intracellular $Ca^{2+}$ and its intracellular receptor calmodulin (CaM) in cell cycle regulation is unquestionable (reviewed in (Kahl and Means, 2003; Takuwa et al., 1995)). It has been shown that during the early G1 and near G1/S transition, the cells are most sensitive to the inhibition of the $Ca^{2+}$/CaM pathway. In terms of hypoxia, the latter is most interesting since the oxygen-dependent cell cycle arrest is observed in late G1.

The concentration of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) as well as the expression of CaM fluctuates as the cell cycle progresses. The peaks of $[Ca^{2+}]_i$ have been detected near G1/S and G2/M transitions and during mitosis before progression from metaphase to anaphase and during cytokinesis (Santella, 1998). The expression of CaM peaks at G1/S boundary and also in early G1 suggesting it regulates the re-entry of quiescent cells into cell cycle (Chafouleas et al., 1984). The levels of $Ca^{2+}$ and expression of CaM seem to be reversed: increase of outer $Ca^{2+}$ ($[Ca^{2+}]_o$) causes a decrease in CaM expression and vice versa (Klug et al., 1994). This phenomenon together with the observation of increased CaM expression in many cancers may explain at least in part the independence of outer $Ca^{2+}$ of many transformed cells.

The mechanisms of CaM-mediated cell cycle control are manifold. One of the early observations of cell cycle regulation by CaM was seen as changes in phosphorylation of pRb (Takuwa et al., 1993). Later on, the CaM has been shown to regulate cyclin D/cdk4 in early G1 and to interact with cyclin E and regulate the activity of cyclin E/cdk2 complex in late G1 (Choi et al., 2006; Kahl and Means, 2004a, b; Taulés et al., 1998). Additionally, CaM has been shown to be involved in DNA replication (López-Girona et al., 1995; López-Girona et al., 1992; Reddy et al., 1992).

The most important downstream mediators of $Ca^{2+}$/CaM pathway in the regulation of cell cycle are CaMKII (calmodulin kinase II) and protein phosphatase calcineurin (Santella, 1998). The role of calcineurin has been controversial. Earlier studies using inhibition of calcineurin by cyclosporine A suggested that calcineurin regulates cell cycle by affecting the expression of cyclins A and E and CKI p21 thus preventing the activation of cdk2 (Khanna and Hosenpud, 1999; Tomono et al., 1998). More recent studies however, indicate that calcineurin would regulate G1 by affecting

expression of both cyclin D and cdk4 (Kahl and Means, 2003). The regulatory role of CaMKII on the other hand, has been pointed into G1 and G2/M transition. The CaMKII has been linked to cell proliferation and cell cycle control by MAPK kinase MEK1-mediated p27 degradation and by direct regulation of cyclin D levels (Li et al., 2009; Morris et al., 1998). There are, however, results suggesting otherwise and that instead of a reduction in cyclin D levels the inhibition of CaMKII would reduce the activity of cdk4 (Kahl and Means, 2003). The other point of control mediated by CaMKII is at the G2/M transition where CaMKII seems to affect cyclin B/cdk1 activity through phosphorylation of cdc25 (Kahl and Means, 2003; Santella, 1998). The controversy and the lack of agreement in the regulation mediated by these two factors might be explained by the fact that most of the studies on calcineurin as well as on CaMKII have been made using inhibitors not absolutely target-specific.

However, in contrast to the attenuating effect of hypoxia on cell cycle progression, chronic hypoxia may actually induce proliferation in several cell types including smooth muscle cells, fibroblasts and renal tubular endothelial cells. This phenomenon has been established at least in renal disease, atherosclerosis and cardiac ischemia (Bradley et al., 1978; Dempsey et al., 1991; Falanga and Kirsner, 1993; Gao et al., 2014; Porter et al., 2014; Sahai et al., 1999; Sahai et al., 1997). In cancer, the role of this phenomenon is not that pronounced. More importantly, a subpopulation of cancer cells needs to escape the hypoxic cell cycle arrest one way or another in order to maintain growth.

It is clear that in addition to hypoxia, the other microenvironmental factors inside the tumor, especially the pH, determine the fate of the individual cells. The analysis of the hypoxic and acidic areas inside the tumor has revealed interesting local non-correlation between these two parameters (Helmlinger et al., 1997). It is to note that these results measured *in vivo* differ remarkably from the situation created *in vitro* in spheres mimicking the real tumor, since spheroids rely completely on the diffusion of oxygen in the lack of vascularization (Gorlach and Acker, 1994). This observation of more complex relation between tumor acidification and hypoxia makes the conclusions between microenvironment and changes in metabolism and proliferation of the cells diffuse. For example, it has been shown that in transformed cells, hypoxia was unable to cause a cell cycle block in buffered environment, but instead enhanced cell viability, without simultaneous acidosis (Schmaltz et al., 1998).

### 2.7.2 Autophagy and cell cycle

Autophagy is a catabolic process where cytoplasmic material is delivered to lysosomes for degradation. There are three main classes of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Macroautophagy, hereafter called autophagy, is the best characterized from the three. The autophagy process involves formation of double membrane autophagosome and its fusion with lysosome where the degradation itself is taking place (figure 9). At the molecular level, autophagy is regulated by autophagy-related *ATG* genes. Different set of *ATG* genes is involved in each of the five stages of autophagy process: (1) induction, (2) recognition and selection of the cargo material, (3) autophagosome formation, (4) fusion of autophagosome with lysosome and finally (5) degradation of the cargo (reviewed in (He and Klionsky, 2009)). The conjugation of Atg12-Atg5 and modification of microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) to LC3-II activated by Atg7 are essential reactions for autophagosome formation (Kabeya et al., 2000; Mizushima et al., 1998; Tanida et al., 2004). Unmodified LC3-I is cytosolic whereas its modified form LC3-II is located on the membranes of autophagosomes which makes it a widely used marker for autophagy (Kabeya et al., 2000; Klionsky et al., 2016; Mizushima and Yoshimori, 2007).

Another important protein involved in autophagy is p62, also called sequestosome 1 (SQSTM1). It is a scaffold and an adaptor protein responsible for binding ubiquitinylated proteins and LC3 (Pankiv et al., 2007; Vadlamudi et al., 1996). The polyubiquitinylated protein aggregates are selectively bound by p62 which recruits them to the autophagic machinery (reviewed in (Lippai and Low, 2014)). As p62 is also degraded through autophagy, it is a useful marker to monitor autophagy at least in some settings (Klionsky et al., 2016).

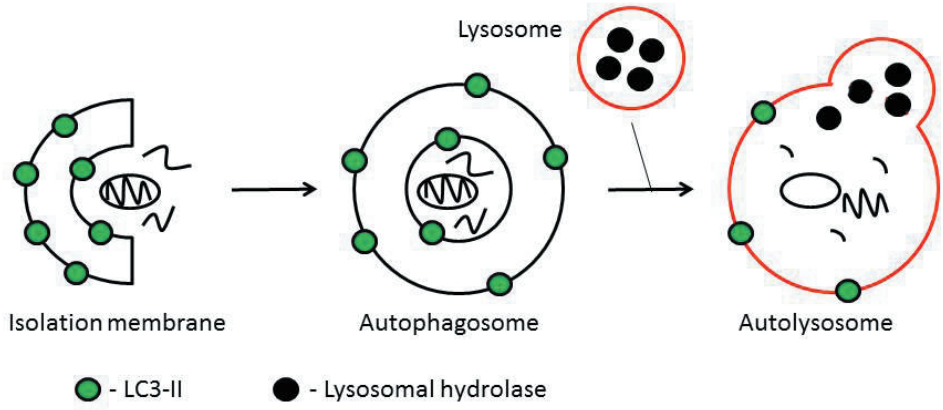


**Figure 9.** Simplified schematic overview of autophagy process.

The role of autophagy in cell survival has been controversial and it was long considered as cell death machinery only. During the last decade, it has become obvious that autophagy is much more complex, and it also serves as a survival mechanism in stressful conditions (Kondo et al., 2005; Menzies et al., 2015; Nixon, 2013; White, 2015). In addition to its physiological functions, autophagy has relevance in many pathological conditions including neurodegenerative diseases and cancer (reviewed in (Nixon, 2013; White, 2015)). The outcome whether autophagy serves as a promoting or an inhibiting factor during carcinogenesis seems to be dependent on the developmental stage of the tumor as well as on the cell type (reviewed in (White, 2012)). Recently it was shown that the expression of *ATG* genes was reduced in clear cell renal cell carcinoma (ccRCC) patients (Liu et al., 2015). The expression levels of these genes correlated to the survival of patients, and the authors suggested that the lower expression of *ATG* genes relate to more aggressive disease. More than 80% of the patients had changes in the gene copy number of *ATG7,* which is a key enzyme in the autophagosome formation. In addition, multiple autophagy-related genes were inactivated by direct loss of gene copy number, by lower expression or by mutations.

Cells at certain phase of the cell cycle are more prominent for autophagy induction (Tasdemir et al., 2007). In addition, many anti-tumoral agents induce autophagy in conjunction with cell cycle arrest (Filippi-Chiela et al., 2011; Kuo et al., 2006; Yao et al., 2003; Zhu et al., 2014). The interplay between autophagy and cell cycle is evident but the mechanisms binding them together are still to be determined.

# 3 AIMS OF THE STUDY

The expression of PHDs is elevated in many cancers but the expression patterns poorly correlate with hypoxic areas of the tumors. During recent years it has become evident that PHD enzymes have broader spectrum of targets than just HIF-1α. As I started my thesis project, our group among others had found the apoptotic function of PHD3 in normoxia. The involvement of PHDs in hypoxic survival mechanisms was to be determined. My work started by studying head and neck squamous cell carcinoma (HNSCC). As the project evolved, the work took into focus also renal cell carcinoma (RCC).

I To determine the role of PHD3 in carcinoma cell survival in hypoxia.

II To study the role and molecular mechanisms of PHD3 in cell cycle regulation.

III To study the role of PHDs on autophagy by cell cycle regulation.

# 4 MATERIALS AND METHODS

The materials and methods used in this study are listed below. Detailed descriptions can be found in the original publications and manuscript.

**Experimental procedures**

| **Methods** | **Used in** |
|---|---|
| cDNA synthesis | I, II, III |
| Cell culture | I, II, III |
| Cell cycle analysis | I, II, III |
| Cell cycle synchronization | I, II |
| Cell fractionation | II |
| Cell imaging | I, II, III |
| Cell proliferation analysis | I |
| Cycloheximide chase | II, III |
| Database searches | I |
| Flow cytometry | I, II, III |
| Hypoxia | I, II, III |
| Immunocytochemistry | I, II, III |
| Immunoprecipitation | II |
| Patient samples | I [Jokilehto et al (2006)] |
| Plasmid transfections | I, II, III |
| qRT-PCR | I, II, III |
| Reoxygenation | II |
| RNA extraction | I, II, III |
| RNA interference | I, II, III |
| Statistical analysis | I, II, III |
| Western blotting | I, II, III |

## Materials

| Cell lines | Used in |
|---|---|
| 786-O | II, III |
| Hela | I, II |
| RCC4 | III |
| UT-SCC2 | I |
| UT-SCC7 | I |
| UT-SCC9 | I |

| Chemicals | Used in |
|---|---|
| Aphidicolin | I, II |
| Bafilomycin A1 | III |
| $CoCl_2$ | II |
| Cycloheximide, CHX | II, III |
| Dimethyloxaloylglycine, DMOG | II, III |
| Glutaraldehyde | III |
| MG132 | II |

## Antibodies

**Table 4.** Antibodies used in immunological applications.

| | Application | | | | |
|---|---|---|---|---|---|
| Antibody | WB | ICC | IP | Manufacturer | Used in |
| Caspase 3 | | x | | Promega | I |
| Cyclin A | x | | | Santa Cruz Biotechnology | I |
| Cyclin B1 | x | | | Abcam | I |
| Cyclin D1 | x | | | Santa Cruz Biotechnology | I |
| Cyclin E | x | | | Santa Cruz Biotechnology | I |
| Flag | | x | | Sigma-Aldrich | II |
| HA | x | | x | Roche Applied Science | II |
| HIF-1α | x | | | BD Transduction Laboratories | I, II |
| HIF-2α | x | | | Novus Biologicals | II, III |
| hKIS | x | | | Santa Cruz Biotechnology/Abgent | unpub. |
| Lamin A/C | x | | | Santa Cruz Biotechnology | II |
| LC3A/B | | x | | Novus Biologicals | III |
| LC3B | x | x | | Novus Biologicals | III |

| | | | | | |
|---|---|---|---|---|---|
| PHD1 | x | | | Novus Biologicals | II, III |
| PHD2 | x | | | Novus Biologicals | I, II, III |
| PHD3 | x | | | Novus Biologicals | I, II, III |
| pRb | x | | | Santa Cruz Biotechnology | I |
| p16 | x | | | Cell Signaling | I, II |
| p16 | x | | | Santa Cruz Biotechnology | III |
| p21 | x | | | BD Transduction Laboratories | I, II |
| p21 | x | | | Cell Signaling | III |
| p27 | x | | x | Santa Cruz Biotechnology | I, II, III |
| pp27(S10) | x | | | Santa Cruz Biotechnology | II |
| pp27(T157) | x | | | R&D Systems | II |
| pp27(T187) | x | | | Santa Cruz Biotechnology | II |
| pp27(T198) | x | | | R&D Systems | II |
| p62 | x | x | | Santa Cruz Biotechnology | III |
| Skp2 | x | | | Santa Cruz Biotechnology | II |
| α-tubulin | x | | | Santa Cruz Biotechnology | II, III |
| β-actin | x | | | Sigma-Aldrich | I, II, III |

Abbreviations: WB – western blotting, ICC – immunocytochemistry, IP – immunoprecipitation

| **siRNAs** | | | **Used in** |
|---|---|---|---|
| LC3 | siRNA pool | Santa Cruz Biotechnology | III |
| p21 | SignalSilence® siRNA | Cell Signaling | III |

**Table 5.** Sequences of the siRNAs used.

| Target | Sequence | Used in |
|---|---|---|
| Scramble ctrl | 5'-CCUACAUCCCGAUCGAUGAUG(dTdT)-3' | I, II, III |
| PHD3 off target | 5'-GUCUAAGGCAUAGGUGGCUUG(dTdT)-3' | I |
| PHD1 | 5'-ACAUUGCUGCAUGGUAGAA(dTdT)-3' | II, III |
| PHD2 | 5'-GACGAAAGCCAUGGUUGCUUG(dTdT)-3' | I, II, III |
| PHD3 | 5'-GUCUAAGGCAAUGGUGGCUUG(dTdT)-3' | I, II, III |
| p27 | 5'-AAGCACACUUGUAGGAUAA(dTdT)-3' | II |
| HIF-1α | 5'-AACUAACUGGACACAGUGUGU(dTdT)-3' | II |
| HIF-2α/EPAS1 | 5'-GCGACAGCUGGAGUAUGAAUU(dTdT)-3' | II |

## RT-PCR primers and probes

**Table 6.** Sequences of the primers and probes used in RT-PCR.

| Target | Primer right | Primer left | Probe #/* | Used in |
|---|---|---|---|---|
| glut1 | ggttgtgccatactcatgacc | cagataggacatccagggtagc | #67 | II |
| hif-2α/epas1 | catctacaaccctcgcaacc | tggagaacaccacgtcattc | #45 | II |
| p27 | tttgacttgcatgaagagaagc | agctgtctctgaaagggacatt | #60 | II |
| phd1 | aagcattggtgccctcat | tgggtaacacgccaccat | #44 | III |
| phd2 | cgacctgatacgccactgt | gttccattgcccggataac | #44 | III |
| phd3 | ttttggcttctgccctttctt | cgaagtgcagccctcttacg | * | I |
| phd3 | atcgacaggctggtcctcta | gatagcaagccaccattgc | #61 | II |

# Universal ProbeLibrary, Roche Diagnostics, * Eurogentec

# 5 RESULTS

## 5.1 PHD3 is induced in head and neck cancer (HNSCC) cell lines in hypoxia and in HNSCC tumors (I)

The expression of PHD3 has been previously shown to be strongly induced in response to hypoxia both at transcriptional and at protein level in several cancer cell lines including cervical cancer cell line HeLa, osteosarcoma cell line U2OS, pancreatic carcinoma cell line BxPC-3, lung carcinoma A549 and several breast cancer (MCF7, T47D and ZR-75–1) and hepatocellular carcinoma (Hep3B, HepG2) cell lines (Appelhoff et al., 2004; Epstein et al., 2001; Marxsen et al., 2004; Metzen et al., 2003) (I figure S6). To study the expression of PHD3 in head and neck squamous cell carcinoma (HNSCC), five different patient derived primary carcinoma cell lines (UT-SCC) were used. Cells were exposed to hypoxia for 6 and for 48 hours and PHD3 mRNA and protein expression was monitored. Compared to normoxia PHD3 mRNA expression was strongly induced in all cell lines studied with maximal induction seen after 48 hours of hypoxia and protein levels were increased in three out of five cell lines (I figure 1A and B). To study the PHD3 expression in human HNSCC tumors, 28 tumor samples were obtained from patients. The expression of PHD3 mRNA was measured and compared to anatomically matching healthy controls (11 samples) (I figure 1C). The expression of PHD3 was found to be 5-fold compared to control ($p < 0.0001$) (I figure 1D). To further study the occurrence of high PHD3 expression in HNSCC database, search using Oncomine® platform was used (Oncomine, 2014). From the five studies found in the database four showed increase in PHD3 expression in HNSCC compared to healthy control three of which were statistically significant ($p < 0{,}002$ to $0{,}005$) (I figure 1E and F).

## 5.2 PHD3 loss causes non-apoptotic cell death in hypoxia (I, unpublished)

In order to study the role of increased PHD3 in HNSCC, the expression of PHD3 was reduced in UT-SCC cell line UT-SCC2 using RNA interference (RNAi) (I figure 2A). After knockdown treatment, the cells were exposed to hypoxia for 48 hours.

The cells treated with PHD3 siRNA showed marked decrease in cell number when grown under hypoxia (I figure 2B). Compared to siScr control, PHD2 knockdown or point-mutated siPHD3 control the amount of surviving cells was reduced almost by 70% in siPHD3 treated samples (I figure 2C, E and F). The result was further validated using independent adenoviral shRNA against PHD3. The plasmid-delivered shRNA showed similar PHD3-dependent inhibition in hypoxic cell growth (II figure S1). Since PHD3 expression was very low in normoxia in the cell lines used, no difference on cell survival was seen under normoxic conditions. To further study the cause of reduced cell number, the proliferation rate of siRNA treated cells was measured both in normoxia and in hypoxia using thymidine analog BrdU and following its incorporation into newly synthetized DNA. In normoxia, there were no marked changes in proliferation rate of PHD3 depleted cells compared to siScr and siPHD2 controls but in 48-hour hypoxia samples the proliferation of siPHD3 cells was reduced by 40% (I figure 2D).

In addition to decreased proliferation, the reduced cell amount could be caused by increased apoptotic rate. To validate the apoptosis in UT-SCC2 cells, the cells were treated with siRNAs and exposed to hypoxia for 6, 24 and 30 hours, fixed and stained for cleaved active caspase 3 which is a widely used apoptotic marker. There was an increase in caspase cleavage in PHD3 depleted cells in early hypoxia time points which was however lost in later time points (I figure 4A and B). As caspase cleavage does not necessary lead to apoptosis (Wilhelm et al., 1998), the flow cytometry was used to further analyze the apoptotic rate by measuring the cell population in sub-G1 phase. As expected, the hypoxia treatment for 24 to 72 hours induced apoptosis detected as an increase in the amount of cells in sub-G1 phase (Shimizu et al., 1996) (I figure 4C and D). The induction was however similar in both control and PHD3 depleted cells (I figure S3). This led to conclusion that although caspase activation was increased in early time points in siPHD3 cells there was no significant change in apoptotic rate in PHD3 depleted cells.

### 5.2.1 PHD3 promotes tumorigenic growth of carcinoma cells (unpublished)

To address the relevance of PHD3-mediated survival, the effect of PHD3 depletion on migration and tumorigenic growth were studied. In addition to reduced proliferation, siPHD3 treatment impaired the migration capacity of HeLa cells in a scratch wound assay both in normoxia and hypoxia (Figure 10).

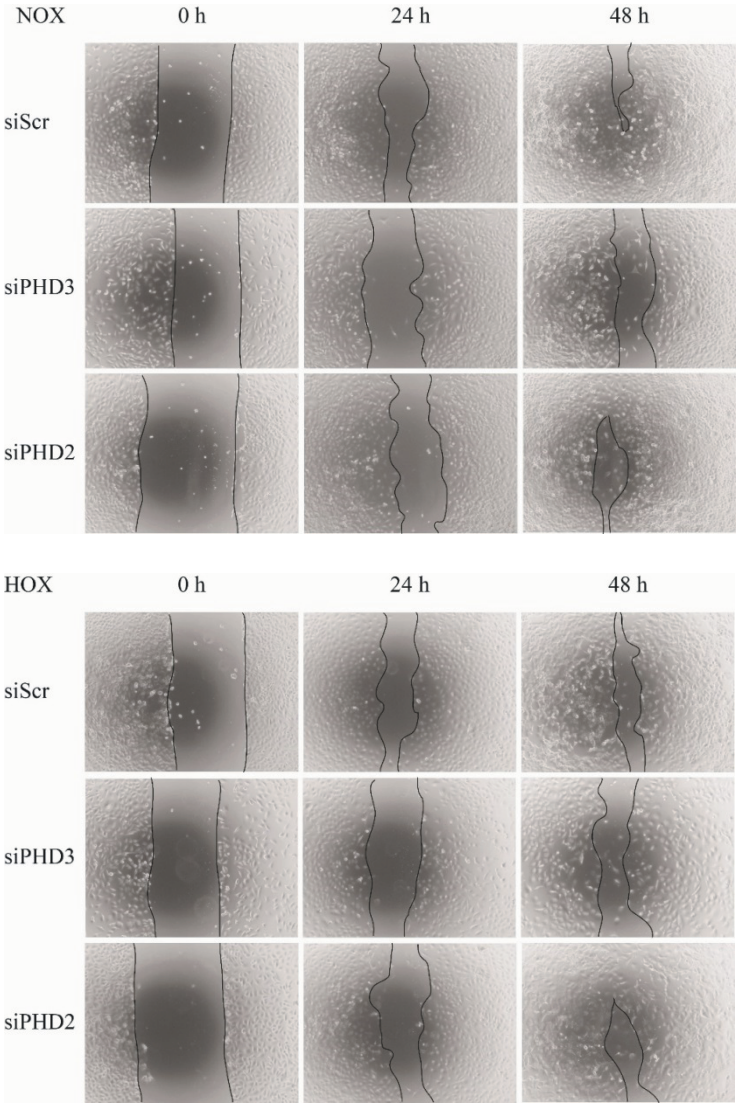


**Figure 10.** Scratch wound assay using siRNA transfected HeLa cells.

To further study the tumorigenic properties of PHD3, colony formation capacity and anchorage-independent growth capability were determined using clonogenic assay, soft agar assay and tumor xenografts. In line with the reduced proliferation rate and the impairment in migration, PHD3 depletion inhibited the colony formation and anchorage-independent growth of HeLa cells both *in vitro* (figure 11, data not shown) and *in vivo* (figure 12).

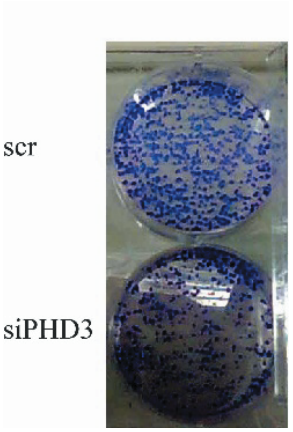


**Figure 11.** The colony formation capability of PHD3 depleted cells is strongly hindered.

For transplantation of tumor xenografts, HeLa cells were transfected with control (siScr) and PHD3 targeted siRNA (siPHD3) and injected subcutaneously in the neck of the immunocompromised athymic nude mice. The RNAi efficiency was confirmed by Western blotting before transplantation (figure 12).

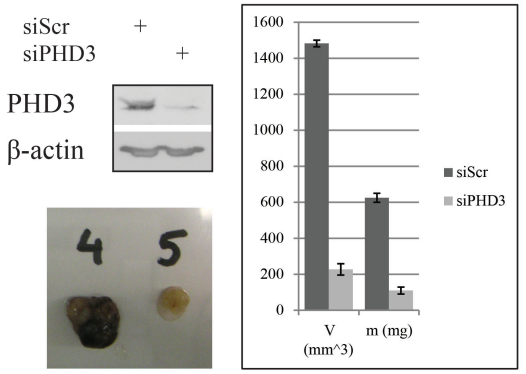


**Figure 12.** Quantification of the volume (V) and mass (m) of tumor xenografts (n=4). The Western blot confirms the effective RNAi. Two representative xenografts: 4 – siScr, 5 – siPHD3.

## 5.3 PHD3 mediated hypoxic cell survival is independent on hydroxylase activity (I)

The rescue experiment was performed to further validate the PHD3-dependence of hypoxic cell survival of UT-SCC2 cells. Cells treated with siRNAs were further transfected with PHD3-EGFP plasmid (I figure S2). After 48 hours of hypoxia, the cell amount was counted. Whereas the cells treated with siPHD3 and vector plasmid showed marked decrease in cell survival after 48-hour hypoxia, the cells treated with PHD3-EGFP demonstrated clear increase in cell survival compared to vector control (I figure 3A and B). Moreover, rescue using hydroxylase-deficient mutant PHD3(R206K)-EGFP (Epstein et al., 2001; McNeill et al., 2002; Mukherji et al., 2001; Myllyharju and Kivirikko, 1997; Rantanen et al., 2008) also showed marked increase in cell survival after hypoxia (I figure 3A and B). This suggests that the PHD3-mediated hypoxic cell survival is independent on its hydroxylase function.

## 5.4 PHD3 depletion halts the cell cycle at the G1/S boundary (I, II, III)

The reduction in proliferation rate and the lack of increased rate of apoptosis of PHD3 depleted UT-SCC2 cells indicated changes in cell cycle progression. The siRNA treated cells were exposed to hypoxia for 24 to 48 hours, fixed with ethanol and stained with propidium iodide. The cell cycle was analyzed using flow cytometer. As expected, the hypoxic treatment caused a block in cell cycle progression of UT-SCC2 cells in G1 (I figure 5A and S4). However, in PHD3 depleted cells the block was more pronounced and even more cells accumulated in G1 (I figure 5A, B and D). Accordingly, and in line with the measured decrease in BrdU incorporation, the amount of cells in S phase reduced by 35% and 25% at 24 and 48-hour hypoxia compared to siScr control (I figure 5C and D).

The hypoxic G1 block was further validated using cervical cancer cell line HeLa and renal cell carcinoma cell lines 786-O and RCC4. Hela cells express low levels of PHD3 in normoxia whereas in hypoxia the expression is strongly induced. In 786-O and RCC4 cells, there is a strong expression of PHD3 both in normoxia and in hypoxia. Interestingly, from these two cell lines HeLa cells halt the cell cycle in hypoxia whereas 786-O or RCC4 cells show no G1 block in hypoxia (II figure 1A, III figure 1A). This kind of difference in hypoxic response of cell cycle progression

is however not uncommon and has been previously described (Box and Demetrick, 2004; Liu et al., 2011). The impairment in G1/S transition in hypoxic HeLa cells was seen also when aphidicolin synchronization on G1/S boundary and subsequent cell cycle release was used. Aphidicolin treatment followed by hypoxia for 2 to 12 hours showed a marked impairment at G1/S transition seen as accumulation of cells in G1 and a decrease in S phase cell amount (I figure S5). The repeated experiment with the use of siRNA transfections showed that the PHD3 depletion further deepened the hypoxic cell cycle block compared to siScr and PHD2 depletion controls (I figure 5E).

Despite the difference in hypoxic cell cycle block, depletion of PHD3 caused an accumulation of cells in G1 in all cell lines used (II figure 1A, III figure 1B). In 786-O and RCC4 cell lines the difference between siPHD3 cells and control cells was even more pronounced and the PHD3 depletion mediated cell cycle block was observed both in normoxia and in hypoxia (II figure 1A, III figure 1B). This led to conclusion that the support of PHD3 on cell cycle progression was more dependent on PHD3 expression than on oxygen availability.

The further experiments on renal cell carcinoma cell lines 786-O and RCC4 also show that the effect is specific for PHD3, since the depletion of PHD1 or PHD2 show no significant G1 accumulation compared to PHD3 depletion (III figure 1B). In line with the previously reported data PHD1 depletion caused accumulation of cells in G2/M phase in normoxia (Moser et al., 2013) (III figure 1B). PHD2 depletion caused a slight increase in G1 cells in normoxia and PHD1 depletion in hypoxia but the increase is not comparable with the cell cycle block caused by siPHD3.

## 5.5 Cell cycle block caused by PHD3 depletion is mediated through pRb dephosphorylation and p27 induction (I, unpublished)

Previous studies have shown that the hyperphosphorylation of retinoblastoma protein pRb is needed for proper cell cycle progression at the G1/S boundary and impairment in pRb phosphorylation could block the cell cycle at G1 (Buchkovich et al., 1989; Chen et al., 1989). UT-SCC2 cells and HeLa cells were treated with siRNAs and exposed to hypoxia for up to 48 hours. In PHD3 depleted cells a marked

reduction in hyperphosphorylated state of pRb was noticed in hypoxia (I figure 6A). As the pRb is phosphorylated by cyclin/cdk complexes in late G1, the expression of cyclins involved in the phosphorylation reactions was studied. In line with the dephosphorylation of pRb, the expression of cyclin D1 was decreased in PHD3 depleted cells (I figure 6A). The expression of cyclin E phosphorylating pRb in late G1 or expression of cyclin A involved in G2 phase progression was not changed in PHD3 depleted cells compared to control (figure 13). However, the expression of cyclin B1 involved in G2/M transition was decreased in PHD3 depleted cells (I figure 6A). This could be a secondary effect caused by a defect in cell cycle progression seen in PHD3 depleted cells. To further validate the effect of PHD3 depletion on cyclin D1 expression independent on cell cycle phase, HeLa cells were synchronized in G0 by serum starvation and exposed to hypoxia for 6 and 24 hours. As expected, there was a marked decrease in cyclin D1 expression which was in line with the observed pRb hypophosphorylation (I figure 6B).

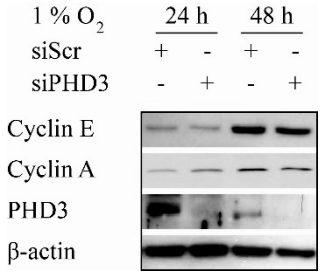


**Figure 13.** The expression of Cyclin A and E is unchanged in PHD3 depleted HeLa cells under hypoxic exposure.

The activity of cyclins and their catalytic cdk counterparts is regulated by CKIs. The expression of three CKIs, p16, p21 and p27, was studied in UT-SCC2 cells, HeLa cells and in 786-O cells. PHD3 depletion had no effect on either p16 or p21 expression whereas the expression of p27 was markedly increased in both normoxic and hypoxic PHD3 depleted cells (I figure 6C-E, II figure 1B, III figure 2A). This was in line with the decreased expression of cyclin D1 in siPHD3 cells and could explain the G1 arrest seen in PHD3 depleted cells. Altogether the data indicated that the cell cycle support by PHD3 is mediated by regulation of p27 expression.

## 5.6 Dephosphorylation of pRb and induction of p27 in PHD3 depleted cells is independent on hydroxylase function of PHD3 (II, unpublished)

Despite the hydroxylase activity of PHD3, it has been shown to have also hydroxylase-independent functions (table 1). Since the hypoxic cell survival was found out to be independent on hydroxylase activity of PHD3 (I figure 3A and B), the dependence of pRb dephosphorylation and p27 induction on PHD3 hydroxylase activity was studied. Two independent nonselective pan hydroxylase inhibitors (DMOG and $CoCl_2$) both targeting the catalytic site of PHD3 were used. Whereas the PHD3 depletion decreased the amount of hyperphosphorylated pRb markedly in HeLa cells also in normoxia, DMOG showed no effect (figure 14). Similarly, when the expression of p27 in response to inhibitors DMOG and $CoCl_2$ was studied in 786-O cells no induction in expression of p27 was seen (II figure S2).

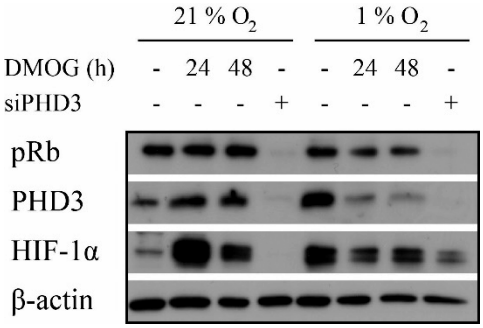


**Figure 14.** The amount of hyperphosphorylated pRb is not dependent on hydroxylase activity of PHD3. The PHD3 depletion showed marked effect on pRb hyperphosphorylation both in normoxic and hypoxic HeLa cells, but no effect was seen with hydroxylase inhibitor DMOG even in 48 hours.

## 5.7 PHD3 regulates hypoxic cell cycle by mediating stability of p27 by HIF- and Skp2-independent mechanism (II, unpublished)

To be sure that the decreased cell survival of siPHD3 treated cells was mediated by increased expression of p27, a rescue experiment was performed. Whereas the HeLa cells treated with siPHD3 only had 20-25% fewer cells after 48-hour hypoxia, the

cells treated with both siPHD3 and sip27 siRNAs showed cell amount comparable with siScr control treated with siScr only or siScr and sip27 siRNAs (II figure 2A and B). The same effect was seen also on cell cycle progression. As the HeLa cells were treated with siPHD3 only, the cell cycle was halted in G1 in hypoxia but when using double knockdown of both PHD3 and p27 the cell cycle proceeded through G1 without arrest (II figure 2C and D). This indicated that the support of PHD3 on hypoxic cell cycle was indeed dependent on p27 expression.

The expression of p27 is regulated at transcriptional and translational level but mainly at posttranslational level (Pagano et al., 1995). As the transcription of p27 has been shown to be induced by hypoxia (Gardner et al., 2001), the effect of PHD3 depletion on p27 mRNA level was studied. Although the hypoxia induced the p27 mRNA production in hypoxic HeLa cells there was no difference between PHD3 depleted and control cells (II figure 3A). In contrast, when using CHX chase to study the stability of p27 a marked increase in p27 stability was noticed in PHD3 depleted cells (II figure 4C and D, S3C and D). This suggested that the increased p27 expression observed in PHD3 depleted cells was due to increased half-life of p27 in these cells.

Even though the transcription of p27 was unchanged in PHD3 depleted cells compared to control, the effect of HIF transcription factors on PHD3 mediated p27 induction was studied by measuring the mRNA and protein levels in hypoxic HeLa cells treated with either siPHD3 only or double knockdown of PHD3 and one of the HIF α-subunits, HIF-1α or EPAS1/HIF-2α. In previous studies the role of HIF-1α in p27 hypoxic induction has been somewhat controversial but p27 does not seem to be a direct HIF-1 target (Carmeliet et al., 1998; Gardner et al., 2001; Goda et al., 2003; Horree et al., 2008; Koshiji et al., 2004). On the contrary, the HIFs have been suggested to co-operate with c-Myc in cell cycle regulation HIF-1α having antiproliferative effects through c-Myc repression and EPAS1/HIF-2α promoting proliferation through c-Myc activation (Gordan et al., 2007b; Koshiji et al., 2004). Thus, HIFs can affect the expression of p27 as the transcription of p27 has been shown to be a direct target and repressed by c-Myc (Yang et al., 2001). The observation of HIF-independence on p27 expression in HeLa cells used in the study was in line with previously reported (II figure 3B and C). In addition, the depletion of PHD3 together with knockdown of either HIF α-subunits showed no effect on p27 expression on mRNA or protein level (II figure 3B-D). The corresponding experiment was performed in 786-O cell line expressing only EPAS1/HIF-2α while

HIF-1α is expressed only as truncated inactive form. In line with the previous experiment performed on HeLa cells, simultaneous depletion of PHD3 and EPAS1/HIF-2α did not abolish the PHD3 depletion mediated p27 increase (figure 15). This further supported the HIF-independence of PHD3 mediated effects on p27 expression.

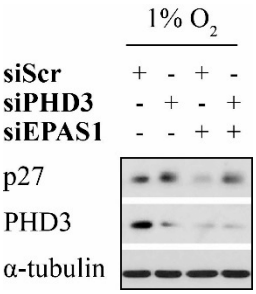


**Figure 15.** The increase in p27 expression is independent on HIF expression. The 786-O cells expressing only functional EPAS1/HIF2-α were transfected with either siScr or siPHD3 only or with double siRNA treatment with simultaneous siEPAS1/HIF-2α siRNA treatment.

The main regulator of p27 stability at G1/S transition is SCF-Skp2 ubiquitin ligase complex which targets the p27 phosphorylated at threonine 187 (T187) for degradation through proteasomes (Carrano et al., 1999; Ganoth et al., 2001; Sutterluty et al., 1999; Tsvetkov et al., 1999). The expression of Skp2 was increased at the G1/S boundary especially in hypoxic HeLa and 786-O cells but there was no significant difference in PHD3 depleted cells compared to siScr control (II figure S5). This was further supported by the unchanged expression of Skp2-target p21 in PHD3 depleted cells (I figure 6C, II figure 1B, III figure 2A and B). In line with Skp2 expression, the phosphorylation of p27 on T187 was not affected by PHD3 depletion (II figure 5A, D and E) and the ubiquitination of p27 was comparable in siScr control and PHD3 depleted cells (II figure S4C). In addition, the blocking of degradation by proteasome inhibitor MG132 had no effect on siPHD3 mediated p27 increase (II figure S4A and B). Together these findings suggested that PHD3 regulates p27 stability independent on the main regulatory pathway mediated by Skp2.

### 5.7.1 Increased stability of p27 in PHD3 depleted cells is not dependent on cell cycle phase (II)

Since the expression of p27 is dependent on cell cycle phase and PHD3 depletion halts the cell cycle in G1, the correlation between these two events was studied. For this purpose, the cell cycle of HeLa and 786-O cells was synchronized to either on G0 by serum starvation or on G1/S boundary by aphidicolin treatment. After synchronization, cell cycle was either released and p27 expression was monitored at indicated timepoints or CHX chase was performed at the cell cycle phase under investigation. In both cell lines used (HeLa and 786-O), the expression of p27 was strongly induced in PHD3 depleted cells at both cell cycle phases studied (II figure 4A-D, S3). At cell cycle release p27, decay was impaired in PHD3 depleted cells especially in HeLa cells (II figure 4A and B, S3A and B). The delay in p27 degradation was even more pronounced after aphidicolin treatment and release, as the p27 level is normally very low or even under detection limit at this point. In PHD3 depleted cells, however, the p27 expression was detectable (II figure S3A and B). The CHX chases performed at both G0 and at G1/S boundary indicated an increased half-life of p27 despite the cell cycle phase (II figure 4C and D, S3C and D).

Since the 786-O cells are not able to exit cell cycle to G0 by serum starvation (Pause et al., 1998), the decay in these cells could not be monitored as in HeLa cells, respectively. However, the increase in p27 expression in response to PHD3 depletion was significant (II figure 4A and B). 786-O is a *PTEN*-null cell line leading to Akt overdrive (Ramaswamy et al., 1999). The constitutively active Akt might balance the impaired decay caused by PHD3 depletion by targeting the excess of p27 to degradation (Shanmugasundaram et al., 2013).

The delay in p27 clearance in PHD3 depleted cells after serum starvation could be due to impairment in cell cycle re-entry although this is unlikely as the effect could be seen also in aphidicolin treated cells and in asynchronously proliferating UT-SCC2, HeLa, 786-O and RCC4 cells (III figure 1A, I figure 5). Together these results indicated that PHD3 mediates the decay of p27 and that the effect is not dependent on cell cycle phase.

## 5.8 PHD3 depletion causes accumulation of p27 phosphorylated on serine 10 (S10) (II, unpublished)

Since the functional activity, accumulation and localization of p27 is determined by phosphorylation, and PHD3 has been implied to regulate protein phosphorylation (Su et al., 2010; Taniguchi et al., 2013; Xue et al., 2010), the phosphorylation of p27 was explored. In addition to threonine 187 (T187), phosphorylation status of three known phosphorylation sites were studied at different cell cycle phases. Whereas only a minor effect on p27 phosphorylation on threonines 157 (T157) and 198 (T198) was seen at G0 in PHD3 depleted cells (II figure 5B, D and E), phosphorylation of serine 10 (S10) was markedly increased by siPHD3 treatment both in HeLa and in 786-O cells at both G0 and G1 phases of the cell cycle (II figure 5C-E, figure 6A and B). Quantification of each phosphorylation site normalized to total p27 expression demonstrated prominently phosphorylated S10, which was comparable to the stabilization by siPHD3 seen on total p27.

Previous studies have implicated p27 phosphorylated at S10 to be the most stable form of p27 (Ishida et al., 2000; Rodier et al., 2001). However, the case seems to be more complex than that. The phosphorylation at S10 takes place as well in resting quiescent cells as in early G1 phase. Whereas the phosphorylation in G0 by Mirk/Dyrk1B serves as a stabilizer (Deng et al., 2004), the S10 phosphorylation in response to mitogen stimulation at G0/G1 boundary by hKIS functions as an export signal from nucleus to cytoplasm (Boehm et al., 2002). When in cytoplasm p27S10 is polyubiquitinylated by KPC1 and KPC2 and targeted for degradation by proteasomes (Kamura et al., 2004). As the most prominent increase in p27 was seen in 786-O cells where serum starvation causes no cell cycle arrest (Pause et al., 1998), the role of mitogen stimulation alone could be studied. The expression of hKIS showed some induction in hypoxic PHD3 depleted cells (figure 16). There was, however, no increase in normoxic hKIS expression even though the p27 was highly induced in these conditions. In hypoxia hKIS expression seemed to be stimulated by mitogens as its expression was increased after 4-hour mitogen treatment (figure 16). In normoxia, this kind of stimulation was not observed. This indicates that change in expression of hKIS is not potential explanation to increased p27 expression in PHD3 depleted cells.

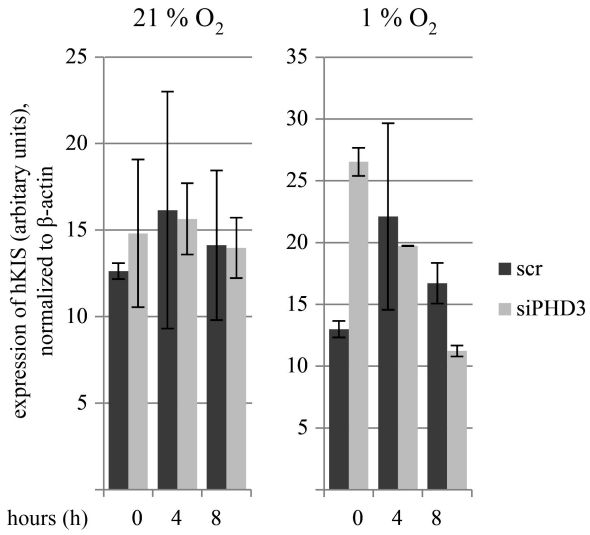


**Figure 16.** Expression of hKIS phosphorylating p27 in response to mitogens at cell cycle re-entry at G0/G1 boundary.

To further study the role of PHD3 depletion, flag-tagged p27 expression plasmids were used. The wild type p27 (p27wt) was expressed mainly in cytoplasm whereas S10 mutated p27 (p27S10A) was trapped into nucleus (II figure 6C). If the phosphorylation on S10 was essential for stabilization of p27 in PHD3 knockdown cells the mutated p27S10A should have been less stable than the p27wt in PHD3 depleted cells. This seemed to be the case, since whereas the p27wt was more stable in PHD3 knockdown cells, the p27S10A was expressed at very low level and also degraded rapidly (II figure 6D).

## 5.9 PHD1 depletion causes cell cycle arrest at G2/M through p21 induction in ccRCC (III)

Since the data by Rocha et al. indicated that PHD1 has a central role in cell cycle regulation (Moser et al., 2013), the effect of PHD1 depletion was studied in ccRCC. The cell cycle progression was determined in two VHL-negative ccRCC cell lines (786-O and RCC4). In both cell lines, the knockdown of PHD1 severely disrupted the cell cycle causing G2/M arrest (III figure 1B).

The cell cycle checkpoint located at G2 prevents the progression of cell cycle into mitosis in case of DNA damage. The arrest at G2 can be p53-dependent or

independent (reviewed in (Taylor and Stark, 2001)). Since ccRCC cell line 786-O has a wild type TP53 allele (Bertout et al., 2009), the role of p53 target cyclin-dependent kinase inhibitor p21 was studied. Indeed, the expression of p21 was strongly increased in PHD1 depleted 786-O cells (III figure 2). As the hydroxylase inhibitor DMOG was unable to induce p21 at early time points, the data suggests that p21 induction could be a secondary effect of Cep192 accumulation caused by PHD1 depletion (Moser et al., 2013). However, the cell cycle arrest of PHD1 depleted cells seemed to be mediated by p21 as simultaneous depletion of PHD1 and p21 restored the cell cycle in PHD1 depleted cells (III figure 3A). At the same time, the cell cycle of the control and PHD2 or PHD3 depleted cells was unchanged in response to p21 depletion (III figure S3). Surprisingly the depletion of p21 in PHD1 knockdown cells decreased the viability of PHD1-p21 double knockdown cells and caused an increase in proportion of sub-G1 cells indicating induced apoptosis or necrosis (III figure 3B). Again, this was specific for PHD1-p21 depleted cells (III figure S3).

## 5.10 Autophagic flux is induced in PHD1 depleted ccRCC cells (III)

The cell cycle arrest mediated by p21 and observed in PHD1 depleted 786-O cells seemed to be protective as simultaneous depletion of p21 caused cell death (III figure 3B). Since p21 has been shown to be involved in regulation of apoptosis and autophagy (Fujiwara et al., 2008; Gartel and Tyner, 2002; Goehe et al., 2012; Luo et al., 2011b), the autophagy in PHD1 depleted cells was determined. Indeed the level of autophagic flux was increased in PHD1 depleted cells measured by increased level of autophagy marker LC3-II, accumulation of LC3 positive puncta in autophagy inhibitor (bafilomycin A1) treatment and decreased level of autophagosomally degraded p62 (III figure 4 and 5). In addition, the formation of autolysosomes was induced illustrated by increased colocalization of lysosomal marker LAMP2 to LC3 positive puncta (III figure 6).

Since the p21 expression could be increased in response to autophagy or it could be the driver of autophagic flux, the autophagy was inhibited using LC3 depletion or chemical inhibitor. None of these treatments was, however, able to restore the p21 expression in PHD1 depleted cells (III figure 7). In contrast, the depletion of p21 in PHD1 knockdown cells restored the LC3-II level to basal level comparable to control or PHD2 or PHD3 depleted cells (III figure 8). This together with

observation of increased cell death of PHD1-p21 double knockdown cells suggested that p21 indeed serves as protective factor in PHD1 depleted ccRCC cells by activating the autophagy in response to stress created by PHD1 depletion.

# 6 DISCUSSION

In my thesis work, I concentrated on studying the role of HIF prolyl hydroxylases (PHDs) in carcinoma cell survival in SCC and also in ccRCC, which has inherently elevated expression of PHDs. The work revealed a central role of PHD1 and PHD3 in cell cycle regulation of carcinoma cells in hypoxia or with elevated PHD expression. The expression of PHD3 protected the cells against hypoxic cell cycle arrest, and depletion of PHD1 or PHD3 caused impaired progression of the cell cycle. PHD3 operated by regulating CKI p27 and PHD1 operated by regulating CKI p21 and macroautophagy.

## 6.1 PHDs as cell cycle regulators

Cell cycle regulation is a delegate mechanism orchestrated by a number of factors. Proper regulation of each phase of the cell cycle ensures the integrity of the genetic material, and halts the cycle if damage is observed by the machinery scanning the DNA. In cancer development, however, the damages also serve as a potential mean to escape from regulation (for a recent review see (Tian et al., 2015)). The tumor microenvironment sets challenge to the carcinoma cells to survive. Fluctuating levels of oxygen create gradients of hypoxia as well as acidification of the tumor. In addition, lack of nutrients predisposes the cells to starvation. These microenvironmental factors change cellular signaling and strongly affect the cell survival. Both cell cycle and cell death are affected by hypoxia, starvation and pH.

To be able to continuously grow and proliferate even in harsh microenvironment, the cancer cells need to break away from physiological restrictions and cell cycle control. The essential parts of cell cycle progression are controlled by cyclin/cdk complexes. The abundancy of the cyclins is dependent on cell cycle phase and its coupling with cdk forms an active complex. The restriction points supervising the quality and completion of each phase of the cell cycle form additional regulatory level of the cell cycle. In cancer, both levels of regulation harbor mutations in the regulatory genes, most of them concentrating on genes involved in G1 control (reviewed in (Collins et al., 1997; Foster et al., 2010; Ho and Dowdy, 2002)).

PHDs were originally found to be regulators of the stability of HIF α-subunits (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). PHD2 has been shown to be the predominant regulator of HIF-1 activity in normoxia (Berra et al., 2003). Since then, particularly PHD1 and PHD3, have been shown to have also HIF-independent functions and other targets besides HIFs (reviewed in (Jaakkola and Rantanen, 2013; Zheng et al., 2014)).

The challenge for many cell types in the hypoxic microenvironment is that the cell cycle is arrested at G1/S transition in response to hypoxia. We have shown that the expression of PHD3 is essential for carcinoma cells to proceed in the cell cycle under hypoxia (Högel et al., 2011). Depletion of PHD3 caused the cell cycle to halt at G1/S transition phase. Of special note is that the arrest was HIF-independent, and was observed also in VHL-negative RCC cell line which otherwise lacks hypoxic cell arrest (Högel et al., 2015; Högel et al., unpublished). In these cells, PHD3 expression is high also in normoxia. In line with this, the depletion of PHD3 caused cell cycle to arrest also in normoxia. Moreover, we found that PHD3 mediated the stability of cyclin-dependent kinase inhibitor p27 (Högel et al., 2015). Depletion of PHD3 caused stabilization of p27 through increased phosphorylation of p27 serine10 residue. This led to cell cycle arrest at G1.

In line with previous data (Moser et al., 2013), I also found that depletion of PHD1 halts the cell cycle at G2/M (Högel et al., unpublished). Interestingly, however, I saw that the arrest was maintained by cyclin-dependent kinase inhibitor p21. Moreover, I demonstrated that PHD1 depletion induced increase in p21 expression led to increased autophagic flux. The role of p21 as a mediator of both transient and permanent cell cycle arrest has been widely studied, and p21 has been strongly linked to cellular senescence as well as to DNA damage induced cell cycle arrest (reviewed in (Campisi and d'Adda di Fagagna, 2007)). Senescence was long considered as irreversible continuum to prolonged G1 arrest but after revealing the role of p21 in G2/M control (Bunz et al., 1998; Dulić et al., 1998), senescence has been found to occur also in cells arrested in G2 (Mao et al., 2012; Wada et al., 2004).

Cell cycle arrest does not, however, always lead to senescence. The depletion of p21 overexpression allows the cell cycle progression after transient G2 arrest but leads to mitotic catastrophe and induction of apoptosis in a subset of cells (Bunz et al., 1998). The recovery from p21 induction is possible if the depletion of p21 is taking place within three days from induction (Chang et al., 2000; Demidenko et al., 2009).

After this point, the cell cycle arrest becomes irreversible and cells lose their capacity to proliferate. In our experiments the duration of p21 expression is difficult to determine. However, since p21 is induced in response to depletion of PHD1, the duration of expression before p21 withdrawal is most likely 24-48 hours. To evaluate the onset of senescence in PHD1 depleted cells a longer period of observation would be necessary. Since hypoxia has been shown to inhibit senescence onset even in the presence of p21, it would be of great interest to study the induction of senescence in PHD1 depleted cells. Interestingly, there are studies showing that hypoxic signaling is involved in the development of colitis and inflammatory bowel disease (IBD). Moreover, the dysfunction of epithelial barrier, which is central to the pathology of the diseases, is mediated through PHD1 (Cummins et al., 2008; Robinson et al., 2008; Tambuwala et al., 2010). Based on their studies conducted on *Phd1*$^{-/-}$ mice, Tambuwala et al. suggest that PHD1 positively regulates apoptosis in intestinal epithelial cells in colitis. Whether or not this is relevant in cancer, it is in any case worth noting, as in both diseases inflammatory response has a central role. The IBD patients have also higher risk of developing colorectal cancer (CRC), and CRC accounts approximately 15% of the deaths of the patients with IBD (reviewed in (Munkholm, 2003; Triantafillidis et al., 2009)).

In addition to its role as mediator of DNA damage response pathway, p21 expression is important for regulator of mitotic entry and progression (reviewed in (Kreis et al., 2015)). The work conducted by Rocha et al. showed that PHD1 depleted cells arrest at prometaphase (Moser et al., 2013). At this same point, p21 is degraded through ubiquitination after its induction during G2 (Amador et al., 2007). The role of p21 in mitotic entry raises a question if the p21 increase is a secondary effect caused by the overlap of the two events. However, since the simultaneous p21 depletion rescues the cell cycle in PHD1 depleted cells, the p21 induction is most probably not due to random overlapping. Interestingly, p21 has been shown to regulate centriole duplication, and p21-deficient cells have abnormally high number of centrioles (Duensing et al., 2006). PHD1 expression, on the other hand, is needed for centriole duplication (Moser et al., 2013).

The most simplified and probable theory is that PHD1 depletion causes disorganization of mitotic spindle resulting in p21 expression as response to damage. Depletion of p21 in these cells causes mitotic catastrophe leading to cell death. However, the role of autophagy in this context is something novel and suggests that PHD1 depleted cells arrested at G2/M survive through autophagy induction.

Whether the phenomenon is more fundamental to cells arrested at G2/M or not, is to be solved. Strikingly, autophagy needs intact microtubules to maximal function (Kochl et al., 2006; Webb et al., 2004) and in PHD1 depleted cells this network is disorganized. It seems that autophagy can still be functional despite the defect in mitotic spindle formation.

## 6.2 Role of PHD3 in regulation of the stability of p27

The stability of p27 is regulated by several phosphorylations and degradation pathways. Many phosphorylations are coordinated by signaling pathways regulating the overall survival of the cell which indicates a central role of p27 in cell cycle regulation.

Since PHD3 depletion caused cell cycle to halt at G1 and p27 functions as a regulator of cdk2 and cdk4 acting on G1, the expression of p27 seemed essential to determine. Indeed, the half-life of p27 was increased in PHD3 depleted cells (Högel et al., 2015). Further studies revealed a significant change in phosphorylation of serine 10 (S10) of p27. When normalized to the total level of p27 the S10 phosphorylated form of p27 was the only phosphorylated form of p27 increased in PHD3 knockdown cells. The S10 phosphorylated form of p27 has been shown to be the most stable form of p27 (Ishida et al., 2000; Rodier et al., 2001). However, this phosphorylation has been shown to have distinct functions depending on cell cycle phase and on localization of p27. In quiescent cells, the S10 stabilizes p27, whereas during cell cycle re-entry at G0/G1 transition it serves as nuclear export signal (Besson et al., 2006; Ishida et al., 2002). Also in G1 the S10 is phosphorylated and p27 stabilized (Kossatz et al., 2006). Especially the work by Kossatz et al. indicates a complex regulation of p27 stability mediated by multiple phosphorylations and their different combinations (Kossatz et al., 2006). It is also not clear how p27 translocation into cytoplasm is regulated by mitogens or other proteins, as some additional signal is necessary to distinguish between the two functions of the same phosphorylation.

I considered the possibility that PHD3 depletion would affect the expression or activity of one of the kinases regulating the abundancy of p27 through S10 phosphorylation. Since PHD3 depletion arrests the cell cycle at G1, the kinase phosphorylating p27 at G0 was not likely to be involved. Regardless, I determined the expression of Mirk/Dyrk1B phosphorylating p27 in quiescence (Deng et al.,

2004). As expected, there was no change in the level of Mirk/Dyrk1B in hypoxia treated PHD3 depleted HeLa cells (figure 17). The simultaneous depletion of the HIF-1 and HIF-2 α-subunits had no effect on the expression either. In addition to Mirk/Dyrk1B, S10 is phosphorylated by hKIS (Boehm et al., 2002). The expression of hKIS was, however, almost unchanged (figure 16).

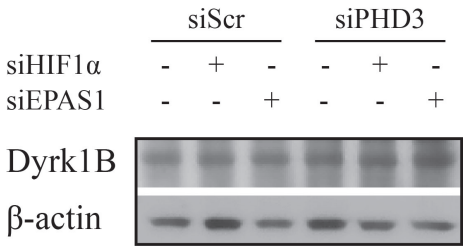


**Figure 17.** Expression of Mirk/Dyrk1B kinase in HeLa cells after 24-hour hypoxia treatment.

The role of Akt in p27 regulation has been shown to be substantial (Fujita et al., 2002; Fujita et al., 2003; Liang et al., 2002; Motti et al., 2004; Shin et al., 2002). In Hela cells the depletion of PHD3 seemed to increase the activity of Akt demonstrated as increase in ser473 phosphorylation of Akt (data not shown). However, since the RCC cell lines (RCC4 and 786-O) used in the studies have constitutively active Akt signaling, the role of increased Akt signaling in our concept was not considered noteworthy. Within the limits of this thesis project, I was unable to determine the kinase responsible for the increased phosphorylation of p27 or alternatively, the phosphatase supposed to dephosphorylate p27.

However, the use of 786-O cells gives some new insight to the problem. The 786-O cells do not respond to serum starvation by halting the cell cycle which indicates mitogen-independent cell cycle control that is very common to cancer cells (Pause et al., 1998). They do still respond to contact inhibition. Interestingly, the depletion of PHD3 caused cell cycle arrest in all cell lines used in the study (Hela, HNSCC, 786-O, RCC4) regardless of the response to hypoxia or the VHL status of the cell line. In all cases the stability of p27 was increased. The previous work by Pause et al. showed that p27 stability was increased in response to serum withdrawal and contact inhibition by VHL-dependent manner (Pause et al., 1998). This together with findings presented in this thesis indicate that PHD3 regulates p27 and cell cycle in a VHL-independent manner.

## 6.3 PHDs in stress response

Since the activity of PHDs is regulated not only by oxygen but also by Krebs cycle intermediate 2-OG, the role of PHDs also as sensors for nutrition status of the cell is a tempting idea (Duran et al., 2013). In their publication they show that amino acid starvation leads to HIF-independent PHD inactivation and disruption in amino acid mediated mTORC1 activation. The inactivation of mTORC1 in response to PHD inhibition, on the other hand, activated autophagy (Duran et al., 2013).

Interestingly, the depletion of PHD3 caused non-apoptotic cell death in HNSCC cells (Högel et al., 2011) whereas in RCC cells the viability of PHD3 knockdown cells was comparable to control cells (Högel et al., unpublished). The cell cycle was arrested in both cases at G0/G1 and the stability of p27 was markedly increased. However, the final outcome was different. There are some fundamental differences between these cell lines. Whereas the HNSCC cells express PHD3 mainly in hypoxic conditions, have functional pVHL and harbor both HIF-1 and HIF-2, the RCC cell lines express PHD3 both in normoxia and in hypoxia, have inactivating mutation on pVHL and thus constitutively active HIF, and might also express just one isoform of HIF. From the RCC cell lines we used in our studies, RCC4 cell line expresses both HIF-1 and HIF-2 but 786-O cell line only HIF-2. The outcome of cell cycle arrest and concomitant p27 increase might be related to the pVHL activity. Preliminary research done on VHL-positive RCC4 cell line indicates that this is the case, since PHD3 depletion in these cells causes an increase in the population of sub-G1 cells and reduction in cell viability especially in hypoxia (data not shown).

In the case of PHD1 depletion, we wanted to look into the mechanisms which could enable the survival of PHD1 depleted ccRCC cells in G2/M arrest. It has been suggested that autophagy would mediate the transition from proliferative to senescent stage (Young et al., 2009). However, others point out that senescent stage can be achieved also when autophagy is inhibited (Goehe et al., 2012). Interestingly, just recently autophagy was shown to maintain proliferative potential of muscle stem cells thus inhibiting senescence (García-Prat et al., 2016). The role of cyclin-dependent kinase inhibitors (CKIs) in autophagy has been previously shown (Jiang et al., 2010; Komata et al., 2003; Liang et al., 2007; Sun et al., 2014). Since the PHD1 depletion increased the expression of p21 and the simultaneous knockdown of p21 caused cell death of a subset of cells presumably in response to mitotic catastrophe described earlier, the role of autophagy was to be determined as survival mechanism

of PHD1 depleted cells. Indeed, we found a strong increase in the level of autophagy markers in PHD1 knockdown cells (Högel et al., unpublished). In addition and most notably, the effect was p21-dependent. The increased stability of p27 in PHD3 depleted cells, however, was unable to activate autophagy in RCC cells. This was verified also with ectopic expression of p27 only.

In PHD1 depleted cells, the autophagy activity is increased as measured by increase in LC3-II level and degradation of p62. However, increased number of p62 granules seen in immunocytochemical staining in PHD1 depleted cells could indicate higher level of degradation but it could also indicate change in mTOR activation as p62 has recently been shown to be necessary for mTORC1 translocation into lysosomes and thus its activation (Duran et al., 2011; Linares et al., 2013; Puissant et al., 2012). Since active mTOR inhibits autophagy, this would be however, inconsistent with the increased autophagic flux observed using other autophagy markers.

# 7 SUMMARY

Tumor microenvironmental factors and especially hypoxia are fundamental mediators of cellular signaling in cancer. Hypoxic response covers all episodes of cellular life from cell division to cell death by affecting cell cycle, cellular metabolism and apoptosis. Many factors involved in hypoxic response have been shown to be involved also in the regulation of cell metabolism and/or cell death pathways. In this thesis work, the role of the HIF prolyl hydroxylases in cell survival was studied. Previously it has been shown that PHDs are important mediators of hypoxic response but that they also regulate the energy metabolism in the cell as well as cell death.

In this study, I demonstrated that PHDs have a fundamental role in cell cycle regulation of carcinoma cells especially in hypoxia. My work implies that the regulation is mediated through cyclin-dependent kinase inhibitors p21 and p27. Disruption of this controlling network by PHD depletion causes cell cycle arrest and predisposes the cells to stress where PHD1 depleted cells avoid death by autophagy activation. This work extends recent knowledge of hypoxic signaling and cell cycle regulation, and especially the interplay between the survival mechanisms in hypoxia.

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