The oxygen sensors fatiga and PHD3 limit pyruvate kinase activity via protein interaction

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THE OXYGEN SENSORS FATIGA AND PHD3 LIMIT
PYRUVATE KINASE ACTIVITY VIA PROTEIN
INTERACTION

A dissertation submitted to
ETH ZURICH

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presented by
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ABSTRACT

Oxygen levels are critical for cellular physiology. Under low oxygen (hypoxia), cells increase glycolytic rates and lactate production, while limiting mitochondrial oxidative metabolism, yet mechanisms to limit glycolysis under these conditions remain poorly understood. Here I show that Drosophila Fatiga and mammalian PHD3, which function as cellular oxygen sensors, interact with the glycolytic enzyme Pyruvate kinase (PK, PyK for Drosophila protein) in vitro and in vivo. For Drosophila larvae, loss of Fatiga caused subcellular localization changes of PyK in the fat body. Over-expression of Fatiga slightly inhibited PyK activities. In mammalian cells (human HeLa cells), down-regulation of PHD3 led to an increase in the highly active, tetrameric form of PK-M2 that was insensitive to allosteric regulation. This resulted in enhanced levels of downstream metabolites including pyruvate and TCA-cycle intermediates. Moreover, PHD3 was required to limit ROS accumulation and to adjust cell proliferation to oxygen availability. My data therefore demonstrate a novel mechanism to regulate glycolytic activity.
ZUSAMMENFASSUNG

LIST OF ABBREVIATIONS

PK: pyruvate kinase
PyK: Drosophila pyruvate kinase
PHD: prolyl hydroxylase domain
HIF: hypoxia-inducible factor
bHLH-PAS: basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS)
HREs: hypoxia response elements
ARNT: Aryl hydrocarbon Receptor Nuclear Translocator
ODDD: oxygen-dependent degradation domain
VHL: von Hippel-Lindau tumor suppressor
CBP: cAMP-response element-binding protein
FIH: factor inhibiting HIF
DMOG: Dimethyloxaloylglycine
BNIP3: BCL2/adenovirus E1B protein-interacting protein
SUMO: Small Ubiquitin-like Modifier
LKB1: STK11: Serine/threonine kinase 11
TOR: target of rapamycin
TSC: tuberous sclerosis complex
PTEN: phosphatase and tensin homolog
PML: promyelocytic leukemia
NF-κB: nuclear factor κB
HPV: Human papillomavirus
RTK: receptor tyrosine kinase

FKBP: FK506 binding protein

MORG1: MAPK organizer 1

ATF-4: activating transcription factor-4

Siah: seven in absentia homolog

eIF: eukaryotic Initiation Factor

TRiC: TCP-1 Ring Complex

PDI: Protein disulfide isomerase

lacZ: β-galactosidase

UAS: Upstream Activation Sequence

LT-MS: liquid chromatography-mass spectrometry

3’-UTR: three prime untranslated region

GST: Glutathione S-transferase

GFP: Green fluorescent protein

BN: blue native

EPO: erythropoetin

VEGF: vascular endothelial growth factor

SDH: succinate dehydrogenase

FH: fumarate hydratase

IDH: isocitrate dehydrogenase

PFK: phosphofructokinase

Glut1: glucose transporter-1

LDH: lactate dehydrogenase
**PDK**: pyruvate dehydrogenase kinase

**PDH**: pyruvate dehydrogenase

**ROS**: reactive oxygen species

**NAD$$^+$$**: Nicotinamide adenine dinucleotide

**TCA**: tricarboxylic acid

**PEP**: phosphoenolpyruvate

**2-OG**: 2-oxoglutarate

**ADP**: Adenosine diphosphate

**ATP**: Adenosine-5'-triphosphate

**FBP**: fructose-1, 6-bisphosphate

**G6P**: Glucose 6-phosphate

**DHAP**: Dihydroxyacetone phosphate

**F6P**: Fructose 6-phosphate

**BGP**: 1,3 bisphospho-Glycerate

**3+2PG**: 3-Phosphoglycerate and 2-Phosphoglycerate

**AcCoA**: Acetyl-CoA

**CIT/ICIT**: Citrate/Isocitrate

**6PG**: glucose-6-phosphate

**Ru5P**: ribulose 5-phosphate

**R5P**: ribose 5-phosphate

**X5P**: xylulose 5-phosphate

**S7P**: sedoheptulose 7-phosphate
CONTRIBUTIONS OF COLLABORATORS

Table 3 and Table 4: Oliver Rinner (Gstaiger Group, IMSB, ETH Zurich) performed LC-MS/MS and searching of database and provided the raw data sets. I compared the lists to identify putative interacting proteins and did the annotations.

Fig 3 was provided by Erich Brunner (University of Zurich).

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Fig 28, Fig 29 and Fig 30: Dominika Czernik (Zamboni Group, IMSB, ETH Zurich) performed LC-MS and quantification of the raw data. I performed normalization and generated all graphs in those figures.
1. INTRODUCTION

1.1 Hypoxia and cellular metabolism

Oxygen has profound effects on most cellular processes. Low oxygen concentration, termed hypoxia, can change the metabolism of a cell or organism in many respects. Multicellular organisms often face the challenge that most internal organs and tissues are exposed to oxygen concentration much lower than that in the air (normoxia). Therefore, cells must be able to sense oxygen concentration, and adapt the metabolism and nutrient oxidation to changes in oxygen supply. This is particularly important in solid tumors, which have a high metabolic demand but often show areas of hypoxia, due to limited vascularization (Brahimi-Horn and Pouyssegur 2007; Aragones, Fraisl et al. 2009).

The most well known regulator of oxygen homeostasis is hypoxia-inducible factor (HIF), a transcriptional factor controlling the expression of broad range of genes that are involved in the adaptation to low oxygen stress. HIF is a hetero-dimer that is composed of one α and one β subunit. Both proteins belong to the basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) family. The hetero-dimer interacts with the hypoxia response elements (HREs) within the enhancer or promoter regions of target genes, and regulates the expression of such genes. HIFβ, also known as Aryl hydrocarbon Receptor Nuclear Translocator (ARNT), is a constitutively expressed nuclear protein, while both the protein level and the activity of α subunit of HIF are highly regulated in response to oxygen (Firth, Ebert et al. 1995). This regulation is achieved through the hydroxylation of particular proline and asparagine residues of HIFα under normoxic conditions. Three isoforms of α subunit have been identified: HIF-1α, -2α and -3α. Both HIF-1α and -2α contain two conserved proline residues (Pro402, Pro564 in HIF-1α) within their oxygen-dependent degradation domains.
Only one proline (analogous to Pro564) is conserved in HIF-3α. Upon prolyl hydroxylation, HIFα is recognized and bound by the von Hippel-Lindau tumor suppressor (pVHL), a component of an E3 ligase complex, and subjected to ubiquitination and subsequent degradation by the 26S proteasome (Bruick and McKnight 2001). In addition, hydroxylation of an asparagine residue by factor inhibiting HIF (FIH) within the C-terminal transactivation domain (Asn803 in HIF-1α) disrupts the interaction between HIF-α and its co-activator CBP/p300. As a result, the transcriptional activity of HIF-α is impaired (Koivunen, Hirsila et al. 2004), providing an additional mechanism to tightly control HIF target gene expression. Since hydroxylation of both proline and asparagine residues requires oxygen, HIF can accumulate and be fully active only under hypoxia. In contrast, HIF’s transcriptional activity is reduced and the protein is degraded under normoxia. Besides, other posttranslational modifications, such as phosphorylation (Richard, Berra et al. 1999), acetylation (Jeong, Bae et al. 2002), SUMOylation (Berta, Mazure et al. 2007), and S-nitrosation (Yasinska and Sumbayev 2003) have been reported to modify the half-life and activities of HIF-1. However, inhibition of hydroxylation caused by hypoxia is by far the most powerful regulatory mechanism for HIF, and hence for target gene expression (Fig. 1A).

HIF is also a target of several oncogenic signaling pathways, and is often over-expressed in tumors independent of oxygen levels. Mutations in several tumor suppressors, such as LKB1, PML, PTEN, and TSC1/TSC2, lead to activation of mTOR, which promotes both transcription and translation of HIF-1α (Brugarolas, Lei et al. 2004). Mutations in pVHL cause accumulation of HIF even under normoxia (Kaelin 2005). Inactivations of succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH) suppress HIF hydroxylation through changing mitochondrial metabolite levels (Selak, Armour et al. 2005; Koivunen, Hirsila et al. 2007; Zhao, Lin et al. 2009). Moreover, it was
shown that nuclear factor κB (NF-κB) is a direct modulator of HIF-1α expression in the presence of normal oxygen concentration (van Uden, Kenneth et al. 2008).

Hypoxia is often a feature of pathophysiological conditions, such as ischemic diseases (stroke and myocardial infarction) and cancer. HIF target genes are involved in diverse cellular and physiological processes, including angiogenesis, erythropoiesis, and glucose metabolism (Kaelin 2005). HIF induced survival responses include the increased production of blood cell (EPO) and formation of blood vessels (VEGF), which increase oxygen delivery to hypoxic tissues (Wang, Jiang et al. 1995). Importantly, recent data also indicate that HIF-1 plays a critical role in the regulation of cancer metabolism.

Most tumors are characterized by a phenomenon termed ‘the Warburg effect’: cells rely on aerobic glycolysis instead of mitochondrial oxidative phosphorylation to generate energy for cellular processes. Up-regulation of glycolysis is a reliable feature of tumor, and is utilized clinically to detect tumors via positron emission tomography using \(^{18}\)F-fluorodeoxyglucose (FDG-PET) (Semenza 2009). This metabolic reprogramming is largely mediated by activation of HIF-1 (Fig. 1B). HIF-1 drives the expression of glucose transporters (Glut-1 and Glut-3), most known glycolytic enzymes and lactate dehydrogenase A (LDH-A), the latter converting pyruvate into lactate (Denko 2008). In addition to up-regulation of glycolysis, HIF-1 also induces the expression of pyruvate dehydrogenase kinase (PDK-1), which phosphorylates and inhibits pyruvate dehydrogenase (PDH), thus blocking the conversion of pyruvate into acetyl-CoA. As a results, pyruvate is shuttled away from mitochondrial oxidative phosphorylation (Kim, Tchernyshyov et al. 2006). Furthermore, HIF-1 activates the BH3 domain protein BNIP3, which induces mitochondria autophagy (Zhang, Bosch-Marce et al. 2008). These adaptations are believed to prevent mitochondrial dysfunction and cellular damage due to inappropriate mitochondrial activity in the absence of oxygen (Kaelin and Ratcliffe 2008; Aragones, Fraisl et al. 2009).
Fig. 1 Regulation of HIF-1 and Metabolism. Pictures are adapted from Brahimi-Horn and Pouyssegur, 2007. (A) Hypoxic stabilization and activation of HIF-1. Under hypoxia, both PHD and FIH are inactive. HIF1α is stabilized, dimerizes with the β subunit and binds to HRE of target genes, which are involved in angiogenesis, anaerobic metabolism, vasodilatation/respiration, erythropoiesis and mitochondrial autophagy. (B) Role of HIF-1 in tumor cell metabolism. HIF-1 increases expression of the glucose transporter (Glut1) and enzymes of glycolysis. Pyruvate is preferably converted into lactate as a result of increased expression of LDH-A and inhibition of PDH caused by increased expression of PDK1.

Importantly, whereas these effects depend on HIF-1 induced transcription, recent data demonstrate that transcription-independent mechanisms also exist to control cellular
metabolism under hypoxia. Firstly, \textit{in vivo} analysis showed that tumors oscillate between high and low oxygen supply, with cycle times varying as little as minutes (Gatenby and Gillies 2004), which is too short for a transcriptional response. Secondly, accumulation of lactate, alanine and acetate levels, which are all derived from pyruvate, was observed within minutes when flies were exposed to acute hypoxia. These effects were reversible, since reoxygenation resulted in a fast recovery (Feala, Coquin et al. 2007; Coquin, Feala et al. 2008). Cellular metabolism must therefore be tightly regulated in response to oxygen sensing, yet factors involved in such a fast adaptation have not been described.

1.2 Pyruvate Kinase as a key regulator of cellular metabolism

The enzyme Pyruvate Kinase (PK; EC 2.7.1.40) performs the last step of glycolysis: The generation of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. In mammals, four isoenzymes of PK are found: type L in liver, R in erythrocytes, M1 in adult and M2 in embryonic cells (Table 1). Pyruvate is an important metabolic control point, as it can either be converted into lactate followed by the secretion pathway, or be decarboxylated to produce acetyl-CoA that feeds into the mitochondrial TCA (tricarboxylic acid) cycle. In the latter situation, energy is produced during oxidative phosphorylation in an oxygen-dependent manner. The different fates of pyruvate represent a feature of cancer cell metabolism.

| Table 1: Four isoenzymes of Pyruvate Kinase in mammals |
|----------------|----------------|----------------|----------------|
| isoforms | Tissue expression | Regulation by FBP | Polymeric form |
| R | Erythrocytes | Yes | Tetramer |
| L | Liver and Kidney | Yes | Tetramer |
| M1 | Muscle and Brain | No | Tetramer |
| M2 | Embryonic cells, Stem cells, Adipose tissue and Pancreatic islets, Cancer cells | Yes | Tetramer/Dimer/monomer |

FBP: Fructose-1, 6-bisphosphate
During tumorigenesis, tissue specific isoforms of PK are replaced by PK-M2 (Christofk, Vander Heiden et al. 2008). The quantification of PK-M2 in plasma is a clinical method for detection of tumors at early stages. Recent studies suggest that PK-M2 plays an important role in the metabolic reprogramming of cancer cells (Mazurek, Boschek et al. 2005). PK-M1 and PK-M2 isoforms result from alternative splicing of a common PKM pre-mRNA and differ in one exon. Recently, three heterogeneous nuclear ribonucleoproteins (hnRNP) have been identified to be responsible for the alternative splicing in favor of PK-M2 isoform. Interestingly, the oncogenic transcription factor c-Myc upregulates the transcription of these hnRNPs, ensuring a high PK-M2/PK-M1 ratio (David, Chen et al. 2009). Therefore the replacement of PK-M1 by PK-M2 is relevant to tumorigenesis.

In contrast to PK-M1 that exists constantly as highly active tetramer, PK-M2 can exist in both tetrameric and less active dimeric/monomeric forms (Kato, Fukuda et al. 1989; Ashizawa, Willingham et al. 1991; Mazurek and Eigenbrodt 2003; Mazurek, Boschek et al. 2005). Dimerization of PK-M2 can be induced by direct interaction of PK-M2 with several oncoproteins, such as pp60v, HPV-16E7 and A-Raf (Mazurek, Zwerschke et al. 2001; Mazurek, Drexler et al. 2007). Association into tetramer is allosterically stimulated by fructose-1, 6-bisphosphotase (FBP), an upstream intermediate of glycolysis. In addition, the oncogenic receptor tyrosine kinase (RTK) pathway is important for the regulation of PK-M2 activities. Phosphorylation on Tyr105 keeps PK-M2 in the less active dimeric form (Hitosugi, Kang et al. 2009). Moreover, phosphotyrosine containing peptides compete with FBP for PK-M2 binding and inhibit its activities (Christofk, Vander Heiden et al. 2008).

PK-M2 functions as a sensor to regulate destinations of glucose carbons, which can choose either pyruvate and energy production, or biosynthetic processes. Similar to the PK-M1 isoform, the more active tetrameric PK-M2 favors glycolysis and pyruvate production, whereas the less active dimeric form causes accumulation of upstream glycolytic...
intermediates, which can be used for synthetic processes, such as lipid and amino acid biosynthesis. The dimeric PK-M2 is found predominantly in cancer cells to meet the increased requirements of biosynthesis and cell growth (Mazurek and Eigenbrodt 2003). Recent study also showed that re-expressing of PK-M1 in cancer cells decreased their proliferation under hypoxia (Christofk, Vander Heiden et al. 2008), suggesting that the PK status (tetramer versus dimer/monomer) determines not only its activities but also the metabolic requirement of oxygen. Yet a possible link to oxygen sensing has not been addressed.

1.3 Proline hydroxylases function as oxygen sensors

Recent studies have shown that the family of prolyl hydroxylase domain (PHD; also called EglN or HPH) proteins function as cellular oxygen sensors (Kaelin and Ratcliffe 2008). These enzymes are members of Fe(II) and 2-oxoglutarate (2-OG) dependent dioxygenases (enzymes that incorporate both atoms of molecular oxygen into their products). In mammals, more than 60 of 2-OG dependent dioxygenases have been predicted via bioinformatic analysis. Although functions of many members in this family are still poorly understood, quite a few dioxygenases have been shown to catalyze a variety of oxidation reactions, including collagen modification, DNA repair, histone modification and cellular oxygen sensing. Thus hydroxylation is an important post-translational modification involved in diverse cellular processes (Pollard, Loenarz et al. 2008).

The $K_M$ of PHDs for the co-substrate oxygen is close to physiological oxygen concentrations, suggesting that it functions as a molecular switch in response to changes in oxygen concentration (Fandrey, Gorr et al. 2006). The best studied PHD target is HIFα, where two proline residues of HIF-1α and HIF-2α are hydroxylated. As mentioned above,
under normoxic conditions, HIFα degradation is mediated in response to hydroxylation by PHDs, while the lack of hydroxylation under hypoxia accounts for the accumulation of HIFα. FIH, which hydroxylates Asn803 of HIF-1α, also belongs to the family of Fe(II) and 2-OG dependent dioxygenases. In vitro studies indicate that the $K_M$ of FIH for oxygen is much lower. Thus it is still active at moderate hypoxic conditions. FIH also has unique catalytic properties when compared with those of PHDs (Koivunen, Hirsila et al. 2004).

Other members of the Fe(II) and 2-OG dependent dioxygenases family include collagen prolyl 4-hydroxylases. These enzymes locate within the lumen of the endoplasmic reticulum and catalyze the formation of 4-hydroxyproline in collagens (Myllyharju 2003). However, these enzymes cannot hydroxylate the proline residues of HIF (Jaakkola, Mole et al. 2001). The $K_M$ of these enzymes is much lower than that of PHDs, and they are functional at quite low oxygen concentrations.

Three mammalian PHDs (PHD1, PHD2 and PHD3) have been characterized. They are all widely expressed but with distinct patterns. PHD3 mRNA levels are high in heart and PHD1 mRNA levels are high in testis (Lieb, Menzies et al. 2002). Sub-cellular localization of GFP-fused PHD proteins has been reported. PHD1 is exclusive nuclear, PHD2 locates predominantly to the cytoplasm and PHD3 is distributed both in the cytoplasm and nucleus (Metzen, Berchner-Pfannschmidt et al. 2003). Interestingly, PHD2 and PHD3 levels are upregulated by hypoxia, which promote the negative feedback regulation of HIF (Epstein, Gleadle et al. 2001; Minamishima, Moslehi et al. 2009). This also implies that PHDs may have functions independent of hydroxylase activity, since their amount is increased whereas the activity is limited under hypoxia.

Of three PHD members, PHD2 is the primary one in respect to HIF hydroxylation in vivo (Berra, Benizri et al. 2003; Appelhoff, Tian et al. 2004). PHD1 and PHD3 contribute to HIF
regulation in particular cells or conditions. For example, PHD3 function becomes limiting for HIF hydroxylation under chronic hypoxia (Ginouves, Ilc et al. 2008). Inactivation of PHD2 resulted in murine embryonic lethality, whereas PHD1+/− and PHD3+/− mice are viable (Takeda, Ho et al. 2006) (Bishop, Gallagher et al. 2008). PHD1-deficient mouse shows better hypoxia tolerance by reprogramming glucose metabolism in the muscle (Aragones, Schneider et al. 2008). Over-expression of PHD3 can induce neuronal cell apoptosis in a HIF independent way (Lee, Nakamura et al. 2005), and PHD3−/− mouse showed defects in neuronal cell number and function (Bishop, Gallagher et al. 2008).

Since PHD2 is the major regulator of normoxic HIF levels, HIF-independent functions of PHD1 and PHD3 have been studied, and new substrates were identified for both enzymes. PHD1 has been implicated in the hydroxylation of the C-terminal domain of RNA polymerase II (Mikhaylova et al., 2008). PHD3 can hydroxylate the β2-Adrenergic receptor (Mikhaylova, Ignacak et al. 2008; Xie, Xiao et al. 2009).

In addition, PHD proteins have been reported to interact with other proteins, although there is no direct evidence whether these proteins are hydroxylated by PHDs. For example, peptidyl prolyl cis/trans isomerase FKBP38 interacts with PHD2 protein and regulates its stability (Barth, Nesper et al. 2007). PHD1 is involved in regulation of hypoxia-induced NF-κB activity (Cummins, Berra et al. 2006). Morg1 interacts with PHD3 and functions as scaffold to negatively regulate HIF activity (Hopfer, Hopfer et al. 2006). PHD3 can bind to, and regulate the stability of ATF-4 (Koditz, Nesper et al. 2007) and myogenin (Fu, Menzies et al. 2007). Additionally, the folding and stability of PHD3 were regulated. PHD3 is a substrate of the TRiC chaperonin complex (Masson, Appelhoff et al. 2004). PHD3 protein level is regulated by the Siah1 and Siah2 ubiquitin ligases (Nakayama and Ronai 2004). Interestingly, PHD3 was assembled into high molecular weight complex, where its catalytic
activity was repressed (Nakayama, Gazdoiu et al. 2007; Rantanen, Pursiheimo et al. 2008). However, the functional relevance of this localization, and whether this reflects hydroxylase-independent functions of PHD3, are not known.

The activity of PHDs are not only determined by oxygen availability, but also regulated by multiple factors that affect cellular redox status and energy metabolism. Like other Fe(II) and 2-OG dependent dioxygenases, PHDs require Fe(II) and ascorbate as co-factors for hydroxylation reaction. Coordination of Fe(II) to the catalytic core is crucial for enzymatic activity. Consistent with this feature, various iron chelators are often used as PHD inhibitors. The function of ascorbate is to keep the catalytic iron in its reduced form (Schofield and Ratcliffe 2004). In contrast, reactive oxygen species (ROS) are believed to cause oxidation of Fe(II) and therefore impair PHD functions (Gerald, Berra et al. 2004). In addition to oxygen, 2-oxoglutarate is a co-substrate and functions as an electron donor in the reaction. The end product, succinate, can inhibit the activity of PHDs. Accumulation of succinate caused by mutations in succinate dehydrogenase (SDH) and fumarate hydratase (FH) was sufficient to inhibit PHDs and stabilize HIFα (Selak, Armour et al. 2005). In contrast, cell-permeating derivatives of 2-oxoglutarate could restore normal PHD activity and HIF1α levels in SDH-suppressed cells (MacKenzie, Selak et al. 2007). Moreover, results from both in vitro and in vivo experiments demonstrated that levels of other tricarboxylic acid (TCA) cycle intermediates, such as citrate, succinate, fumarate, malate, could modulate PHD activities (Pan, Mansfield et al. 2007). Another modulator of PHD activity is nitric oxide (NO). Under normoxia, NO leads to HIF accumulation by interfering the interaction between PHDs and oxygen (Metzen, Zhou et al. 2003). Under hypoxia, NO paradoxically inhibits HIF accumulation. This is because NO can reduce mitochondrial respiration, and thereby increase the available intracellular oxygen for PHDs (Hagen, Taylor et al. 2003).
Fig. 2 Function and regulation of PHD proteins. Pictures are adapted from Kaelin and Ratcliffe, 2008. (A) Catalytic function of the prolyl-hydroxylase-domain proteins. These enzymes need O$_2$ and 2-oxoglutarate as co-substrates, and Fe$^{2+}$ and ascorbate as co-factors. The hydroxylation reaction forms hydroxyl-proline and succinate. (B) Central role of PHD and HIF in response to hypoxia.
1.4 Hypoxic response pathway is conserved in *Drosophila*

Most knowledge of the hypoxic signaling pathway is established in mammalian system. Some recent data has revealed that this pathway is also conserved in *Drosophila*, where a single prolyl hydroxylase, Fatiga, has been characterized *in vivo* (Lavista-Llanos, Centanin et al. 2002). The bHLH-PAS domain containing proteins Sima and Tango are *Drosophila* homologues of mammalian HIFα and HIFβ, respectively. A lacZ reporter, controlled by the hypoxia response element (HRE-lacZ), could be induced by hypoxia in *Drosophila* embryo. Fatiga mutant flies (embryos or larvae) showed increased Sima protein levels and ectopic HRE-lacZ activation. Additionally, RNAi against Fatiga in *Drosophila* Kc167 cells could induce expression of the HIF target gene LDH (Bruick and McKnight 2001). Thus the function of prolyl hydroxylase is conserved in *Drosophila*. A *Drosophila* homologue of pVHL was also identified (Aso, Yamazaki et al. 2000). In addition to the conservation of main components of hypoxic response pathway (Table 1), there is another advantage for using *Drosophila* as a model system to study this pathway: mammals encodes 3 isoforms of HIFα and 3 forms of PHD while *Drosophila* has only one gene for each component. Less redundancy makes it much easier to use some systematic tools.

<table>
<thead>
<tr>
<th>Mammalian proteins</th>
<th><em>Drosophila</em> proteins</th>
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<tr>
<td>HIF-1α, HIF-2α, HIF3α</td>
<td>Sima (Similar)</td>
</tr>
<tr>
<td>HIF-1β</td>
<td>Tango</td>
</tr>
<tr>
<td>PHD1, PHD2, PHD3</td>
<td>Fatiga (previously called HPH)</td>
</tr>
<tr>
<td>pVHL</td>
<td>dVHL</td>
</tr>
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</table>

Table 2: *Drosophila* homologues to mammalian members of the HIF pathway.

*Drosophila* are much more resilience to oxygen deprivation compared to mammals, both adult flies and larvae can survive for hours at extremely low oxygen levels (Haddad, Wyman et al. 1997). Despite this difference, the function of *Drosophila* Sima is similar to mammalian HIF. Sima is critical for survival and development in hypoxia (Centanin,
Ratcliffe et al. 2005). The remodeling of *Drosophila* tracheal system, which is analogous to mammalian angiogenesis, is Sima and Fatiga dependent (Centanin, Dekanty et al. 2008). Similar to mammals, amount and activity of Sima are increased by the TOR pathway. Interestingly, our lab has previously reported that *Drosophila* Fatiga regulates cellular growth in a HIF-independent manner, yet mechanisms remained elusive (Frei 2004; Frei and Edgar 2004). Therefore, we are interested in discovery of HIF-independent regulation of growth and metabolism in response to oxygen sensing.
1.5 Aim of Project

The aim of this study was to uncover novel mechanisms controlling growth and metabolism in response to oxygen sensing. To address this question, we first aimed to identify new interacting proteins of PHD. Since the PHD/HIF pathway is well conserved between Drosophila and mammals, we chose Drosophila as a simplified model to start the project. Pyruvate kinase, a key enzyme of glycolysis, was identified as a Fatiga/PHD3 interacting protein via a proteomic approach. Second part of my work was mainly carried out using human HeLa cells to elucidate the functional link between PHD3 and PK-M2, and the implication in metabolic adaptation under hypoxia in tumor cells.

1.6 Significance

We have demonstrated that Drosophila Fatiga and mammalian PHD3 bind directly to PK, both in vitro and in vivo. This interaction was previously unknown. Further study in HeLa cells indicates that under hypoxia, binding of PHD3 leads to inhibition of PK activities in vivo. It is a novel function of PHD3 because it is HIF independent, and does not rely on the hydroxylase activity of PHD3. In addition, we have addressed mechanism that controls this regulation. Upon down-regulation of PHD3, PK-M2 shifts towards the more active tetrameric form independent of FBP stimulation, leading to increased pyruvate production and oxidative phosphorylation. Our data suggest a novel mechanism of cellular metabolic control under hypoxia.
2. RESULTS

2.1 Drosophila Pyruvate kinase is an interacting protein of Fatiga

2.1.1 Affinity pull down and mass spectrometry

To uncover novel interacting proteins of Fatiga, we performed a mass spectrometry based proteomic analysis in collaboration with Oliver Rinner (Gstaiger Group, IMSB, ETH Zurich). Triple HA tagged Fatiga was constructed into a copper inducible vector and stably transfected into Drosophila S2 cells. HA tagged Fatiga was induced by CuSO$_4$ and immunoprecipitated by anti-HA antibody conjugated agarose beads. Precipitated proteins were eluted and subjected to trypsin digestion. Purified peptides were then injected for LTQ-MS/MS analysis. MS results were searched against Drosophila peptide database and corresponding proteins were identified. Untransfected S2 cells were used as a control. Pull-down experiments and MS analysis were performed in triplicates. A filtered list was generated for each experiment, containing candidate proteins that appeared only in the bait sample but not in the control sample. Finally, three filtered lists were compared and proteins identified twice or three times are shown in Table 3.

In filtered lists, peptides from Fatiga were always most abundant in bait samples but were not found in control samples. Identified peptides covered $\sim$50%-60% sequence of Fatiga, suggesting a specific enrichment of the bait protein on beads. Sima (HIF$\alpha$ homolog), the only known substrate of Fatiga, was detected twice out of three experiments. Since Sima is constantly degraded and exists in low abundance under normal oxygen concentration, our method is sensitive enough to detect low abundant proteins, and can be used to identify novel hydroxylation targets of Fatiga. In addition, several components of the TRiC chaperonin-containing complex were identified. It was reported that this complex specifically bound to
human PHD isoform 3 (Masson, Appelhoff et al. 2004) and my results suggest that this interaction is conserved in *Drosophila*.

**Table 3. Proteins indentified by MS more than twice**

<table>
<thead>
<tr>
<th>CG number</th>
<th>Protein name and short description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG31534</td>
<td>Fatiga, PHD, BAIT</td>
</tr>
<tr>
<td>CG7070</td>
<td>Pyruvate kinase activity</td>
</tr>
<tr>
<td>CG7340</td>
<td>Cytoplasmic amino peptidase, RCC1 domain containing protein</td>
</tr>
<tr>
<td>CG3074</td>
<td>Peptidase, protein metabolism</td>
</tr>
<tr>
<td>CG17464</td>
<td>vacuolar ATP synthase subunit b</td>
</tr>
<tr>
<td>CG9946</td>
<td>eIF2A</td>
</tr>
<tr>
<td>CG8905</td>
<td>SOD2, super-oxidize dismutase,</td>
</tr>
<tr>
<td>CG7951</td>
<td>Sima, homolog of HIF1α</td>
</tr>
<tr>
<td>CG1516</td>
<td>mitochondrial protein, pyruvate metabolism</td>
</tr>
<tr>
<td>CG9075</td>
<td>eIF4A</td>
</tr>
<tr>
<td>CG11958</td>
<td>protein folding/transport, ER or plasma membrane</td>
</tr>
<tr>
<td>CG9629</td>
<td>aldehyde dehydrogenase (NAD) activity, metabolism</td>
</tr>
<tr>
<td>CG5525</td>
<td>TRiC, chaperonin-containing T complex</td>
</tr>
<tr>
<td>CG8258</td>
<td>TRiC, chaperonin-containing T complex</td>
</tr>
<tr>
<td>CG6699</td>
<td>coatamer beta, Golgi to ER transportation</td>
</tr>
<tr>
<td>CG6988</td>
<td>PDI, containing a proline which can be hydroxylated</td>
</tr>
<tr>
<td>CG7109</td>
<td>pp2a</td>
</tr>
<tr>
<td>CG10527</td>
<td>farnisoic acid O-methyltransferase activity</td>
</tr>
<tr>
<td>CG1345</td>
<td>aminotransferase</td>
</tr>
<tr>
<td>CG8439</td>
<td>Cct5, TRiC, chaperonin-containing T complex</td>
</tr>
<tr>
<td>CG11963</td>
<td>TCA cycle, succinate-coA ligase</td>
</tr>
<tr>
<td>CG6692</td>
<td>cysteine-type endopeptidase activity</td>
</tr>
<tr>
<td>CG9124</td>
<td>eIF3</td>
</tr>
<tr>
<td>CG5330</td>
<td>Nap1, nucleosome assembly protein</td>
</tr>
<tr>
<td>CG16944</td>
<td>sesB, mitochondrial protein, stress related</td>
</tr>
<tr>
<td>CG2158</td>
<td>nuclear pore transporter function, RanBP1 domain</td>
</tr>
<tr>
<td>CG7111</td>
<td>homolog to mammalian RACK1, in PKC signaling</td>
</tr>
<tr>
<td>CG2331</td>
<td>TRE94, ER organization and biogenesis</td>
</tr>
<tr>
<td>CG5012</td>
<td>mRPL12, mitochondrial protein, genetic upstream of Fatiga</td>
</tr>
<tr>
<td>CG6948</td>
<td>clathrin light chain</td>
</tr>
<tr>
<td>CG33303</td>
<td>membrane protein, protein amino acid glycosylation</td>
</tr>
<tr>
<td>CG2674</td>
<td>SAM synthase</td>
</tr>
<tr>
<td>CG8863</td>
<td>unfolded protein binding, defense, stress</td>
</tr>
<tr>
<td>CG12775</td>
<td>RPL21, ribosome protein</td>
</tr>
<tr>
<td>CG5732</td>
<td>polynucleotide adenyltransferase activity</td>
</tr>
<tr>
<td>CG1404</td>
<td>Ran GTPase</td>
</tr>
<tr>
<td>CG3936</td>
<td>membrane protein, notch protein</td>
</tr>
<tr>
<td>CG5374</td>
<td>TRiC, chaperonin-containing T complex</td>
</tr>
<tr>
<td>CG8759</td>
<td>nascent polypeptide associated complex alpha unit, protein binding</td>
</tr>
<tr>
<td>CG12233</td>
<td>mitochondrial protein, TCA cycle</td>
</tr>
<tr>
<td>CG1782</td>
<td>ubiquitin activity enzyme Uba1</td>
</tr>
<tr>
<td>CG8073</td>
<td>monosaccharide metabolism</td>
</tr>
<tr>
<td>CG33297</td>
<td>PP2a-29B, regulatory subunit</td>
</tr>
<tr>
<td>CG6912</td>
<td>Unknown protein</td>
</tr>
</tbody>
</table>
Among the list of potential Fatiga partners, several proteins were particularly interesting. Firstly, mRpL12 (CG5012), a mitochondrial ribosome protein, was shown to genetically associate with and function upstream of Fatiga in growth control (Frei, Galloni et al. 2005). Secondly, the protein disulfide isomerase PDI (CG6988) has been found to form a dimer with the pro-collagen prolyl hydroxylase α subunit inside the ER. Notably, several translation initiation factors, eIF2A (CG9946), eIF4A (CG9075) and eIF3 (CG9124) appeared in the list, suggesting a possible role for Fatiga in translational regulation. Recently, hypoxia has been shown to suppress protein synthesis through the regulation of the initiation step of mRNA translation (van den Beucken, Koritzinsky et al. 2006). Therefore, it will be interesting to find out whether oxygen sensors directly regulate those translation initiation factors. In addition, Pyruvate kinase (CG7070) and several other metabolic enzymes were also identified, consistent with a role for Fatiga in growth control. Proteins mentioned above were chosen for further verification. Detailed mass spectrometry information of these confirmed interacting proteins are listed in Table 4.

Table 3. Shown are proteins identified twice or three times as Fatiga interacting proteins in pull-down experiments. CG numbers and a short description of each protein are included.

<table>
<thead>
<tr>
<th>CG5474</th>
<th>signal sequence receptor beta, ER protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG4389</td>
<td>fatty acid beta-oxidation</td>
</tr>
<tr>
<td>CG3195</td>
<td>RpL12, ribosome protein</td>
</tr>
<tr>
<td>CG7340</td>
<td>Granny Smith, proteolysis</td>
</tr>
</tbody>
</table>
Table 4. MS information of confirmed Fatiga interacting proteins

<table>
<thead>
<tr>
<th>Name/Function</th>
<th>CG number</th>
<th>Pull-down Round</th>
<th>Coverage</th>
<th># unique peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatiga (bait)</td>
<td>CG31543</td>
<td>1</td>
<td>63.40%</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>60.90%</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>50.25%</td>
<td>29</td>
</tr>
<tr>
<td>Sima (Hif1α)</td>
<td>CG7951</td>
<td>1</td>
<td>5.80%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>4.55%</td>
<td>3</td>
</tr>
<tr>
<td>Cct5/T-complex</td>
<td>CG5525</td>
<td>1</td>
<td>12.40%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>6.90%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>12.40%</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>CG7070</td>
<td>1</td>
<td>42.40%</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>25.20%</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>12.10%</td>
<td>5</td>
</tr>
<tr>
<td>eIF2A</td>
<td>CG9946</td>
<td>1</td>
<td>6.50%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>12.90%</td>
<td>3</td>
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<td></td>
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<td>eIF3</td>
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<td></td>
<td></td>
<td>2</td>
<td>8.50%</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4. Mass spectrometry information of selected proteins that were confirmed as Fatiga interacting proteins. Number of identified unique peptides and coverage of protein sequence are shown.
2.1.2 Study of Ran as a potential prolyl hydroxylation substrate

2.1.2.1 Proline$^{160}$ of Ran is a hydroxylation target site

The Ran GTPase (CG1404) was identified as one of the initial hits in my pull-down experiments. Ran was chosen for further characterization in priority, because another piece of evidence suggested that it could be a hydroxylation target. In collaboration with Erich Brunner (University of Zurich), we performed a database search against the experimental Drosophila peptide library established by the C-MOP center (Brunner, Ahrens et al. 2007). Peptides containing a hydroxylated proline (indicated by a 16 Da mass-shift) were indentified. This search did not complete the whole database because of limited searching power, huge and increasing size of the database. However, we did identify some peptides containing hydroxylated prolines. In addition to a large amount of peptides from pro-collagen, three peptides showed a 16 Da m/z increase on the proline residue. One of the peptides was assigned to Ran (Fig 3). The other two peptides were assigned to PDI (CG6988) and super coiling factor Scf (CG9148).

Fig. 3 Proline$^{160}$ of Ran protein can be hydroxylated. Two different forms of MS/MS results of Ran peptide (153-166) containing Proline$^{160}$ were found: Left panel is the non-hydroxylated form of the peptide. In the right panel, all ions bearing Proline$^{160}$ showed a 16 Da shift, which is predicted as a hydroxylation modification. Numbers stand for the calculated m/z values. Highlighted color means the peak corresponding to this value is experimentally detected. This data was kindly provided by E. Brunner.
2.1.2.2 Pro\textsuperscript{160} is relevant to Ran function

The small nuclear GTPase Ran controls the directionality of macromolecular transport between the nucleus and the cytoplasm. Components of HIF-PHD-VHL pathway are subjected to the regulation by nucleo-cytoplasmic trafficking. It is known that *Drosophila* Sima and human HIF-1α are imported into the nucleus under hypoxic conditions (Centanin, Ratcliffe et al. 2005; Depping, Steinhoff et al. 2008). The role of Ran in this process is less explored. Furthermore, pVHL can be exported to the cytoplasm in a Ran-dependent manner (Groulx, Bonicalzi et al. 2000). Therefore we wanted to investigate whether Pro\textsuperscript{160} hydroxylation of Ran affected its function during nucleo-cytoplasmic transportation. To address this question, I utilized an established hypoxia reporter, a firefly luciferase reporter under the control of a hypoxia response element (HRE) (Dekanty, Lavista-Llanos et al. 2005). Both hypoxia and DMOG (Dimethyloxaloylglycine), an inhibitor of prolyl hydroxylases, could induce the activities of this HRE reporter in S2 cells (Fig 4B and data not shown). Co-expression of exogenous Ran protein with the HRE reporter increased its activity (Fig 4B). Next, I knocked down Ran protein by RNAi in S2 cells. Two different double stranded RNA were used: dsRan1 targets the coding sequence of Ran and dsRan2 targets the 3’-UTR. Both dsRNAs could down regulate Ran protein levels and reduce the HRE reporter activities (Fig 4C-D). These experiments suggested that the protein level of Ran is positively related to the HRE reporter activity. Next, wild type or Pro160Ala mutant forms of Ran were re-expressed to rescue the knockdown of endogenous Ran. In the rescue experiment, dsRan2 was used. Since these over-expression constructs do not contain the 3’-UTR of Ran, they are not targeted by the dsRNA. Wide type but not the P160A mutant Ran could rescue the activity of HRE reporter (Fig 4E). Similarly, over-expression of wild type Ran is more active than the Pro160Ala mutant in stimulating the HRE reporter (Fig 4F),
suggesting that hydroxylation of Pro$^{160}$ of Ran GTPase is relevant to its function in hypoxic response.

Fig. 4 Ran protein level affects HRE luciferase reporter activity in S2 cells. (A) Schematic representation of HRE-firefly luciferase reporter. (B, D-F) HRE-firefly luciferase activity was measured and normalized to tubulin-Renilla luciferase activity. (B) DMOG and over-expression of exogenous Ran protein enhanced HRE reporter activity. DMOG: 1mM, 20hrs. (C) Down-regulation of Ran protein level by both dsRNA was confirmed by western blots. (D) Down-regulation of Ran reduced HRE reporter activity. Same samples as in (C) (E) wild type (Ran) but not P160A mutant (RanMut) can rescue RNAi caused inhibition of HRE reporter. (F) Over-expression of wild type (Ran) but not P160A mutant (RanMut) can enhance HRE activity. Both RNAi and over-expression were performed 48 hrs before assay.

Since Ran GTPase is also required for the transportation of components of other signaling pathways, it is important to clarify whether the observed effects by Ran is specific to the HIF
pathway. Therefore a reporter that responded to the Wnt signal (wf reporter) was used as a control (Schweizer and Varmus 2003). Similar to my observation with the HRE reporter, over-expression of Ran increased the wf reporter activity, while RNAi against Ran reduced it. The wf reporter activity could be rescued by expressing wild type protein but not P160A mutant (Fig 5A-B). This experiment indicated that the activity change caused by the modification of Pro$^{160}$ of Ran is not specific to the hypoxic pathway. The Wnt pathway is regulated in the same manner. Therefore, this observed effect by Ran is more likely a general consequence of altered capacity of nucleo-cytoplasmic transportation.

On the other hand, there was a difference between wild type and P160A mutant Ran in regulating both reporters. This suggests that the hydroxylation of Pro$^{160}$ is relevant to Ran function. This proline is conserved from Drosophila to human, and it does not reside within a domain of defined function. We therefore addressed whether Fatiga was responsible for Pro$^{160}$ hydroxylation of Ran.

**Fig. 5 Ran affects on wf-reporter activity in S2 cells.** wf-firefly luciferase activity was measured and normalized to tubulin-Renilla luciferase activity. Indicated transfection and RNAi were performed 48 hrs before assay. (A) Expression of exogenous wild type (Ran) but not P160A mutant (RanM) could enhance wf-reporter activity. (B) RNAi (dsRan2) against Ran reduced HRE reporter activity. Wild type (Ran) but not P160A mutant (RanM) can rescue RNAi caused inhibition of wf-reporter.
2.1.2.3 Ran is probably not a Fatiga substrate

To investigate whether Ran is a substrate of Fatiga, I performed an *in vitro* hydroxylation assay. Synthetic Ran peptide (153-166) containing Pro^{160} was incubated with relevant enzymes, co-substrates and co-factors, followed by mass spectrometry analysis. If the proline was hydroxylated, it would show a 16Da m/z shift by MS detection. The peptide failed to be modified by either GST-Fatiga or endogenous Fatiga from S2 cell extracts. Considering substrate binding may require amino acids outside of the core peptide, I also used bacterial expressed GST-Ran as a substrate in the same assay. In this experiment, we could detect both hydroxylated and non-hydroxylated forms of Pro^{160}. Surprisingly, both forms were also detected in untreated GST-Ran control samples, suggesting that certain bacterial enzymes were responsible for the hydroxylation of Pro^{160} on Ran. Fatiga/PHD belongs to the protein family of Fe^{2+} and 2-OG dependent oxygenases. Members of this family were also found in prokaryotes (van der Wel, Ercan et al. 2005), although they are not closely related to Fatiga/PHD. My data therefore suggest that the hydroxylation of Pro^{160} found on GST-Ran or endogenous Ran is most likely not caused by Fatiga. Besides, direct interaction between Fatiga and Ran could not be confirmed by either *in vitro* GST pull-down (Fig. 6A) or co-immunoprecipitation assays in S2 cells. It will be interesting to find out the enzyme responsible for Ran hydroxylation and the function of this modification. However, I did not continue this study in my following work because we are more interested in the function of Fatiga/PHDs in metazoans.
2.1.3 Interaction between PyK and Fatiga

2.1.3.1 Interaction between Fatiga and candidate proteins in vitro

Before further characterization of a particular protein as an interacting partner of Fatiga, it is critical to confirm that the interaction detected by mass spectrometry is specific. For this purpose, I performed in vitro pull-down experiments. Recombinant GST-Fatiga or GST control protein was immobilized on glutathione beads, and incubated with in vitro translated $^{35}\text{S}$-Met labeled proteins. Sima and Cct5 (one of the components of TRiC Complex) were included as positive controls. Both proteins interacted with GST-Fatiga but not GST. In addition, Pyruvate kinase (PyK), translation initiation factors eIF2$\alpha$ and eIF3 interacted with GST-Fatiga specifically. Several candidates like eIF4A, Ran and mRpL12 showed no interaction with bait proteins (Fig. 6A). Both eIF2$\alpha$ and eIF3 are confirmed as positive hits, implying that Fatiga might play a role in protein translational control in response to oxygen availability. It would be very interesting to test this hypothesis. However, I did not continue investigating the functional relationships between Fatiga and these proteins. Instead, I decided to focus on the interaction between Fatiga and Pyruvate kinase because of our interest in metabolic regulation.

2.1.3.2 Interaction between PyK and Fatiga in S2 cells

Next, I confirmed the interaction between PyK and Fatiga in S2 cells. HA tagged Fatiga and V5 tagged PyK or V5 tagged GFP were co-transfected into S2 cells. Fatiga was co-immunoprecipitated with V5-PyK but not V5-GFP (Fig. 6B). In addition, both HA-Fatiga and endogenous PyK are localized in the cytoplasm as shown by immunofluorescence (Fig. 6C). Interestingly, the localization of HA-Fatiga monitored by anti-HA antibody is rather
speckled. Since we do not have an antibody to detect endogenous Fatiga, we cannot address whether the speckled localization reflected endogenous Fatiga or it was an artifact caused by its terminal tag.

**Fig. 6 Interaction between PyK and Fatiga** (A) *in vitro* translated proteins were pulled down by GST or GST-Fatiga and detected by autoradiography. Luciferase was included as a negative control. 10% input was used as loading control. (B) Co-immunoprecipitation of tagged proteins from S2 cells. Cell lysates over-expressing HA-Fatiga and either V5-PyK or V5-GFP were incubated with anti V5 antibody and immunoblotted with HA antibody. 10% input was used as loading control. (C) Immunostaining of HA-Fatiga (green) and endogenous PK (Red) in S2 cells.

In summary, I indentified *Drosophila* Pyruvate kinase as a novel interacting protein of Fatiga using a proteomic method. In addition, the interaction was confirmed both *in vitro* and in S2 cells. My following work focused on the characterization of the functional link between Pyruvate kinase and Fatiga/PHD proteins, both in *Drosophila* and cultured mammalian cells.
2.2 Characterization of Fatiga-PyK interaction in *Drosophila*

2.2.1 Fatiga-PyK interaction is different from that of Fatiga-Sima

To investigate the functional link between *Drosophila* Fatiga and PyK, I first examined whether PyK could be a novel substrate of Fatiga. Different from other post-translational modifications such as phosphorylation, there is no well-established method to detect prolyl hydroxylation due to the lack of specific antibodies. The most straightforward method is to monitor a 16Da m/z shift by mass spectrometry (Bruick and McKnight 2001). However, this method has its limitations. Most proteins contain many candidate proline residues, and it is difficult for MS analysis to monitor all of them. I analyzed all peptides data that we received from our mass spectrometry results. PyK contains 511 amino acids in total and 18 proline residues. Our mass data covered ~55% of the whole protein sequence and 8 proline residues, none of which was hydroxylated (Fig. 7). There are other methods to detect prolyl hydroxylation like $^{14}$C labeled succinate measurement. However, this assay is established for peptide substrates instead of whole protein substrates.

```
AAGADTQLEHMCRLQFDSPVHVRLSGIVCTIGPASSSVEMLEKMMATGMNIA
RMNFSGSHYHAATVANVRQAVKNYSAKLGYEHPVAIALDTKGEIRTGLIG
GSRTAEIELKGEKIKLTNKEFLEKGSLEIVYDNEYIVNVPGLRKFVDD
GLISLIVREVGKDSLTCVENNGGSLSRKGVNLPGFVIFDLPAVSEKDLDLLF
GVEQEVDMIFASFIRNAALTEIRKVLGEKGKNIISKIQIQHNLDEII
EADGIMVARGDGIEIPAEKVFLOAQAICNPKAGKPVICATQMLESMVKK
PRPRAEISDVANVLGADCVMLSGETAKGEYPLECVLTMAKTCKEAEALW
HQNLNDLVRGAGTIDASHAAIAAVEAATKASAIIVVTTSKQSAQVSKY
RPRCPFIAVTRFAQTARQAHLYRGLVPLIYKEPGGGLDVLKDVDVRVQPGLQVG
KKNFIFKTGDSVVVTGWKQSGFTNTIRIVTVE
```

**Fig. 7 PyK peptides identified by Mass spectrometry.** Whole protein sequence of PyK is shown. Peptides identified by mass spectrometry are shown in blue and Proline residues are shown in red.
Since Sima is a known substrate of Fatiga, I compared the conservation between Sima and PyK in interacting with Fatiga. Three conserved residues of Fatiga (H\textsuperscript{169}, D\textsuperscript{171} and H\textsuperscript{230}) are known to be critical for iron coordination and catalytic activity (Bruick and McKnight 2001). I constructed a Fatiga mutant by changing all three amino acids into alanine, and used GST-Fatiga-mut in pull-down experiment. Whereas the Fatiga-Sima interaction was completely lost by these mutations, PyK still interacted with the mutant form of Fatiga (Fig. 8B). Similarly, interactions between Fatiga and eIF2A and eIF3 were not changed by the mutations. Moreover, when I mapped the domains in Fatiga that were sufficient for Sima or PyK binding, I found that only full length Fatiga interacted with Sima. In contrast, the N-terminus (amino acids 1-51), as well as the C-terminus (amino acids 150-325) of Fatiga, were sufficient for PyK binding (Fig. 8C). Since these domains do not contain an entire catalytic domain, I concluded that PyK is most likely not an enzymatic target of Fatiga. However, I cannot rule out the possibility that the regions outside of the catalytic core of Fatiga provide a site for substrate recognition and binding.

**Fig. 8** PyK-Fatiga interaction is different from Sima-Fatiga. (A) Coomassie staining of wild type and mutant GST-Fatiga proteins used in pull-down assay. (B, C) in vitro translated samples were pulled down by indicated GST fusion proteins and detected by autoradiography. 10% input was used as loading control. (B) Pull-down by GST-Fatiga-wt (wild type) or GST-fatiga-mut (H169A, D171A and H230A triple mutations). (C) PyK or Sima was pulled down by indicated GST fusion protein of full length or truncated Fatiga. FgaN (1-51aa), FgaC (249-325aa), FgaΔN (151-325aa).
2.2.2 *Drosophila* Pyruvate kinase (PyK) gene

According to Fly Base, 6 genes are predicted to have Pyruvate kinase activities due to sequence similarity. For 5 out of these genes (CG2964, CG7069, CG7362, CG11249, CG12229), there is no experimental evidence or mutant phenotype to support this prediction. The remaining gene, PyK (CG7070), has functional data showing that it encodes an active enzyme (Rust and Collier 1985). Several fly lines carrying different insertions into the genomic region of PyK were acquired from Bloomington stock center for further characterization (Fig. 9). Two strong alleles (*pyk*\(^{EY10213}\), *pyk*\(^{f06019}\)) that are homozygous lethal before reaching the adult stage, and two weak alleles (*pyk*\(^{d05514}\), *pyk*\(^{d08171}\)) that are homozygous viable, were identified. Quantitative PCR indicated that PyK mRNA levels in homozygous larvae of *pyk*\(^{EY10213}\), and trans-heterozygous larvae of *pyk*\(^{EY10213}\), *pyk*\(^{f06019}\), were only 3-5% compared to that of wild type larvae. Similarly, extracts from these mutant larvae contain less than 5% Pyruvate kinase activities (see below Fig 14D, E). Therefore PyK (CG7070) is the major Pyruvate kinase in *Drosophila*.

![Fig. 9 Representation of *Drosophila* PyK (CG7070) locus. Shown is the genomic map of CG7070 (PyK) (modified from Flybase: [http://flybase.org/](http://flybase.org/)). There are two predicted PyK isoforms. All data](image-url)
refer to the PB isoform. Element insertions of various alleles are also shown. Highlighted are alleles used in following experiments.

2.2.3 Reduced hypoxia reporter activity in pyk mutant larval fat body

To reveal the functional link between Fatiga and PyK, I first examined the effect of PyK on the hypoxia pathway in Drosophila. To monitor hypoxic activity in vivo, a reporter (Ldh-Gal4, UAS-lacZ) that can be induced by hypoxia treatment was used in our lab (Lavista-Llanos et al., 2002). The intensity of LacZ staining in larval fat body tissue (functionally equals to mammalian adipose tissue and liver) was used as a read out of hypoxia signaling activity. I generated a fly line carrying both pyk heterozygous mutant (pyk\textsuperscript{FY10213}) and the reporter (Ldh-Gal4, UAS-lacZ). This fly line was then crossed to either wild type or pyk mutant flies. LacZ staining was performed in the fat body of 5-day old larvae. Under both normoxic and hypoxic (9\%, 20hrs) conditions, larvae carrying two copies of pyk mutant showed reduced hypoxia activities compared to the control (Fig 10). This observation implies that normal PyK function is required for HIF activity. However, it is unclear whether physical interaction between PyK and Fatiga is required for this regulation. In mammals, biochemical evidence showed that PHD activity could be modified by various cellular metabolites, including pyruvate (Lu, Dalgard et al. 2005). Therefore, the observed effect on Sima/HIF activities in the pyk mutants could be mediated independently of the PyK –Fatiga interaction. In addition, I examined the activities of the HRE reporter in S2 cells either over-expressing or knocking down PyK, and I could not observe a significant effect on the HRE reporter. Furthermore, this observation does not agree with our results from HeLa cells that down-regulation of PK did not change the expression level of several HIF targets including LDH-A (see below Fig. 22). Taken together, I do not have evidence that the effect on HRE activities upon deregulation of PyK is mediated by direct binding of PyK to Fatiga.
**Fig. 10 Reduced hypoxia reporter activities in pyk mutant larval fat body.** Shown are fat bodies of 5-day-old larvae. Red: anti-lacZ staining (Ldh-Gal4, UAS-lacZ as hypoxia reporter). Blue: DAPI. Upper: Normoxia, 21% O₂, lower: Hypoxia, 9% O₂, 20 hrs. Genotype used in the left: yw; Ldh-gal4, UAS-lacZ/+; pyk<sup>EY10213</sup>/+ and in the right: yw; Ldh-gal4, UAS-lacZ/+; pyk<sup>EY10213</sup>/pyk<sup>d05514</sup>.

**2.2.4 Different sub-cellular localization of GFP-PyK in fga mutant larval fat body**

Next, I investigated whether loss of Fatiga function affected PyK levels or activities. It is difficult to test this hypothesis in S2 cells, because I could not effectively knock down Fatiga by RNAi treatment in cultured cells. Thus, I compared PyK protein levels, localizations and activities in wild type and fga mutant larvae. Since there is no antibody available to detect PyK in larvae, I used a fly line expressing a GFP tagged PyK from the endogenous genomic site (Clyne, Brotman et al. 2003). This fly line is homozygous viable, fertile and has no obvious growth defect. We first confirmed proper expression of GFP-PyK protein by western blot (Fig. 11A). We also examined GFP-PyK expression pattern in *Drosophila*
larvae. GFP signal was strong in the brain and proventiculus, and was also detected at a lower level in the fat body and imaginal discs (Fig. 11B). We could show that the GFP-PyK protein is catalytically active by comparing the activities of immunoprecipitated GFP-PyK and GFP proteins (Fig. 11C, D). In addition, I noticed that extracts from GFP-PyK larvae showed lower activities than wild type larvae (Fig. 11E), implying that the GFP tag interfered partially with the catalytic activity of PyK.

Next we generated a GFP-PyK fly line carrying a fga mutant allele. In total larval extracts, there is no change in GFP-PyK mRNA or protein levels between wild type and fga mutant

Fig. 11 Characterization of GFP-PyK larvae. (A) Western blot of total larval extracts confirmed GFP-PyK expression. (B) GFP signal in the brain tissue (left) or fat body (right). (C) GFP-PyK or GFP was immunoprecipitated from larval extract by anti-GFP antibody. (D) Enzymatic activity of precipitated proteins in (C). (E) PK activities of wild type and GFP-PyK larvae. 4-day-old larvae of were used in (A-E). Genotype: yw; +; + or yw; +; GFP-PyK. Pictures in (B) were kindly provided by Christian Frei.
However, we observed a difference in sub-cellular localization of GFP-PyK between wild type and fga mutant larvae. In wild type larval fat body cells, GFP-PyK localized both to the cytoplasm (perinuclear), where it overlapped with mitochondria, and to the plasma membrane. In addition, some GPF-PyK localized to the nucleoplasm. In fga mutant larval fat body, GFP signal on the plasma membrane was reduced, and strong, specific GFP signal was detected in the nucleus (Fig. 13). This difference in sub-cellular localization may reflect a functional regulation of PyK activities. It is believed that PyK around the nucleus is associated with glycolytic enzyme complex and has higher activity (Sullivan, MacIntyre et al. 2003).

Fig. 12 PyK level did not change in fga mutant larvae. 4-day-old larvae of were used in (A) mRNA levels determined by qRT-PCR. (B) Western blot of total larval extracts. Genotype: yw; +; GFP-PyK and yw; +; GFP-PyK, fga1/+ and yw; +; GFP-PyK, fga1/fgaS030304.
Fig. 13 Different sub-cellular localization of GFP-PyK in *fga* mutant larval fat body. Shown are fat bodies of 4-day-old larvae. Mitochondrion was stained by MitoTracker red. Genotype: yw; +; GFP-PyK (upper) and yw; +; GFP-PyK, *fga*¹⁷¹⁰⁵⁰⁴ (lower). Pictures are kindly provided by Ch. Frei.

2.2.5 Measurement of PK activities

Finally, I set up an enzymatic assay to measure PK activities. This assay is coupled to a LDH reaction, and the principle is shown in Fig. 14A. Whole cell lysates or homogenized larval extracts were used as enzymatic sources. PK activities were normalized to total protein amount. Over-expression or knocking down of PyK in S2 cells caused corresponding activity changes (Fig. 14B, C). A Larval extract of the homozygous *pyk*¹⁰²¹³ mutant shows very low activity, correlating with reduced PyK mRNA levels (Fig. 14D, E). Next, I measured
activities of larvae over-expressing Fatiga or fga mutant. Fatiga over-expressing larvae showed about 10% less PK activity, implying that Fatiga negatively regulated PyK (Fig. 15A, D). However, PK activities of fga mutant showed no significant change (Fig. 15B, E). Noticeably, the fga mutants used in this assay (yw; +; fga<sup>1</sup>/fga<sup>5030304</sup>) were not strong alleles and retained ~50% of Fatiga mRNA levels (Fig. 15E). It is possible that such down-regulation was not sufficient to cause a phenotype on PK activity. Further reduction of Fatiga with stronger fga alleles killed these larvae in early developmental stages. It has been reported that fga/sima double mutant can rescue the lethality of fga mutant (Centanin, Ratcliffe et al. 2005). Therefore we also measured the PK activities in fga/sima double mutants (yw; +; fga<sup>1</sup>/sima<sup>07607</sup>). PK activities were reduced in fga/sima double mutant larvae (Fig. 15C), compared to sima single mutants (yw; +; sima<sup>07607</sup>). However, PyK mRNA levels were also reduced in the double mutant (Fig. 15F). Thus the experiment of fga/sima double mutant was not conclusive: the lower PK activities could just reflect less PyK protein levels in the double mutant.

To summarize our findings with Drosophila PyK: activities of a hypoxia reporter were reduced in pyk mutant larval fat bodies. Sub-cellular localization of GFP-PyK was changed in fga mutants. However, we could not observe corresponding PK activity changes under this situation. Over-expression of Fatiga inhibited PyK activities.
Fig. 14 PyK activity assays of cell lysates or larval extracts. (A) Principle of enzymatic reaction. (B-E) PyK activities normalized to protein amount. (B) Over-expressed V5-PyK in S2 cells was highly active. (C) dsRNA against PyK but not LUC (Luciferase) treatment in S2 cells decreased PyK activity. (D) Larval extracts from homozygous *pyk*<sup>EY10213</sup> mutants showed little activity. (E) PyK mRNA levels of larvae used in (D). All samples were measured in triplicates.
Fig. 15 Fatiga levels and PyK activities in larval extracts. (A-C) Around twenty 4-day-old larvae of indicated genotype were homogenized and total extracts were used for enzymatic assays. (D-F) Corresponding mRNA levels of larval samples used for activity measurements in (A-C). All samples were measured in triplicates. Genotype: (A, D) flp; +; + and flp; UAS-fga/+; UAS-GFP (B, E) yw; +; + and yw; +; fga^S036304 (C, F) yw; +; + and yw; +; sima^07607 and yw; +; fga^sim^07607. * equals P<0.05; ns: not significant.

2.2.6 Drosophila PyK does not respond to FBP stimulation

This part of work was done after I discovered that mammalian PHD3 regulates PK-M2 activities by regulating its distribution between tetramer and dimer (See below 2.5). We wondered whether Fatiga regulates Drosophila PyK activities in a similar manner. As mentioned in the introduction, mammals have PK-M1 and PK-M2 isoforms that differ in only one exon, while PK-M1 is not regulated by FBP and always exists as highly active tetramer. Sequence alignment cannot reveal which PK isoform is more homologous to Drosophila PyK. However, the Lysine 433 residue that is important for FBP binding in PK-M2 is not conserved in PyK (Fig. 16A).

In the PK activity assays shown in 2.2.5, I only used 5mM PEP as a standard substrate concentration, which could not distinguish tetramer/dimer forms. Here I measured PyK activities with both high (5mM) and low (0.2mM) concentration of PEP. As shown below (see 2.5 for more details), PK activity measurement with 0.2mM PEP reflects the tetramer form of PK. Furthermore, I used FBP, which functions as a allosteric activator to promoting tetramer formation. I first measured activities of endogenous or over-expressed PyK in S2 cells. Activities of both samples could not be stimulated by FBP at 0.2mM PEP concentration (Fig. 16B, C). This is more similar to PK-M1 that constantly exists as highly
active tetramer (See below, Fig. 24). Next, I examined larval extracts. The result was similar to that in S2 cells. Reduction of PEP concentration from 5mM to 0.2mM did not strikingly reduce PK activities, implying that the $K_M$ of *Drosophila* PyK is more similar to that of PK-M1 (Fig. 16D). Furthermore, neither *fga* mutant nor hypoxia treatment (9% O$_2$, overnight) changed PyK activities, or its sensitivity to FBP stimulation. Therefore, I concluded that *Drosophila* PyK exists in a highly active tetrameric form. Although there is no allosteric regulation of *Drosophila* PyK, our previous observations suggest that Fatiga affects PyK activities and localization. The mechanism under this regulation needs to be further elucidated.

**Fig. 16** *Drosophila* PyK did not respond to FBP stimulation. (A) Sequence alignment of alternatively spliced exon 9 and 10 between human PK-M1 and PK-M2 and *Dm* PyK (CG7070). Identical and similar residues were highlighted. Position of Lysine 433 in PK-M2 was labeled with an arrow. (B-C) Enzymatic assay of (B) S2 cells (C) S2 cells over-expressing PyK at indicated PEP/FBP conditions. PK activity was shown in original format (dOD/min/mg). (D) Activity of 4-day-old larvae of indicated genotype: *yw*; +; + and *yw*; +; *fga*'/+ and *yw*; +; *fga*'/*fga*803084. N: Normoxia, H: Hypoxia, 9% O$_2$, 18hrs. All samples were measured in triplicates.
2.3 Mammalian Pyruvate kinase interacts with PHD3

2.3.1 Human PHD3 interacts with PK-M2 in vitro

As mentioned above, I was interested in identifying interactions that are conserved between *Drosophila* and mammals. Therefore, I tested whether the Fatiga-PyK interaction is conserved in mammals using *in vitro* pull-down experiments. There are three isoforms of HIF prolyl hydroxylase and four isoforms of Pyruvate kinase in mammals. I cloned tumor-related isoform PK-M2 from cDNA library of HeLa cells, and used GST-PK-M2 as a bait to pull down *in vitro* translated human PHD proteins. The results showed that GST-PK-M2 specifically interacted with PHD3 but not PHD1 or PHD2 (Fig. 17A).

I performed a sequence alignment between Fatiga and three human PHD proteins. Within the catalytic domain, *Drosophila* Fatiga is ~50% identical in amino acid sequence to mammalian PHDs. Fatiga and PHD3, however, lack the large N-terminal domains found in PHD1 and PHD2 (Fig. 17B). Since PHD2 is the major isoform that controls HIFα abundance, Fatiga function is more similar to PHD2 with respect to HIF regulation. However PHD3 is the evolutionary conserved homologue with respect to the interaction with PK, which is novel and probably independent of HIF regulation. The additional N-terminal extension in PHD1 and PHD2 might impede the interaction with PK. Indeed, specific protein interactions with PHD3 have been reported: TRiC chaperonin complex associated with PHD3 but not the other two PHDs (Masson, Appelhoff et al. 2004). In addition, PHD3 but not PHD1/2 was found in high-molecular-mass complexes, where its activity on HIF was inhibited (Nakayama, Gazdoiu et al. 2007).
Fig. 17 human PHD3 interacts with PK-M2 in vitro. (A) Three human PHD proteins were pulled-down by GST or GST-hPK-M2 and detected by autoradiography. 10% input was used as loading control. (B) Schematic representation of sequence alignment between Drosophila Fatiga and human PHD1-3.

2.3.2 PHD3 interacts with PK-M2 in cells

To confirm the PHD3-PK-M2 interaction in vivo, I performed co-immunoprecipitation assays with HeLa cells expressing V5 tagged PHD3 and HA tagged PK-M2. PK-M2 is co-precipitated by V5-PHD3 but not GFP control (Fig. 18A). Furthermore, I examined whether endogenous PHD3 and PK-M2 proteins interacted with each other in mouse embryonic fibroblast cells (MEFs) (Also see below 2.8). Both wild type (PHD3+/+) and PHD3 knock out (PHD3−/−) MEFs were pretreated overnight at 1% O₂ to induce PHD3 protein expression. Co-immunoprecipitation was performed using a specific PHD3 antibody. PK-M2 was pulled down in wild type but not PHD3−/− MEFs (Fig. 18B). I also analyzed sub-cellular localization of PHD3 and PK-M2 in MEFs by immunofluorescence microscopy. The PHD3 antibody used for co-IP and western blot does not function in immunostaining. Instead, the localization of over-expressed GFP-PHD3 was analyzed in PHD3−/− MEFs. GFP-PHD3 was localized in both the nucleus and the cytoplasm, which is consistent with a previous report (Metzen, Berchner-Pfannschmidt et al. 2003). PK-M2 was predominantly localized in the cytoplasm.
Cytoplasmic GFP-PHD3 overlapped with PK-M2 (Fig. 18C). Taken together, these results demonstrated that mammalian PHD3 does interact with PK-M2 in cultured cells.

**Fig. 18 PHD3 interacts with PK-M2 in cells.** (A) Co-immunoprecipitation of tagged proteins from HeLa cells. Lysates over-expressing HA-PK-M2 and V5-PHD3 or GFP were incubated with anti V5 antibody and immunoblotted with HA. (B) Co-immunoprecipitation of endogenous proteins. MEFs from PHD3\(^{-/-}\) or wild type mice were treated with 1% O\(_2\) for 16h. PHD3 was immunoprecipitated from cell lysates. 10% input was used as loading control. (C) Immunofluorescence of PHD3\(^{-/-}\) MEFs transfected with GFP-PHD3 (green) and stained with antibodies against PK (red).

### 2.3.3 Both PK-M1 and PK-M2 interact with PHD3

One critical regulation of PK in tumorigenesis is the replacement of tissue specific PK isoforms by PK-M2. After confirming the interaction between PK-M2 and PHD3, we wondered whether PHD3 also bound to the highly homologous PK-M1 protein. Therefore, I cloned PK-M1, and compared GST-PK-M1 and GST-PK-M2 in pull-down experiments. PHD3 interacted with both PK-M1 and PK-M2 with equal intensity (Fig. 19), suggesting that PHD3 binds to a sequence that is conserved between PK-M1 and PK-M2. However, it does not necessarily mean that PHD3 regulates two PK isoforms in the same manner. The following results indicate that in the context of HeLa cells, where only PK-M2 exists, PHD3
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regulates the transition between tetrameric and dimeric PK-M2 (See below 2.5 and 2.6). Such regulation is not possible for PK-M1, because it exists only as tetramer. Similarly, a recent publication showed that phosphorylation of Tyr 105 in PK-M2 shifted it from tetramer to dimer (Hitosugi et al, 2009). Tyr 105 and flanking region is completely conserved in PK-M1, while the regulation mechanism is specific to PK-M2.

Fig. 19 PHD3 interacts with both PK-M1 and PK-M2. *in vitro* translated human PHD2 and PHD3 proteins were pulled-down by GST-PK-M1 or GST-PK-M2 and detected by autoradiography. 10% input was used as loading control.
2.4 PK-M2 is not a substrate of PHD3

2.4.1 Analysis of possible hydroxylation motif

To elucidate a functional link between PK-M2 and PHD3, I first examined an obvious possibility that PK-M2 is hydroxylated by PHD3. Through sequence alignment, I found one ‘LXXLAP’ motif in the alternatively spliced exon that is specific to PK-M2 (Fig. 20A). This motif was found in HIFα prolyl hydroxylation sites, and was used by bioinformatics to search for novel PHD substrates (Cummins, Berra et al. 2006). Interestingly, a structural study on Pyruvate kinase showed that this particular proline (Pro\textsuperscript{403}) is localized at the interface of two PK-M2 dimers (Wooll, Friesen et al. 2001). Mutation of Pro\textsuperscript{403} to Serine, as it is at the same position of PK-M1, caused a conformational change. This change resembled to some extent of allosteric regulation by FBP. Thus Pro\textsuperscript{403} was an interesting potential site to be hydroxylated by PHD3. Such modification will link oxygen sensing to allosteric regulation of PK-M2. To test this hypothesis, I introduced similar mutation (P403S) into human PK-M2, over-expressed the mutant protein in HeLa cells and compared its activities to wild type PK-M2. A kinase dead mutant of PK-M2 (R270A) was included as negative control. At comparable expression levels (Fig. 20D), P403S mutation had no effect on PK activities, and did not change its sensitivity to FBP stimulation (Fig. 20C), suggesting that Pro\textsuperscript{403} is not involved in allosteric regulation of PK-M2. In contrast, PK-M2 R270A mutant did not, as expected, change PK activity. Very recently, β\textsubscript{2}-adrenergic receptor was identified as a substrate of PHD3, and this protein did not contain a ‘LXXLAP’ motif (Xie, Xiao et al. 2009). Therefore, the ‘LXXLAP’ motif is not strictly required for hydroxylation by PHDs, and the functional relevance of this motif in PK-M2 is still not known.
Fig. 20 Analysis of ‘LXXLAP’ motif. (A) Sequence alignment of alternatively spliced exon 9 and 10 between human PK-M1 and PK-M2 and Dm PyK (CG7070). ‘LXXLAP’ motif was underlined. (B) View of tetramer structure of rabbit PK-M2. Pro\textsuperscript{403} is shown with colored balls and highlighted with black arrow. Adapted from Wooll, Friesen et al. 2001. (C) Relative PK activities of lysates over-expressing indicated HA-PK-M2 or mutant. 2mM FBP was added when indicated. (D) Detection of endogenous and exogenous HA-tagged PK proteins by anti-PK antibody in lysates from (C).

2.4.2 in vitro hydroxylation assay

Next, I collaborated with Katarzyna Nytko (Wenger group, Institute of Physiology, Zurich University) and performed in vitro hydroxylation assays. This assay uses a HIF1α peptide (556-574) containing Pro\textsuperscript{564} as a substrate. Baculoviral expressed GST-PHD3, together with 2-oxoglutarate, Fe\textsuperscript{2+} and ascorbate, was incubated with the HIF peptide. The hydroxylated peptide was subsequently quantified by thioredoxin-tagged pVHL/elongin B/elongin C.
(VBC) complex binding. The same HIF1α peptide containing a P564A mutation was used as a negative control. This assay can be used to monitor PHD activity changes caused by additional factors (Nytko, Spielmann et al. 2007). Incubation of PK-M2 with the reaction mixture had no effect on hydroxylation status of the HIF peptide (Fig. 21A), demonstrating that PK-M2 does not affect PHD3 activity on HIF peptide in vitro. Besides, it suggests that PK-M2 is not likely a PHD3 substrate, since it does not compete with the Pro$^{564}$ substrate.

**Fig. 21** rPK-M2 has no effect on PHD3 activity on HIF in vitro. (A) The hydroxylation activity of PHD3 was measured in microtiter plate-based VBC binding assays in the absence or presence of recombinant PK-M2 or control GST. (B) PHD2 was used as a control in the same assay as (A).

### 2.4.3 Down-regulation of PK-M2 did not change HIF activities

Furthermore, we tested whether PHD3 or HIF activities might be altered in response to deregulation of PK-M2 in cells. For this purpose, I established a HeLa cell line stably expressing a small hairpin RNA targeting PK-M2. This shRNA targets a sequence that is conserved between PK-M1 and PK-M2, and is indicated as shRNA$^{PKM}$. shRNA$^{PKM}$ led to strong down regulation of PK-M2 mRNA and protein levels, as well as PK enzymatic activities (Fig. 22A, B and C). However, shRNA$^{PKM}$ did not affect mRNA or protein levels of PHD3 (Fig. 22A), and did not affect the expression of some well-established HIF target
genes LDH-A, PDK-1, PFK (Phosphofructokinase), Glut1 (Glucose transporter-1) and VEGF (Vascular endothelial growth factor), both under normoxia and hypoxia (Fig. 2C).

Taken together, I concluded that PHD3 levels and the activities on HIFα were not altered upon PK-M2 deregulation.

![Graph A](image1)
![Graph B](image2)
![Graph C](image3)

**Fig. 22 Down-regulation of PK-M2 did not change HIF activity.** (A-C) HeLa cells stably transfected with shRNA\textsuperscript{control} or shRNA\textsuperscript{PKM} were treated under Normoxia (N) or Hypoxia (H: 1% O\textsubscript{2} for 18hrs). (A) The protein levels of PHD3 and PK-M2 were measured by western blotting. Tubulin was used as loading control. (B) Pyruvate kinase activities of cell lysates were measured, using 5mM PEP as a substrate. (C) mRNA levels of indicated genes were measured by qRT-PCR and normalized to Actin.
2.5 PK-M2 activity is increased upon down-regulation of PHD3

2.5.1 Stable down-regulation of PHD3

To investigate whether PHD3 regulates PK activity, I generated a stable pool of HeLa cells targeting PHD3 with specific hairpins (shRNA^{PHD3}). As observed before, (Appelhoff, Tian et al. 2004; Nakayama, Gazdoiu et al. 2007; Bishop, Gallagher et al. 2008; Ginouves, Ilc et al. 2008; Rantanen, Pursiheimo et al. 2008) hypoxia treatment led to a strong increase in PHD3 mRNA and protein levels. Western blots showed that under normoxic condition, PHD3 protein levels are very low, indicating that PHD3 function might be more relevant under hypoxic condition. In shRNA^{PHD3} cells, I observed a strong down-regulation of endogenous PHD3 mRNA and protein levels under both normoxia and hypoxia (Fig. 23A, B). shRNA^{PHD3} did not change the transcriptional levels of PK-M2 itself and other tested HIF target genes LDH-A, PDK-1, PFK, Glut1 and VEGF, both under normoxia and hypoxia (Fig. 23B). In addition, western blots indicated equal PK-M2 abundance in shRNA^{Control} and shRNA^{PHD3} cells (Fig. 23A). These observations agree with previous reports that PHD3 is not the major hydroxylase required for HIF inhibition in vivo (Ginouves, Ilc et al. 2008; Minamishima, Moslehi et al. 2009). It also implies that PHD3-PK interaction could be a novel mechanism how glycolytic rates are controlled in response to oxygen levels independently of HIF.
Fig. 23 Stable down-regulation of PHD3. HeLa cells stably transfected with shRNA\textsuperscript{control} or shRNA\textsuperscript{PHD3} were treated with Normoxia (N) or Hypoxia (H: 1% O\textsubscript{2} for 18hrs). (A) The protein levels of PHD3 and PKM were measured by western blotting. Tubulin was used as loading control. (B) mRNA levels of indicated genes were measured by qRT-PCR and normalized to Actin.

2.5.2 Effect of PEP concentrations and FBP on PK-M2 activities

As mentioned above, the PK-M2 isoform is expressed in rapidly dividing cells such as embryonic cells, stem cells and tumor cells. PK-M2 exists as tetramer in the presence of FBP, showing a high affinity to its substrate PEP (K\textsubscript{M} 0.17mM). In the absence of FBP, its affinity to PEP is much lower (K\textsubscript{M} 2.1mM) (Dombrauckas, Santarsiero et al. 2005). PK-M2 exists mainly as dimer/monomer in tumor cells, and is believed to be inactive under physiological conditions (Mazurek, Boschek et al. 2005). To distinguish different PK-M2 forms, I measured PK activities using different PEP concentrations. I included PK-M1 as a control that always exists as tetramer. Firstly, activities of recombinant proteins were compared. GST-PK-M1 and GST-PK-M2 have comparable activities at 5mM PEP. Activities of GST-PK-M1 were less sensitive to reduction in PEP concentrations (from 5mM to 0.2mM) than that of GST-PK-M2 (Fig. 24A). Similarly, over-expressed PK-M2 was more sensitive to decreased PEP concentrations than over-expressed PK-M1 in HeLa cells (Fig. 24B and C). Furthermore, whereas activities of PK-M1 were insensitive to FBP stimulation, activities of PK-M2 could be stimulated by FBP at low PEP concentration (0.2mM) but not at high PEP concentration (5mM) (Fig. 24D). These data demonstrated that PK activities in response to different PEP concentrations could be used to distinguish between the M1-like behavior (constant tetramer form) and the M2-like behavior (equilibrium between tetramer and dimer/monomer form) of PK-M2. When measured at 5mM PEP concentration, which is much higher than physiological condition, activities of all forms of PK-M2
(tetramer/dimer/monomer) are assayed, and PK activities cannot be further stimulated by FBP. Thus PK activity at 5mM PEP reflects total PK-M2 amount. When measured at 0.2mM PEP concentration, which is similar to physiological concentration, only tetramer activities are measured. Furthermore, adding FBP into the reaction can stimulate the activities of dimeric/monomeric PK-M2 but not tetramer.

**Fig. 24** PK-M1 and PK-M2 respond differently to various PEP concentration and FBP stimulation. (A) Relative PK activities of GST-PK-M1 and GST-PK-M2 at indicated PEP concentrations. (B) Relative PK activities of HeLa lysates over-expressing either HA-PK-M1 or HA-PK-M2 at indicated PEP concentrations. (C) Western blot of cell lysates from (B), the higher band is the HA tagged PK protein. Tubulin was used as loading control. (D) Relative PK activities of lysates from (B) at 5mM or 0.2mM PEP. 2mM FBP was added when indicated. PK activities at 5mM PEP were normalized to 100.
2.5.3 PHD3 inhibits PK-M2 activity under low PEP concentration

Next, I measured PK activities of different HeLa cell lines using the conditions established above. shRNA\textsuperscript{PHD3} had no effect on PK activities at high PEP concentration (5mM), which reflects the activities of all PK-M2 forms (Fig. 25A). This is consistent with the observation that PK-M2 protein levels are comparable in shRNA\textsuperscript{Control} and shRNA\textsuperscript{PHD3} cells (Fig. 23A). Next, PK enzymatic assays were performed using low PEP (0.2mM), reflecting the activity of tetrameric PK-M2 only. Strikingly, shRNA\textsuperscript{PHD3} cells showed higher PK activities, especially under hypoxia. Moreover, adding FBP into the reaction did stimulate PK activities in shRNA\textsuperscript{Control} cells, but not in shRNA\textsuperscript{PHD3} cells (Fig. 25B). Thus upon down-regulation of PHD3, PK-M2 showed an increase in activity under physiological PEP concentration (0.2mM), and was insensitive to FBP stimulation. These results can be explained by a shift of PK-M2 from dimer/monomer to tetramer. Alternatively, PHD3 could control the alternative splicing between the M1 and M2 isoforms. The latter possibility can be excluded, because shRNA\textsuperscript{PHD3} cells exclusively expressed the M2 isoform, as confirmed by isoform-specific antibodies (data not shown). Since PHD3 protein is more abundant under hypoxia, it is not surprising that the effect by down-regulation of PHD3 is more pronounced under hypoxia. For further characterization, we therefore focused on hypoxic conditions.

I also measured PK activities of shRNA\textsuperscript{PKM} cells. As shown above (Fig. 22), at high (5mM) PEP concentration, PK activities in shRNA\textsuperscript{PKM} cell lysates were ~10% compared to that in shRNA\textsuperscript{control} cells, reflecting the reduced protein levels (Fig. 25C). However, reduced PEP concentration (0.2mM) did not affect PK activities as strikingly as the control cells. Moreover, FBP could not stimulate PK activities at 0.2mM PEP, both under normoxia and hypoxia (Fig. 25D). Since the total amount of PK-M2 is greatly reduced in shRNA\textsuperscript{PKM} cells, it is not surprising that the enzyme exists as highly active tetramer as a compensatory mechanism for cells to survive.
Fig. 25 Knockdown of PHD3 increases PK-M2 activity under hypoxia. Pyruvate kinase activities were measured in the presence of (A, C) 5mM PEP or (B, D) 0.2mM PEP. 2mM FBP was added when indicated. PK activity of shRNAcontrol cells under normoxia at 5mM PEP was normalized to 1.
2.6 Increased tetrameric PK-M2 upon down-regulation of PHD3

2.6.1 Gel filtration failed to separate different forms of PK-M2

Based on our results showing the regulation of PK activities by PHD3 levels, we proposed that down-regulation of PHD3 under hypoxia caused a shift of PK-M2 from dimer/monomer to tetramer, and increased its activities. To look for direct evidence that supports this hypothesis, we utilized biochemical approaches. It has been published that tetrameric and dimeric PK-M2 can be separated by gel filtration (Spoden, Mazurek et al. 2008). With the kind help of Roland Scholz (Neumann Group, ICB, ETHZ) and Reinhard Dechant (Peter group, IBC, ETHZ), I tried to separate recombinant rPK-M2 or endogenous PK-M2 in whole cell lysates by gel filtration using size exclusion columns Sephacryl™ 200 or Superose™ 6, respectively. Fractions from gel filtration were harvested. PK activities of each fraction were measured to plot the separation pattern of PK-M2. Unfortunately, both columns failed to separate PK-M2 into more than one broad peak. Calculated by the elution time, the PK activity peak corresponds to the molecular weight of dimeric PK-M2 (Fig. 26). Upon FBP stimulation, the activity peak of recombinant PK-M2 slightly shifted towards higher molecular weight (Fig. 26A). A similar shift was observed in shRNA^{PHD3} cell lysate compared to shRNA^{control} cell lysate (Fig. 26B). It is not known whether this shift was caused by increased PK-M2 tetramer. Alternatively, it could reflect a conformational change instead of molecular weight differences. Since we had limited access to these facilities, and extensive effort and time were required to optimize the conditions, we decided to use an alternative method.
Fig. 26 Gel filtration failed to separate different PK-M2 forms. PK activities of each gel filtration fraction (0.25ml) from Superose™ 6 column were measured and plotted to volume. (A) gel filtration of recombinant PK-M2 in the presence or absence of 2mM FBP. (B) gel filtration of cell lysates of shRNA<sub>control</sub> or shRNA<sub>PHD3</sub> cells. Position of expected tetramer (240kDa, ~14.5ml) and dimer (120kDa, ~16.5ml) were shown in red or yellow, respectively.

2.6.2 Separation of PK-M2 by 2D BN-SDS-PAGE

To directly monitor monomer, dimer and tetramer forms of PK-M2 with good separation, I used two-dimensional Blue Native-SDS-PAGE (BN-SDS-PAGE). The principle of this method is shown in Fig. 27A. The negatively charged dye Coomassie blue, which binds nonspecifically to all proteins, is added into the sample before first dimensional BN-PAGE. Coomassie blue does not act as a detergent. When used at proper concentration, it preserves the association between proteins. Proteins were separated in the first dimension according to size under native conditions (shown horizontally using gradient native gels). The second dimensional separation with SDS-PAGE (shown vertically) improves the resolution, and is often necessary for following detection. The gels were then transferred to a membrane and processed by western blotting. As a proof of principle, I used this method to separate recombinant PK-M2 protein. As expected, western blots revealed three populations of PK-M2 corresponding to the molecular weight of tetramer (~240KDa), dimer (~120KDa), and
monomer (~60KDa). Furthermore, composition of PK-M2 showed a shift towards the tetrameric form in the presence of 2mM FBP (Fig. 27B). After validating our method, I analyzed lysates from hypoxia-treated shRNA\textsuperscript{Control} or shRNA\textsuperscript{PHD3} cells. In shRNA\textsuperscript{Control} lysate, monomeric and dimeric PK-M2 were predominant, and only ~3-5% of PK-M2 existed as tetramer. Incubation with 2mM FBP stimulated tetramer formation (~24%). In contrast, 15% of PK-M2 existed as tetramer in shRNA\textsuperscript{PHD3} lysate (Fig. 27C). These results agree with the increased PK enzymatic activities described above. Thus the increase of PK activities in shRNA\textsuperscript{PHD3} cells can be explained by increased tetrameric PK-M2.

**Fig. 27** Knockdown of PHD3 increases tetrameric PK-M2. (A) Principle of 2D BN-SDS-PAGE. (adapted from Swamy, Siegers et al. 2006) Proteins are separated under native conditions in a first-dimension gradient native gel, then denatured by SDS in the gel strip and applied to a second-dimension SDS gel. (B) Recombinant rPK-M2 was separated by 2D BN-SDS-PAGE. 2mM FBP was added when indicated. (C) Hypoxia-treated samples were separated by 2-D BN-SDS-PAGE. (B-C) Monomeric (M, 60kD), dimeric (D, 120kD) and tetrameric forms (T, 240kD, indicated by square boxes) of PK-M2 were detected by western blotting.
2.7 Down-regulation of PHD3 changed cellular metabolism under hypoxia

2.7.1. Alteration of intracellular metabolite levels

We have shown that down-regulation of PHD3 led to increased PK-M2 activities under hypoxia at a physiological PEP concentration. We then continued to examine the effect on cellular metabolism by loss of PHD3. In collaboration with Dominika Czernik (Zamboni Group, IMSB, ETHZ), we measured the metabolome of HeLa cells stably expressing different shRNAs. Compared to traditional methods that measure limited number of metabolites, metabolomics allow us to have a global view of cellular metabolism, and acquire large amount of information from one experiment.

Cultured cells were incubated at 1% O₂ for 18 hours before total cellular metabolites were extracted. Relative levels of each metabolite were acquired by LC-MS/MS (Buscher, Czernik et al. 2009). Metabolite levels were normalized to protein amount of individual samples and averaged for triplicates. We successfully identified most intermediates in central carbon metabolism, and we could observe changes in metabolites clustered in certain metabolic process (Fig. 28).

shRNA_PKM cells were included as control. As expected, levels of most intermediates in glycolysis were increased in these cells, suggesting that reduction in PK activities caused accumulation of upstream glycolytic intermediates (Fig. 29A). In shRNA_PHD3 cells, pyruvate levels showed a 3-fold increase, which was consistent with higher PK activities observed in these cells. In contrast, glycolytic intermediates upstream of PK were not significantly changed (Fig. 29B). Remarkably, the increase in pyruvate did not lead to enhanced synthesis of lactate, but led to more acetyl-CoA and the TCA-cycle intermediates 2-oxoglutarate, succinate, fumarate and malate (Fig. 29D). Similar effects were observed in cells re-expressing PK-M1, with enhanced mitochondrial oxygen consumption rather than producing
lactate (Christofk, Vander Heiden et al. 2008). Therefore, PK-M2 not only produces pyruvate, but also determines the next step: either synthesis of lactate, or mitochondrial respiration. In agreement with the data shown above, this provides further evidence that down-regulation of PHD3 leads to increased tetrameric PK-M2 that behaves similarly as PK-M1. However, it is surprising that the lactate levels are unchanged, because enhanced oxidative phosphorylation usually is coupled to reduced production of lactate. To confirm this, I also measured levels of secreted lactate, which was also not changed in shRNA^{PHD3} cells (Fig. 29D). It will be interesting to find out whether shRNA^{PHD3} cells uptake more glucose and increase total pyruvate production.
Fig. 28 **Overview of metabolome.** Relative levels of detected metabolites were shown. (A) Metabolites were grouped in glycolysis, TCA cycle and pentose phosphate pathway.

In addition to changes in glycolysis and oxidative phosphorylation, we also observed striking up-regulation of metabolites in pentose phosphate pathway upon down-regulation of PHD3 (Fig. 30). This is very surprising and difficult to explain at this moment. The main function of pentose phosphate pathway is to provide reducing power (NADPH) and ribose for biosynthesis of nucleotides. Its primary role is anabolic rather than catabolic. The biological relevance and the underlying mechanism have not been addressed.
Fig. 29 Relative metabolites levels in shRNA<sup>PKM</sup> and shRNA<sup>PHD3</sup> cells. (A-B) Metabolites in glycolysis were compared between shRNA<sup>Control</sup> and (A) shRNA<sup>PKM</sup> (B) shRNA<sup>PHD3</sup> cells. (C) Secreted lactate levels in shRNA<sup>Control</sup> and shRNA<sup>PHD3</sup> cells. (D) Metabolites in TCA cycle in shRNA<sup>Control</sup> and shRNA<sup>PHD3</sup> cells. G6P: Glucose 6-phosphate; DHAP: Dihydroxyacetone phosphate; F6P: Fructose 6-phosphate; FBP: Fructose 1,6-bisphosphate; BGP: 1,3 bisphospho-Glycerate; 3+2PG: 3-Phosphoglycerate and 2-Phosphoglycerate; AcCoA: Acetyl-CoA; CIT/ICIT: Citrate/Isocitrate; * equals to P<0.05; *** equals to P<0.001.

Fig. 30 Up-regulation of Pentose phosphate pathway in shRNA<sup>PHD3</sup> cells. Shown are relative levels of metabolites in pentose phosphate pathway. 6PG: 6-phosphogluconate, Ru5P: ribulose 5-phosphate, R5P: ribose 5-phosphate, X5P: xylulose 5-phosphate, S7P: sedoheptulose 7-phosphate.
2.7.2 Measurement of oxygen consumption

Our metabolites measurement indicated more pyruvate entered oxidative phosphorylation in shRNA^{PHD3} under hypoxia, suggesting that these cells have more active mitochondria, and therefore consume more oxygen. To confirm this, I tried to measure oxygen consumption of these cells. Under normoxic condition, there is no significant difference between shRNA^{Control} and shRNA^{PHD3} cells. This is not surprising because the effect we observed is largely specific to hypoxic cells. However, there is a technical limitation to examine O₂ consumption of hypoxic cells, because the measurement has to be performed outside of the hypoxic chamber. Re-oxygenation caused large variations, and I could not receive reliable results from hypoxic samples.

2.7.3 Increased ROS accumulation in shRNA^{PHD3} cells

Instead of oxygen consumption, I measured intercellular reactive oxygen species (ROS), which are mostly derived from mitochondrial oxidative phosphorylation, and are commonly assumed to correlate with mitochondrial dysfunction. The assay is based on using 5-(&-6)-carboxy-2’, 7’-dichlorodihydrofluorescein diacetate (carboxyl-HDCFDA), a reliable fluorogenic marker for ROS in live cells. Marker incubation can be performed in the hypoxic hood, and generated fluorescence signals are stable for more than an hour. The DCF fluorescence was determined and normalized to a mitochondrial signal (MitoTracker Red).

To validate the method, H₂O₂ was used as a positive control to induce oxidative stress and ROS production. DCF fluorescence was strikingly induced by H₂O₂ (Fig. 31A). When cells were exposed to hypoxia, down-regulation of PHD3 led to a small but significant increase in intracellular ROS (Fig. 31B). This is consistent with our hypothesis that shRNA^{PHD3} cells have more active mitochondria.
Increased ROS accumulation in shRNA$^{\text{PHD3}}$ cells. Reactive oxygen species (ROS) levels were measured by DCF fluorescence and normalized to MitoTracker Red fluorescence. (A) 100uM or 200uM $\text{H}_2\text{O}_2$ were used to induce ROS production. (B) ROS levels in hypoxia treated shRNA$^{\text{Control}}$ and shRNA$^{\text{PHD3}}$ cells. *** equals to P<0.001.

2.7.4 Loss of PHD3 reduces proliferation under hypoxia

Finally, to further test whether the effect seen on PK-M2 activities would alter the cell’s capacity to respond properly to hypoxia, I performed proliferation assay at both 21% O$_2$ and 3% O$_2$. Upon down-regulation of PHD3, cells divided at a lower rate when grown at 3% O$_2$. In contrast, we did not observe such defects when cells were grown under normoxia (Fig. 32), demonstrating that PHD3 is critical for cells to adapt properly to hypoxia. Again this agrees with the observation that PK-M1 expressing cells proliferate slower (Christofk, Vander Heiden et al. 2008).

Loss of PHD3 reduces proliferation under hypoxia. Proliferation curves of control or PHD3 knockdown cells under normoxia (21% O$_2$) or hypoxia (3% O$_2$).
2.8 PK activities in MEFs

During my study of PHD3 function in HeLa cells, papers charactering PHD3 knockout mice were published. These knockout mice are viable, but show defects in neuronal apoptosis (Takeda, Ho et al. 2006; Bishop, Gallagher et al. 2008). The authors did not comment how these mice respond to stress conditions like hypoxia. We acquired both wild type and mutant mouse embryonic fibroblasts (MEFs), the latter derived from PHD3 knockout mice. The co-IP experiment described above (Fig. 18B) and the following experiments were done using immortalized MEFs transformed with SV40 large T antigen.

Loss of PHD3 in PHD3−/− MEFs was confirmed by western blot. PK-M2 protein levels were also examined (Fig. 33A). In contrast to HeLa cells, both hypoxia and knockout of PHD3 increased PK-M2 protein levels by ~20-40%. Hypoxic knockout MEFs have twice the amount of PK-M2 compared to normoxic wild type MEFs (Fig. 33B). This is different from the observation in Hela cells, where down-regulation of PHD3 has no effect on PK-M2 protein levels. Next I performed similar studies on monitoring PK activities in MEFs using various PEP concentrations. Compared to wild type cells, PHD3−/− MEFs showed slightly enhanced PK activities, both under normoxia and hypoxia (Fig. 33C). This effect is most likely due to the up-regulation of PK-M2 protein. However, unlike shRNA^{PHD3} HeLa cells, which have strikingly higher PK activity than the control cells at low PEP concentration, PHD3−/− MEFs showed no difference in PK activities compared to wild type MEFs. Remarkably, in all samples and conditions, PK activities of MEFs do not respond to FBP stimulation, which is completely different from our observations in HeLa cells. To confirm this, I measured PK activities in wild type MEFs using titrated PEP concentrations in parallel with HeLa cells. Purified PK-M1 was also included as control. Compared to HeLa cells, which showed a 7.5-fold drop in PK activities under low PEP concentration, MEFs had only a 2-fold drop in PK activities under same condition. In this aspect, MEFs behaved identically.
to PK-M1, which is constantly in a tetramer form. However, western blots using isoform-specific antibodies showed that MEFs express exclusively PK-M2, but not M1 (data not shown). This result suggests that in these MEFs, PK-M2 always exists as tetramer. Removal of PHD3 does not have an effect since PK-M2 is already in its tetrameric form.

Fig. 33 PK activities of MEFs do not respond to FBP stimulation. (A) Western blot showed no PHD3 protein in PHD3−/− MEFs. (B) Blots and quantification of PK-M2 protein levels. (C) Relative PK activity at indicated PEP concentration for cell lists from (B). 2mM FBP was added when indicated. (D) Relative PK activity of MEFs, HeLa cells and PK-M1 at indicated PEP concentration.

In summary, experiments described above showed that the interaction between PHD and Pyruvate Kinase is conserved from Drosophila to mammals. In Drosophila larvae, loss of Fatiga caused changes in sub-cellular localization of PyK in the fat body. Fatiga over-expression slightly inhibited PyK activities. In HeLa cells, down-regulation of PHD3 led to an increase in the highly active, tetrameric form of PK-M2. This resulted in enhanced pyruvate production and oxidative phosphorylation. Moreover, PHD3 was required to limit ROS accumulation and to adjust cell proliferation to oxygen availability.
3. DISCUSSION AND OUTLOOK

The long-term aim of this project is to understand the mechanisms that underlie metabolic control by oxygen sensing. My PhD study focused on the characterization of a novel interaction between Prolyl hydroxylase domain proteins PHD3/Fatiga and the glycolytic enzyme Pyruvate kinase (PK/PyK). Prior to our research, it had been well established that PHD proteins function as cellular oxygen sensors and post-transcriptionally control the abundance of HIF, the master regulator of oxygen homeostasis (Kaelin and Ratcliffe 2008). However, whether these PHD proteins have other targets, or are involved in HIF-independent functions, are less studied. To investigate these possibilities, we chose Drosophila as a conserved but less redundant model organism, and utilized a systematic approach to identify unknown interacting partners of Fatiga. Many putative Fatiga-binding proteins were identified this way. Among these candidates, we characterized PyK and several other proteins as genuine interacting partners of Fatiga in vitro. We chose Pyruvate kinase for further functional studies because of its potential roles in metabolism and growth. Using Drosophila larvae as an in vivo model, we could observe interesting effects on either sub-cellular localizations or activities of PyK, upon loss-of-function or gain-of-function of Fatiga. However, the mechanism responsible for these phenomena is not fully understood yet. We found that human PK-M2 interacted with PHD3 as well, demonstrating a conserved interaction from Drosophila to human. The second part of my study was carried out in HeLa cells because of the critical role of PK-M2 in tumor cell metabolism. To investigate functional consequences of the interaction between PHD3 and PK-M2, and the mechanism that regulate this process, we carried out PHD3 loss-of-function studies by stably knocking down PHD3 in HeLa cells. In these cells, we observed increased PK activities under hypoxia and corresponding changes in cellular metabolism. Our results suggested a novel mechanism
in which PHD3, through its interaction with PK-M2, inhibits the latter and regulates cellular metabolism under hypoxia.

**Discovery of novel protein interactions by mass spectrometry**

The starting point of this study was the discovery of Pyruvate kinase and several other proteins as Fatiga binding partners through a proteomic approach. We have demonstrated that affinity pull-down, followed by mass spectrometry analysis, is an effective and powerful tool to uncover novel protein interactions, which provide clues for uncharacterized functions. Like other systematic approaches, every identified candidate still needs further confirmation. In addition, it is important to include a proper control sample. In my experiment, I used untransfected S2 cells as a control. However, a better control would be a S2 cell line stably over-expressing an independent HA-tagged protein, such as GFP. Such kind of control would help to minimize the difference in pull-down samples caused by over-expression.

The method combining pull-down and mass spectrometry analysis is unbiased. On the other hand, it can be designed for more targeted searching. For example, we can modify our experiment to focus on novel Fatiga substrates. My results showed that the interaction between Fatiga and its known substrate Sima is very sensitive to mutations in critical residues within the catalytic center. It is reasonable to speculate that Fatiga requires these residues for interacting with other substrates as well. Therefore, by using a catalytically dead Fatiga mutant and a wild-type Fatiga for paralleled pull-downs, we can distinguish real Fatiga substrates, which only bind to wild type but not mutant Fatiga, from other binding proteins. In this set-up we can reduce the number of candidates that need to be tested as potential substrates. I have already established stable S2 cell lines over-expressing HA-
tagged Fatiga mutant, which will be a starting point of another project for identifying novel targets of prolyl hydroxylases.

My results also indicate that *Drosophila* is a valid model organism for such systematic approach. The conserved interaction between PHD3/Fatiga and PK/Pyk was initially identified in *Drosophila*. Nevertheless, much of my study was carried out in mammalian cells, and the mechanism was better elucidated with mammalian proteins. It suggests that certain regulating mechanisms may not be identical during the evolution from *Drosophila* to mammals, but the protein-protein interaction can still be conserved because of sequence consensus.

**Regulation of PK-M2 by PHD3 and its implication in tumors**

My data have demonstrated that PHD3 interacts with PK-M2, which leads to the inhibition of PK activities *in vivo*. Since PHD3 protein is less abundant under normoxia and increases drastically under hypoxia, the effects observed are largely hypoxia-specific. Regulation of PK-M2 activity is achieved by shift of equilibrium between tetramer and dimer/monomer forms. Herein we propose that the binding of PHD3 to PK-M2 either leads to dissociation of the PK-M2 tetramers, or impedes the monomer/dimers associating into tetramers (Fig. 34).
**Fig. 34 PHD3 limits PK-M2 activity via interaction.** Shown is our current model of the role of PHD3 in the regulation of PK-M2 and cellular metabolism. Under hypoxia, expression of PHD3 is induced. In control cells (right), it binds to and keeps PK-M2 in the dimeric/monomeric form. Other factors indicated by question marker may be involved in this regulation. When PHD3 was knocked down (left), more PK-M2 existed as highly active tetramer and produced more pyruvate, which prefer to be converted into acetyl CoA rather than lactate.

Since tumors are often hypoxic and predominantly express the PK-M2 isoform, the PHD3-PK-M2 interaction might be particularly important in tumors. Accordingly, recent data showed that PHD3 protein levels were up-regulated in pancreatic tumors, and increased PHD3 levels correlated with aggressive tumor behavior (Couvelard, Deschamps et al. 2008). Tumors take up large amounts of glucose for energy production and biosynthesis, yet the accumulation of certain metabolites during this process, such as lactate, might be deleterious. Thus tumor cells should also be able to limit glycolysis when necessary, however
mechanisms underlying this regulation were previously not known. Here we show that PHD3 limits PK activities and pyruvate production. Besides, PHD3 is also required to prevent improper mitochondrial activation and ROS accumulation under low oxygen. Tumors are facing complicated and fast changing environmental stress. For example, the oxygen levels within tumors varied frequently (Kimura, Braun et al. 1996). Since the regulation of PK-M2 by PHD3 does not require transcription but rather direct protein-protein interaction, it can therefore give more immediate response, and presumably in a reversible way to adjust metabolism according to oxygen availability. Such quick adaptation should provide survival and growth advantages for tumors. In the future, pharmacological interference with the PHD3/PK-M2 interaction could therefore be used as a novel target to reduce tumor growth.

Regulation of PK-M2 is independent of PHD3 activities

Importantly, our data also suggest that regulation of PK-M2 is independent of the hydroxylase activities of PHD3. When PK was identified as a Fatiga/PHD3 binding protein, we examined the possibility whether PK is a novel substrate for prolyl hydroxylation. As for Drosophila proteins, several critical residues in the catalytic domain of Fatiga are absolutely required for the interaction with Sima, but not necessary for binding to PyK. Although it is possible that Fatiga interacts with Sima and PyK using different domains, it is unlikely that mutations in catalytic residues have no effect on PyK binding if it is a substrate. These amino acids at the active site are conserved through the family of Fe$^{2+}$ and 2-OG dependent oxygenases. They are essential for Fe$^{2+}$ incorporation, which is a prerequisite for substrate binding. Crystal structural study of human PHD2 also confirmed the importance of these
residues for Fe$^{2+}$ and substrate incorporation (McDonough, Li et al. 2006). Therefore, they should also be important for binding of other Fatiga substrates.

As for mammalian proteins, PK-M2 does not compete with HIF1α for PHD3 binding, or affect PHD3 activities, both in vitro and in vivo. Since PK-M2 is more abundant than HIF or PHD3, it should have interfered with the PHD3-HIF1α interaction if it is another substrate of PHD3. However, we need more direct evidence to support these assumptions. As one of our plans to better address this question, we would like to perform a ‘rescue’ experiment. In shRNA$^{PHD3}$ cells, we can re-introduce an shRNA resistant PHD3, either wild type or catalytically dead mutant. Changes in PK activities will be monitored, through which we can clarify whether the catalytic activity or the physical interaction is required for regulation of PK-M2. I have not performed that ‘rescue’ experiment in HeLa cells. Instead I have constructed and over-expressed a wild type or an inactive PHD3 mutant in PHD3$^{-/-}$ MEFs. My preliminary results showed that there was no difference between wild type and mutant PHD3 in regulation of PK activities. Over-expression of both wild type and mutant constructs caused a reduction in PK activities that was independent of PEP concentrations. The mechanism under this effect is unclear. However, as described in 2.8, the PK activities of MEFs responded to PEP and FBP differently from HeLa cells. Thus the ‘rescue’ experiment in HeLa cells is important to clarify the requirement of catalytic activities of PHD3.

On the other hand, it is not surprising that PHD3 has functions independent of its prolyl hydroxylase activity. Our results indicate that the regulation of PK-M2 by PHD3 is mainly specific to hypoxic conditions. Under hypoxia, PHD3 activity is restricted by low O$_2$ availability, and such regulation is important for adaptation of cellular metabolism. A recent study showed that re-activation of PHD3 and PHD2 by a 2-OG homologue under hypoxia...
resulted in metabolic catastrophe and cell death (Tennant, Frezza et al. 2009). Interestingly, PHD3 is named ‘oxygen sensor’ because it utilizes oxygen as its substrate. In our model, this activity is probably not required. However, PHD3 still ‘senses’ oxygen levels because itself is upregulated by hypoxia. Under normoxia, PHDs (mainly the PHD2 isoform) inhibit HIF activity. Under hypoxia, PHD3 further limits oxidative phosphorylation by inhibiting PK-M2 and pyruvate production in the presence of HIF. Therefore the function of PHDs as oxygen sensors is more complicated than just targeting HIF for degradation.

**PHD3 and other regulators of PK-M2**

Prior to our research, many studies have characterized the regulation of PK-M2, especially the transition between tetrameric and dimeric/monomeric form. Plenty of evidence showed that in tumor cells, PK-M2 mainly existed as low active dimers to ensure the channeling of glucose carbon into biosynthesis for cell growth. The formation of dimeric PK-M2 can be induced by interaction with various oncoproteins (Mazurek, Zwerschke et al. 2001; Mazurek, Drexler et al. 2007), as well as phosphotyrosine containing peptides (Christofk, Vander Heiden et al. 2008) and certain synthetic peptides (Spoden, Mazurek et al. 2008). Here we have identified PHD3 as a novel regulator that binds to PK-M2 and keeps it as low active dimer. It will be interesting to investigate potential competition between PHD3 and other PK-M2 regulators, such as phosphotyrosine containing peptides. To examine whether PHD3 interferes with phosphotyrosine binding to PK-M2, I treated control or PHD3 knockdown cells with pervanadate. Pervanadate can increase the levels of phosphotyrosine containing peptides, which prevent formation of PK-M2 tetramer and reduce PK activities at low PEP concentration. However, I could not observe any reduction in PK activities by pervanadate in control cells as described in literature (Christofk, Vander Heiden et al. 2008). Thus I cannot
draw any conclusion from this experiment. Nevertheless, it is unlikely that PHD3 competes with phosphotyrosine peptides for PK-M2 binding. PK-M2 interacted with phosphotyrosine via an alternatively spliced exon that is different from PK-M1, whereas PHD3 interacted with both PK-M1 and PK-M2. Thus PHD3 and phosphotyrosine should interact with different domains of PK-M2. Similarly, PHD3 should not compete with FBP binding because FBP also binds to the specific exon. Since FBP could stimulate PK-M2 activities in hypoxic shRNA\textsuperscript{Control} cells, where PHD3 is present, allosteric regulation by FBP seems to be dominant over the regulation by PHD3. It will be interesting to find out whether the conformational change of PK-M2 caused by FBP is enough to dissociate PHD3 from PK-M2.

In addition, it is possible that other factors are involved to regulate the interaction between PHD3 and PK-M2. For example, it has been reported that PHD3 aggregated and assembled into large molecular complexes, where its catalytic activity was repressed (Nakayama, Gazdoiu et al. 2007; Rantanen, Pursiheimo et al. 2008). PK-M2 has also been shown to form complexes with other glycolytic enzymes (Mazurek, Boschek et al. 2005), yet more experiments are required to investigate whether the PHD3-containing complexes associate with glycolytic enzymes.

**Function and mechanism of Fatiga-PyK in *Drosophila***

Our studies in HeLa cells indicate that PHD3 interacts with PK-M2 and inhibits its activities by changing the equilibrium of PK-M2 between tetramer and dimer/momomer. However, for their *Drosophila* homologues Fatiga and PyK, the functional consequences of this interaction are less clear. Although we observed that over-expression of Fatiga reduced PK activities in *Drosophila* larvae, we don’t know whether this inhibition is caused by a mechanism similar
to that in HeLa cells. It is still unknown whether *Drosophila* PyK also exist in both tetrameric and dimeric forms. Our enzymatic assays showed that *Drosophila* PyK was not allosterically stimulated by FBP, suggesting it stays constantly as tetramer, similar to PK-M1. On the other hand, it is possible that *Drosophila* PyK can exist in both forms, but the metabolic environment is so different in *Drosophila* tissues and cells that endogenous FBP concentration is high enough to keep PyK as tetramer. If it is true, adding extra FBP may not further stimulate PyK activities, which can be an alternative explanation to our observation. Thus there are different possibilities to explain the inhibition of PyK by Fatiga over-expression. Firstly, if *Drosophila* PyK can be allosterically regulated, Fatiga might change the balance in a similar way as in HeLa cells. Alternatively, Fatiga may interfere with the association between PyK and other proteins, for example glycolytic complex, and inhibited its activities. The latter possibility is consistent with our observation that the sub-cellular localization of GFP-PyK changes in larval fat body of Fatiga mutant.

To further elucidate the regulation of PyK by Fatiga binding, biochemical assays, such as BN-SDS-PAGE, need to be performed with *Drosophila* samples. A technique limitation, however, is that there are no antibodies available to detect endogenous *Drosophila* Fatiga or PyK. At the moment, another Ph.D. student in our lab is constructing tagged genomic rescues of *Drosophila* PyK and Fatiga genes. Once these transgenic fly lines are ready, they can be analyzed to provide an insight view of the regulation between PyK and Fatiga *in vivo*. 
4. MATERIALS AND METHODS

Plasmid constructions

Constructs for *Drosophila* Proteins

Cu\(^{2+}\) inducible pMT-3HA-Fatiga construct was a kind gift from M. Gstaiger. cDNA of *Drosophila* PyK, Sima, eIF2A, eIF3, eIF4A, Ran, mRPL12 and Cct5, were obtained by PCR amplification from total cDNA isolated from S2 cells, and inserted into pET20 vectors for *in vitro* transcription and translation. cDNA of Ran, PyK and GFP were inserted into pAc5.1/V5-His vector (Invitrogen) for expression in S2 cells. PyK cDNA were inserted into pGEX-6p-1 vector (GE Healthcare) for GST-fusion protein expression. P. Wappner kindly provided the HRE-firefly construct. *wf*-firefly and Tubulin-Renilla constructs were acquired from Basler group. Mutations of Fatiga and Ran were introduced using QuikChange® Site-Directed mutagenesis kit (Stratagene).

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<th>Primers for site-directed mutation</th>
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Constructs for Mammalian Proteins

Human PK-M1 cDNA clone was ordered from imaGenes. Human PK-M2 cDNA was obtained by PCR amplification from total cDNA isolated from HeLa cells. Both cDNA were inserted into pcDNA3-3HA vector (Invitrogen) and pGEX-6p-1vectors. pcDNA3-V5-PHDs
constructs were kind gift from G. Camenisch. GFP tagged PHD3 was inserted into pcDNA3 vector. Constructs for lentivirus production were kindly provided by Krek group.

**Cell culture**

*Drosophila* S2 cells were cultured in GIBCO Schneider's Drosophila liquid Medium (1X) from Invitrogen, supplemented with 10%(v/v) FCS and penicillin/streptomycin. Transfection in S2 cells was performed using jetPEI™ reagent (Brunschwig AG) according to manufacturer’s instruction. pMT-3HA-Fatiga plasmid bearing a blasticidin resistant gene was transfected into *Drosophila* S2 cells and selected using 25uM blasticidin S (Invitrogen) for 14 days. Cells were then kept in 10uM blasticidin S for experiment. dsRNA was produced using MEGAscript® T7 kit (Ambion). For RNAi treatment in S2 cells, cells were incubated with dsRNA in serum free Medium for 1-2 hours before replacing with complete medium.

<table>
<thead>
<tr>
<th>PCR Primers for dsRNA generation</th>
<th>F</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFPRNAi</td>
<td>TTAATACGACTCTATAGGGAGAAACGTAACGCGCCAAGATTTTC</td>
<td>TTAATACGACTCTATAGGGAGATGCCATGCTAGGGTGGAGCAACAGTGGAT</td>
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<tr>
<td>LucRNAi</td>
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<td>TTAATACGACTCTATAGGGAGATGCCATGCTAGGGTGGAGCAACAGTGGAT</td>
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<tr>
<td>RNAiPyK</td>
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<td>TTAATACGACTCTATAGGGAGAAGATCAAGCGCCACAGTGGAT</td>
</tr>
<tr>
<td>RNAiRanF</td>
<td>TTAATACGACTCTATAGGGAGAAGATCAAGCGCCACAGTGGAT</td>
<td>TTAATACGACTCTATAGGGAGAAGATCAAGCGCCACAGTGGAT</td>
</tr>
<tr>
<td>RNAiRanF</td>
<td>TTAATACGACTCTATAGGGAGAAGATCAAGCGCCACAGTGGAT</td>
<td>TTAATACGACTCTATAGGGAGAAGATCAAGCGCCACAGTGGAT</td>
</tr>
</tbody>
</table>

HeLa cells and MEFs were cultured in DMEM Medium (Invitrogen) supplemented with 10%(v/v) FCS and penicillin/streptomycin. Transfection experiments were conducted using the FuGENE 6 Transfection Reagent from Roche according to manufacturer’s instruction.
Chemicals and Antibodies

If not specified, chemicals were obtained from Sigma. The following antibodies were used: mouse anti-V5 (1:3000, Invitrogen), rabbit anti-PKM (1:1000 for Blot and 1:100 for staining, Cell signaling), mouse anti PHD3 (1:500, kindly provided by Cockman M. and Ratcliffe P.), mouse anti-tubulin (1:5000, Sigma), mouse anti-HA (HA.11, 1:1000 for Blot and 1:250 for staining, Covance Research Products), mouse anti-lacZ (1:1000), goat anti-rabbit conjugated with Alexa fluor 568 (1:500, Molecular Probes), goat anti-mouse conjugated with Alexa fluor 488 (1:400, Molecular Probes), goat anti-mouse conjugated with Alexa fluor 680 (1:1000, Molecular Probes, for Blot), and goat anti-rabbit IRDye 800 CW (1:5000, for Blot, LI-COR biosciences).

Fly stocks and handling

The following fly lines were used: yw; +; +; For mutant alleles of PyK (CG7070): yw; +; pykd05514; and yw; +; pykDG05605; and yw; +; pykEY10213/TM6B; and yw; +; pykf06019/TM6B; For GFP-PyK expression: yw; +; GFP-PyK; and yw; +; fga1 GFP-PyK/TM6B; and yw; +; fgaS030304 GFP-PyK/TM6B; For hypoxia reporter: yw; LDH-Gal4 UAS-lacZ/+; pykEY10213/TM6B; For over-expression of Fatiga: w; +; Act>CD2>Gal4, UAS-GFP; and hs-Flp122; UAS-Fatiga11.1; +; For double mutant: yw; +; fga1/TM6B; yw; +; fgaS030304/TM6B; yw; +; sima07607; .

Flies were kept at 25°C, 60% humidity and a 12 hours day-night light-cycle. For experiment, flies were kept at a density of 50 larvae/vial. We use feeding, mid-3rd instar larvae (4 or 5 days after egg deposition as indicated). For RNA isolation and protein extracts, larvae were frozen in liquid nitrogen and stored at -80°C before homogenization.
**Hypoxia treatment**

*Drosophila* larvae were incubated at 9% O$_2$ in a tightly sealed Modular incubator chamber (Billups-Rothenber, Inc.) at room temperature. Cells were incubated at 37°C, 5% CO$_2$, 1% O$_2$ in an Invivo2 400 Hypoxia Workstation (Ruskinn).

**Affinity pull-down and Mass spectrometry**

1X10$^9$ S2 cells were used for each pull-down experiment. 200uM CuSO$_4$ was added to induce expression of HA-Fatiga 40 hours before harvest. Cells were lysed in 10 ml cold TNN buffer (50mMTris PH7.5, 5mM EDTA, 250mM NaCl, 0.5% NP40, 1mM DTT, 1mM PMSF, 50mM NaF, 1.5mM Na$_3$VO$_4$, and mixed Protease inhibitor from Roche) and cleared by centrifugation. Supernatant was incubated with 200ul anti-HA Affinity Matrix from Roche. After washing (3X10min), protein was eluted with 0.2M Glycine PH2.5, neutralized with 1M NH$_4$CO$_3$, PH8.8, reduced with 5mM TCEP (tris (2-carboxyethyl) phosphine) and alkylated with 10 mM iodoacetamine before trypsin (Promega) digestion. Digested peptides mixture were then acidified with 1% FA and purified by using Ultra-micro-spin columns (Harvald Apparatus). LC-MS/MS was performed to identify proteins in each sample.

**Luciferase assay**

1X10$^5$ S2 cells were plated in 96 well plates in triplicates. Luciferase plasmids (0.1 ug HRE-firefly or 0.1 ug wf-firefly together with 0.01 ug Tubulin-Renilla) were transfected into each well. For over-expression, 0.1 ug pAc-Ran plasmid (wild type or P160A mutant) was co-transfected with Luciferase plasmids. For RNAi, cells were incubated with 2ug dsRNA in serum-free medium for 1 hour before transfection. Cells were harvested 48 hours after
transfection or RNAi. Luciferase assay was performed using Duo Luciferase Assay kit (Promega). Briefly, 100 ul cold 1XPLB buffer were added into each well. Cells were lysed in for 30 min by rotating. 10ul of lysate was used for Luciferase assay using MicroLumatPlus LB96V luminometer (EG&G Berthold).

**Recombinant protein production**

Recombinant GST-Fatiga protein was expressed in DH5α cells. GST-PK-M1 or GST-PK-M2 were expressed in BI21 (DE3) cells. Cells were grown to an absorbance of 0.6 at 600 nm, induced with 0.2 mM IPTG for 16 hrs at 18°C. Cells were then lysed by lysozyme in cold lysis buffer (50 mM Tris PH8.0; 500 mM NaCl; 1mM EDTA; 1mM DTT; 1mM PMSF; 1.5mM Na3VO4; Protease inhibitor) and cleared by centrifugation. Supernatant was incubated with Glutathione sepharose beads (GE healthcare). The resin was washed extensively before pull-down experiment. Otherwise GST fusion proteins were eluted with 20mM glutathione in elution buffer (50 mM Tris PH7.5; 100 mM NaCl; 2mM DTT). To purify rPK-M2, glutathione was removed by dialysis overnight at 4°C. GST was cleaved by 3C protease and removed by Glutathione sepharose.

**Protein interaction studies**

In vitro translated [S35] methionine-labeled proteins were generated using TNT® Coupled Reticulocyte Lysate Systems (Promega) and incubated in binding buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5% glycerol, 0.25% Nonidet P-40, 1 mM dithiothreitol and protease inhibitor cocktail) with glutathione beads bearing GST or GST fusion proteins on rotator for
3 h at 4°C. After washing (4X5min), the bound proteins were eluted in SDS loading buffer, resolved by SDS–polyacrylamide gel electrophoresis before analyzed by autoradiography.

**Immunoprecipitation and blotting**

For co-immunoprecipitation, cells were lysed in buffer containing 50mM HEPEs pH 7.4, 150mM NaCl, 0.3% Nonidet P-40, 1mM EDTA, 1 mM dithiothreitol, 1.5mM Na$_3$VO$_4$ and protease inhibitor. Supernatant of cell lysate was then incubated with protein A Sepharose (GE Healthcare) and indicated antibodies (10ug anti-V5 antibody or 10ul anti-PHD3 antibody) at 4°C, 3hrs. After washing (3X3min.), SDS sample buffer was added to the beads and boiled before SDS-PAGE and Blot. Western blots were developed using Odyssey Infrared Imagines System (LI-COR Biosciences).

**Staining and microscopy**

For *Drosophila* fat body samples, 4-day-old larvae were inverted and fixed in 8% paraformaldehyde for 2 hrs. After washing with PBS containing increased concentration of Triton 100 (0.2%-1%), samples were incubated with 1% milk in PBS (1% Triton) at 4°C overnight. The larvae were washed again with PBS containing decreasing concentration of Triton 100 (1%- 0.2%) and then incubated with anti-mouse lacZ antibody in PBS (0.2% Triton 100) at room temperature for 2 hrs. After washing for 3X30min, second antibody and DAPI (1:1000) were added and incubated for 1 h. Samples were then washed over night, the fat bodies were mounted on slides in Vectashield solution (Vector Labs). Images were taken on a Deltavision Olympus K70 microscope or an Apotome Zeiss microscope using 40X objectives.
For cell samples, cells were cultured on coverslips. Cells were fixed in 4% paraformaldehyde for 15 min. After washing by PBS (3X3min), cells were incubated with blocking solution (2% NGS in PBS/0.3% Triton 100) for 30min. First antibody was then added and incubated for 2 hrs at room temperature. After washing by PBS (3X3min), second antibody together with DAPI (1:1000) were added and incubated for 45min. Coverslips were then washed again (4X5min), and mounted using Vectashield solution. Images were taken on a Zeiss Lsm-710 confocal microscope using 63X objectives.

**in vitro prolyl-4-hydroxylation assays**

Enzymatic activity of recombinant PHD3 was determined as described (Nytko, Spielmann et al. 2007). Biotinylated peptides (100 ng/well) derived from human HIF-1α aa 556 to 574 (either wild-type or P564A mutant) were bound to Avidin-coated 96-well plates (Pierce). Baculoviral purified recombinant PHD proteins were used to hydroxylate the peptides in the presence of 0.5 mM 2-oxoglutarate, 2 mM ascorbate, and 10 uM FeSO4 in buffer containing 20 mM Tris-HCl pH 7.5, 5 mM KCl, and 1.5 mM MgCl2 for 1 h at room temperature. A polycistronic expression vector for His6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was expressed in bacteria. VBC complex was purified and bound to the hydroxylated peptides. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies, followed by secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma), using the TMB (3,3’, 5,5’-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding 1M H2SO4 and absorbance was determined at 450 nm in a microplate reader.
shRNA constructs and lentiviral production

shRNA constructs in lentiviral cassettes were ordered from Sigma (Mission shRNA). An shRNA with high PK knockdown efficiency was used as shRNA^{PKM} (5’-CCGGGCTGTGGCTCTAGACACTAAACTCGAGTTAGTCTAGAGCCACAGCTTTTTG-3’). An shRNA with high PHD3 knockdown efficiency was used as shRNA^{PHD3} (5’-CCGGCACCTGCATCTACTATCTGAACCTCGAGTTAGTAGATGCAGGTGTGTTTTT-3’). An shRNA with no effect was used as shRNA_{Control} (5’-CCGGCGCAAGCTGGTTGAAACTTCTCGAGTTGAACTTCTCAACAGCTTTGTTTTG-3’). Lentivirus was made using a three-plasmid packaging system. Briefly, shRNAs in the pLKO.1-puro vector were co-transfected into 293T cells along with expression vectors containing the gag/pol, rev and vsvg genes. Lentivirus was harvested 48 h after transfection, and 5μg/ml polybrene was added. Subconfluent HeLa cells were infected with harvested lentivirus, and were selected using 2μg/ml puromycin for 10 days.

Quantitative PCR

Total RNA were isolated using NucleoSpin RNA II (Macherey-Nagel). For cDNA synthesis, Read-To-Go You-Prime First-Strand beads (GE healthcare) were used. Quantitative real time PCR was performed using the Light Cycler 480 (Roche).

<table>
<thead>
<tr>
<th>qPCR Primers for mammalian Proteins</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPHD3</td>
<td>ATCGACAGGCTGGCTCTCTC</td>
<td>GATAGCAAGCCACCATTGC</td>
</tr>
<tr>
<td>hPK-M2</td>
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<tr>
<td>hPFKP</td>
<td>CTTGTGAGGTGAGGGATG</td>
<td>CATGGGGAGACAGAGACTCCT</td>
</tr>
</tbody>
</table>
Measurement of Pyruvate Kinase activities

For *Drosophila* larvae samples, twenty 4-day-old larvae were homogenized in 300ul lysis buffer (50mM HEPEs pH 7.4, 150mM NaCl, 0.5% Triton 100, 1mM EDTA, 1 mM dithiothreitol, 1.5mM Na$_3$VO$_4$ and protease inhibitor). For cells, samples were lysed in cold CelLytic™ M (Sigma) supplemented with protease inhibitor and 2mM DTT with gentle shaking. Pyruvate Kinase activities were measured by a kinetic assay coupled to lactic dehydrogenase (LDH) reaction. Continuous change in absorbance at 340nm was measured using a SpectraMAX 190 spectrophotometer (Molecular Devices). Enzymatic assay mixture contained indicated recombinant protein (1-5ng) or cell lysate (1–2 mg), HEPES pH 7.2 (50 mM), KCl (70 mM), MgCl$_2$ (15 mM), ADP (3 mM), NADH (0.2 mM), LDH (6.7 units), and PEP (5 mM or 0.2mM, as indicated). Activities were then normalized to protein amount and indicated as arbitrary unit.

Gel filtration

1X 10$^7$ Cells were lysed in 500 ul lysis buffer containing 50mM HEPEs pH 7.4, 150mM
NaCl, 0.3% Nonidet P-40, 1mM EDTA, 1 mM dithiothreitol, 1mM PMSF, 1.5mM Na$_3$VO$_4$ and protease inhibitor. After centrifuge at 30,000 rpm for 1hr, 200ul supernatant was injected into Superose$^\text{TM}$ 6 column (GE healthcare). The elution buffer contains 50mM HEPES pH 7.4, 200mM NaCl, 1 mM dithiothreitol, 10uM PMSF. The elution rate was 0.2 ml/min. Fraction volume was 0.25ml.

**Blue Native PAGE and 2D Electrophoresis**

Cells were lysed in cold CelLytic$^\text{TM}$ M (Sigma) supplemented with protease inhibitor and 2mM DTT with gentle shaking and cleared by centrifugation. Supernatant containing 30ug protein was loaded to NativePAGE Novex 4-16 % Bis-Tris Gel (invitrogen) and electrophoresis was performed according to manufacturer’s instructions. After performing the first-dimension BN-PAGE, gel slice was cut out and incubated in SDS Sample Buffer (Tris 12.5 mM, PH 6.8; SDS 4%; Glycerol 20%; Bromophenol blue 0.02%) for 20 min before SDS-PAGE. Second dimension SDS-PAGE was performed according to standard protocols.

**Measurement of metabolites levels**

5 X 10$^7$ subconfluient cells were incubated for 18 hours at 1 % O$_2$. Cells were quickly trypsinized inside hypoxia hood (1% O$_2$) and pellets were frozen in liquid nitrogen. Metabolites were extracted in 1 ml 70% methanol at -20$^\circ$C and were centrifuged at 5000 rpm for 5 min at -9$^\circ$C. Supernatants were dried at 0.14 mbar to complete dryness in a sped vac composed of an Alpha 2-4 LDplus cooling trap, RVC2-33 rotational vacuum concentrator, and RC- vacuum chemical hybrid pump (Christ, Osterode am Harz, Germany). The dry
metabolite pellet was resuspended in 100 ul H2O. Metabolite were measured on an Agilent 1100 series high-performance liquid chromatography system coupled to an Applied Biosystems/MDS SCIEX 4000 Q TRAP mass spectrometer. Chromatographic separation was performed using a Waters Atlantis T3 150- by 2.1-mm by 3-µm column at 40°C (Buscher, Czernik et al. 2009). The injected volume was 8 µl. Samples were measured in triplicates and normalized to protein amount.

**Measurement of lactate production**

Lactate production was measured using Lactate Assay Kit II (BioVision). 5 ml fresh media was added to 10 cm plate of subconfluent cells, and aliquots of media were assessed after 1.5 hrs. Lactate levels were then normalized to cell numbers, which were determined using a Coulter particle analyzer.

**Measurement of intracellular ROS**

Cells (5 X 10^5) were plated into 60mm plates and incubated at 1 % O₂ for 20 hrs. 1 hour before measurement, cells were incubated with DMEM containing 50 uM Carboxy-H2DCFDA (5-6-carboxy-2’,7’-dichlorodihydro- fluorescein diacetate, Molecular Probes) and 1uM Mito Tracker Red (Molecular Probes). Cells were quickly washed and lysed in 200 ul lysis buffer (25mM Tris, PH7.5; 2mM dithiothreitol; 10 % glycerol; 1 % Triton X-100). DCF fluorescence (excitation, 500 nm; emission, 530nm) was measured in the supernatant of cell lysate using a Spectra GeminiXS spectrofluorimeter (Molecular Devices). DCF data were normalized to mitochondria content estimated with Mito Tracker Red fluorescence (excitation, 580nm; emission, 610nm).
Cell Proliferation analysis

5 X 10^4 cells were seeded in 60mm Plates in triplicates, accurate cell counts were obtained every 24 hrs using a Coulter particle analyzer for 4-day period.

Statistical analysis

In all experiments, significance was determined using the Student’s t-distribution (two-tailed; two-sample equal variance). *** equals P<0.001; ** equals P<0.01; * equals P<0.05; ns: not significant.
5. REFERENCES


