Metabolic regulation of prolyl hydroxylase domain-containing protein and hypoxia-inducible factor in glioblastoma cells in-vitro

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1 Introduction

1.1 Glioblastoma multiforme

Glioblastoma multiforme (GBM), the most common primary brain tumor in adults, is considered to be one of the most lethal diseases in modern medicine. In the 20th century, Percival Bailey and Harvey Cushing first provided a description of GBM as an entity, separating the clinically most malignant and histopathologically atypical tumor from other neoplastic entities arising in the central nervous system (Stoyanov and Dzhenkov 2018). Unlike other advances made in modern oncology, however, no breakthrough has been achieved to improve the clinical outcome in GBM. The overall survival of GBM patients did not extend significantly since over a hundred years and still stays at a dim 11-15 months after diagnosis (Hanif, Muzaffar et al. 2017, Sasmita, Wong et al. 2018, Stoyanov, Dzhenkov et al. 2018, Zygogianni, Protopapa et al. 2018). The complex nature of GBM has been the subject of a great body of research over the past decades. The following sections will provide an introduction to our current understanding of GBM, from its clinical features to the biology of one of man's most lethal tumors.

1.1.1 Clinical features of glioblastoma multiforme

The clinical presentation of GBM can be diverse depending on its location, as is the case with all space-occupying lesions in the central nervous system. Usually, the tumor presents itself with a short clinical history of 3 – 5 months, except when it develops from a lower grade glioma, in which case symptoms can slowly develop over the course of several years. Leading symptoms include headache (30 – 50%), focal neurological deficits (40 – 60%) and seizures (20 – 40%) (Hanif, Muzaffar et al. 2017). The incidence rate is less than 10 per 100,000 people, with an age peak of around 55 years (Hanif, Muzaffar et al. 2017). The most important diagnostic procedure is contrast-enhanced MRI, where GBM usually presents itself as lesions with irregular contrast-enhanced margins (corresponding with perilesional oedema) and central heterogeneous signals (composing of necrosis or haemorrhage at the core) (Hanif, Muzaffar et al. 2017, Zygogianni, Protopapa et al. 2018). The definite diagnosis of GBM requires histopathological confirmation in tumor samples attained by surgery or stereotactic excision.

Even though GBM remains one of the most challenging brain cancera to treat, therapeutic strategies have seen many changes and updates over the years. As of the

present, the standard treatment approach consists of complete surgical resection, combined with chemotherapy with temolozomide and radiation following the Strupp regimen (Zygogianni, Protopapa et al. 2018). Meanwhile, a lot of clinical studies are investigating alternative therapy options. Among the most promising novel approaches are nanoparticle therapy, immunotherapy and especially the Tumor-Treating-Field therapy recently approved by the Food and Drug Administration, which uses alternating electric fields generated by a wearable helmet to non-invasively disrupt the mitosis process of tumor cells (Zygogianni, Protopapa et al. 2018). The evaluation and possible establishment of such therapies will be much anticipated in the years to come.

However, despite therapeutic advancements, the five-year survival rate still remains at a poor 5 % (Dolecek, Propp et al. 2012). Therapeutic failure can mostly be attributed to the high recurrence rate. At the time of diagnosis, most glioblastomas have already spread to sites distant from the primary tumor mass in the form of micrometastases, consisting of highly resilient cells (Aderetti, Hira et al. 2018). So far, neither surgical excision, nor radiochemotherapy or any novel approach has been able to fully eliminate those remnant neoplastic cells. Those cells then give rise to recurrent tumor masses that are often elusive to current treatment options and result in the patient's death within an average of six months (Zygogianni, Protopapa et al. 2018).

In order to advance therapeutic success, it is crucial to dissect and understand the biology of GBM, from tumorigenesis to mechanisms of tumor maintenance, metastasis and therapy resistance.

1.1.2 The biology of glioblastoma multiforme

Similar to the majority of tumors of the central nervous system, GBM arises from glial cells, the cell population crucial for forming the supportive network for neuronal function. Historically, de-novo GBM are called primary GBM, whereas tumors developing from less malignant astrocytoma are called secondary GBM (Louis, Perry et al. 2016, Zygogianni, Protopapa et al. 2018). This is reflected in the molecular finding of severe trigger mutations in primary GBM, whereas the accumulation of a high number of less prominent mutations seem to drive the tumorigenesis of secondary GBM (Ohgaki and Kleihues 2007).

Table 1.1: Verhaak classification of GBM based on distinct genetic profiles.

	Absent mutations	Genetic markers	Cell type with associated gene signature	Therapy response	Survival mean	Average patient age
Classic	• p53 • PDGFRA • IDH1 • NF1	• Chromosome 7 amplification • LOH chromosome 10 • EGFR mutations CDKN2A deletion (Rb Pathway)	Murine astrocytes	Aggressive treatment shows greatest benefit on survival	12.2 months	55.7 years
Mesenchymal	• IDH1 • PDGFRA	• Mesenchymal markers CHI3L1, MET • High expression of TNF and NF-kB pathway genes NF1 mutations	Immortalized astrocytes, microglia	Clear effect of aggressive treatment	11.8 months	57.7 years
Proneural	-	• IDH1 mutation • p53 mutation • PDGFRA mutation high expression of oligodendrocytic development genes	Oligodendrocytes	Aggressive treatment not as effective	11.3 months	51.8 years
Neural	• PDGFRA • EGFR	• Expression of neuron markers (NEFL, GABRA1) similar to the expression pattern of normal brain tissue	Oligodendrocyte, astrocytes, neurons	-	13.3 months	63.8 years

EGFR = epithelial growth factor receptor, LOH = loss of heterozygosity, IDH1 = isocitrate dehydrogenase 1, NF1 = neurofibromin 1, Rb = retinoblastoma protein, NEFL = neurofilament light, GABARA1 = gamma-aminobutyric acid receptor alpha 1, CHI3L1 = chitinase-3-like protein 1, MERTK = MER tyrosine kinase, TNF = tumor necrosis factor, NF-kB = nuclear factor kappa B, PDGFRA1 = platelet derived growth factor receptor A1.

In contrast to the meaning of the term "multiforme" in the 20th century that was mainly based on the complexity of histopathological findings, the multiformity of GBM nowadays is apparent in its molecular heterogeneity. Using next-generation sequencing techniques, the cancer genome atlas was established and enabled the classification of GBM into subtypes with distinct mutation and genetic profiles, correlating with different disease courses in regard to progression, treatment response and outcome. One of the most prominent molecular classification system is the Veerhak classification, which introduces four subtypes of GBM: classic, mesenchymal, proneural, and neural (Verhaak, Hoadley et al. 2010).

The classic subtype features the most common genetic aberrations in GBM: epithelial growth factor receptor (EGFR) mutations, loss of heterozygosity of chromosome 10 (LOH 10), and chromosome 7 amplification. Additionally, it shows a lack of mutations in the gene loci of isocitrate dehydrogenase 1 (IDH1), neurofibromin 1 (NF1), tumoric protein p53 and retinoblastoma protein (Rb) (Verhaak, Hoadley et al. 2010). The neural subtype expresses neuronal markers like neurofilament light (NEFL) and GABA receptor alpha 1 (GABARA1), and is the most similar to the transcriptional profile of normal brain tissue, although their histopathological phenotype undoubtedly fulfills the diagnostic criteria for GBM (Verhaak, Hoadley et al. 2010). The mesenchymal subtype expresses mesenchymal markers chitinase-3-like protein 1 (CHI3L1) and MET proto-oncogene next to astrocytic markers CD44 and MER tyrosine kinase (MERTK), which is reminiscent of an epithelial-to-mesenchymal transition (EMT). The high expression of genes acting in the tumor necrosis factor (TNF) and nuclear factor kappa B (NF-kB) pathway correlates with the high overall necrosis and inflammatory response associated with this subtype (Verhaak, Hoadley et al. 2010). Lastly, the proneural subtype harbours platelet derived growth factor receptor subunit A (PDGFRA) and IDH1 mutations in characteristically high rates. In addition, it's high expression of oligodendrocytic development genes deems it a rather atypical GBM type, suggesting a possible origin from progenitor or neural stem cells that could also give rise to oligodendrogliomas (Verhaak, Hoadley et al. 2010). **Table 1.1** outlines the most important characteristics of each subtype.

This classification also differentiates between varying clinical presentations and treatment responses, thus helping to guide clinical management. For instance, the proneural subtype - corresponding to secondary GBM - is often found in younger patients and does not benefit from aggressive treatment with chemotherapy and radiation. The more malignant classic or mesenchymal subtypes, on the other hand, require fast and aggressive chemotherapy to attain better results on an already poor prognosis (Verhaak, Hoadley et al. 2010).

With the emerging importance of targeted therapy, molecular characteristics thus begin to be integrated into clinical classifications. The most important clinical classification system is the WHO classification of tumors of the central nervous system, which has been revised in 2016 to include molecular markers (**Table 1.2**). It declares three new subgroups of GBM based on the mutation status of isocitrate dehydrogenase (IDH): GBM with IDH-wildtype, GBM with IDH-mutation and GBM not otherwise specified (in case IDH mutation status cannot be determined) (Louis, Perry et al. 2016). This classification underlines the clinical significance of IDH mutation, a strong prognostic factor for outcome and treatment response. Notably, GBM with IDH-mutation in the WHO classification show a high overlap with the proneural subtype according to Verhaak, as well as the historical definition of secondary GBM. A concept widely believed is that IDH mutation is the starting point for the tumorigenesis of lower grade gliomas, which then gain malignancy by accumulating minor genetic aberrancies over time and develop into secondary GBM of the proneural subtype (Stoyanov and Dzhenkov 2018).

Table 1.2 Current WHO-grading of selected astrocytic and oligodendroglial tumors.
The prognosis of each tumor type depends on the grading (higher grading correlates with poorer prognosis), but also the mutation status of IDH, H3K27M and/or 1p/19q-deletion.

Selected brain tumors	**WHO Grading**			
	I	**II**	**III**	**IV**
Pilocytic astrocytoma	x			
Diffuse astrocytoma, IDH-mutant		x		
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted		x		
Anaplastic astrocytoma, IDH-mutant			x	
Anaplastic oligodendrogliomas, IDH-mutant and 1p/19q-codeleted			x	
Diffuse midline glioma, H3K27M-mutant				x
Glioblastoma, IDH-mutant				x
Glioblastoma, IDH-wildtype				x

IDH: isocitrate dehydrogenase

Regardless of possible different origins, all GBM subtypes show a similar pattern of tumor progression. The malignancy of GBM partly lies in its fast growth, macroscopically apparent in the "butterfly" formation, which describes the trespassing growth from one brain hemisphere into the other. This enormous cell proliferation speed outgrows the

blood supply and creates hypoxic and necrotic regions. Hypoxic regions then induce the formation of new blood vessels, a process called neovascularisation. Histopathologically, both pseudopalisadic necrosis and neovascularisation are very characteristic features of GBM and belong to the list of its diagnostic criteria (Stoyanov, Dzhenkov et al. 2018). On a molecular level, the genetic instability of such prolific growth results in an increasing number of mutations over time. Different molecular subtypes of the Verhaak classification could be found in multiple samples of the same tumor, although one subtype usually dominates (Ho and Shim 2017). Notably, with disease progression, the dominant molecular subtype often switches to the aggressive mesenchymal subtype. This, in turn, is reflected in the high prevalence of the latter subtype in recurrent tumors (Ho and Shim 2017).

In the characteristic hypoxic regions in GBM, a number of distinct cell subpopulations emerges, such as cells expressing stem cell markers like CD133, which may be a driving force of tumor recurrence (Aderetti, Hira et al. 2018). Hypoxia-inducible factor (HIF) plays a central role in orchestrating the cellular response to hypoxia, amongst others promoting the expression of CD133, and thus fostering the tumor stem cell phenotype. HIF is shown to contribute to disease progression in many tumor types (Aderetti, Hira et al. 2018). This work aims to deepen the understanding of the metabolic regulation of HIF and its related pathways in GBM.

1.2 Hypoxia-inducible factors in tumor biology

1.2.1 Hypoxic tumor microenvironment in GBM

Next to the molecular diversity, the heterogeneous microenvironment within GBM adds to the complex nature of the tumor's biology. The tumor microenvironment is the entirety of extra-cellular fluid, matrix, biomolecules and non-cancerous cells surrounding the tumor cells. On one hand, changes in the microenvironment like hypoxia, nutrient starvation, acidosis, as well as chemotherapy and radiation toxicity, act as external signals that modulate cancer cell behavior (Schiffer, Annovazzi et al. 2018). On the other hand, signals from the cancer cells themselves actively shape the microenvironment, often in a complex crosstalk with non-cancerous cells (Wu, Wei et al. 2010). For example, GBM cells secrete transforming growth factor beta 1 (TGF-β1) and macrophage-inhibitory cytokine 1 (MIC-1) into the microenvironment to polarize microglia and macrophages into an immunosuppressive phenotype, inhibiting immune surveillance function by T cells (Wu, Wei et al. 2010). Understanding the mechanisms in the tumor

microenvironment will therefore provide a framework for a more efficient therapeutic approach to GBM and other tumors.

One factor known to influence the behavior of nearly all solid tumors is the presence of hypoxia in the tumor microenvironment. Physiological oxygen concentration in adult tissue ranges between 2 – 9%, much lower than the atmospheric level of 21% (Colwell, Larion et al. 2017). In all solid tumors, hypoxia is omnipresent, distributed only with varying intensity (Schiffer, Annovazzi et al. 2018). In GBM, intratumoral oxygen levels can reach as low as 1.21%, as compared to 2.35% in peritumoral areas (Beppu, Kamada et al. 2002). This shortcoming is mainly attributed to insufficient oxygen delivery within the tumor. One reason is the rapid growth of the tumor mass, which outpaces vascularization and creates areas undersupplied with oxygen and nutrition. Even in neovascularized areas, the formed vessels lack the structural support of pericytes and are characteristically leaky. This faulty vascularization impedes perfusion in large parts of the tumor. Additionally, microvascular thromboses occlude vessels, further promoting intratumoral hypoxia (Colwell, Larion et al. 2017). Importantly, tumors with large hypoxic regions are associated with a higher WHO tumor grade and thus a worse outcome, as well as more frequent and faster recurrence after standard therapy (Evans, Judy et al. 2004).

1.2.2 The function of hypoxia-inducible factor

The molecular response of cells upon hypoxia is mediated by hypoxia-inducible factor (HIF). The transcription factor HIF consists of an α- and a β-subunit. Both subunits are members of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor family. The β-subunit is constitutively expressed and oxygen-insensitive, whereas the α-subunit is oxygen-labile and continuously degraded in normoxic conditions. If hypoxia occurs, the α-subunit is rapidly stabilized and relocates itself from the cytoplasm into the nucleus where it forms a heterodimer with the β-subunit (Kaluz, Kaluzova et al. 2008). This heterodimer recruits p300/CBP coactivators and binds to the hypoxic-response element (HRE) of target genes, thereby activating the transcription of those genes (Keith, Johnson et al. 2011, Huang, Lin et al. 2017) (**Figure 1.1**). The activated genes then start the machinery of adaptation to ensure the cell's survival under unphysiologically low oxygen levels.

There are three isoforms of the α-subunit, named HIF-1α, HIF-2α, and HIF-3α, respectively. The earlier discovered isoforms HIF-1α and HIF-2α are mainly responsible

for the hypoxic adaptive mechanisms known to date. HIF-1α and HIF-2α have many overlapping functions but also possess distinct, sometimes even opposing roles in tumor biology (Keith, Johnson et al. 2011). The role of HIF-3α, on the other hand, remains largely unknown (Huang, Lin et al. 2017). Some splice variants of HIF-3α are found to inhibit the activity of the other two isoforms. There are also reports of HIF-3α's own transcription activity, but the significance of this in the context of tumor biology remains to be elucidated (Yang, Wu et al. 2015).

Multiple metabolic signaling pathways activated by HIFs converge into a program that encourages tumor cell survival and proliferation. HIFs modify glucose and glutamine utilization, angiogenesis, apoptosis, the stemness of tumor cells and the immune response (Semenza 2010, Keith, Johnson et al. 2011). The following paragraphs will review the different processes one by one.

Angiogenesis is a hallmark of cancer that is strongly susceptible to regulation by HIF. Vascular endothelial growth factor (VEGF), the common target of both HIF-1 and HIF-2, is the most important pro-angiogenic factor for the initiation of sprouting angiogenesis. Furthermore, HIF-2 has been shown to upregulate angiopoetin 2 (ANGPT2) and ephrin A1 to support vessel formation and maturation (Keith, Johnson et al. 2011).

Metabolic reprogramming is another hallmark of cancer. In an altered state of metabolism called the Warburg effect, tumor cells shift their energy generation away from oxidative phosphorylation towards less-efficient glucose fermentation (Liberti and Locasale 2016). By doing so, they depend less on the availability of oxygen to generate ATP. HIF-1 in particular supports this shift by increasing the expression of enzymes involved in glycolysis such as hexokinase 1 and 2 (HK1, HK2), phosphofructokinase 1 (PFK1), lactate dehydrogenase A (LDHA), and upregulating transporters GLUT1 and GLUT3 to increase glucose uptake (Keith, Johnson et al. 2011, Colwell, Larion et al. 2017). Meanwhile, oxidative phosphorylation is reduced by the upregulation of pyruvate dehydrogenase kinase 1 (PDK1) expression, which actively shunts pyruvate away from the tricarboxylic acid (TCA) cycle. As a result, lactate accumulates in the cytoplasm and is released into the extracellular space by HIF-inducible plasma membrane monocarboxylate transporters (MCT-1 and MCT-4), thereby acidifying the surrounding microenvironment (Colwell, Larion et al. 2017).

Next to enhanced glucose utilization, tumor cells also unphysiologically switch to glutamine as a main carbon source for macromolecular synthesis (DeBerardinis and Cheng 2010, Metallo, Gameiro et al. 2011). HIF-targeted enzymes involved in this shift

are, among others, membrane glutamate transporters SLC1A1 and SLC1A3 (Hu, Takano et al. 2014) and glutamate dehydrogenase (GDH) (Jiang, Wang et al. 2017).

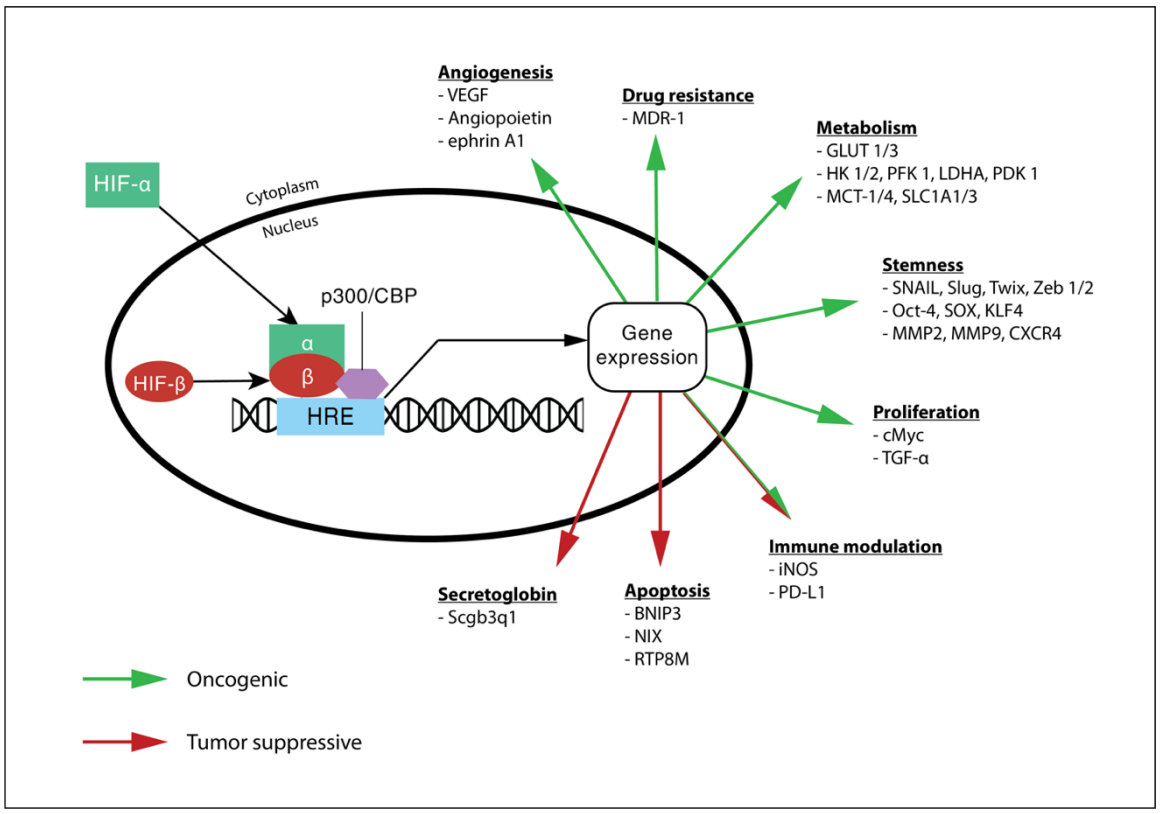

Figure 1.1: The role of HIF in tumor biology.
Under hypoxia, the stable HIF-α subunit translocates itself into the nucleus. Together with the HIF-β subunit and p300/CBP coactivators, it activates the expression of genes modulating many processes of the tumor, including angiogenesis, drug resistance, metabolism, stemness, proliferation, immune modulation and apoptosis.

Furthermore, HIF is responsible for the activation of pluripotent factors Octamer-binding factor 4 (Oct4), sex-determining region box 1 (Sox-1), Homeobox NANOG, and Kruppel-like factor 4 (Klf-4) to induce and maintain the stemness of cancer stem cells like the previously mentioned GBM stem cells (Li, Bao et al. 2009, Colwell, Larion et al. 2017, Ho and Shim 2017). This subpopulation, which is particular prone to metastasizing, expresses HIF targets that are master regulators of the epithelial-to-mesenchymal transition (EMT) like zinc finger E-box-binding homeobox (ZEB)1, ZEB2, Snail, Slug, as well as supporters of migration and invasion such as matrix metalloproteases MMP-2 and MMP-9, lysyl oxidase (LOX), and chemokine receptor type 4 (CXCR4) and its ligand stromal cell-derived factor (SDF)1 (Semenza 2012, Zhang, Huang et al. 2013, Muz, de la Puente et al. 2015).

HIF is also implicated in proliferation via the upregulation of the master regulator cMyc and TGF-α (Keith, Johnson et al. 2011), as well as immune modulation via the expression of inducible nitric oxide synthase (iNOS) and programmed death-ligand 1 (PD-L1) (Colwell, Larion et al. 2017). Finally, HIF-1 in particular has been described to induce the expression of the multidrug resistance-1 (MDR-1) gene encoding P-glycoprotein transporters, thereby contributing greatly to chemotherapy resistance (Ho and Shim 2017).

It is important to highlight the differences between HIF-1 and HIF-2. As mentioned above, several genes are distinct targets of either HIF isoforms. In general, metabolism-related genes are preferentially regulated by HIF-1, whereas HIF-2 has been found to have a greater role in promoting the stemness phenotype. Notably, HIF-1α stabilization occurs only at lower oxygen levels (< 2%), as contrasted with HIF-2α already accumulating in moderate hypoxia (2 - 5%) (Keith, Johnson et al. 2011). Hence, HIF-1 is also the isoform primarily active in the case of acute severe hypoxia. If hypoxia prevails, however, the cellular response becomes more dependent on HIF-2. This signalling switch from HIF-1-dependence to HIF-2-dependence is especially found in advanced stages of cancer, which is proposed to ensure tumor proliferation and stemness maintenance in chronic hypoxia (Keith, Johnson et al. 2011). Other triggering factors for this switch include the accumulation of onco-miRNA, oncometabolites, and chemokines (Murugesan, Rajajeyabalachandran et al. 2018). From a clinical perspective, HIF-1 and HIF-2 expression levels are sometimes associated with opposing prognosis (favorable and poor, respectively) in certain types of tumors such as neuroblastoma and renal cell cancer. In GBM, most evidence point to HIF-2, rather than HIF-1, to correlate with an aggressive tumor phenotype and poor prognosis (Keith, Johnson et al. 2011).

Interestingly, in recent years, some evidence for antioncogenic effects of HIFs have emerged. In neuroblastoma and renal cell carcinoma, elevated HIF-1 levels correlate with favorable outcome (Keith, Johnson et al. 2011). Several HIF knock-down experiments report accelerated tumor growth in lung, breast, pancreatic cancer and sarcoma models, but also in GBM (Keith, Johnson et al. 2011, Huang, Lin et al. 2017, Acker, Till et al. 2005). The mechanisms behind these observations remain largely unclear. However, some aspects of antioncogenic properties of HIFs are slowly gaining a foothold, such as the HIF-2-driven expression of the putative tumorsuppressor secretoglobin 3A1 (Keith, Johnson et al. 2011). Even though there are still many questions left concerning possible tumorsuppressing effects of HIF, the presented facts highlight the complexity of the cellular response mediated by such a master gene

regulator and stresses the importance of careful assessment in developing HIF-targeting anti-cancer therapy.

In summary, HIF is the transcription factor responsible for rewiring the cellular machinery to adapt to hypoxia. In tumor biology, HIFs modulate many tumor-promoting processes including angiogenesis, invasion and metastasis, metabolism, immune surveillance, and the maintenance of cancer stem cells. However, HIF function is complex in the sense that it is isoform- and cell type-dependent, exhibiting many oncogenic and some tumorsuppressive effects. These findings explain the difficulties in establishing therapeutic strategies targeting HIF, even though the discovery of the protein and its significance already dates back to several decades ago.

1.2.3 The regulation of hypoxia-inducible factors

Similar to the majority of proteins, HIF can be controlled on the transcriptional, translational, and post-translational level. In contrast to the relatively stable HIF-β subunit, the HIF-α subunit is more susceptible to and thus the principal subject of HIF activity regulation. The transcription and translation of HIF-α is mainly regulated by the mTOR, MAPK and NF-κB pathways, which are themselves subject to modulation by a number of cytokines and growth factors (Lee, Bae et al. 2004, van Uden, Kenneth et al. 2008, Keith, Johnson et al. 2011).

However, it is at the protein level that HIF is controlled most tightly. Among all mechanisms, post-translational hydroxylation has the greatest effect (**Fig. 1.2**). The key proteins concerting this action belong to the class of prolyl hydroxylase domain (PHD) enzymes. In the presence of oxygen, PHD continuously hydroxylates proline residues (P402 and/or P564 in HIF-1α, P405 and/or P531 in HIF-2α) in the oxygen-dependent degradation (ODD) domain of HIF-α (Epstein, Gleadle et al. 2001). The hydroxylation of either proline residue is sufficient for the specific recognition by the von Hippel-Lindau (vHL) protein, a component of the E3 ubiquitin ligase, which leads to the rapid proteosomal degradation of HIF-α (Kaluz, Kaluzova et al. 2008). Since PHD uses oxygen as a substrate, hypoxia inhibits its enzymatic activity and puts a halt to HIF-α degradation. This regulatory mechanism mediated by PHD/vHL therefore ensures a low HIF-α steady state level under normoxia, and reacts with quick HIF-α stabilization upon the onset of hypoxia (**Fig. 1.2**).

Another oxygen-dependent regulation of HIF-α activity is mediated by the factor inhibiting HIF (FIH), an asparaginyl-hydroxylase enzyme closely related to PHDs. FIH hydroxylates

an asparagine residue (N803 in HIF-1α and N847 in HIF-2α) under the utilization of oxygen. The asparagine residues are located in the C-terminal activation domain of HIF-α and are responsible for the interaction with the coactivators p300/CBP. FIH hydroxylation blocks HIF-α interaction with p300/CBP, therefore decreasing HIF-α transactivation activity (Bardos and Ashcroft 2005, Kaluz, Kaluzova et al. 2008).

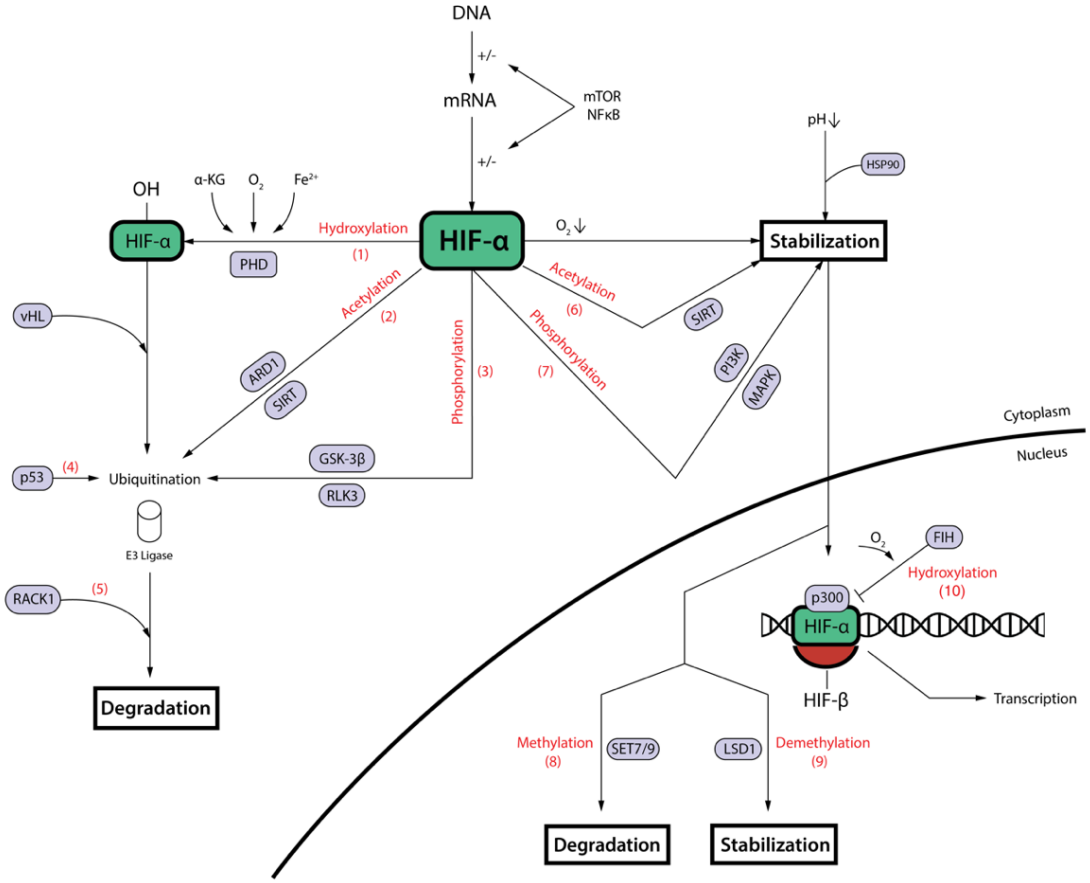

Figure 1.2: The complex regulation of HIF-α.
While HIF protein transcription and translation are influenced by the mTOR and NF-kB pathway, the main regulation of HIF activity occurs at the post-translational level on the subunit HIF-α. PHD is the principal enzyme initiating HIF-α ubiquination and degradation via oxygen-dependent hydroxylation (1). Other processes leading to ubiquitination include acetylation by arrest-defective-1 protein (ARD1) and sirtuins (SIRT) (2), phosphorylation by glycogen synthase kinase 3β (GSK-3β) and polo-like-kinase 3 (RLK3) (3), as well as p53 binding (4). RACK1 augments this degradation process (5). On the other hand, HIF-α stabilization is supported by acetylation by other SIRTs (6) or phosphorylation by phosphatidylinositol kinase 3 (PI3K) and mitogen activated protein kinase (MAPK) (7). Inside the nucleus, methylation by histone-lysine methyltransferases (SET7/9) destabilizes (8), whereas demethylation by histone-lysine demethylase (LSD1) stabilizes HIF-α (9). Factor-inhibiting HIF (FIH) hydroxylases HIF-α in an oxygen-dependent manner and thereby blocks HIF-α interaction with coactivators p300/CBP (10).

In addition to hydroxylation and ubiquitination, HIF-α is also subject to acetylation, another posttranslational modification (**Fig. 1.2**). The protein acetyltransferase arrest-defective 1 (ARD1) negatively regulates HIF-1α stability by acetylating lysine residue K532 in the HIF-α oxygen-dependent degradation (ODD) domain, thereby accelerating

the proteosomal degradation process under normoxic conditions (Jeong, Bae et al. 2002). This process is dependent on the FIH-mediated hydroxylation of ARD1, which is also oxygen-sensitive, thus further finetuning the cellular response to hypoxia (Kang, Chun et al. 2018). Moreover, acetylation by the family of sirtuins (SIRT1, SIRT3, SIRT6) was reported to have both positive and negative effects on the stability of HIF-α (Keith, Johnson et al. 2011).

Other posttranslational modifications include phosphorylation and methylation. Mitogen activated protein kinases (MAPK/ERK), Src kinase and phosphatidylinositol kinase 3/protein kinase B (PI3K/Akt) are reported to increase HIF-α stability and transactivation activity by direct phosphorylation, while glycogen synthase kinase 3β (GSK-3β), polo-like kinase 3 (PLK3), and casein kinase Iδ (CKIδ) have the opposite effects (Bardos and Ashcroft 2005, Mennerich, Dimova et al. 2014). Inside the nucleus, methylation by the histone-lysine methyltransferases SET7/9 and demethylation by histone-lysine demethylase LSD1 induce proteosomal degradation and HIF-α stabilization, respectively (Baek and Kim 2016). Moreover, S-nitrosylation and SUMOylation are two other posttranslational modifications described to influence both the transcriptional activity and protein stability of HIF-α. However, different research groups have presented contradicting reports on the effects of these two modifications on HIF-α stability (Dimova and Kietzmann 2010). More investigations into the detailed mechanisms of S-nitrosylation and SUMOylation are thus needed.

Lastly, direct protein-protein interactions also play an important role in the regulation of HIF-α. Among them, attention has been directed at the tumor suppressor protein p53. Upon direct physical contact with HIF-α, p53 recruits mouse double minute 2 homolog (Mdm2), which initiates the proteosomal degradation of HIF-1α (Masoud and Li 2015). In accordance with this, upregulated levels of HIF-1α have been observed in tumors containing a loss of p53 gene. Another factor that controls HIF-α stability is the interaction with the chaperone heat shock protein Hsp90, activated by microenvironmental acidosis. Hsp90 has been shown to competitively inhibit the binding of scaffold protein RACK1 to HIF-α, which leads to HIF-α proteosomal degradation independent from the PHD/vHL axis (Liu, Baek et al. 2007, Filatova, Seidel et al. 2016). Furthermore, the transactivation capability of HIF-α can be enhanced by binding to pyruvate kinase M2 (PKM2), whereas the binding of fructose-1,6-bisphosphatase 1 (FBP1) with the HIF-α inhibitory domain inhibits HIF-α nuclear function (Xie and Simon 2017). Finally, the inhibitory PAS protein (IPAS), a splice variant of the *hif-3α* locus, binds to HIF-1α to inhibit its dimerization and binding to DNA (Bardos and Ashcroft 2005).

Taken together, the presented mechanisms show the complexity of the regulation of HIF-α, a transcription factor fundamental to the cellular response to hypoxia. This tight regulation highlights the role of HIF-α in tumor biology, where the dysregulation of HIF-α is associated with tumorigenesis, tumor growth, and tumor aggressiveness. Despite being embedded in and regulated by many cellular processes, HIF-α is mainly governed by the key protein PHD, which is the subject of interest in the following section.

1.3 PHDs as a key regulator of HIF-α

Prolyl hydroxylase domain (PHD) enzymes belong to the family of αKG-dependent dioxygenases. Characteristically, PHDs require oxygen and αKG as cosubstrates, as well as Fe^{2+} and ascorbate as cofactors in a reaction to hydroxylate proline residues in the HIF-α oxygen-dependent degradation (ODD) domain. Products of this reaction include succinate, CO_2 and oxidized Fe^{3+} (Epstein, Gleadle et al. 2001, Schofield and Ratcliffe 2005) (**Figure 1.3**). The hydroxylation of the ODD domain commences the cascade for HIF-α degradation as described in the sections above. Importantly, PHDs have a particularly high K_m for oxygen, ranging from 100 to 250 μmol depending on the isoform (Schofield and Ratcliffe 2005, Nguyen and Duran 2016). Given the distinctly lower physiological intracellular oxygen concentrations of 30 – 50 μmol, PHDs are thus responsive oxygen sensors and therefore highly suitable HIF-α regulators.

There are three PHD isoforms relevant in tumor biology known to date: PHD1, PHD2, and PHD3. Despite displaying some overlapping tumor-related functions, the three isoforms are found to be non-redundant due to the differences in tissue specificity, substrate preference, cellular localization, and regulation. The most abundant isoform in most mammalian cells is PHD2, which is mainly present in the cytoplasm. PHD1, on the other hand, is exclusively localized in the nucleus and primarily expressed in testes, brain, kidney and heart. Lastly, PHD3 is equally found in both cell compartments and shows the highest expression in heart tissue (Metzen, Berchner-Pfannschmidt et al. 2003). While HIF-α is the main substrate of all isoforms, PHD2 exhibits a clear preference for HIF-1α, whereas PHD3 preferentially regulates HIF-2α. Additionally, all three isoforms can hydroxylate the highly conserved P564 residue, but only PHD1 and PHD2 can hydroxylate the more recently evolved Pro402 residue in HIF-1α (Appelhoff, Tian et al. 2004, Yang, Su et al. 2014, Huang, Lin et al. 2017). Knockdown experiments of each isoform result in a wide variety of defects, suggesting rather complex functions of PHDs

that go beyond regulating HIF-α stability, depending on cell type and isoform (Yang, Su et al. 2014).

1.3.1 The role of PHD goes beyond HIF regulation

The main function of PHD is the hydroxylation of HIF-α. Its biological role is therefore closely coupled to HIF-α function. For instance, the loss of PHD activity goes hand in hand with HIF-α accumulation and is linked to tumorigenesis and tumor growth in several types of neoplasia, such as endometrial, renal, and germline cancers (Nguyen and Duran 2016). Functional PHD can control the glycolytic rate by dampening the HIF-α-mediated Warburg effect, thereby reinforcing the tumorsuppressive role of PHD in cancer metabolism (Nguyen and Duran 2016).

Interestingly, in recent years, a growing body of evidence suggests an involvement of PHDs in orchestrating hydroxylation of non-HIF targets. In conditions of normoxia and high nutrient availability, PHD3 hydroxylation activity has been found to take part in finetuning metabolism by negatively regulating Acetyl-coA-transferase and positively regulating pyruvate kinase M2 (PKM2) in a HIF-independent manner (Ivan and Kaelin 2017). In addition to the mentioned enzymes, PHDs also target signalling cascades that have even greater relevance in tumor biology. Among them is nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), an important mediator of the innate immune response. The inhibitor of κB kinase (IKKβ) is part of the NFκB signaling, participating in the translocation of NFκB into the nucleus. PHD1 and PHD3 have been proposed to negatively regulate this translocation process by hydroxylating IKKβ, thereby dampening the inflammatory response. Alternatively, PHD3 has also been suggested to block the interaction between IKKβ and HSP90, thereby inactivating IKKβ function (Yang, Han et al. 2014, Nguyen and Duran 2016).

Mammalian target of rapamycin mTOR is a serine/threonine kinase which promotes proliferation and reacts to growth factor signals, stress, oxygen and nutrient availability. Increasing evidence points towards an involvement of PHDs as a metabolite sensor in the mTOR pathway, in particular for sensing αKG. The depletion of αKG leads to PHD inactivation, which inhibits mTOR activity and reduces tumor proliferation and growth (Boulahbel, Duran et al. 2009, Duran, MacKenzie et al. 2013, Nguyen and Duran 2016). Next to mTOR, proliferation and growth is also regulated by EGFR. The isoform PHD3, specifically, promotes growth inhibition by causing EGFR internalization. In high-grade gliomas, PHD3 is often significantly downregulated in hypoxia, resulting in impairment of

this internalization process. Consequently, EGFR is hyperactivated, promotes proliferation and enables the tumor cell to escape growth inhibition by hypoxia (Garvalov, Foss et al. 2014, Henze, Garvalov et al. 2014).

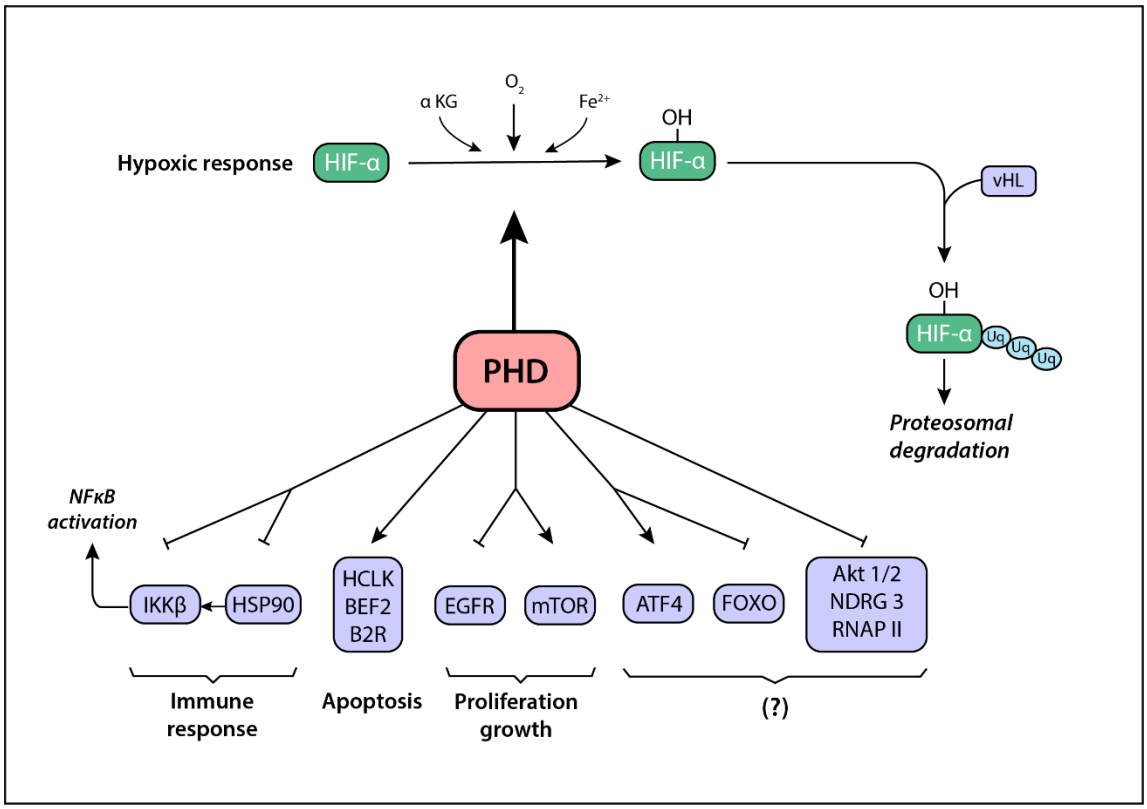

Figure 1.3: The role of PHD in tumor biology.
The main function of PHD is found in the hydroxylation of HIF-α, thereby inducing the latter's ubiquitination and proteosomal degradation. However, many more functions that go beyond HIF-α regulation have emerged in the recent years. PHD dampens the immune response by reducing NFkB activation, enhances apoptosis, and modules proliferation and growth through EGFR and mTOR pathways. The effect of PHD regulation of the transcription factors ATF4 and FOXO, as well as other enzymes with an oncogenic potential, in the context of tumor biology remains largely unclear. *Uq: Ubiquitin*.

The hydroxylation capability of PHDs further constrains the activity of several enzymes possessing an oncogenic potential. PHD3, for example, induces apoptosis through the hydroxylation of homolog of the *Caenorhabditis elegans* biological clock 2 protein (HCLK2), eukaryotic elongation factor 2 (eEF2), and β_2-receptor (B2R) (Nguyen and Duran 2016, Ivan and Kaelin 2017). PHD also downregulates transcription factors like Forkhead box O3a (FOXO3a), whose aberrant expression is frequently observed in malignancies of breast, liver, and colon. PHD also suppresses activating transcription factor 4 (ATF4), a transcription factor promoting tumor survival (Yang, Su et al. 2014, Ivan and Kaelin 2017).

Among the three isoforms, evidence points towards PHD2 as the prominent one associated with tumorsuppressive effects. It negatively regulates metabolic protein kinases Bα and β, also called Akt1 and Akt2, which have been found to be overexpressed

in several tumor types (Ivan and Kaelin 2017). N-Myc Downstream-Regulated Gene 3 Protein (NDRG3), an oncogenic protein that activates the Raf/ERK axis (Ivan and Kaelin 2017), is hydroxylated by PHD2, leading to its ubiquitination and proteosomal degradation. Interestingly, an oncogenic effect of PHD1/PHD3-mediated hydroxylation of the widely distributed RNA polymerase II (RNAP II) is inhibited by PHD2-mediated hydroxylation by mechanisms that are still unknown (Yang, Su et al. 2014).

Taken together, it is evident that PHDs exhibit a range of functions that go beyond the regulation of HIF-α abundance, including cellular proliferation, immune response, and apoptosis. The different effects governed by different isoforms emphasize the non-redundant function of PHD1, PHD2 and PHD3 in the cellular machinery. Even though HIF-independent PHD functions are still in the early stage of discovery and many molecular mechanisms are poorly understood, many studies have emphasized the tumorsuppressive contributions of PHD in tumor biology.

1.3.2 The regulation of PHD

The enzymatic activity of PHD is mainly controlled by product inhibition and substrate availability. Reduced oxygen levels, i.e. hypoxia, potently inhibit PHD activity. In a similar manner, the cosubstrates αKG and/or the cofactors Fe^{2+} and ascorbate can also limit PHD activity. This opens up the possibility of a metabolite-sensing function for PHDs. In fact, intracellular αKG depletion caused by amino acids starvation led to PHD inactivation (Duran, MacKenzie et al. 2013). In accordance, αKG supplementation has been shown to boost PHD activity even under hypoxia, consequently suppressing tumor growth through both HIF destabilization and HIF-independent, PHD3-mediated apoptosis (Tennant, Frezza et al. 2009, Tennant and Gottlieb 2010).

However, the low K_m reported for αKG puts into question the significance of this substrate in modulating PHD activity. Both PHD1 and PHD2 have a K_m of 1 – 2 µmol for αKG, while PHD3's is around 12 µmol, all values much lower than the measurable αKG levels *in vivo* (Yang, Su et al. 2014). Further investigation revealed that the effect on PHD activity was less mediated by intracellular αKG concentrations alone than the altered αKG-to-succinate ratio in favor of succinate (Carey, Finley et al. 2015). Next to αKG depletion, the accumulation of the enzymatic product succinate also inhibits PHD activity. This finding corresponds with the clinical finding of mutations of the TCA cycle enzyme succinate dehydrogenase in hereditary paraganglioma and pheochromocytoma, which results in accumulated succinate levels (Yang, Su et al. 2014). Similarly, fumarate and

oxaloacetate – structural analogues of succinate – also inhibit PHD activity (**Figure 1.4**). Mutations of the TCA cycle enzyme fumarate hydratase, which lead to the accumulation of fumarate, are also frequently found in leiomyomata and renal cell cancer (Schofield and Ratcliffe 2005).

The depletion of the cofactor iron by iron chelators, or the competitive inhibition of Fe^{2+}-binding by the artificial addition of Co^{2+}, Mn^{2+}, or Ni^{2+} also reduces PHD activity. Furthermore, ascorbate is used for the reduction of Fe^{3+} to Fe^{2+}. Its availability therefore indirectly regulates PHD activity through the availability of Fe^{2+} (Schofield and Ratcliffe 2005).

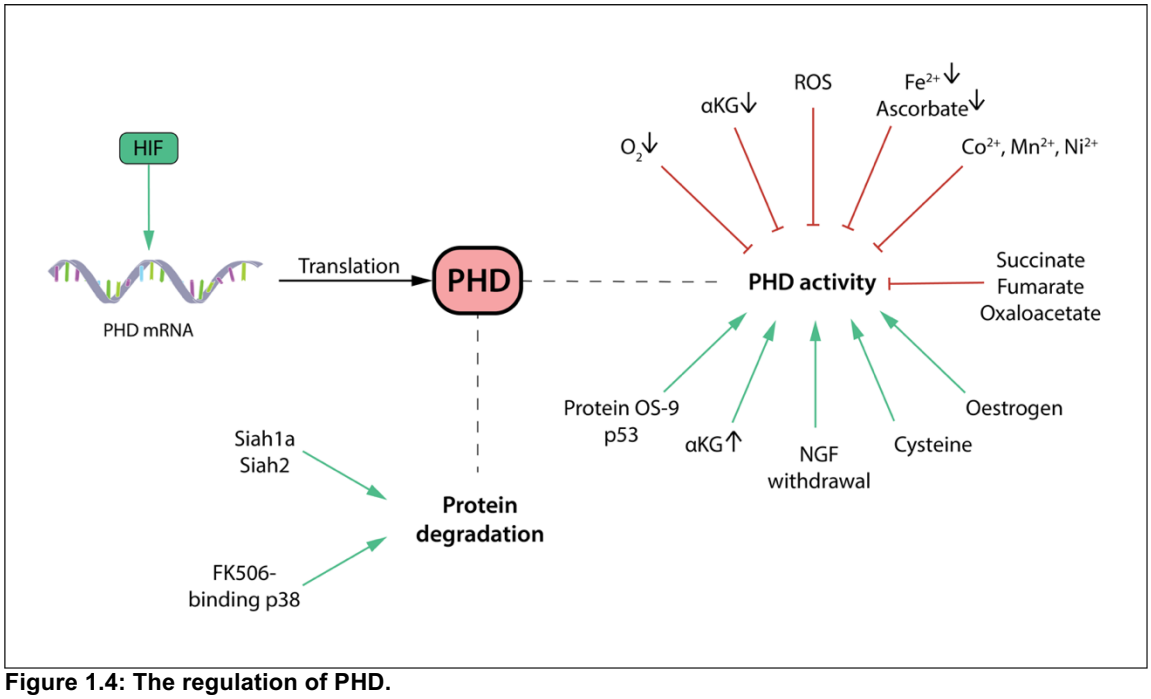

Figure 1.4: The regulation of PHD.
PHD activity is inhibited by a shortage of its most important substrates O_2 and αKG, as well as its necessary cofactors Fe^{2+} and ascorbate. By competitive inhibition, succinate, fumarate and oxaloacetate take up αKG binding sites, whereas Co^{2+}, Mn^{2+}, and Ni^{2+} compete with Fe^{2+}. Furthermore, reactive oxygen species (ROS) inhibit PHD activity. Conversely, factors enhancing PHD activity include oestrogen, nerve growth factor (NGF) withdrawal, protein OS-9, p53, and the metabolite cysteine. On the transcriptional level, PHD expression is upregulated by HIF. Protein degradation is driven by ubiquitin ligases Siah1a and Siah2, as well as FK506-binding protein 38.

There are a number of other factors regulating PHD gene expression, protein level and activity (**Fig. 1.4**). Many factors, including protein OS-9, p35, nerve growth factor withdrawal, cysteine and the hormone oestrogene, the influence of which was in particular found for PHD2 in breast cancer cells, are positively enhancing PHD activity by mechanisms that are still unclear (Baek, Mahon et al. 2005, Briggs, Koivunen et al. 2016). Interestingly, PHD2 and PHD3 genes possess a HIF-response element for HIF-1α and HIF-2α, respectively, and are therefore hypoxia-inducible. This transcriptional upregulation of PHD is proposed to be part of a negative feedback loop for HIF to self-

limit the hypoxic response and prevent excessive HIF-α levels under chronic hypoxia (Henze, Riedel et al. 2010).

Less is known about negative regulators of PHDs. The ubiquitin E3 ligase seven in absentia homology Siah1a and Siah2 is responsible for the proteosomal degradation of PHD (Schofield and Ratcliffe 2005). At the same time, PHD2 degradation is also mediated by FK506-binding protein 38 in a ubiquitin-independent proteosomal pathway (Yang, Su et al. 2014). There are also reports of an inhibitory effect of reactive oxygen species (ROS) and NO on PHD activity. One proposed mechanism for ROS is that it oxidizes Fe^{2+} to Fe^{3+}, whereas NO is thought to compete with oxygen for binding the active site. However, PHD activity is reconstituted after prolonged exposure to both ROS and NO by mechanisms yet unknown (Yang, Su et al. 2014, Nguyen and Duran 2016).

In conclusion, PHD regulation mainly occurs by the modulation of its activity. Besides reacting to the availability of its most important substrate oxygen, PHD activity is also sensible to oxidative stress, growth factors, hormones, and abnormal levels of trace elements and intracellular metabolites. Among those factors, αKG is of particular interest, not only because of its high regulatory potential for PHDs, but also due to its central role in cellular metabolism. Therefore, the susceptibility of PHDs to changes in αKG homeostasis underlines PHDs' involvement in tumor metabolism as a metabolite-sensor.

1.4 The role of α-ketoglutarate in tumor biology

α-Ketoglutarate, also known as 2-oxoglutarate, is a pleiotropic metabolite that plays an essential role in cellular metabolism. Next to being a key component of the fundamental tricarboxylic acid cycle (TCA), αKG also serves as a nitrogen transporter and is involved in amino acid biosynthesis (Zdzisinska, Zurek et al. 2016). However, the significance of αKG in tumor biology is not only limited to metabolism (**Fig. 1.5**). By being the necessary co-substrate for the family of over 60 αKG-dependent dioxygenases, αKG attains multiple extrametabolic functions, among which is its involvement in the regulation of the hypoxic response through PHD as introduced in the previous chapter.

α-Ketoglutarate-dependent dioxygenases are a subset of non-heme, iron-containing enzymes that consume αKG and oxygen to catalyze post-translational hydroxylation of proline, aspartate, asparagine and lysine residues. Representatives of this enzyme family include the already mentioned PHDs and FIH (Bardos and Ashcroft 2005). Not

less relevant in tumors are the epigenetic regulators of the Jumonji-C-containing histone demethylator family (KDM1-7) and the ten-eleven translocator family (TET1-3), which mediate histone demethylation and DNA hydroxymethylation, respectively. An inactivation of KDM or TET enzymes upon decreased αKG levels, or the accumulation of αKG's competitive inhibitors succinate and fumarate has been shown to epigenetically promote a more stem-cell-like phenotype of tumor cells, resulting in therapy resistance and more frequent metastasis (Kaelin 2011, Hojfeldt and Helin 2016, Pan, Reid et al. 2016).

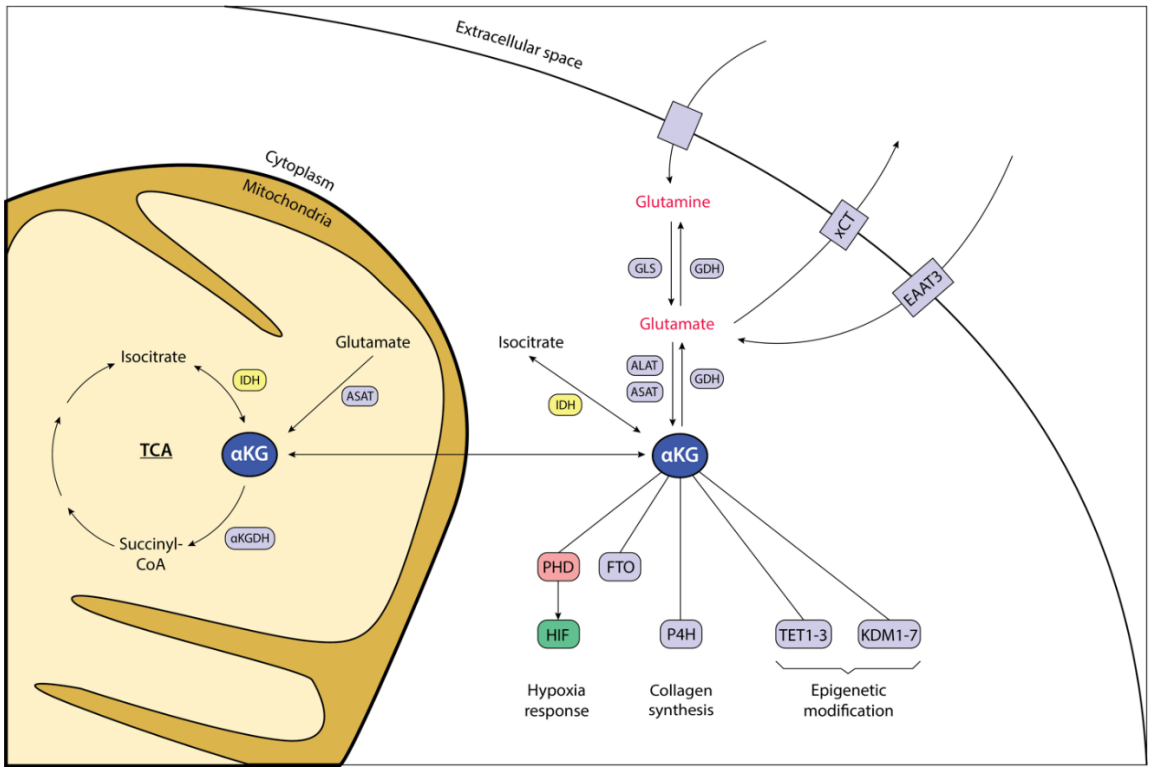

Figure 1.5: The position of αKG in the cell.
The intracellular level of αKG is sustained firstly by the tricarboxylic acid cycle (TCA) transformation of isocitrate into αKG, catalyzed by IDH2 in the mitochondria. IDH1 drives the same reaction in the cytoplasm. Secondly, glutaminolysis generates αKG with glutamate in an interim stage. Involved in this process are the enzymes GLS (glutaminase), ALAT (alanine transferase) and ASAT (aspartate transferase). Glutamine and glutamate enter the cell via diverse Na^+-dependent and Na^+-independent transporters. For glutamate transport, the xCT antiporter system with cysteine, as well as EAAT3 (excitatory amino acid transporter) seem to play a significant role. αKG can reversely be transformed into its precursor isocitrate by IDH, or glutamate by GDH (glutamate dehydrogenase) under certain conditions that remain to be elucidated. As for the metabolite itself, αKG plays a major role as the regulator of αKG-dependent enzymes PHD, P4H (prolyl-4-hydroxylase), TET (ten-eleven translocator) and KDM (JmjC-histone demethylator) in their respective governance of the hypoxic response, collagen synthesis and epigenetic modification. The significance of fat mass and obesity-associated protein (FTO) in tumor biology remains unclear.

Further important dioxygenases are prolyl-4-hydroxylases (P4H), fat mass and obesity-associated protein (FTO) and a number of ribosomal hydroxylases (Yang, Su et al. 2014). The significance of those enzymes in the context of tumor biology is only in the early stages of discovery. There have been recent reports of P4H enhancing cancer cell

stemness and chemoresistance in breast cancer (Xiong, Stewart et al. 2018), while FTO displays oncogenic properties in acute myeloid leukemia (Chen and Du 2019).

Aside from regulating αKG-dependent dioxygenases, αKG also possesses anti-proliferative effects by interfering in the cell cycle. An increasing expression of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, as well as decreased protein levels of cyclin D1 and inhibited phosphorylation of the Rb protein are all associated with increasing αKG levels. Tumor cells experiencing these effects will be arrested in the G1 phase and prevented from initiating cell division (Zdzisinska, Zurek et al. 2016). In addition, αKG was shown to exert antioncogenic activity by enhancing the effect of the chemotherapeutic 5-FU in lung cancer (Matsumoto, Imagawa et al. 2006).

In short, it is evident that αKG is an important metabolite displaying various anti-cancerous effects. It wields its influence on tumors primarily through the regulation of αKG-dependent dioxygenases, among which HIF-regulating PHD and epigenetic regulators KDM1-7 and TET1-3 are the most relevant ones discovered to date. By governing the activity of these enzymes, αKG links tumor metabolism with tumor epigenetics and the hypoxic response.

1.4.1 Factors influencing endogenous αKG production

To have a better understanding of αKG's position in cellular metabolism and tumor biology, it helps to look at the pathways involved in its production, the associated oncogenic abnormalities and the consequences thereof.

Being an endogenous metabolite, αKG physiologically originates from within the cell. The intracellular formation is dependent on two main pathways. The first one is the TCA cycle, which produces αKG from isocitrate through isocitrate dehydrogenase (IDH) and utilizes it to form succinyl-CoA via αKG dehydrogenase (αKGDH) (**Fig. 1.5**). The second pathway is αKG's participation in glutamine metabolism, which generates αKG through the oxidative deamination of glutamate by glutamate dehydrogenase (GDH). An alternative way to yield αKG from glutamate is additionally provided by the transaminases alanine transferase (ALAT) and aspartate transferase (ASAT), which at the same time catalyze the reaction to form pyruvate from alanine and oxaloacetate from aspartate, respectively (**Fig. 1.5**).

Among all those factors and enzymes involved in sustaining αKG homeostasis, three factors will be the focus of this work. They are the αKG producing enzyme IDH and the

amino acid precursors glutamine and glutamate, all of which will be discussed in the following sections.

1.4.2 IDH mutation and IDH wildtype in tumors

Isocitrate dehydrogenase (IDH) is best known for being one of the nine enzymes running the fundamental TCA cycle. In a two-step reaction, it catalyzes the oxidative decarboxylation of isocitrate to αKG while generating reducing equivalent NADH/NADPH in the process. Of the three isoforms known in mammalian cells, IDH1 and IDH2, which are 70% identical in structure, function in a homodimer and are $NADP^+$-dependent, whereas IDH3, consisting of three different subunits forming a tetraheterodimer, utilizes NAD^+ as a cofactor. IDH3, located in the mitochondria, is the principal isoform taking part in the TCA cycle. IDH2 is also exclusively present in the mitochondria, aiding the maintenance of the redox balance. IDH1 is found in the cytoplasm and peroxisomes to take part in lipid synthesis, glucose sensing and defense against oxidative stress. Interestingly, IDH1 and IDH2 are also able to catalyze the reversed reaction to form isocitrate from αKG under certain conditions of substrate and co-factor availability, which are not seldom found in tumors (Corpas, Barroso et al. 1999, Mullen, Wheaton et al. 2011, Megova, Drabek et al. 2014, Bogdanovic 2015).

The interest surrounding IDH in the context of tumor research has been kindled ever since the discovery of frequent IDH mutations in glioma and acute myeloid leukemia. Recurrent mutations in the *IDH1* and *IDH2* gene, but not *IDH3*, are found in up to 70% of low-grade gliomas and in around 10% of acute myeloid leukemia cases (Dang, Jin et al. 2010). Genomic studies have confirmed IDH mutations, albeit less frequently, in other hematopoietic malignancies, chondrosarcoma, prostate cancer and colon cancer (Megova, Drabek et al. 2014). The mutations consist of a single amino acid substitution for an arginine residue that is either R132 or R100 in IDH1 and either R172 or R140 in IDH2 (Parsons, Jones et al. 2008, Yan, Parsons et al. 2009, Dang, Jin et al. 2010).In gliomas, the majority of mutations are IDH1 mutations, with the arginine residue R132 being replaced by histidine in 90% of cases (Megova, Drabek et al. 2014).

All mutated IDH enzymes display two common features: the reduced ability to catalyze the decarboxylation of isocitrate into αKG, and a gain of function in the ability to convert αKG into the oncometabolite 2-hydroxyglutarate (2HG) (Dang, White et al. 2009, Zhao, Lin et al. 2009). Notably, IDH mutations are in most cases heterozygous, and the

complete loss of wildtype IDH has been shown to suppress 2HG production (Jin, Reitman et al. 2013).

The clinical significance of IDH mutation for brain tumors lies in its excellent prognostic value. Better outcome is reported for IDH-mutated gliomas compared to non-mutated ones by multiple independent research groups, even though the product of mutated IDH is the highly oncogenic metabolite 2HG. Accumulated 2HG is found to competitively inhibit αKG-dependent dioxygenases like PHD, thereby driving tumor progression by stabilizing HIF-α. The same inhibition applies to KDM and TET, leading to tumorigenicity promoted by epigenetic modification (Chowdhury, Yeoh et al. 2011, Borodovsky, Seltzer et al. 2012). It is worth noting that competitive inhibition of αKG-dependent dioxygenases by the oncometabolite 2HG, as well as the TCA cycle intermediates fumarate and succinate, is alleviated by increasing intracellular αKG levels. This, in turn, was found to negatively affect tumor growth in a number of *in vitro* studies, thus further highlighting αKG's potency as a tumorsuppressive agent (MacKenzie, Selak et al. 2007, Tennant, Frezza et al. 2009, Zhao, Lin et al. 2009, Xu, Yang et al. 2011). Interestingly, a recent study discovered that HIF-1α negatively regulates IDH1 expression in osteosarcoma, resulting in increased tumor cell proliferation (Liu, Zheng et al. 2017).

In contrast to IDH mutations, comparably less is known about the significance of wildtype IDH in tumor biology. However, gliomas consist of 90% non-mutated IDH tumors. This highlights the need for more investigation into the role of wildtype IDH in glioma, as well as in tumors in general.

Wildtype IDH1 activity is shown to be crucial for maintaining intracellular αKG levels, consequently negatively affecting tumor proliferation, invasion and metastasis through HIF-α destabilization (Zhao, Lin et al. 2009, Hu, Liu et al. 2014, Yang, Du et al. 2015). Also, a pro-apoptotic role of IDH1 has been described in melanoma cells upon endoplasmatic reticulum (ER) stress. ER stress induced by the drug tunicamycin upregulates IDH1 through the transcription factors C/EBP homology protein (CHOP) and C/EPBβ, leading to HIF-1α degradation and survival inhibition of melanoma cells (Yang, Du et al. 2015). It is possible that the effects of IDH1 on HIF-α stability in all the mentioned studies are mediated by PHD and the dependence of its activity on αKG, but there is a lack of evidence establishing a direct link between wildtype IDH1 and PHD to date.

Furthermore, a study showed IDH2 to be crucial for maintaining the methylation status of melanoma through the activity of another αKG-dependent dioxygenase, the DNA demethylator TET. In mouse models, active IDH2 ensures TET activity and high DNA

hyroxymethylation at the 5 position of cytosine (5-hmC), consequently promoting growth inhibition and prolonged tumor-free survival in melanoma (Lian, Xu et al. 2012). Lastly, FOXO, a transcription factor considered to be tumorsuppressive, is found to upregulate IDH expression (Molenaar, Maciejewski et al. 2018).

Conversely, in an attempt to explain the worse outcome of IDH-wildtype tumors compared with IDH-mutated tumors, an oncogenic effect of IDH has been proposed. IDH1 and IDH2 contribute to the cell's protection from oxidative stress by generating the reducing agent NADPH. Reports of increased sensitivity to radiotherapy and chemotherapeutic drugs in IDH1-knockdown tumors speculate that IDH1 supports resistance to therapy-induced reactive oxygen species (Molenaar, Maciejewski et al. 2018). Additionally, IDH1 and IDH2 are shown to participate in the reductive carboxylation of glutamine in hypoxic tumor cells (Metallo, Gameiro et al. 2011, Wise, Ward et al. 2011). This reversed reaction to produce isocitrate from αKG is part of glutaminolysis and *de novo* lipid synthesis, which provides the macromolecules required for tumor cell proliferation (Filipp, Scott et al. 2012). The idea of tumor promoting effects of IDH is supported by the detection of upregulated IDH1 expression in certain cohorts of glioma and lung cancer (Tan, Jiang et al. 2012, Calvert, Chalastanis et al. 2017).

In summary, the discovery of IDH1 and IDH2 mutations especially in glioma and acute myeloid leukemia has proved to be a milestone in cancer research. The role of wildtype IDH, in contrast, remains remarkably understudied. Studies in the last couple of years suggest both oncogenic and tumorsuppressive traits of wildtype IDH. On the one hand, the oncogenic effect derives from the participation of IDH in stocking up the NADPH pool and in the production of macromolecules, thereby protecting tumor cells from oxidative stress and promoting proliferation. On the other hand, tumorsuppressive characteristics are displayed by IDH's role in maintaining intracellular αKG levels, consequently reducing tumor progression through αKG-dependent dioxygenases. Even though there is evidence of underlying epigenetic changes mediated by the αKG-dependent dioxygenase TET, little is known about how IDH advances HIF destabilization, especially whether or not αKG-dependent PHD is involved in this chain of effects.

1.4.3 Microenvironmental glutamine and glutamate

Another source for intracellular αKG is presented by its amino acid precursors glutamine and glutamate. In tumors, TCA cycle anaplerosis is altered towards being mainly maintained by glutaminolysis, in which glutamine is first converted to glutamate and then,

following its deamination, enters the TCA cycle in the form of αKG. It has been proposed that the intracellular αKG pool in tumors primarily derives from glutamine instead of glucose (Wise, Ward et al. 2011, Carey, Finley et al. 2015). This metabolic rewiring is especially employed under hypoxia and is also part of the tumor-associated metabolic reprogramming, mediated by none other than HIF-1 (Metallo, Gameiro et al. 2011, Wise, Ward et al. 2011, Filipp, Scott et al. 2012).

The delivery of glutamine into the cell is straightforwardly ensured by several Na^+-dependent and Na^+-independent membrane transporters (Xiao, Zeng et al. 2016). However, despite being the most abundant amino acid in the plasma, glutamine deficiency can occur in a manner likewise to oxygen deficiency in core regions of solid tumors, which then induces a significant decrease in cell proliferation (Wise, Ward et al. 2011). However, the accompanying αKG shortage in glutamine-deficient tumor regions is also shown to inhibit αKG-dependent histone demethylases, thereby promoting a dedifferentiated, aggressive tumor phenotype (Hojfeldt and Helin 2016, Pan, Reid et al. 2016). Whether or not microenvironmental glutamine deficiency, a condition frequently occurring in fast-growing solid tumors, affects the PHD/HIF axis in a similar way has not been addressed so far.

Comparatively less is known about the role of the other αKG precursor glutamate in the glioblastoma tumor microenvironment. Unlike glutamine, the extracellular levels of glutamate are extremely low due to its cytotoxicity, fluctuating only in the µM level (Yao, Kang et al. 2014). The transport of glutamate into the cell depends on the xCT antiporter in exchange with cysteine (Briggs, Koivunen et al. 2016), or on Na^+-dependent transporters, with excitatory amino acid transporter EAAT3 being the subtype predominantly present in GBM cells (Dunlop, Lou et al. 1999). However, high-grade tumors show an impairment in glutamate uptake (Ye and Sontheimer 1999). On the contrary, peripheral glioma cells tend to secrete glutamate into the microenvironment. Toxic glutamate concentrations sustained in peritumoral areas aid glioma expansion by inducing apoptosis of neighboring neurons.

High glutamate concentration in the microenvironment also inhibits the xCT antiporter and, interestingly, leads to PHD inactivation and HIF1α stabilization (Briggs, Koivunen et al. 2016). This effect, however, is less mediated by elevated intracellular glutamate or altered αKG levels than the reduced availability of intracellular free cysteine. Briggs and colleagues showed that free cysteine is needed to prevent the oxidation of cysteine residues in PHD. This oxidation is sufficient to inhibit PHD activity and to stabilize HIF1α even under normoxic conditions (Briggs, Koivunen et al. 2016). To what extent this

microenvironmental glutamate–PHD–HIF1α connection applies to glioblastoma remains to be elucidated.

All in all, microenvironmental changes of αKG precursors glutamate and glutamine have an effect on αKG-dependent dioxygenases, through which tumor progression or suppression is mediated. However, our knowledge is still limited in respect to the effects on the PHD-HIF pathway, and the sometimes contradicting pre-existing data calls for more investigation into this area.

1.5 Purpose of the study

Glioblastomas are tumors with one of the worst prognosis. Despite modern treatment strategies with aggressive surgical, chemo- and radiation therapy, a complete elimination of tumor cells is a state rarely achievable. Especially the subset of highly evasive and resilient glioblastoma stem cells is thought to be the cause of regular tumor recurrence, which then leads to death within an extremely short time span. In the face of such a lethal tumor, understanding the underlying biological processes lays the foundation to developing better treatment options.

This work focuses on the link between hypoxia and metabolism in GBM, particularly the mechanisms centering around the metabolite αKG. Hypoxia is known to be an omnipresent trait in fast-growing solid tumors, on the one hand causing cell necrosis, on the other hand inducing a resilient phenotype such as the aforementioned glioblastoma stem cells. The hypoxic response is mediated by the transcription factor HIF, which is tightly regulated by the αKG-dependent enzyme PHD. In recent years, the role of PHD was found to go beyond the hypoxic response and extend its significance especially in the field of tumor metabolism. This involvement in metabolism is majorly attributed to its ability to sense changes in and consequently react to intracellular αKG levels. Changes in intracellular αKG levels, in turn, play an important part in glioblastoma progression through mechanisms of hypoxic response modulation, epigenetic modification and cell cycle interference.

This work aims to investigate the potential factors affecting intracellular αKG levels and to assess their effect on the hypoxic response, particularly on the PHD-HIF axis. In this context, our focus lies on the microenvironmental presence of αKG precursors glutamine and glutamate, as well as the principal enzyme producing intracellular αKG: IDH.

2 Materials and methods

2.1 Materials

2.1.1 Cell lines

Table 2.1: Cell lines

Name	Type/Description	Origin
G55TL	Human glioblastoma	M. Westphal, K, Lamszus (Hamburg, Germany)
U87	Human glioblastoma	ACTT No. HTB-14
G55 pOR 4-7	G55TL transduced with the ODD-luciferase-construct (pOR, Omelyan Trompak), clone 4-7 Maintenance of transgene expression with Blasticidin (6 µg/ml)	Omelyan Trompak, Nuray Bügürcü, Rebecca Seibel (Giessen, Germany)
G55 pOR 4-7 shIDH G55 pOR 4-7 nsc	G55 pOR 4-7 transduced with a short-hairpin RNA silencing IDH1 (pGIPZ-shIDH1, Thermo Scientific) and non-silencing empty control vector (nsc, Thermo Scientific) Selection with Puromycin (2 mg/ml)	Omelyan Trompak, Nuray Bügürcü, Rebecca Seibel (Giessen, Germany)
U87 pOR 1-3	U87 transduced with the ODD-luciferase-construct (pOR, Omelyan Trompak), clone 1-3 Maintenance of transgene expression with Blasticidin (6 µg/ml)	Omelyan Trompak, Nuray Bügürcü, Rebecca Seibel (Giessen, Germany)
MDA-231 pOR	Human breast adenocarcinoma transduced with the ODD-luciferase-construct (pOR, Omelyan Trompak) Maintenance of transgene expression with Blasticidin (6 µg/ml)	M. Mazzone (Leuven, Belgium); Omelyan Trompak (Giessen, Germany)

2.1.2 Antibiotics

All solutions were filtered to sterility.

Table 2.2: Antibiotics used for maintenance of transgene expression

Name	Stock solution	Concentration for maintenance of transgene expression
Blasticidin S HCL (#R201-01, Invitrogen)	6 mg/ml in distilled water	6 µg/ml
Puromycin (#P9620, Sigma-Aldrich)	10 mg/ml ready-to-use solution	2 µg/ml

2.1.3 Antibodies

2.1.3.1 Primary Antibodies

Table 2.3: Primary antibodies for Western Blots

Name	Company	Dilution
anti-HIF-1α, rabbit	Cayman Chemical, #10005421	1:5000
anti-HIF-2α, rabbit	Novus Biologicals, #NB100-102	1:750
anti-Tubulin-α, mouse	Dianova Jackson ImmunoResearch, #DLN-09992	1:8000
anti-IDH1, goat	Santa Cruz, #sc-49996	1:1000
anti-ZEB 1, rabbit	Santa Cruz, #sc-25388	1:1000
anti-T-cadherin, mouse	Santa Cruz, #155857	1:500
anti-CD44, mouse	Cell Signaling, #3570	1:5000

2.1.3.2 Secondary Antibodies

Table 2.4: Secondary antibodies for Western Blots

Name	Company	Dilutions
donkey-anti-goat, HRP-conjugated	Dianova Jackson ImmunoResearch, #705-035-147	1:5000

goat-anti-rabbit, HRP-conjugated	Dianova Jackson ImmunoResearch, #111-035-144	1:5000
goat-anti-mouse, HRP-conjugated	Dianova Jackson ImmunoResearch, #115-035-146	1:5000

2.1.4 Protein Ladders

Spectra Multicolor High Range Protein Ladder: #SM1851, Fermentas Life Science

Page Ruler Prestained Protein Ladder: #SM0671, Fermentas Life Science

2.1.5 Media, Buffer and specific reagents

2.1.5.1 Western Blot

30 % Acrylamid/bis 29:1: #1610156, Bio-Rad

8 % Acrylamid seperating gel: 2,7 ml 30 % Acrylamid, 100 µl 10 % APS, 2,6 ml Lower Buffer, 4,65 ml aqua dest., 5 µl TEMED

8 % Acrylamid stacking gel: 0,65 ml 30 % Acrylamid, 50 µl 10 % APS, 1,3 ml Upper Buffer, 3,05 ml aqua dest., 5 µl TEMED

APS (**Ammonium persulfate):** 1 g powdered APS in 100 ml aqua dest. 1 ml aliquots stored at -20 °C. #9592.1, Roth

Blocking buffer: 5 % Milk powder in 1x PBS and 0,1 % Tween 20

Laemmli buffer: 10 mM Tris Base with pH adjusted to 7,5 with 5 M HCl, 2 % SDS, 2 mM EGTA, 20mM NaF

Loading buffer: 4 % SDS, 16 ml 1 M Tris Base pH 6,8, 20 ml glycerol, 19 ml aqua dest. Stored in 800 µl aliquots. For usage, add 200 µl 1 % bromphenol blue and 50 µl β-mercaptoethanol

Lower buffer: 1,5 M Tris Base (121,6 g/mol), 0,4 % SDS (storage at 4°C), pH adjusted to 8,8 with 5 M HCl

20x PBS: 140 mM NaCl (58,44 g/mol), 2,7 mM KCl (74,55 g/mol), 10 mM Na_2HPO_2 x 2 H_2O (177,99 g/mol), 1,8 mM KH_2PO_4 (136,09 g/mol), pH adjusted to 7,4

10x Running buffer: 250 mM Tris Base (121,1 g/mol), 2 M glycine (75,07 g/mol), 1 % SDS

Stripping buffer: 200 mM glycine (pH 2,5) and 0,05 % Tween 20

TEMED: #1610801, Bio-Rad

Tween 20: #A4974,0100, Applichem

10x Transfer buffer: 60,6 g Tris Base (121,1 g/mol), 288,0 g glycine (75,07 g/mol) in 2 l aqua dest.

Upper buffer: 0,5 M Tris Base (121,6 g/mol), 0,4 % SDS (storage at 4°C), pH adjusted to 6,8 with 5 M HCl

Washing buffer: 50 ml 20x PBS in 1 l aqua dest., 0,05 % Tween 20

2.1.5.2 Cell Culture

CHX (Cycloheximide): 50 mg/ml, dissolved in ethanol (#C1988, Sigma-Aldrich)

D-glutamine: #G9003, Sigma-Aldrich

D-MEM (Dulbecco's Modified Eagle Medium) High Glucose (with GlutaMAX™, 4,5 g/l D-Glucose, Sodium Pyruvate): #31966-021, Gibco

D-MEM High Glucose without L-glutamine: #21969-035, Gibco

D-MEM/F-12 (1:1) (with GlutaMAX™): #31331-028, Gibco

DMSO (Dimethyl sulfoxide): #A994.1, Roth

EGF (epidermal growth factor): Stock solution 200 µg/ml (#C-10015-SS, Peprotech)

FCS (Fetal Calf Serum): #10270-106, Gibco

bFGF (fibroblast growth factor): Stock solution 200 µg/ml (#C-10018-BF, Peprotech)

L-glutamate: #G8415, Sigma-Aldrich

L-glutamine, 200 mM: #25030081, Gibco

Medium, standard growth (adherent condition): 500 ml D-MEM High Glucose, 10 % FCS

Medium, tumor sphere: 500 ml D-MEM/F-12, 10 ml B-27 Serum Free Supplement (#17504-044, Invitrogen), 5 ml Amphotericin B solution (#A2942, Sigma-Aldrich), 2,5 ml 1 M HEPES (#1563-056, Gibco), 0,5 ml gentamicin (50 mg/ml) (#15750037, Invitrogen)

MG-123: 10 mM, dissolved in DMSO (#474790, Merck Chemicals)

1x PBS: #10010-056, Gibco

Trypsin/EDTA: #L11-004, PAA Laboratories GmbH

2.1.5.3 Luciferase Assay

Reagent A: 50 ml glycylglycin (#A-1068, AppliChem), 15 ml KH_2PO_4 (pH 8,0) (#1048730250, Merck Chemicals), 8 ml EGTA (#0878-0005, AppliChem), 40 ml ATP (#A1348-0010, AppliChem), 2 ml Dithiothreitol (#111474-005, Merck Chemicals), 15 ml $MgSO_4$ (#105886, Merck Chemicals). Adjust pH to 8,0 with 2 M KOH, then add 2 ml Coenzyme A (#A0813-0100, AppliChem) and 3,75 ml Beetle Luciferin (#E1602, Promega). Fill up to 1 l with aqua dest. and protect reagent from light.

Reagent B: 220 ml NaCl, 4,4 ml Na_2EDTA (#A1104, AppliChem), 220 ml KH_2PO_4 (pH 5,0) (#1048730250, Merck Chemicals), 44ml BSA (Roth), 2,6 ml NaN_3 (Merck Chemicals), 200 µl Coelenterazine (#S2001, Promega) in 10 ml ethanol. Adjust to pH 5,0 with 2 M KOH and fill up to 1 l with aqua dest.

2.1.5.4 Others

All reagents, solvents and chemicals, if not mentioned differently, were purchased from Sigma-Aldrich, Roth, Merck Chemicals or AppliChem.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culturing conditions

Under normal conditions, all cell lines were cultured in D-MEM supplemented with 10 % FCS on 10 cm tissue culture dishes. Cells were put in a 5 %-CO_2 incubator (HERAEUS, Hanau) with 95 % relative humidity at 37 °C.

For L-glutamine deprivation experiments, D-MEM High Glucose without L-glutamine was used. L-glutamine was added manually to create 1 mM L-glutamine media, supplemented with 10 % FCS. Cells were cultured on 10 cm tissue culture dishes.

In some experiments, tumor sphere conditions were provided with tumor sphere media (see 4.1.5.2) and 20 ng/ml EGF and 20 ng/ml bFGF. Cells were grown in 10 cm non-adhesive culture dishes to be able to free-float and form spheres in the media suspension.

2.2.1.2 Subculturing

Only cells kept in adherent conditions were subject to subculturing.

The first step consisted of washing the cells with PBS. After adding 3 ml Trypsin-EDTA, cells were incubated at 37 °C for about 3 minutes. This enabled them to detach from the culture dish. To inactivate trypsin activity, 5 ml standard growth medium was added. This cell suspension was aspired by gentle pipetting into Eppendorf ® tubes and centrifuged at 1000 round-per-minute (rpm) for 3 minutes. The supernatant was discarded, the cells were resuspended in standard growth medium and subcultured in ratios from 1:10 to 1:40.

2.2.1.3 Cell counting

The CASY Cell Counter and Analyzer System Model TT (# 05651697001, Roche Diagnostics GmbH) was used to determine cell number, size distribution and viability of a sample based on electronic pulse area analysis. 100 µl cell suspension was mixed with

10 ml CASY ton solution (#05651808001, Roche Diagnostics GmbH) and analysed according to the manufacturer's instructions.

2.2.1.4 Freezing and thawing

Since cells are prone to genetic drift in continuous culture and unexpected events like bacterial contamination could always occur, it is crucial to have established cell lines frozen for long-term storage. The effort was made to cryopreserve cells at a passage number as low as possible.

Cells in culture dishes with 70 – 90 % confluence were taken for cryopreservation. Cells were washed and trypinized in a manner similarly to subculturing, then centrifuged at 900 rpm for 3 minutes. The supernatant was discarded and the cell pellet resuspended in 2 - 3 ml of D-MEM with 20 % FCS and 10 % DMSO. 1 ml of the new suspension was pipetted into each cryo vial, which was in turn put into commercially available containers used to control the freezing rate of cells (#5100-0001, Thermo). Those cryo-containers contained isopropanol at room temperature and ensured a temperature reduction rate of 1 °C/min when placed in -80 °C. Cryo vials were transferred to liquid nitrogen containers on the following day.

Cryopreserved cells were rapidly thawed by putting cryo vials in a 37 °C water bath. Cells were mixed with 10 ml warm complete growth medium and seeded in 10 cm culture dishes. The medium was changed on the day after and antibiotics added for selection or the maintenance of transgene expression if needed.

2.2.1.5 Hypoxic incubation

For hypoxic treatment, cells were placed in the Hypoxic Workstation Invivo2 500 (Ruskin Technology) at 37 °C, 5 % CO_2 and 1 % oxygen for the timespan indicated in each experiment.

2.2.2 Western Blot

2.2.2.1 Protein extraction

Growth medium was aspired from adherent culture dishes. Cells were washed with 5 ml 4 °C PBS. About 60 µl – 80 µl Laemmli lyse buffer was added, the cells then lifted from

the culture dish with the help of a cell scratcher. The suspension was pipetted into Eppendorf ® tubes and complete cell lysis was secured by sonication for 30 s with the following settings: amplitude 90 %, pulse 0,5 s/ 0,5 s (Bandelin Sonoplus). Samples were always kept on ice until transferred to -80 °C.

Cells grown in tumor sphere conditions were pipetted with their medium into Falcon® tubes that were kept on ice. The tubes were centrifuged at 4 °C and 2500 rpm for 1 minute. The supernatant was discarded, leaving the cell pellet to be mixed with 60 µl – 80 µl Laemmli lyse buffer. The suspension was transferred to Eppendorf ® tubes and sonicated akin to the adherent cells treatment. The samples were also kept on ice until transferred to -80 °C.

2.2.2.2 Measuring protein concentration

The determination of protein concentration is based on the Lowry method, which measures the colour change of the sample solution based on a two-step reaction. Under alkaline conditions, copper ions react with peptide bonds to form violet-coloured coordination complexes (Biuret test). This reaction combined with the redox reaction of the Folin–Ciocalteu reagent and aromatic residues of amino acids, mainly of tryptophan and tyrosine, turns the sample colour into blue. The absorbance can be reliably measured at 650 nm – 750 nm.

According to the manufacturer's instructions, protein concentration was measured using the colorimetric DC Protein Assay Reagent Package (Bio-Rad #500-0116), which consisted of Reagent A (alkaline copper tartrate solution), Reagent B (diluted Folin reagent) and Reagent S. In a microtiter plate, 5 µl of the protein sample were mixed with 25 µl Reagent A' (2 % Reagent S in Reagent A) and 200 µl Reagent B. 5 µl Laemmli buffer was used as a blank sample for standardisation. After a 15 minute incubation in the dark, bubbles, if formed, were popped with a clean and dry needle. Absorbance was read at 750 nm and the protein concentration calculated based on a calibration curve.

2.2.2.3 SDS-PAGE (Sodium-Dodecyl-Sulfate-Poly Acrylamide Gel Electrophoresis) / Western blot

This method is based on the separation of proteins according to their size. By binding to SDS, proteins receive the same charge per unit mass, so that the only the thing fractionizing them in gel electrophoresis is their size.

Standard discontinuous SDS-polyacrylamide denaturing gel electrophoresis was carried out in Mini-Protean Tetra cells (Bio-Rad). Depending on the size of the proteins of interest, 8 % or 12 % acrylamide gels were used (see Materials, 4.1.5.1). Electrophoretical transfer of separated proteins to PVDF membranes (Hybond ECL, Amersham) was carried out using the Wet Blot System from Bio-Rad at 125 mA per gel for 2 hours. The membranes were incubated for 1 hour at room temperature with 5 % milk blocking buffer to reduce unspecific binding. Respective primary antibodies diluted in 5 % milk blocking buffer were added to the membranes. After overnight incubation at 4 °C, membranes where washed 3 times with 1x PBS/0,1 Tween 20 for 15 minutes at room temperature. Respective secondary antibodies with HRP-conjugation were diluted in blocking buffer and added to membranes for 1 – 1,5 hours. Three times washing with 1x PBS/0,1 % Tween 20 and one time washing with 1x PBS was carried out before producing chemiluminescent signal with the ECL Western Blotting Detection System (Thermo Scientific) or the ECL Plus System (Perkin Elmer). Membranes were put in developing cassettes and the luminescent signal developed on X-ray films with the help of an imager (Thermo Scientific) for timespans ranging from 10 seconds to 1 hour, depending on the signal strength.

2.2.2.4 Stripping

To detect another protein on the same membrane, existing antibodies were washed out with stripping buffer, which was incubated with the membrane for 1 hour at room temperature. The membrane was blocked again with blocking buffer for 1 hour. New primary antibody was added and incubated overnight at 4 °C, and the rest of the procedure carried out like the protocol described above.

2.2.3 Luciferase reporter assay

This method is based on the expression of the luciferase reporter gene, which is coupled with a regulatory gene region of interest. Changes in luciferase activity have a strong correlation with changes in the activity surrounding the gene region being analysed. This can be measured by quantifying luminescent signal in a chemical reaction that the luciferase enzyme catalyses. The Dual-Luciferase® Reporter Assay System (Promega) contains two luciferase reporters to heighten the reliability of measurements: firefly luciferase and Renilla luciferase. One of the luciferase reporters is typically linked to the experimental conditions, while the other one is expressed constitutionally to serve as an internal control. The addition of respective reagents (reagent A for firefly luciferase and

reagent B for Renilla luciferase) starts the creation of luminescent signal by each luciferase, which is measured in relative luminescence units (RLU). The quantified experimental luciferase reporter activity is then normalized to the activity of the control reporter, which effectively minimizes variability caused by differences in cell viability, cell lysis effectivity and so on.

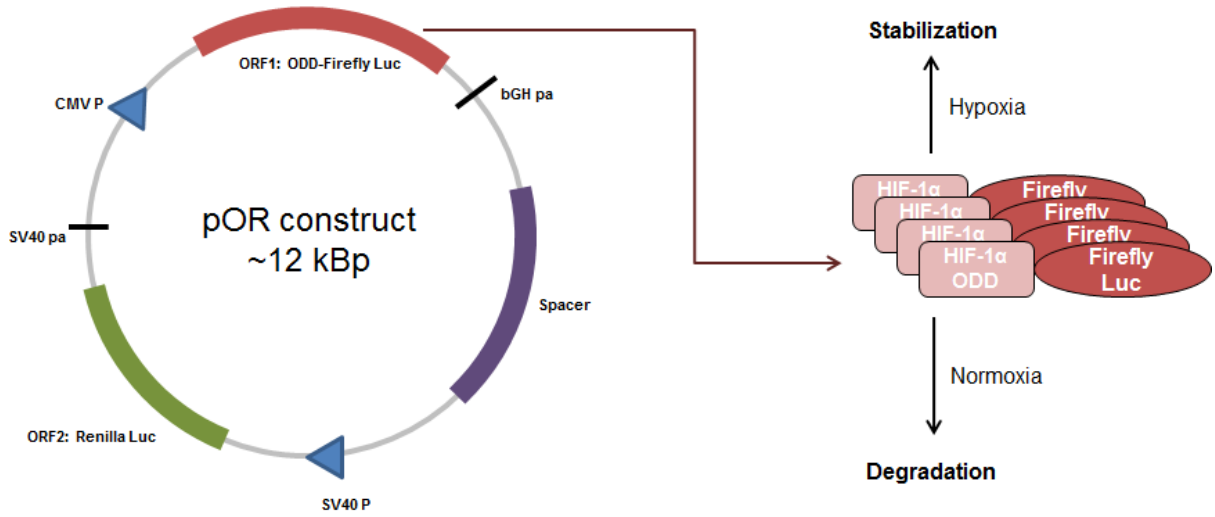

Figure 2.1: pOR reporter construct created by Omelyan Trompak.
One open reading frame (ORF1) is the Firefly Luciferase (Luc) gene coupled to the HIF-2α oxygen-dependent degradation (ODD) domain. Under normoxia, firefly luciferase is marked for degradation by PHD hydroxylation at the ODD domain. Under hypoxia, firefly luciferase is stable and its luminescence producing activity can be measured. The second vector contains the pSV40-Renilla luciferase gene, which produces Renilla luciferase as the internal control of transfection efficiency. Figure modified from Omelyan Trompak.

A vector construct containing the firefly luciferase reporter gene coupled with the gene coding for the oxygen-dependent degradation (ODD) domain of HIF-2α was kindly provided by Omelyan Trompak. The expression of luciferase fusion protein with an ODD domain therefore enables luciferase activity to correspond with HIF stabilization in dependence of PHD activity. For internal control, pRL-SV40 was used to ensure constitutive expression of Renilla luciferase. Nuray Bügürcü carried out the transfection of U87 and G55 cells with this cloning vector. A modified limiting dilution assay was performed and single cell clones selected by Rebecca Seibel, thus lowering transfection variability and ensuring the creation of cells with the most stable HIF induction under hypoxia. In this work, U87 pOR 1-3 and G55 pOR 4-7 clones were used. Omelyan Trompak created an MDA pOR cell line for his work and was so kind as to provide those cells for luciferase assays in this work, too.

2.2.4 Modified Boyden chamber assay

To filter and select cells with high invasive and migratory potential, a modified Boyden chamber assay protocol was constructed. 24-well Transwell® plates with polycarbonate membranes and a pore size of 8 µm (# 3422, Corning Life Sciences) were used.

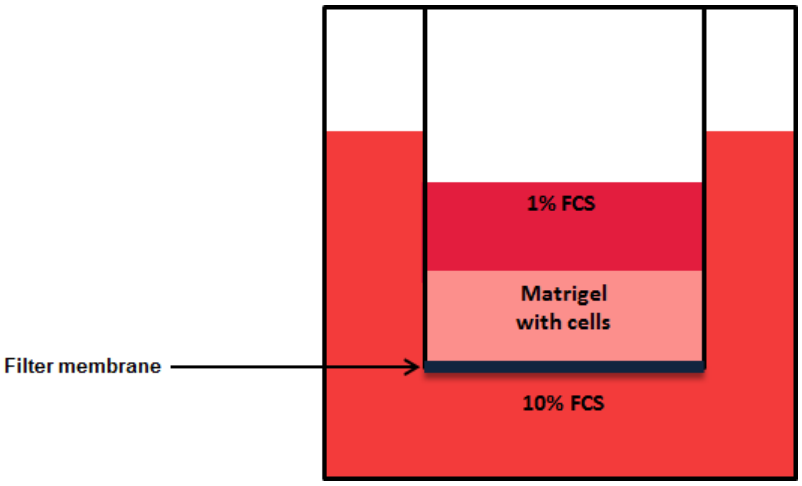

Figure 2.2: Schematic setup of the modified Boyden chamber.
The upper compartment contains a Matrigel-cell mixture, topped by 1 % FCS medium. The lower compartment is filled with normal 10 % FCS growth medium to create a FCS gradient, thus stimulating cell migration towards the filter membrane.

One Boyden chamber well consists of one upper and one lower compartment. For one well, either 200 000 cells of U87 or G55 cells were suspended in 400 µl 1 mg/ml Matrigel-D-MEM mixture (Matrigel #356234, BD Biosciences). The cell suspension was pipetted into the upper compartment and incubated at 37 °C for one hour. 600 µl 10 % FCS D-MEM was added to the lower compartment, whereas the upper compartment was filled with 100 µl 1 % FCS D-MEM. An FCS gradient was hereby generated, which should stimulate cell migration from the upper compartment to the lower compartment. The filter membrane strengthened the selection of cells with the strongest invasion tendency. After an incubation time of 18 h – 24 h at 37 °C, the content in the upper compartment was aspired and discarded. The medium in the lower compartment was replaced with Trypsin-EDTA for 3 minutes. Trypsin-EDTA was aspired and the bottom side of the filter membrane washed with normal 10 % FCS D-MEM to collect the filtered cells. Cells were transferred to 10 cm culture dishes and normal growth medium was (10 % FCS D-MEM) added.

For each condition of an experimental setting, a triplicate of wells was prepared.

2.2.5 Statistical analysis

Results were displayed as mean + standard error mean (SEM) in graphs created in Microsoft Excel©. Statistical analysis was carried out using the two-tailed Student's t-test. Statistical significance was defined as $p < 0,05$ (*), $p < 0,01$ (**), $p < 0,001$ (***).

3 Results

As an essential cosubstrate for several enzymes that regulate important pathways in GBM and other tumors, αKG and its utilization stand in the focus of this work. Factors influencing αKG availability were investigated, including the αKG precursors glutamine and glutamate, as well as IDH1, the principal enzyme incorporating αKG into cell metabolism. The goal of our investigation was to assess the effect of these factors on PHD enzyme activity and HIF protein levels.

This work was done in close collaboration with Nuray Bögürcü. In order to maintain the conclusiveness of the results, some of their data will be mentioned or presented in the following sections.

3.1 Maintenance of PHD activity in different tumor types

PHD, the most potent HIF regulator, functions as sensitive oxygen sensors. Reduction in oxygen availability inhibits PHD activity, resulting in HIF-α accumulation and activation of a number of adaptive responses. Interestingly, PHD has been shown to be HIF-1/2α target itself as part of a negative feedback loop. This negative regulation leads to an increase in PHD levels in response to prolonged hypoxia (Henze et al., 2010). We questioned whether PHD protein abundance under such circumstances also translates into higher PHD activity.

The two GBM cell lines U87 and G55 and breast adenocarcinoma cells MDA underwent luciferase reporter assays in this experiment setting. The luciferase construct reports changes in PHD-mediated hydroxylation of the oxygen-dependent degradation domain HIF-2α-ODD. Increased hydroxylation by PHD marks the HIF-2α-ODD-containing luciferase protein for degradation in a manner similar to HIF-2α, which results in a reduced luminescent signal. Our results showed a clear reduction of PHD hydroxylation activity in all three cell lines in the first 18 hours of hypoxia (1% oxygen) (**Figure 3.1**). After 96 hours of hypoxic exposure, however, PHD activity in both GBM cell lines increased again, although it remained significantly lower than under normoxia (21% oxygen). In contrast, PHD activity in MDA cells underwent continuous suppression for the entire duration of the hypoxic treatment.

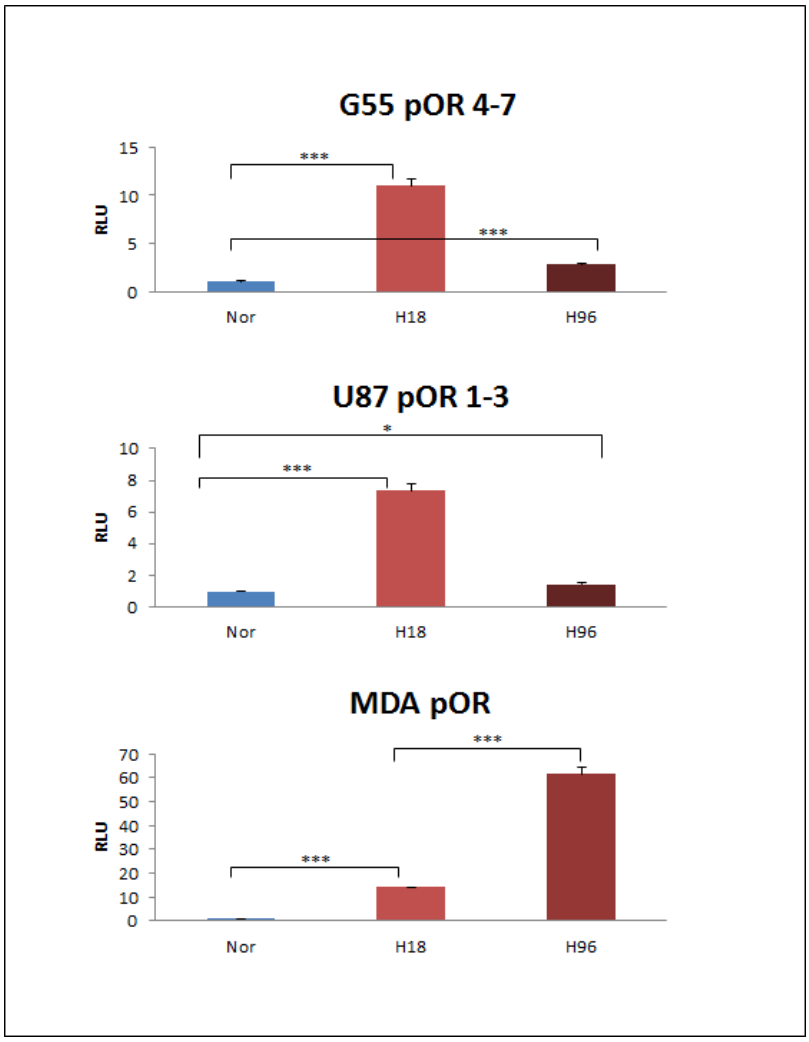

Figure 3.1: Hypoxia inhibits PHD activity.
G55, U87 and MDA cells were stably transduced with the pOR plasmid containing HIF-2α-ODD-firefly luciferase reporter and the internal control Renilla luciferase. This reporter construct (**pOR**) was created to indirectly measure the hydroxylation activity of PHD. Firefly luciferase readings were normalized to renilla readings and the data represented in relative luminescence units (**RLU**) as mean (n=3) + SEM. ****p < 0.001.* The hypoxic incubation of cells in 1% O_2 led to a significant decrease in PHD activity and hence increased luciferase readings. This phenomenon can be seen in both glioblastoma cell lines (**G55, U87**) and breast adenocarcinoma cells (**MDA**) compared to their respective normoxic control (**Nor**, 21% O_2). However, after 96 hours (**H96**), PHD activity in glioblastoma cells increased again while the activity in MDA cells remained suppressed.

These findings confirm the negative feedback of the HIF-PHD pathway by which GBM cells react to prolonged exposure to hypoxia. Previous data showed PDH protein levels to increase again after an initial downregulation under hypoxia, if given enough time to adapt (Henze et al., 2010). The current results confirm that the protein abundance ensured sufficient enzymatic activity to hydroxylate HIF-α-ODD despite the scarcity of oxygen as a necessary co-substrate. Interestingly, this feature is distinct for glioblastoma and could not be observed in breast adenocarcinoma cells.

3.2 The influence of α-ketoglutarate precursors on the PHD/HIF pathway

The results above showed that glioblastoma cells can overcome PHD inhibition under extended exposure to hypoxia. This raises the question how PHD activity can be regulated when its strongest limiting factor, oxygen scarcity, is already present. We investigated other factors that are essential for the enzymatic activity of PHD, among which is the co-substrate αKG. Glutamate and glutamine – two known precursors of αKG – are two amino acids of interest within this context. The following experiments investigate the indirect regulatory effect of microenvironmental glutamate and glutamine on PHD activity and HIF protein levels.

3.2.1 L-glutamate regulates PHD activity and HIF levels

Glutamate concentrations can reach toxic levels especially in peritumoral areas in GBM (Briggs, Koivunen et al. 2016). To mimic this condition *in vitro*, we added L-glutamate to U87 and G55 cells grown in standard adherent culture conditions and assessed PHD activity in luciferase assays.

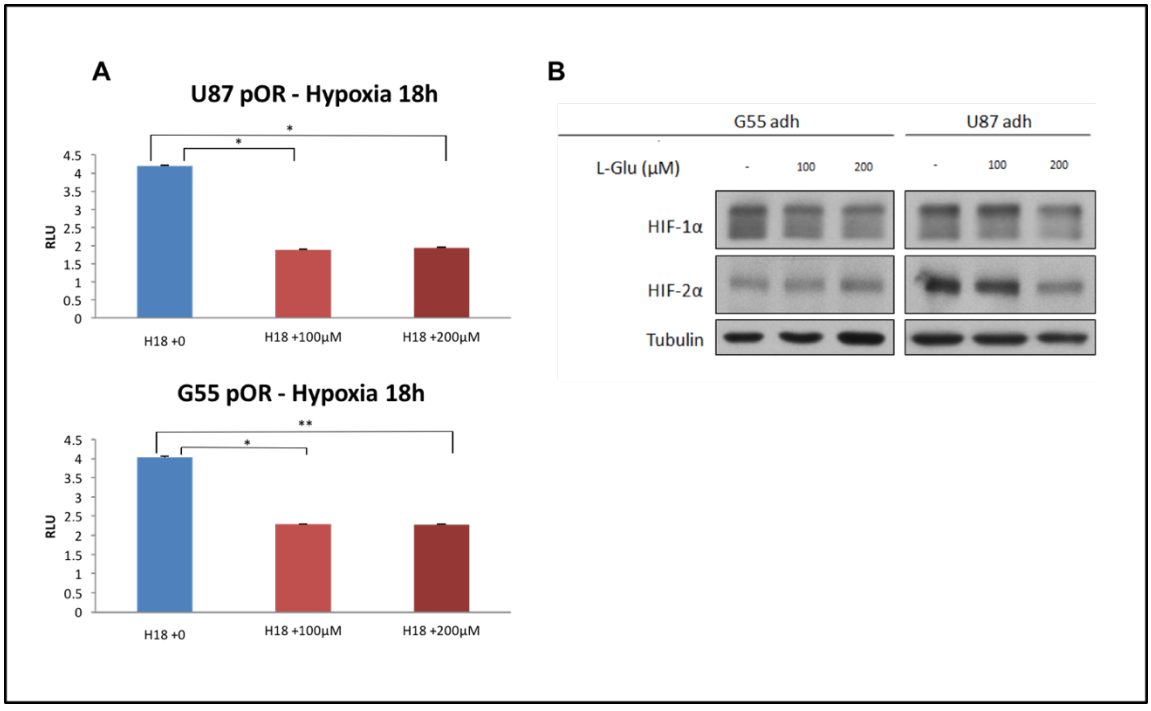

Figure 3.2: L-glutamate increases PHD activity and downregulates HIF levels under hypoxia.
A, U87 and G55 cells were transfected with the pOR reporter construct. PHD activity increases after the addition of 100 µM or 200 µM L-glutamate. Data are displayed in relative luminescence units (**RLU**) as mean (n=3) + SEM. *$p < 0.1$, **$p < 0.01$. **B**, HIF-1α and HIF-2α levels were detected by western blotting under L-glutamate (**L-Glu**) treatment in G55 and U87 cells. Cells were incubated in the hypoxic chamber (1% O_2) for 6 hours. L-Glutamate addition led to decreased HIF-1α protein levels in both cell lines. HIF-2α also clearly decreased in U87 cells, whereas a decrease in G55 cells cannot be definitely determined due to uneven tubulin levels. Tubulin served as the loading control. One representative western blot is presented among at least three repetitions.

GBM cells react to glutamate in the microenvironment with an increase in PHD activity despite the hypoxic treatment, as indicated by the decreased luciferase readings (**Figure 3.2A**). This increase in activity was sufficient to reduce HIF-1α, and less distinctly HIF-2α protein levels (**Figure 3.2B**). These results suggested that elevated concentrations of the αKG precursor glutamate could overcome the hypoxia-mediated inhibition of PHD activity, to the point that HIF-α degradation could still be ensured under hypoxia.

3.2.2 L-glutamine regulates HIF levels

The other αKG precursor, glutamine, is an essential nutrient taking part in various critical cell processes like protein synthesis, cell growth and lipid metabolism (Xiao 2016). Similar to the experiments above, we assessed to what extent microenvironmental changes of glutamine take part in the regulation of HIF-α.

Even though it is usually available in abundance, glutamine can reach critical limits in certain regions in solid tumours (Pan, 2016). To mimic this environment, a medium containing 1 mM L-glutamine instead of the usual 4 mM was generated. We first investigated the effect of this nutrient deprivation on GBM cells, then examined how L-glutamine re-supplement could alter HIF-α expression. U87 cells were first grown in standard adherent conditions in 4 mM L-glutamine medium. The medium was replaced by 1 mM L-glutamine medium for up to 14 days. On the last day, a sample representing each deprivation period was incubated in hypoxia for 6 hours before undergoing western blotting (**Figure 3.3A**).

Our results showed two interesting effects of L-glutamine deprivation in the short-term and in the long-term. After one day of L-glutamine deprivation, HIF-1α was clearly upregulated compared to the non-deprived control cells, especially in samples experiencing hypoxia (**Figure 3.3A**). This short-term effect could be caused by a sudden decrease in αKG levels, which in turn inhibits PHD activity and stabilizes HIF-α. In contrast, after a L-glutamine deprivation period beyond 7 days that most likely enabled different adaptation mechanisms, the HIF-α stabilizing effect was slowly revoked.

In a separate experiment, U87 cells were pre-treated with 1 mM L-glutamine for 24 hours, then received a gradual re-supplement of L-glutamine (**Figure 3.3B**). Interestingly, the HIF-stabilizing effect after short-term L-glutamine deprivation was gradually abrogated by increasing L-glutamine concentrations in the medium. This result strongly suggested that GBM cells are sensible to microenvironmental changes of glutamine. Similar to

glutamate, glutamine in the microenvironment seems to be utilized by GBM cells to enable HIF-α degradation even under hypoxia.

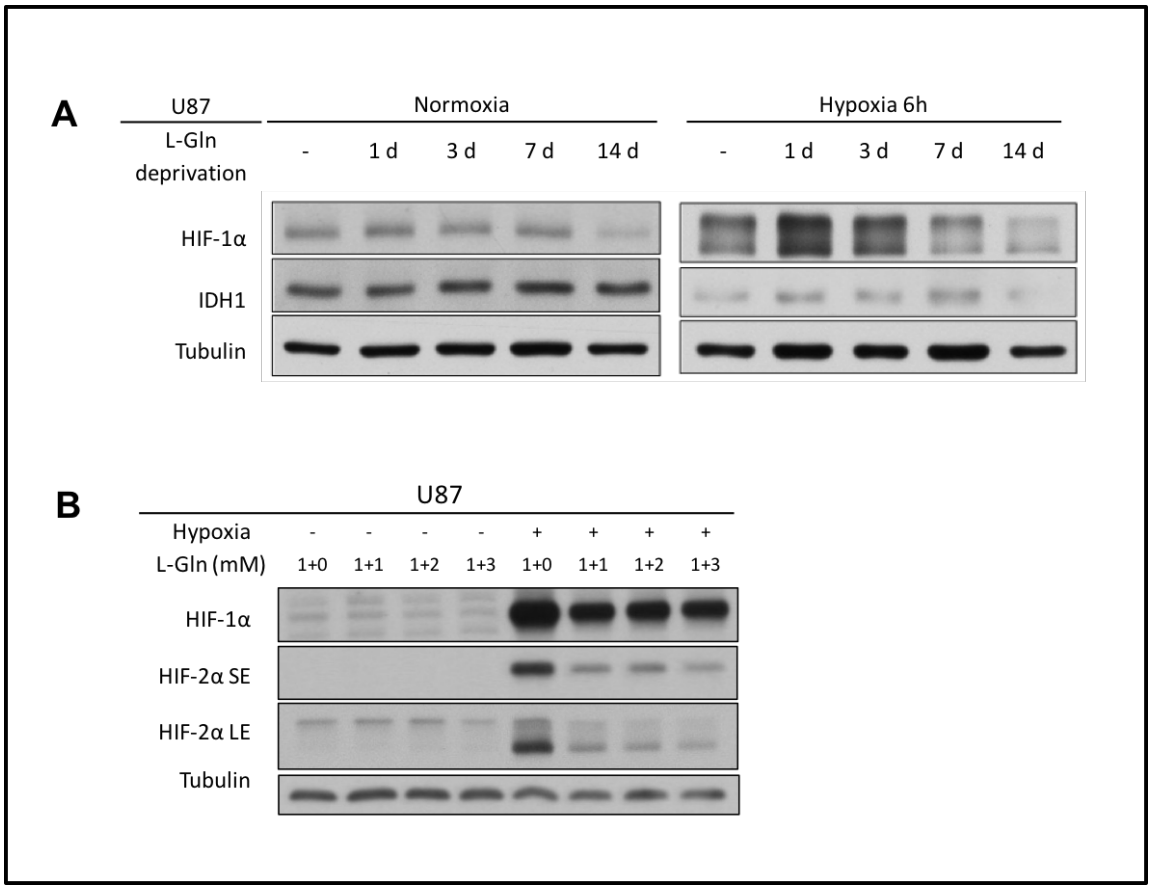

Figure 3.3: L-glutamine influences HIF-α levels.
A, U87 cells were grown in medium containing 4 mM L-glutamine (L-Gln). This medium was replaced with 1 mM L-glutamine medium for up to 14 days. On the last day, one sample of cells undergoing each L-glutamine deprivation period was incubated in hypoxia for 6 hours. Immunoblots of HIF-1α show elevated protein levels after 1 day of deprivation, with the most striking change under hypoxia. Beyond 7 days, HIF-1α levels significantly decreased in both normoxia and hypoxia. IDH1 expression showed no relevant changes. B, U87 cells were in 1 mM L-glutamine (L-Gln) medium for 24 hours. The control cells remained in 1 mM L-glutamine medium (1+0), while others received additional 1, 2 or 3 mM L-glutamine (+1/+2/+3). Samples were either incubated under normoxia or hypoxia for 6 hours. L-glutamine addition caused a decrease in HIF-1α and especially HIF-2α levels under both normoxia and hypoxia. (SE, short exposure; LE, long exposure). Tubulin served in all figures as loading control. One representative western blot is presented among at least three repetitions.

Taken together, we have shown that an increase in concentration of both αKG precursors L-glutamate and L-glutamine in the microenvironment could abrogate the HIF-stabilizing effect of hypoxia. This phenomenon is most likely caused by an increase in αKG concentration, which in turn increases the enzymatic activity of PHDs. Reversely, a sudden decrease of the usually abundant glutamine in the microenvironment stabilizes HIF-α. Given enough time to adapt, however, GBM cells activate alternative mechanisms to decrease HIF-α levels again.

3.3 The central role of IDH1 in the PHD/HIF pathway

IDHs are the principal enzymes to produce αKG in the cell by converting isocitrate to αKG. α-Ketoglutarate, in turn, is an essential co-substrate for various αKG-dependent enzymes. Among those enzymes, PHDs are the most important for the regulation of the hypoxic response mediated by HIF-α. It therefore stands to reason that IDH, as an αKG-producing enzyme, can regulate PHD activity by modulating intracellular αKG levels. Here, the importance of the cytoplasmic isoform IDH1 is shown. IDH1 is further characterized in the later sections, as well as its possible role in promoting the invasive phenotype of glioblastoma cells.

3.3.1 IDH1 knockdown inhibits PHD activity and stabilizes HIF-α

IDH1 produces αKG in the cytoplasm – the same cell compartment where PHDs hydroxylate HIF-α for ubiquitination. The mutation of IDH1 in cancer cells has been shown to produce the oncometabolite 2-HG instead of αKG, and by reduction of the wildtype activity also shifts the αKG-to-succinate ratio towards succinate (Bogdanovic, 2015). Consistent with those findings, we showed that knockdown of wildtype IDH1 significantly reduced intracellular αKG levels (**Figure 3.4A**). This reduction was sufficient to inhibit PHD activity in both normoxia and hypoxia, as confirmed in luciferase assays (**Figure 3.4C**). The stabilizing effect on both HIF-1α and HIF-2α resulting from this phenomenon was gradually reversed by increasing concentrations of αKG again, further proving the crucial role of the metabolite αKG in the regulation of HIF-α protein levels (**Figure 3.4B**).

All in all, we demonstrated the importance of wildtype IDH1 function in limiting the hypoxic response in glioblastoma cells. A reduction of its ability to produce αKG results in increased HIF-α stabilization, an effect mediated by the inhibition of PHD activity.

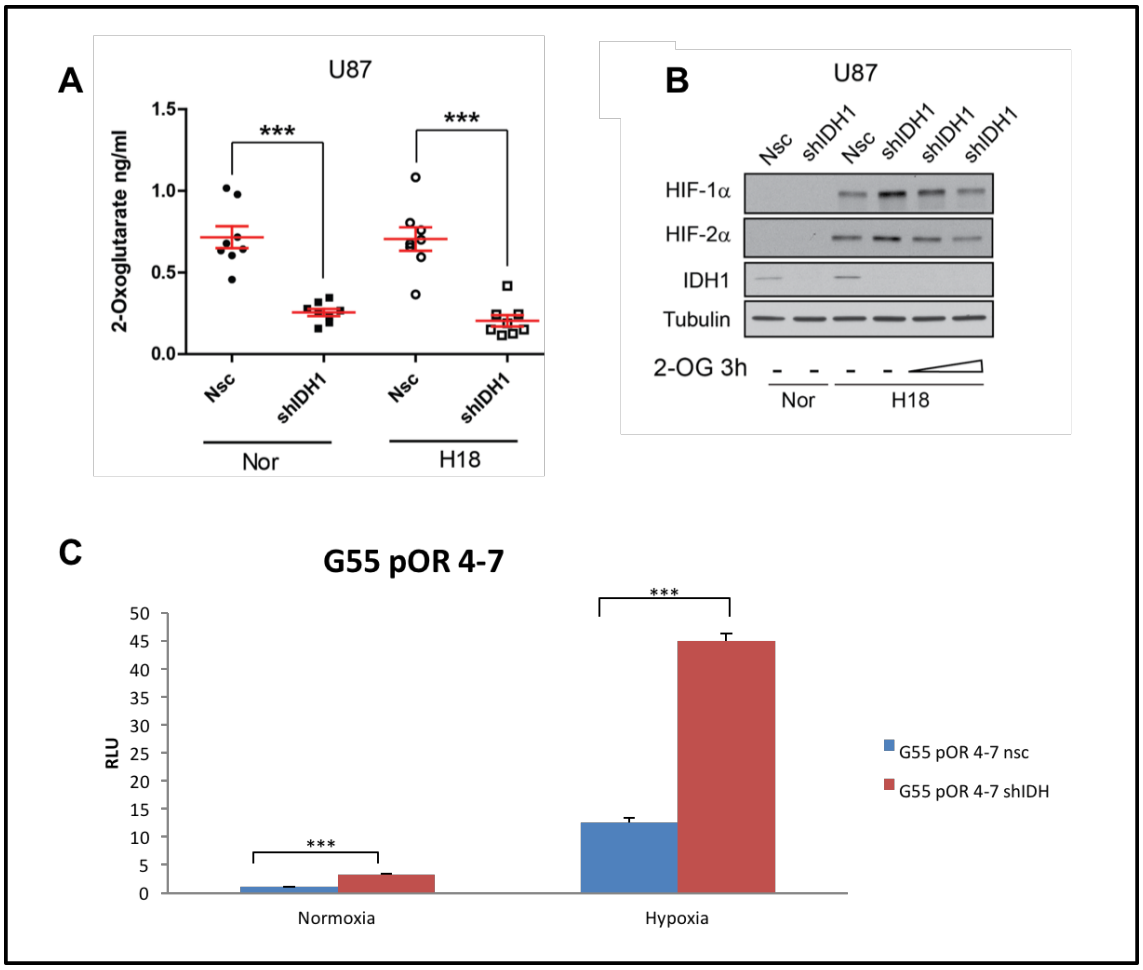

Figure 3.4: The effect of IDH1 silencing on the PHD/HIF pathway.
U87 and G55 cells were transduced with lentiviruses carrying a short-hairpin RNA responsible for silencing IDH1 (**shIDH1**) or a non-silencing (**nsc**) control shRNA. The data in **A** and **B** are kindly provided by Nuray Bögürcü. **A**, α-Ketoglutarate (also called 2-oxoglutarate, **2-OG**) levels were measured in mass spectrometry and values, as well as the mean (n=8) +/- SEM are shown. α-Ketoglutarate decreased in IDH1 knockdown cells in both normoxia (**Nor**) and after 18 hours of hypoxic incubation (**H18**). **B**, Immunoblotting of HIF-1α, HIF-2α, IDH1 and loading control Tubulin are shown. IDH1 silencing caused an increase in HIF-α levels under hypoxia. This effect could be gradually reversed by the addition of α-ketoglutarate (**2-OG**) in increasing concentrations. **C**, G55 cells containing the ODD-luciferase plasmid (**pOR**) underwent IDH1 silencing as described above. Single-cell clones were selected for both IDH1-silenced (**shIDH**) and non-silencing (**nsc**) cells. Luciferase readings in IDH1-knockdown cells were significantly higher than in the non-silencing control, indicating decreased PHD activity, in both normoxia and hypoxia. All values are displayed in relative luminescence units (**RLU**) as mean (n=3) + SEM. ****p < 0.001.*

3.3.2 IDH1 knockdown sensitizes tumor cells to L-glutamine

So far, our results have proposed that wildtype IDH1 activity and sufficient glutamine concentrations in the microenvironment are crucial for the maintenance of HIF-α degradation. A disturbance of both conditions can be found in cancer cells and is associated with tumour progression (Semukunzi, 2017; Pan, 2016). We aimed to analyse the interplay of IDH1 and glutamine with regards to their regulation of PHD activity,

bearing in mind that they are both involved in maintaining intracellular αKG concentrations.

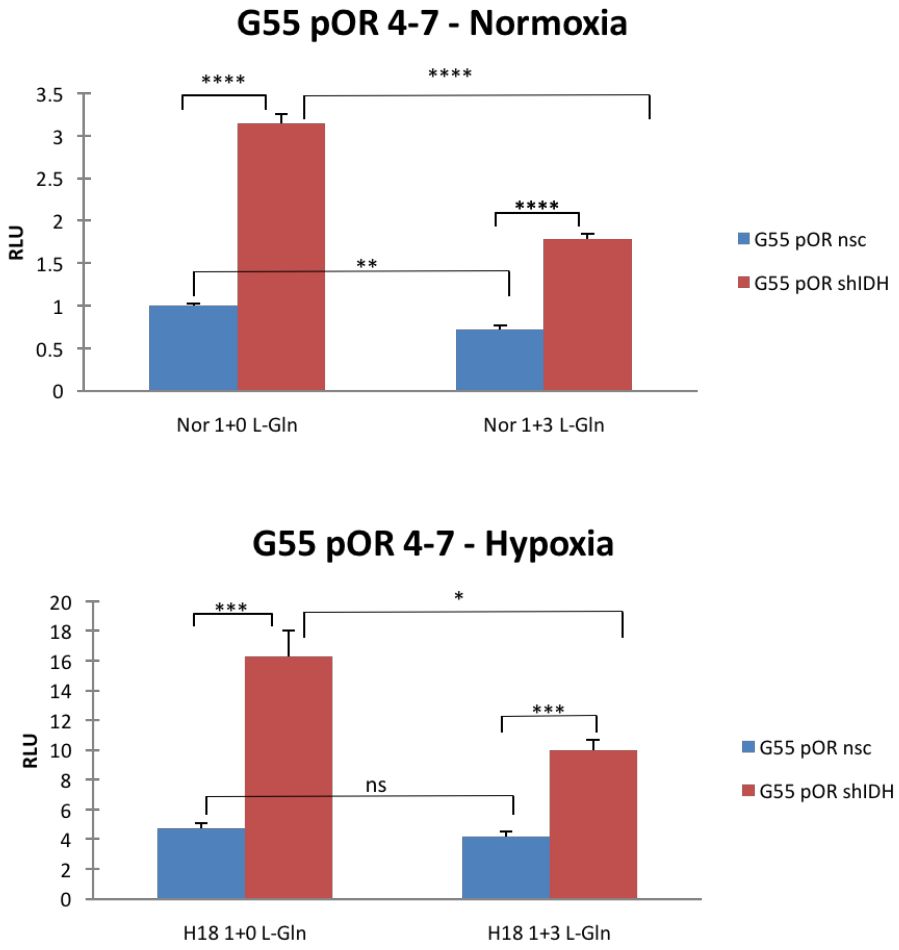

Figure 3.5: IDH1 knockdown sensitizes tumor cells to L-glutamine.
G55 pOR clone 4-7 were grown in 1 mM L-glutamine (**L-Gln**) medium for 24 hours. A subsequent addition of 3 mM L-glutamine was carried out and samples incubated either in normoxia (**Nor**) or hypoxia for 18 hours (**H18**). PHD activity in IDH1-silenced cells (**shIDH**) was significantly lower than non-silenced cells (**nsc**). L-glutamine supplement increased PHD activity, an effect that was more pronounced in IDH1 knockdown cells. All values are represented in relative luminescence units (**RLU**) as mean (n=3) + SEM. ***p < 0.01, ***p < 0.001, ****p < 0.0001.*

Our findings confirmed the complementary effects of IDH1 and glutamine. As evident in luciferase assays, both IDH1 knockdown and L-glutamine deprivation reduced PHD activity in glioblastoma G55 cells (**Figure 3.5**). In IDH1 knockdown cells, L-glutamine re-supplementation led to a highly distinct increase in PDH activity. Conversely, in the IDH1 non-silenced control cells, the increase in PHD activity upon L-glutamine supplement was less striking and even non-significant under hypoxia. This suggested that tumor cells with reduced IDH1 levels, in particular, are susceptibe to altered L-glutamine levels, as

most likely, the intracellular αKG deficit caused by L-glutamine deprivation becomes a limiting factor for PHD activity. Taken together, our results underline the importance of wildtype IDH1 function on maintaining HIF-α degradation by PHDs.

3.3.3 Fetal calf serum reduces IDH1 levels

Following the realization of the importance of wildtype IDH1 on the PHD/HIF pathway, we aimed to analyse whether or not IDH1, in turn, is affected by microenvironmental changes. The modulation of glutamine and glutamate concentrations, however, do not seem to have a huge impact on IDH1 levels (**Figure 3.3A**, other results not shown). Therefore, a screening of whether or not IDH1 levels react to microenvironmental signals at all was implemented. In order to do so, the microenvironment was modified by stimulating GBM cells with foetal calf serum (FCS), which contains a composition of soluble factors, among them cytokines and growth factors that have yet to be characterised in detail. A downregulation of IDH1 could be clearly observed after FCS addition, confirming a response of IDH1 to microenvironmental stimuli in GBM cells. It is already known that in the tumor microenvironment, a number of inflammatory cytokines like TGF-β, TNF-α, and IL-1β are secreted to regulate tumour cell mechanisms (Christofides, 2015). Which factors exactly are involved in IDH1 regulation remain to be elucidated.

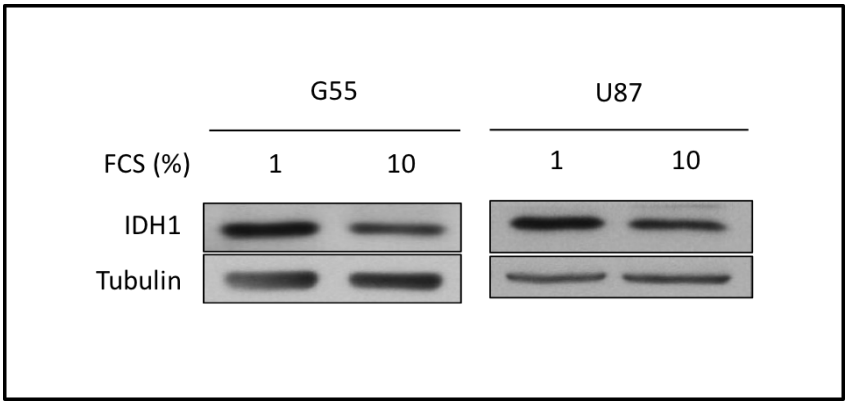

Figure 3.6: FCS causes a downregulation in IDH1 levels.
Immunoblotting of IDH1 in G55 and U87 cells grown in D-MEM medium supplemented with either 1 % or 10 % foetal calf serum (**FCS**). Higher concentrations of FCS led to a decrease in IDH1 levels. Tubulin served as loading control. One representative western blot is presented among at least three repetitions.

3.3.4 The stability of IDH1 at protein level

Following the observation that IDH1 was downregulated upon FCS stimulus, we aimed to investigate whether and how this effect was mediated on the protein level. So far, the

mechanisms behind IDH1 regulation are not well-understood. We treated glioblastoma cells G55 and U87 with the protein translation inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132. In western blots, the positive control HIF-1α quickly vanished upon the halt of protein translation and accumulated greatly under proteosomal inhibition, whereas IDH1 signals were only slightly decreased by blocking protein translation and remained unaffected by proteosomal inhibition within the analyzed timeframe (**Figure 3.7**). These results confirmed that unlike HIF-1α, IDH1 did not have a high protein turnover, and any change in protein concentrations did not primarily take place on the level of protein stability.

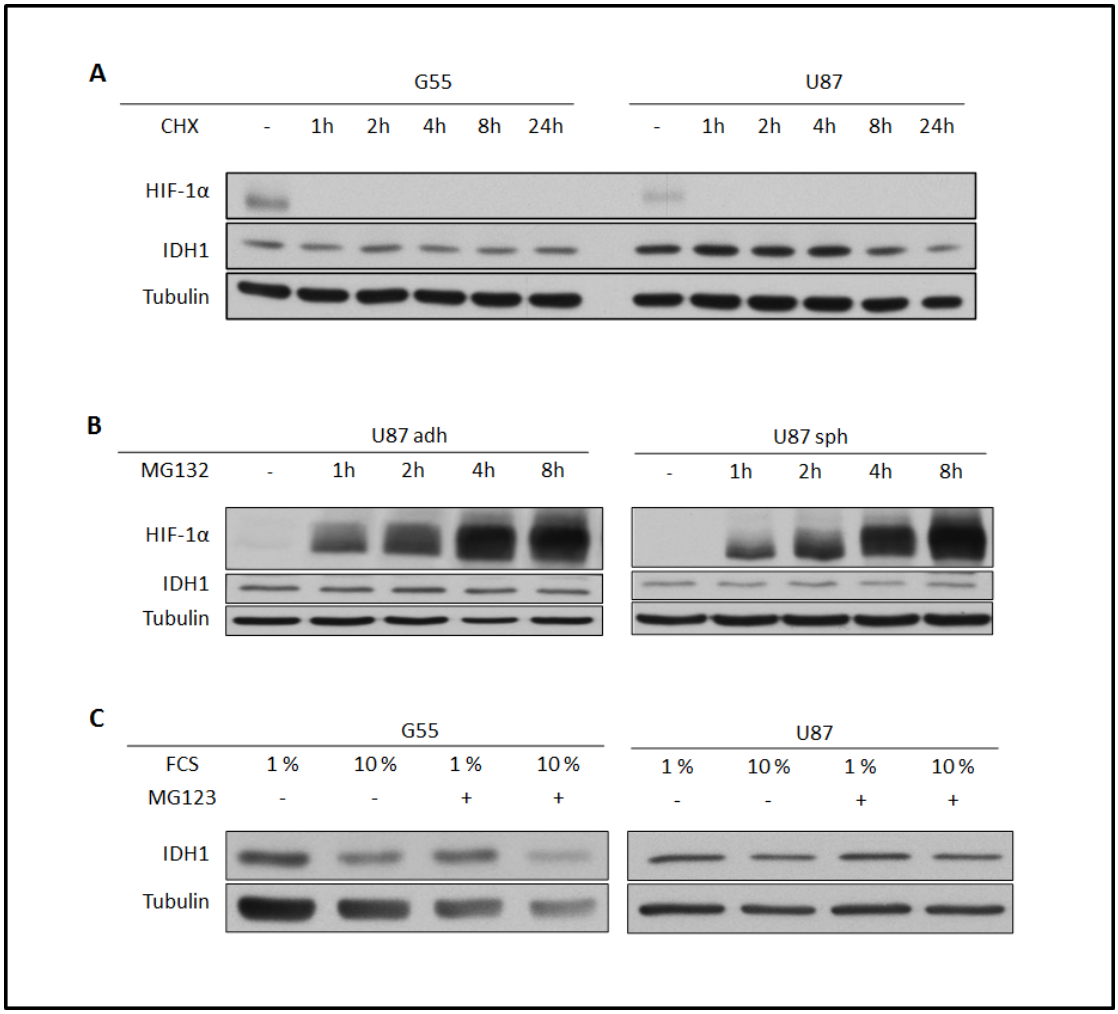

Figure 3.7: IDH1 protein turnover.
G55 and U87 cells were treated with the protein translation inhibitor cycloheximide (CHX) or proteasome inhibitor MG123. Immunoblotting of IDH1, the positive control HIF-1α and the loading control Tubulin is shown. **A**, In contrast to the positive control HIF-1α, IDH1 levels showed only a slight decrease within the experimental timeframe of 24 hours under CHX treatment. **B**, IDH1 levels in U87 cells in both adherent (**adh**) and tumour sphere (**sph**) conditions did not accumulate within 8 hours, which indicates IDH1's lack of short-term dependency on the proteasomal degradation pathway. HIF-1α, on the other hand, displayed a steady accumulation, fitting to its known regulatory proteosomal degradation. **C**, G55 and U87 cells were supplemented with either 1 % or 10 % foetal calf serum (**FCS**) and treated with MG123. A downregulation of IDH1 under 10% FCS occurred, but no significant difference between treatment or no treatment with MG123 was discernible. Tubulin served as loading control. One representative western blot is presented among at least three repetitions.

3.3.5 IDH1 expression in cells with an invasive phenotype

One important feature of growth in highly malignant tumors like GBM is their strong invasive capacity. The transcriptional factor HIF-α induces the expression of many gene products which play critical roles for invasion such as Snail, Twist, CXCR4, and ZEB1 (Mimeault and Batra 2013, Siebzehnrubl, Silver et al. 2013). Consequently, our hypothesis that IDH1 regulates HIF-α levels opens up interesting questions regarding IDH1's involvement in the promotion of tumour invasion.

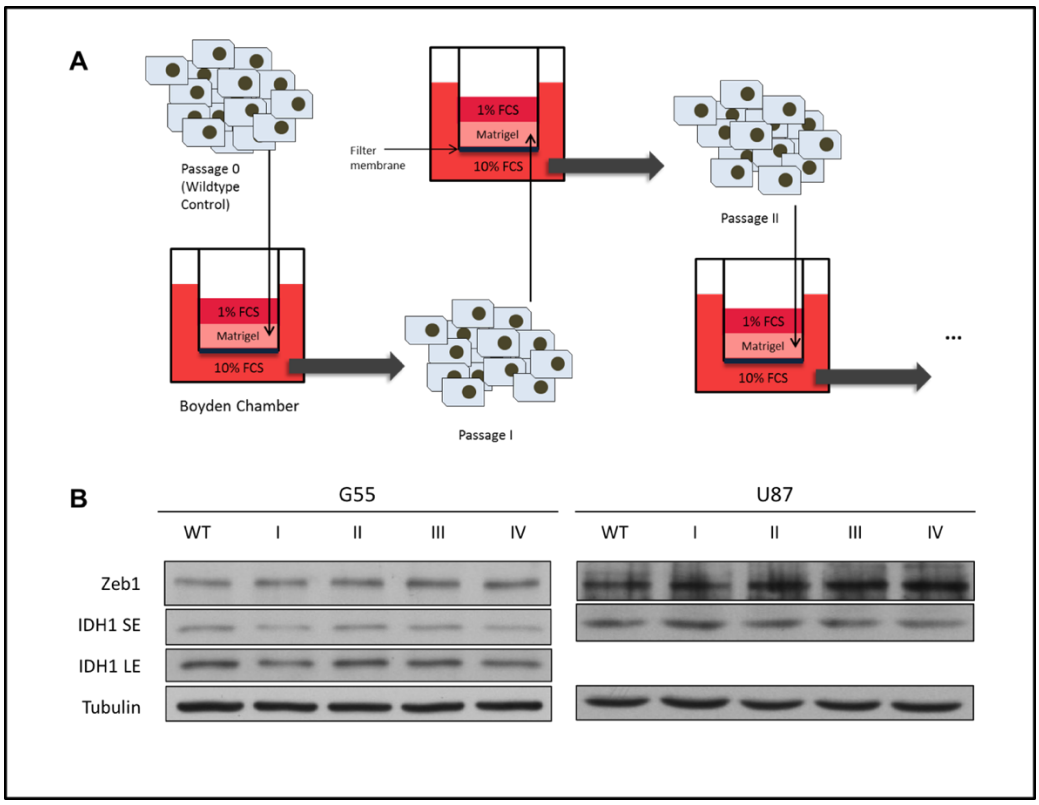

Figure 3.8: IDH1 expression in cells with an invasive phenotype.
A, The modified Boyden chamber assay was deployed to create cell pools with increasing invasive tendency. Invasive cells were harvested from the bottom side of the filter membrane and used for another passage through the modified Boyden chamber assay. Each passage contains a pool of cells from n=3 Boyden chambers. **B**, Zeb1 and IDH1 were assessed in the wild type (**WT**) of G55 and U87 cells and their filtered pools (passaged up to 4 times – I to IV) in Western blots (**SE**, short exposure; **LE**, long exposure). While Zeb1 levels increased with each passage, indicating a stronger invasive phenotype, IDH1 levels showed no consistent changes.

Using modified Boyden chamber assays, which allowed cells to migrate through a porous filter membrane, the invasive capacity of G55 and U87 cells was assessed. We run wildtype cells through several passages of the modified Boyden chamber, thereby collecting cell pools with different levels of invasiveness, confirmed by the increasing pro-invasive Zeb1 signal in western blots (**Figure 3.8 A, B**). It is interesting to note that IDH1 signal showed no consistent changes in relation to the invasive capacity, suggesting that even though an IDH1 deficit could promote a more invasive phenotype, persistent IDH1 reduction is not selected for during tumor invasion.

4 Discussion

4.1 The role of α-ketoglutarate precursors in the PHD/HIF pathway

PHD belongs to the family of αKG-dependent dioxygenases, which contains enzymes that require a sufficient amount of oxygen, Fe^{2+}, and αKG to carry out their catalytic activity (Schofield and Ratcliffe 2005). While the role of PHD as an oxygen sensor and thus its involvement in the hypoxic response has been studied for over a decade, PHD's ability to act as a metabolite-sensor due to its dependency on αKG has only received greater attention in the recent years. αKG has also emerged as a new point of focus in cancer research, given its participation in numerous metabolic processes that are reprogrammed in various cancer types.

In osteosarcoma cells, Duran and colleagues have shown that amino acid starvation leads to the depletion of intracellular αKG, followed by complete PHD inactivation (Duran, MacKenzie et al. 2013). In this study, we investigated the effect of the αKG precursors glutamine and glutamate on PHD activity and HIF protein levels in GBM cells. The modulation of extracellular glutamine and glutamate concentrations to mimic *in vivo* microenvironmental conditions is shown to affect the PHD/HIF axis in a way that underlines their role as the main sources for intracellular αKG.

4.1.1 Glutamate increases PHD activity and HIF degradation

As one of the main excitatory transmitters in the brain, *in vivo* glutamate concentration in non-excited brain tissue is kept as low as 50 μM in perisynaptic regions and 1-3 μM in the remaining brain tissue (Ye and Sontheimer 1999, de Groot and Sontheimer 2011). Astrocytes absorb this potent excitatory neurotransmitter to prevent unwanted neuronal activity and protect the rest of the brain from excitotoxic damage. *In vitro*, GBM cells secrete glutamate into the extracellular space with reported concentrations of around 30 μM (Yao, Kang et al. 2014). Notably, the glutamate distribution is uneven *in vivo*, with up to 4-fold elevated concentrations in peritumoral areas compared with the remaining tumour (de Groot and Sontheimer 2011). We mimicked the extreme GBM microenvironment *in vitro* by adding L-glutamate in concentrations of 100 – 200 μM to cells cultured in standard, glutamate-free medium.

Our findings display a clear effect of glutamate in enhancing PHD activity and downregulating HIF protein levels in glioblastoma cell lines G55 and U87 (**Figure 3.2**).

While it seems plausible that an elevated concentration of glutamate could increase intracellular αKG levels and thus enhance PHD activity, the complex utilization of glutamate in the brain suggests there can be other ways by which the effect on PHD activity is mediated.

The following points need to be taken into account. First, gliomas display a pathological glutamate transporting behavior. In contrast to astrocytes, they secret a notable amount of glutamate into the extracellular space while also exhibiting an impaired glutamate uptake (Ye, Rothstein et al. 1999, Ye and Sontheimer 1999). An elevation in the extracellular glutamate concentration therefore does not necessarily correlate with elevated intracellular tumor glutamate levels. Second, even if glutamate is transported into the cell, its intracellular fate takes on various routes. Next to the conversion into αKG, glutamate can be directly incorporated into protein biosynthesis, used for the production of the antioxidant glutathione or transformed into glutamine, especially when the cell experiences glutamine deprivation (Takeuchi, Nakayama et al. 2018). Unfortunately, data on glutamate's heterogeneous utilization within tumors and GBM, specifically, remains limited.

A direct link between extracellular glutamate and the PHD-HIF axis has so far only been established to a limited extent. Briggs et al. show that elevated glutamate levels in triple-negative breast cancer inhibits one of the main glutamate transporters, the xCT antiporter system, which drives glutamate secretion in exchange with cysteine uptake. The inhibition of this antiporter system causes intracellular depletion of free cysteine, a condition which inactivates PHD by allowing oxidation of its cysteine residues, and which ultimately stabilizes HIF-1α (Briggs, Koivunen et al. 2016). On the other hand, Hu and colleagues found HIF to augment glutamate secretion and signaling under hypoxia, which results in the progression of hepatocellular and clear cell renal carcinoma (Hu, Takano et al. 2014). In accordance with those findings, mounting evidence suggests extracellular glutamate to promote tumor progression. Glutamate acts either as an excitotoxin inducing apoptosis in surrounding neurons and clearing the path for tumor expansion (Ye and Sontheimer 1999), or it activates proliferation and grwoth through the ionotropic receptors of the AMPA family and metabotropic receptors mGlrR (DeBerardinis and Cheng 2010, de Groot and Sontheimer 2011, Hu, Takano et al. 2014).

The results presented in the current study, which showed that the tumor-promoting factor HIF is reduced and the activity of tumor-suppressing PHD is increased, would be the first to suggest anti-tumor effects of extracellular glutamate. However, this is not sufficient evidence for a general anti-tumor attribution of extracellular glutamate in GBM biology.

At concentrations as high as 200 μmol glutamate in the microenvironment, it is possible that GBM cells increase PHD activity and reduce HIF protein levels to limit HIF-mediated glutamate secretion in the context of a negative feedback (**Figure 4.1**). This feedback prevents exorbitantly high glutamate concentrations that are toxic to the tumor itself. This hypothesis, in turn, would stand in accordance with existing literature data ascribing a general tumor-promoting role to glutamate.

4.1.2 Transient glutamine deprivation stabilizes HIF-1α

Next to glutamate, glutamine is the other well-known precursor of αKG. The fact that glutamine is the most abundant amino acid in human plasma suggests its key role in metabolism. Indeed, it is the major vehicle to transport nitrogen between organs and adopts a range of functions on the cellular level that go far beyond the creation of polypeptides. Serving as a major provider of energy and intermediates for the synthesis of macromolecules, glutamine is utilized to a great extent in rapidly dividing cells, including tumor cells. Glutamine also participates in the formation of glutathione, the main scavenger for reactive oxygen species (ROS) in the cell. Next to protecting cells from oxidative stress, it also facilitates cell growth by activating the mTOR and ERK signaling pathways (DeBerardinis and Cheng 2010, Shanware, Mullen et al. 2011).

Despite the mentioned pro-tumor effects of aiding proliferation and survival, growing evidence suggests that regional glutamine deficiency, especially in tumor core regions, promotes an aggressive tumor phenotype (Hojfeldt and Helin 2016, Pan, Reid et al. 2016). Core regions of solid tumors usually experience limited blood supply, which results in hypoxia and the starvation of glutamine among other nutrients. Similar to the way by which hypoxia supports the formation and maintenance of cancer stem cells, glutamine deficiency seems to commence an intrinsic program for cells to express a more stem-cell-like phenotype (Pan, Reid et al. 2016).

At this point, it is important to note that the majority of αKG in cancer cells derives from glutamine (Xiao, Zeng et al. 2016). In contrast to glutamate, glutamine transport into the cell is straightforwardly ensured by a number of Na^+-dependent and Na^+-independent transporters (Xiao, Zeng et al. 2016). The effective mechanisms of transport suggest a strong correlation between alterations in extracellular and intracellular glutamine concentrations, and thus intracellular αKG concentrations as well. One mechanism behind the more aggressive phenotype in glutamine-deficient cells is based on a depleted intracellular αKG pool, which inhibits the activity of αKG-dependent histone

demethylases and consequently promotes an epigenetically dedifferentiated state (Hojfeldt and Helin 2016, Pan, Reid et al. 2016).

This work showed a connection between glutamine deficiency and the adaptive hypoxic response in glioblastoma cells G55 and U87. Especially under hypoxia, the sudden deprivation of glutamine led to significantly higher levels of stabilized HIF-1α (**Figure 3.3A**). This effect is mediated by αKG-dependent PHD, the activity of which diminishes with reduced glutamine supply to sustain αKG levels. Accordingly, addition of glutamine restored PHD activity and reduced HIF-1α levels in the same hypoxic setting (**Figure 3.3B**). Interestingly, prolonged glutamine starvation reduced HIF-α protein levels after an initial increase (**Figure 3.3A**). This observation could be owed to the fact that prolonged starvation of a nutrient as essential as glutamine restricts the viability of every cell, resulting in a reduced production of a variety of proteins. Supporting this, Kwon et al. showed that in cells chronically deprived of glutamine, HIF-α downregulation results from translational inhibition rather than accelerated degradation (Kwon and Lee 2005).

Taken together, we introduced a new pathophysiological aspect of microenvironmental glutamine deficiency in glioblastoma. Limited perfusion in rapidly growing solid tumors not only causes hypoxia but also deficient nutrient availability, among which glutamine is critical. Transient but not prolonged glutamine starvation triggers a cellular response mediated by PHD and HIF. We showed that the hypoxic response is aided by decreased αKG-dependent PHD activity and higher HIF-α stability. In accordance with our findings, glutamine deficiency has also been reported to reduce the activity of αKG-dependent KDM and TET, resulting in epigenetic modifications (Hojfeldt and Helin 2016, Pan, Reid et al. 2016). Both effects subsequently promote the glioblastoma stem cell phenotype and result in more aggressive tumor characteristics.

4.1.3 Glutamine and glutamate – the conclusion

The link between the hypoxic response and metabolic abnormalities in tumors is seen in a new light with the emerging significance of PHD as an αKG sensor. The known precursors of αKG, glutamine and glutamate, are two proteinogenic amino acids that experience striking abnormalities in certain tumor conditions and therefore possess a possible significance in tumor biology.

High extracellular glutamate concentrations are found in tumors of the CNS compared to other tumor types. This high glutamate level is thought to promote tumor progression,

among others by inducing excitotoxic cell death in surrounding healthy tissue (Ye and Sontheimer 1999), which in turn could create necrotic and hypoxic regions in peritumoral regions. The findings of this work are the first to show extracellular glutamate to dampen the hypoxic response by increasing PHD activity and reducing HIF-α levels in GBM. However, taken into account that HIF promotes glutamate secretion into the extracellular space, this effect could be part of a self-protective, negative feedback mechanism to limit glutamate-associated excitotoxicity (Hu, Takano et al. 2014) (**Figure 4.1**). The increase of microenvironmental glutamate could be controlled to induce apoptosis in healthy surrounding tissue to a degree that does not harm tumor cells themselves at the same time.

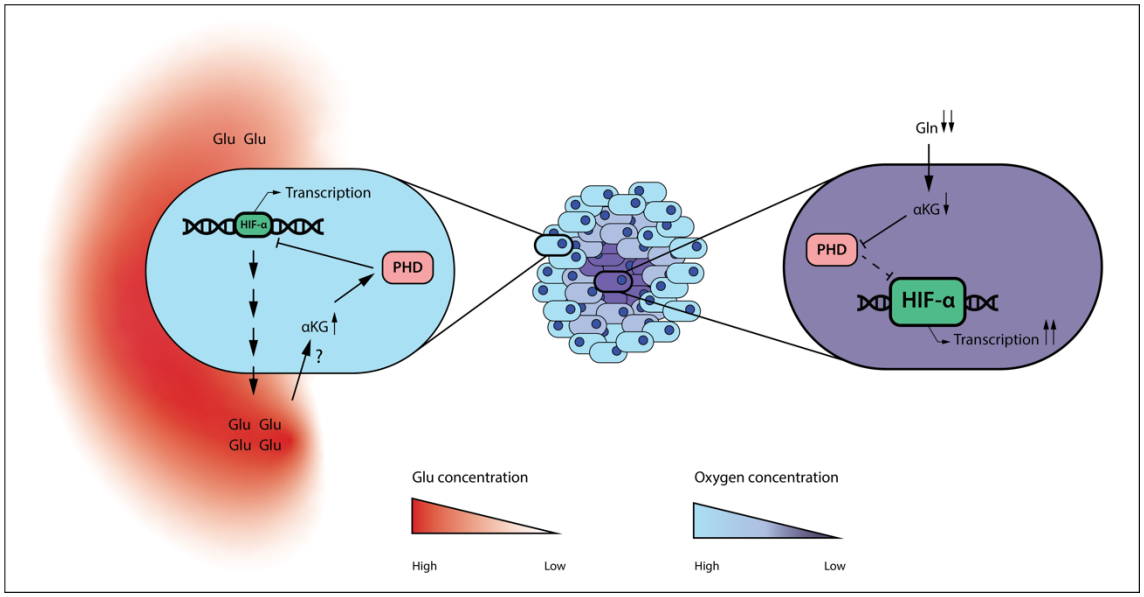

Figure 4.1: The role of microenvironmental glutamine and glutamate in GBM biology.
Glutamate (Glu) concentration is usually elevated in peritumoral areas and promotes tumor expansion. HIF aids the secretion of Glu into the microenvironment, thus further raising Glu concentration in the tumor periphery. However, when reaching a certain threshold, high Glu concentration becomes toxic to the tumor itself. GBM cells might sense this threshold and register it as a negative feedback. In consequence, PHD is activated and HIF is degraded, putting a halt to augmented Glu secretion. Glutamine (Gln), on the other hand, is scarce in tumor core regions. Microenvironmental Gln deprivation leads to intracellular depletion of αKG, which in turn inhibits PHD activity and stabilizes HIF. In that way, Gln reinforces the adaptive response of GBM cells to hypoxia in tumor core regions.

Glutamine, on the other hand, is physiologically available in abundance. Abnormalities in glutamine availability occur in core regions of solid tumors, similarly to the deficiency of oxygen and other nutrients. In this work, *in vitro* models mimicking this condition showed glutamine deprivation to augment the hypoxic response via PHD inactivation and HIF-α stabilization. These results stand in line with existing data about the promotion of a dedifferentiated tumor phenotype through glutamine deficiency (Hojfeldt and Helin 2016, Pan, Reid et al. 2016). This effect of glutamine on the PHD/HIF axis seems to

largely rely on the reduction of intracellular αKG levels following glutamine deprivation (**Figure 4.1**).

In summary, with the knowledge that αKG precursors glutamate and glutamine shape the glioblastoma microenvironment, we could show that both amino acids have a regulatory potential on HIF protein levels through the modulation of PHD activity. Whether or not this effect is based on changes of intracellular αKG levels is unclear in the case of glutamate but highly suggestive for glutamine, warranting further analysis.

4.2 IDH1 as a central enzyme in the PHD/HIF pathway

IDH is the principal enzyme producing endogenous αKG, catalyzing the reaction to transform isocitrate to αKG. Among the many enzymes linked to αKG metabolism, IDH is of special interest not least due to its clinical relevance. Frequent mutations in the isoforms IDH1 and IDH2 in low-grade gliomas and a number of other tumors raised the question of how IDH and its mutant forms are involved in tumorigenesis and tumor progression. In gliomas, mutations of IDH1 are much more common than of IDH2 (Zhang, Moore et al. 2013).

While much attention has been brought upon mutated IDH, not much is known about the role of wildtype IDH in tumor biology. This current study investigated the wildtype isotype IDH1 and the consequences of its inactivation in GBM *in vitro* models. We found that IDH1 significantly affected intracellular αKG levels and subsequently PHD activity and HIF levels. On the other hand, GBM cells with an invasive phenotype did not show a consistent IDH1 downregulation, suggesting that IDH1 is not a predominant factor driving invasion. Further investigation in GBM cell lines G55 and U87 revealed IDH1 itself to be subjected to regulation by FCS stimulation. The results in this work suggested that the regulation of IDH1 does not take place at the protein level.

4.2.1 IDH1 affects HIF levels by regulating αKG concentrations and PHD activity

Contrary to the interest surrounding IDH mutations and its role in tumorigenesis, not much attention has been given to the involvement of wildtype IDH in tumors. The fact that mutated IDH1 dimerizes with wildtype IDH1 to inhibit wildtype activity, thereby leading to HIF-1α stabilization, suggests the significance of proper wildtype IDH1 function on maintaining low HIF levels (Zhao, Lin et al. 2009). Knockdown experiments

in this work confirm this dependency of HIFα levels on wildtype IDH1 in the glioblastoma cell lines U87 and G55, and reveal that this dependency is indeed mediated by IDH1's significant influence in stabilizing αKG levels and thus PHD activity (**Figure 3.4**). Supporting this notion was the fact that IDH1 knockdown strengthened the effect of glutamine addition in increasing PHD activity in our cells (**Figure 3.5**). It is likely that because IDH1 inactivation downsized the intracellular αKG pool, cells were more sensitive to microenvironmental addition of glutamine, which could be quickly utilized to replenish the intracellular αKG shortage. This suggests a synergistic effect of IDH1 and glutamine in regulating PHD activity through the maintenance of αKG levels.

It is notable that the enzymatic conversion of isocitrate to αKG by IDH1 and IDH2 can be reversed under specific conditions. Previous studies have mentioned this reversed flux through IDH to be an indispensable factor for lipogenesis and thus cell proliferation under hypoxia in lung cancer, GBM and melanoma cell lines (Metallo, Gameiro et al. 2011, Wise, Ward et al. 2011, Filipp, Scott et al. 2012). Interestingly, this effect is suggested to be part of a HIF-mediated metabolic reprogramming (Wise, Ward et al. 2011). However, the presented knockdown experiments prompt a critical evaluation of approaching IDH1 as a target to inhibit tumor growth. While IDH1 could indeed partly carry out the carboxylation of αKG to isocitrate, this activity appears to be outweighed by the TCA cycle-conform decarboxylation of isocitrate to αKG. The decreased αKG levels instead of αKG accumulation upon IDH1 knockdown, followed by decreasing PHD activity and ultimately HIF-1α stabilization (**Figure 3.4**), underlines this hypothesis. An inactivation of IDH1, even if it slows down tumor growth, can drive tumor progression by stabilizing HIF-1α and promoting a more aggressive tumor phenotype.

4.2.2 IDH1 is not a predominant driver of invasion

One hallmark of cancer, which is also a characteristic that particularly marks the aggressiveness of glioblastoma, is the capacity for invasion. Given HIF's known role in promoting invasion and metastasis (Filatova, Acker et al. 2013, Zhang, Huang et al. 2013, Liu, Liu et al. 2014, Muz, de la Puente et al. 2015) and IDH1's involvement in HIF regulation, we investigated the relationship between IDH1 and an invasive phenotype of GBM. Zeb1 is a transcription factor that is associated with the epithelial-to-mesenchymal transition (EMT) in carcinomas, but is also linked to invasion and chemoresistance in GBM (Siebzehnrubl, Silver et al. 2013, Nesvick, Zhang et al. 2016). Unpublished results from our lab showed that IDH1 knockdown upregulated Zeb1 and increased the invasive capacity of GBM. However, the current study revealed that in a selected pool of invasive

cells, shown by elevated Zeb1 levels, IDH1 was not downregulated compared to the pool of wildtype controls (**Figure 3.8**). This suggests that although a loss of IDH1 activity can contribute to the cell's invasive potential, IDH1 is not the only, or at least a persistent IDH1 downregulation is not the predominant driver of invasion in glioblastoma. This idea is reflected in the widely-accepted hypothesis of IDH1 mutation (and along with it the inactivation of wildtype IDH1) to contribute to the malignant transformation of glial cells and thus tumorigenesis (Zhang, Moore et al. 2013, Turkalp, Karamchandani et al. 2014), rather than invasion and metastasis.

4.2.3 Microenvironmental effects on IDH1 protein levels

While the intratumoral heterogeneity of GBM is characterized on multiple molecular levels (Aubry, de Tayrac et al. 2015), a possible heterogeneous distribution of IDH1 depending on microenvironmental signals has not been assessed so far. There are scarce reports of IDH1 regulation in non-tumor cells in response to microenvironmental stimuli, like the upregulation of IDH1 in neurons following hemorrhagic stroke (Chen, Wang et al. 2017) or the downregulation of IDH1 in chondrocytes in response to pro-inflammatory cytokines TNF-α and IL-1β (Haseeb, Makki et al. 2014). Our results show a similar effect of IDH1 downregulation upon the stimulation with FCS in GBM cells (**Figure 3.6**), although it remained to be determined which factor of the unknown composition of FCS, which contains many cytokines and growth factors, mediated this effect.

Unpublished data by Nuray Bügürcü has identified inflammatory cytokines TGF-β and TNFα to be able to suppress IDH1 protein levels, the latter of which was also described in the aforementioned chondrocytes. Haseeb and collegues showed TNFα to lower IDH1 activity in chondrocytes by reducing its gene expression, thereby leading to decreased αKG levels and the inhibition of αKG-dependent TET enzymes (Haseeb, Makki et al. 2014). Similarly, a decreased intracellular αKG pool in GBM would be able to inhibit PHD and stabilize HIF-α. TGF-β, on the other hand, has been suggested to synergize with HIF in activating VEGF gene transcription and thereby promoting tumor angiogenesis (Sanchez-Elsner, Botella et al. 2001, Han, Alvarez-Breckenridge et al. 2015). Even though it was shown that the TGF-β-activated transcription factor Smad and HIF both have binding sites within the VEGF promotor region, a cross-stimulation between the two pathways could not be found (Sanchez-Elsner, Botella et al. 2001). Our results in this study (together with Nuray Bügürcü's unpublished results) suggested for the first time that the intersection of cytokine-mediated signaling and the HIF pathway might lie in the

modulation of IDH1 expression. It is our goal for upcoming studies to further characterize the effect of TNFα and TGF-β on IDH1 levels, the αKG pool, and thereby PHD activity and HIF-α stability in GBM. Interestingly, hypoxia or modulated extracellular glutamine or glutamate concentrations did not have an effect on IDH1 levels (**Figure 3.3A**, other data now shown).

The changes in IDH1 protein levels in our GBM cells was not a result of an inhibition in protein translation or increased proteosomal degradation, as our experiments with cycloheximide, a translational inhibitor, and MG123, a proteosomal inhibitor, could show (**Figure 3.7**). This affirms that IDH1 is barely controlled on the level of protein degradation, which is the mechanism by which proteins with a high turnover, like HIF-α, are regulated. It can be assumed that IDH1 in glioblastoma is rather regulated in earlier steps of gene expression, which stands in line with literature data described for other cell lines. An upregulation of IDH1 expression, measured by increased IDH1 mRNA, is attributed to the transcriptional factor FOXO in HeLa cells (Charitou, Rodriguez-Colman et al. 2015), to CHOP and C/EBPβ upon ER-stress (Yang, Du et al. 2015), to SERBP-1/2 within lipogenesis (Shechter, Dai et al. 2003) and to HuR in pancreatic cancer (Zarei, Lal et al. 2017). Interestingly, the inhibition of IDH1 transcription, on the other hand, is shown to be targeted by HIF-1α in osteosarcoma cells (Liu, Zheng et al. 2017). Whether FCS or the mentioned cytokines TNFα and TGF-β mediate IDH1 downregulation on the transcriptional level is also an aim for further studies.

Taken together, we have shown that IDH1 can directly respond to microenvironmental stimuli in GBM. These stimuli are likely to come from growth factors and cytokines, particularly TGF-β and TNF-α, rather than hypoxia or extracellular metabolic signals.

In summary, our data have shown IDH1 to be of great significance in GBM biology. The reduction of wildtype IDH1 activity, either due to IDH1 mutation or modulating factors from the tumor microenvironemnt, depletes intracellular αKG levels to inhibit PHD activity and stabilize HIF-α. Wildtype IDH1 loss regulates HIF and the hypoxic response, as well as HIF-independent PHD functions, and thus can be considered a metabolic driver of several hallmarks of cancer (Hanahan, Douglas et al. 2011), including angiogenesis, invasion, reprogrammed energy metabolism, sustained proliferation and apoptosis resistance (**Figure 4.2**).

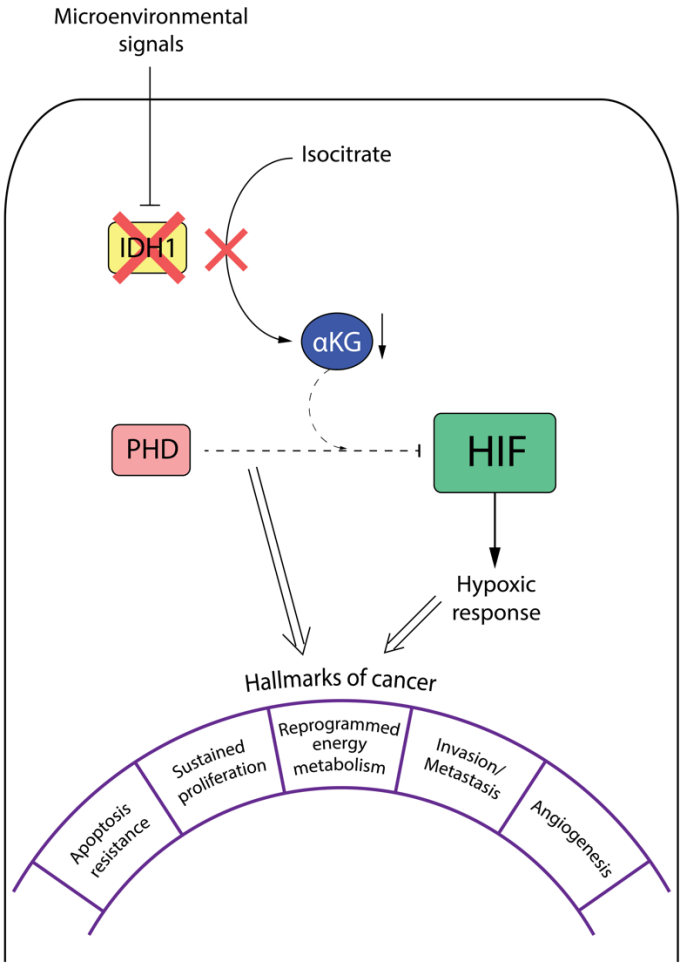

Figure 4.2: Schematic illustration of IDH1 as the link between metabolism, the hypoxic response and the hallmarks of cancer.
In this hypothesis, the loss of IDH1 wildtype activity in GBM cells, which either results from inhibitory signals from the tumor microenvironment or from IDH1 mutations, depletes intracellular αKG. Intracellular αKG shortage inhibits PHD activity, leading to the stabilization of HIF and subsequently the activation of the hypoxic response. IDH1 is therefore a crucial metabolic factor that, through the tight control of PHD and HIF, regulates biologic processes which could drive several hallmarks of cancer, including apoptosis resistance, sustained proliferation, reprogrammed energy metabolism, invasion, and angiogenesis.

5 Summary

Glioblastoma multiforme (GBM) is one of the most lethal tumors known to man. This lethality is partly attributable to its rapid growth, a characteristic feature of GBM, which creates intratumoral regions scarce of oxygen and nutrients. Tumor cells in these hypoxic regions adopt an aggressive phenotype that drives tumor progression, therapy resistance and tumor recurrence. The adaptive mechanisms to hypoxia are orchestrated by the transcription factor hypoxia-inducible factor (HIF). HIF, in turn, is tightly regulated by prolyl-hydroxylase domain-containing protein (PHD). PHD uses the cosubstrates oxygen and alpha-ketoglutarate (αKG) to hydroxylate specific HIF residues, thereby marking HIF for degradation. Under hypoxia, the hydroxylation activity of PHD is inhibited and HIF is stabilized to mediate the hypoxic response.

This work aims to characterize the role of the cosubstrate αKG in regulating PHD activity under different oxygen availabilities. First, certain microenvironmental conditions of the αKG precursors glutamine and glutamate were modelled in cultures of GBM cells *in vitro*. A sudden reduction of glutamine – a condition that is often present in GBM tumor core regions – inhibited PHD hydroxylase activity and subsequently stabilized HIF even in the absence of hypoxia. By contrast, elevated glutamate levels in the microenvironment – as found in peritumoral areas – increased PHD activity and led to HIF degradation. Furthermore, we investigated the central enzyme involved in the endogenous αKG production: isocitrate dehydrogenase (IDH). IDH1 proved to be essential for maintaining the intracellular αKG level. The knockdown of IDH1 led to a significant reduction in αKG, a striking inhibition of PHD activity and subsequently HIF stabilization even under normoxic conditions. Further characterization of the role of IDH1 in GBM biology revealed its protein levels to be susceptible to microenvironmental signals that are likely to be cytokines and growth factors.

In summary, this work showed that the adaptive response of GBM cells mediated by HIF can be mitigated or intensified by metabolic changes. Among those metabolic changes, αKG plays a central role, as it is the necessary co-substrate of PHDs, the most potent regulator of HIF. Microenvironmental changes in glutamine and glutamate, as well as the functionality of the enzyme IDH1 are prominent factors that can influence the PHD/HIF axis by regulating αKG levels. Accordingly, these factors are interesting subjects for future studies investigating possible therapeutic approaches to interrupt the adaptive hypoxic response in GBM.

6 Zusammenfassung

Glioblastoma multiforme (GBM), der häufigste primäre Hirntumor, ist eine der tödlichsten Erkrankungen der Humanmedizin. Das rasche Wachstum des Tumors erzeugt intratumorale Regionen, die einen Mangel an Sauerstoff und Nährstoffe aufweisen. Gerade diese hypoxischen und Nährstoff-unterversorgten Regionen unterhalten jedoch einen besonders aggressiven Tumorzellphenotyp, der für die Tumorprogression, Therapieresistenz und für Tumorrezidive verantwortlich ist. Die adaptiven Mechanismen an die Hypoxie, auch Hypoxieantwort der Zelle genannt, werden von dem Hypoxie-induzierbaren Faktor (HIF) koordiniert. HIF wird engmaschig durch Prolyl-hydroxylase domain-containing (PHD) Proteine reguliert. Unter Nutzung von Sauerstoff und alpha-Ketoglutarat (αKG) katalysiert PHD die Hydroxylierung von bestimmten Prolinresten von HIF. Hierdurch wird HIF für die Ubiquitinierung markiert und anschließend abgebaut. Unter Hypoxie fehlt PHD das Cosubstrat Sauerstoff, sodass die Hydroxylaseaktivität blockiert ist. HIF wird demzufolge nicht mehr markiert und abgebaut, und kann die Hypoxieantwort der Zelle einleiten.

In dieser Arbeit wurde die Rolle des zweiten Cosubstrats – αKG – auf die enzymatische Aktivität von PHD unter verschiedenen Sauerstoff-Verfügbarkeiten untersucht. Wir imitierten bestimmte Konzentrationsbedingungen der αKG-Vorläufer Glutamin und Glutamat im Tumormikromilieu von GBM-Zellen. Es konnte gezeigt werden, dass eine abrupte Reduktion von Glutamin im Mikromilieu – wie es oft in GBM Kernregionen aufzufinden ist – zu einer Inhibition der PHD-Aktivität und zur HIF-Proteinstabilisierung führte. Glutamat akkumuliert dagegen besonders in den peripheren Bereichen der Tumorränder. Eine *in vitro* Akkumulation von Glutamat konnte in unseren Experimenten die PHD Aktivität erhöhen und den HIF-Abbau befördern. Im zweiten Teil untersuchten wir das Schlüsselenzym Isocitrat-Dehydrogenase 1 (IDH1), welches eine zentrale Rolle für die endogene Produktion von αKG spielt. Die IDH1-Aktivität ist essentiell für die Aufrechterhaltung des intrazellulären αKG-Pools, denn ein IDH1-Knockdown führte zu einer signifikanten Inhibition der PHD-Aktivität und demzufolge zur HIF-Stabilisierung unter normalen Sauerstoffkonzentrationen. Dieser maßgebliche Einfluss von IDH1 auf die PHD/HIF-Achse veranlasste weitere Untersuchungen zu der Rolle des Proteins in der GBM Tumorbiologie. So fanden wir heraus, dass IDH1 Protein Level sensibel auf bestimmte Veränderungen im Tumormikromilieu, mutmaßlich Zytokine und Wachstumsfaktoren, reagiert.

Zusammengefasst konnte in dieser Arbeit gezeigt werden, dass die durch HIF koordinierte Hypoxieantwort in GBM Zellen durch metabolische Veränderungen beeinflussbar ist. Veränderungen der Konzentration von Glutamin und Glutamat im

Mikromilieu, sowie auch der Aktivität des Proteins IDH1 modulieren die PHD/HIF-Achse und damit die Hypoxieantwort der Zelle maßgeblich. Diese Ergebnisse eröffnen neue Ansätze für die weitere Erforschung und therapeutische Beeinflussung der Hypoxieantwort in Tumorzellen durch metabolische Faktoren.

7 List of tables and figures

7.1 Tables

7.2 Figures

8 List of abbreviations

αKG	alpha-ketoglutarate
αKGDH	alpha-ketoglutarate dehydrogenase
2HG	2-hydroxyglutarate
5-hmC	5 hydroxymethylcytosine
AFT4	activating transcription factor 4
ALT	alanine transferase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANGPT2	angiopoetin 2
APS	ammonium persulfate
ARD1	arrest-defective 1
ASCT2	alanine, serine, cysteine transporter 2
AST	aspartate transferase
bHLH-PAS	basic helix-loop-helix Per-Arnt-Sim
CHI3L1	chitinase-3-like protein 1
CHX	cycloheximide
CKIδ	casein kinase Iδ
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EAAT3	excitatory amino acid transporter 3
EDTA	Ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
EGFR	epithelial growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	endoplasmatic reticulum
FBP1	fructose-1,6-biphosphatase 1
FCS	fetal calf serum
FGF	fibroblast growth factor
FIH	factor inhibiting HIF
FOXO3	forkhead box O3
FTO	fat mass and obesity-associated protein
GABARA1	gamma-aminobutyric acid receptor alpha 1
GBM	glioblastoma multiforme
GDH	glutamate dehydrogenase
Gln	glutamine
Glu	glutamate
GLUT	glucose transporter
GSK-3β	glycogen synthase kinase 3β
HCLK2	homolog of the *Caenorhabditis elegans* biological clock protein 2
HIF	hypoxia-inducible factor
HK	hexokinase
HRE	hypoxic response element
Hsp90	heat shock protein 90kD
IDH	isocitrate dehydrogenase
IPAS	inhibitory Per-Arnt-Sim protein
IREBP1	iron response element binding protein 1
KDM	histone lysine demethylase
Klf4	Kruppel-like factor 4
LDHA	lactate dehydrogenase A
LOH	loss of heterozygosity
LOX	lysyl oxidase
LSD1	lysine-specific histone demethylase 1
MAPK	mitogen activated protein kinase
MCT	monocarboxylate transporters

Mdm2	mouse double minute 2 homolog
MDR-1	multidrug resistance 1
MERTK	MER tyrosine kinase
mGlrR	metabotropic glutamate receptors
MIC-1	macrophage-inhibitory cytokine 1
MMP	matrix metallo protease
mTOR	mammalian target of rapamycin
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NDRG3	N-myc downstream-regulated gene 3 protein
NEFL	neurofilament light
NF-kB	nuclear factor kappa B
NF1	neurofibromin 1
NGF	nerve growth factor
NOS	not otherwise specified
Oct4	octamer-binding factor 4
ODD	oxygen-dependent degradation
P4H	prolyl-4-hydroxylase
PBS	phosphate-buffered saline
PD-L1	programmed death-ligand 1
PDGFRA1	platelet derived growth factor receptor A1
PDK1	phosphate dehydrogenase kinase 1
PFK	phosphofructokinase
PHD	prolyl-hydroxylase domain containing protein
PKM2	pyruvate kinase M2
PLK3	polo-like kinase 3
RACK1	receptor for activated C kinase 1
Rb	retinoblastoma protein
ROS	reactive oxygen species
SDF-1	stromal cell-derived factor 1
Siah1/2	seven in absentia homology 1/2
SIRT	sirtuins
SLC	solute carrier
Sox-1	sex determining region box 1
TCA	tricarboxylic acid (cycle)
TCGA	the cancer genome atlas
TEMED	tetramethylenediamine
TET	ten-eleven translocator
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
vHL	von Hippel-Lindau
WHO	world health organization
xCT	cysteine-glutamate antiporter
Zeb1/2	zinc finger E-box-binding homeobox 1/2

9 Literature

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