Oxygen sensing by the prolyl-4-hydroxylase PHD2 within the nuclear compartment and the influence of compartmentalisation on HIF-1 signalling

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Summary

Hypoxia-inducible factors (HIFs) regulate more than 200 genes involved in cellular adaptation to reduced oxygen availability. HIFs are heterodimeric transcription factors that consist of one of three HIF-α subunits and a HIF-β subunit. Under normoxic conditions the HIF-α subunit is hydroxylated by members of a family of prolyl-4-hydroxylase domain (PHD) proteins, PHD1, PHD2 and PHD3, resulting in recognition by von-Hippel–Lindau protein, ubiquitylation and proteasomal degradation. It has been suggested that PHD2 is the key regulator of HIF-1α stability *in vivo*. Previous studies on the intracellular distribution of PHD2 have provided evidence for a predominant cytoplasmic localisation but also nuclear activity of PHD2. Here, we investigated functional nuclear transport signals in PHD2 and identified amino acids 196–205 as having a crucial role in nuclear import, whereas amino acids 6–20 are important for nuclear export. Fluorescence resonance energy transfer (FRET) showed that an interaction between PHD2 and HIF-1α occurs in both the nuclear and cytoplasmic compartments. However, a PHD2 mutant that is restricted to the cytoplasm does not interact with HIF-1α and shows less prolyl hydroxylase activity for its target HIF-1α than wild-type PHD2 located in the nucleus. Here, we present a new model by which PHD2-mediated hydroxylation of HIF-1α predominantly occurs in the cell nucleus and is dependent on very dynamic subcellular trafficking of PHD2.

Key words: Hypoxia, Prolyl-hydroxylase, HIF, NLS, NES, Importin, CRM1

Introduction

Due to an imbalance in oxygen supply and demand, solid tumours commonly evolve hypoxic regions associating with malignant cancer phenotype and poor patient survival. As a result, hypoxia stimulated pathways, including the hypoxia-inducible factor (HIF) pathway, are commonly activated in tumours. The role of HIF in gene expression induced by reduced oxygen availability in tumours has been well established (Maxwell et al., 1997; reviewed by Moeller and Dewhirst, 2006). HIFs are heterodimeric basic-helix-loop-helix Per/Arnt/Sim (bHLH-PAS) transcription factors composed of a constitutive β-subunit (ARNT; arylhydrocarbon receptor nuclear translator) and one out of three O_2-labile α-subunits (HIF-1α, -2α or -3α) (Lee and Percy, 2011; Semenza, 2010). In normoxia, HIF-α subunits are hydroxylated at two crucial proline residues, leading to recognition by von-Hippel–Lindau tumour suppressor protein (pVHL) and proteasomal degradation. This process is initiated by specific prolyl-4-hydroxylase domain (PHD) proteins, namely PHD1, PHD2 and PHD3 (Epstein et al., 2001). Despite similar *in vitro* enzymatic properties and the fact that overexpression of each PHD isoform suppresses HIF-dependent transcriptional activity differences among the three PHDs could be identified (Huang et al., 2002). Each PHD isoform is expressed in a tissue and cell type specific manner, has its distinct subcellular localisation pattern and shows differential activities towards HIF-1α and HIF-2α (Appelhoff et al., 2004; Metzen et al., 2003; Steinhoff et al., 2009). Inhibition of PHD2 by RNA interference was shown to be sufficient to upregulate HIF-1α in normoxia, suggesting PHD2 as the key regulator of HIF-1α *in vivo* (Appelhoff et al., 2004; Berra et al., 2003).

Investigations on intracellular localisation showed that PHD1 is present exclusively in the nucleus, PHD2 is mainly cytoplasmic and PHD3 is located both in the nucleus and the cytoplasm (Metzen et al., 2003; Steinhoff et al., 2009). In line with this data Wotzlaw and colleagues demonstrated that PHD1 and HIF-1α interact within the nuclear compartment (Wotzlaw et al., 2010). Recently, the expression patterns, as well as the subcellular localisation of PHDs, have been linked with tumorigenesis. Different studies demonstrated the promoting role of PHD2 nuclear localisation for carcinoma cell growth. Jokilehto and colleagues (Jokilehto et al., 2006; Jokilehto and Jaakkola, 2010) demonstrated that increased levels and nuclear translocation of PHD2 associate with tumour aggressiveness and that high nuclear expression of PHD2 increases anchorage-independent carcinoma cell growth (reviewed by Jokilehto and Jaakkola, 2010). Moreover, it has been shown that low nuclear staining of PHD2 associates

with increased treatment success in radiotherapy, suggesting PHD2 expression as a marker for radiation resistance (Luukkaa et al., 2009). Accordingly, increased PHD expression and nuclear PHD translocation have been associated with poor survival in pancreatic endocrine tumours (Couvelard et al., 2008). However, impacts of PHD intracellular localisation on tumour development, the precise localisation of cellular oxygen sensing and molecular mechanisms underlying nuclear import and export of the three PHDs, are hitherto hardly known.

In order to cross the nuclear pore complex (NPC), proteins larger than ~40 kDa require binding to specific nuclear transport receptors. The 'classical' nuclear import is mediated by the importin α/β receptor family and depends on short peptide motifs within cargo proteins, known as nuclear localisation signals (NLS) (Fontes et al., 2000). Importin α forms a heterodimer with importin β, which in turn mediates docking and translocation through the nuclear pore complex (NPC). A couple of proteins can directly bind the importin β subunit and do not utilise the adaptor protein importin α (Riddick and Macara, 2007). Classical NLS consensus sequences have short clusters of basic amino acids in common. Nuclear export is mediated by leucine rich nuclear exclusion signals (NES) that are recognised by nuclear export receptors of which CRM1 (chromosome region maintenance1) is the most abundant exportin (reviewed by Ossareh-Nazari et al., 2001).

Recently, we and others studied the involvement of different nuclear transport receptors in regulation of the oxygen sensing pathway (Chachami et al., 2009; Depping et al., 2008). Moreover, we could demonstrate that nuclear import of PHD1 is importin-α/β-dependent and relies on a nuclear localisation signal (NLS) (Steinhoff et al., 2009).

In this study, we wanted to characterise the mechanism and functional effects of the regulated nuclear translocation of PHD2, because the import and export of large molecules from the nucleus is a very important regulatory step for many cellular processes (Vandromme et al., 1996). Here we have studied the localisation of several PHD2 deletion constructs, as well as the impact of PHD2 intracellular localisation, on the HIF signalling pathway. We identified functional targeting signals in PHD2 mediating nuclear import and export of the protein and resolved the contribution of PHD2 intracellular localisation to the interaction with its target HIF-1α, to its enzymatic activity for HIF-α hydroxylation and to HIF-1α protein expression. These results indicate that nuclear–cytoplasmic trafficking of PHD2 is vitally important for interaction with and hydroxylation of HIF-1α and subsequent degradation. Our data provide evidence for observations of a crucial role of PHD2 nuclear localisation in tumour biology and the pathophysiology of ischaemic disease.

Results

Nuclear import of PHD2 is mediated by a non-classical localisation signal

EGFP-fused PHD2 was expressed in U2OS cells and cellular distribution was analysed by fluorescence microscopy. In order to study intracellular transport mechanisms, cells were treated with the nuclear export inhibitor LMB which specifically inhibits the export receptor CRM1. PHD2–EGFP appeared mainly in the cytoplasm in untreated cells but distributed throughout the whole cell after inhibition of CRM1 (Fig. 1A). To identify intracellular targeting sequences in the primary sequence of PHD2, we analysed intracellular localisation of different PHD2 deletion constructs (PHD2Δ181–220, PHD2Δ188–195, PHD2Δ191–195, PHD2Δ191–192 and PHD2Δ196–205) expressed in U2OS cells and compared the localisation to wild-type PHD2–EGFP (Fig. 1A). Deletion mutant PHD2Δ181–220 was restricted to the cytoplasm and was not translocated to the nucleus before as well as after LMB treatment. However, deletion of amino acids 191–192 had no effect on intracellular localisation of PHD2. In contrast, nuclear import of the deletion mutants PHD2Δ188–195, PHD2Δ191–195 and PHD2Δ196–205 was inhibited. These results strongly suggest that the region 188–205 is involved in nuclear import of PHD2. However, the deletion constructs PHD2Δ188–195 and PHD2Δ191–195 were still imported into the nucleus albeit to a small degree after inhibition of CRM1. Deletion mutant PHD2Δ196–205 generally resided in the cytoplasm, indicating that amino acids 196–205 can be regarded as a component of a putative non-classical NLS. To determine whether the identified nuclear targeting signal is sufficient to mediate nuclear import of PHD2, amino acids 180–220 of PHD2 were fused to EGFP–EGFP–GST (EEG, kindly provided by D. Doenecke). Expression of EEG in mammalian cells results in cytoplasmic localisation because it is too large to enter the nucleus by passive diffusion (Kahle et al., 2009). Fusion of PHD2 amino acids 180–220 to EEG resulted in nuclear import of the fusion protein, indicating functional NLS activity of PHD2 amino acids 180 to 220 (Fig. 1B).

The N-terminal part of PHD2 is essential for nuclear export

In order to clarify the nuclear shuttling mechanism we analysed the presence of a putative NES in the PHD2 sequence. The N-terminal part of the protein is essential for nuclear export as the deletion mutant PHD2Δ1–100 showed balanced nuclear and cytoplasmic localisation without inhibition of nuclear export (Steinhoff et al., 2009). Therefore, the EGFP-tagged N-terminal deletion mutant PHD2Δ6–20 was constructed and expressed in U2OS cells. Translocation of this protein to the nucleus was observed. The transfected cells (mutant PHD2Δ6–20) showed equally distributed cytoplasmic and nuclear fluorescence intensities before LMB treatment (Fig. 2A). Localisation of PHD2Δ6–20 did not change after inhibition of CRM1 by leptomycin B. These results strongly suggest amino acids 6–20 as an essential component of the NES of PHD2. In order to analyse the involvement of phosphorylation sites in nuclear export, we generated serine point mutants PHD2S5A, PHD2S12A, PHD2S14A and PHD2S39D and analysed their subcellular localisation (Fig. 2B). Comparable to wild-type PHD2, the PHD2 point mutants were localised to the cytoplasm before LMB treatment indicating that nuclear export of PHD2 does not depend on serine residues located in the N-terminal part of the primary sequence.

Expression of the EGFP tag does not impact on the intracellular localisation of PHD2

In order to exclude side effects caused by the expression of the EGFP tag, indirect immunofluorescence analyses were performed using plasmids encoding non-tagged PHD2 constructs (Fig. 3). Wild-type PHD2 as well as PHD2 deletion mutants PHD2Δ196–205 and PHD2Δ6–20 showed subcellular expression patterns comparable to those of EGFP-tagged fusion proteins indicating that the EGFP tag itself does not have an impact on the specific PHD2 intracellular localisation.

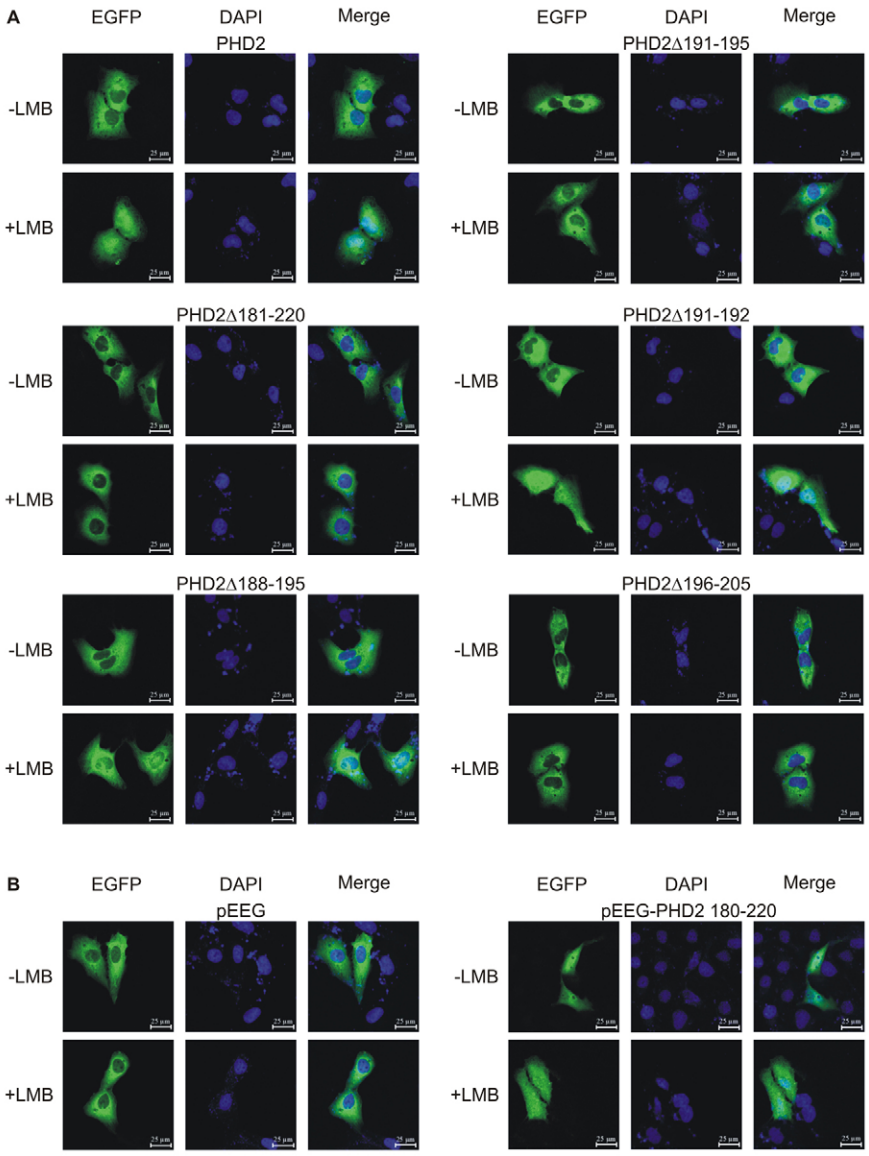

Fig. 1. Identification of the molecular determinants for nuclear import of PHD2. (**A**) U2OS cells were transiently transfected with EGFP-tagged wild-type PHD2, PHD2Δ188–195, PHD2Δ196–205, PHD2Δ191–192 or PHD2Δ181–220. Experiments were performed without (upper panel) and with (lower panel) addition of the nuclear export blocker leptomycin B (LMB). LMB treatment results in nuclear accumulation of PHD2. NLS deletion (PHD2Δ196–205, PHD2Δ181–220) inhibits nuclear import of PHD2. Shown are representative microscopic images (Leica TCS SP5, Leica, Germany), taken at a final magnification of 1000 fold. (**B**) EGFP–EGFP–GST (EEG) or EEG–PHD2-180–220 was transiently expressed in U2OS cells. Fusion of the PHD2 sequence motif to EEG enables nuclear import of the construct. Scale bars: 25 μm.

Oxygen sensing by PHD2 is localised within nuclear compartment

To further analyse the compartmentalisation of PHD2, the interaction of PHD2 and HIF-1α was analysed by FRET measurements. To this aim, HEK293 cells were co-transfected with the donor molecule ECFP–HIF-1α and PHD2–EYFP as the acceptor molecule. Addition of DMOG provided stabilisation of HIF-1α in the nucleus and to a lower level in the cytoplasm. After HIF-1α stabilisation, high FRET efficiencies were detected in both compartments indiciating an interaction between ECFP–HIF-1α and PHD2–EYFP in the nucleus as well as the cytoplasm (Fig. 4A,B, FRET efficiency 20%). The importance of PHD2 intracellular localisation was further investigated by cotransfection of ECFP–HIF-1α and the deletion constructs PHD2Δ195–205–EYFP and PHD2Δ6–20–EYFP. As shown in Fig. 4A,B, transfection of PHD2Δ195–205–EYFP, a mutant that is not imported into the nucleus, did not reveal any interaction between HIF-1α and PHD2 (FRET efficiency 0%). In contrast, high FRET efficiencies were detected in cell nuclei after transfection with ECFP–HIF-1α and PHD2Δ6–20–EYFP (Fig. 4A,B), a mutant deficient for the putative nuclear export signal (FRET efficiency 23%). However, co-transfection of the different PHD2 variants with the cytoplasmic localised HIF-1α mutant (HIF-1α-mut–ECFP, K719AK753A) revealed high FRET efficiencies in the cytoplasm (Fig. 4A,C, FRET efficiencies 25% [PHD2Δ196–205), 24% (PHD2Δ6–20) and 22% (PHD2)]. In case of co-transfection of HIF-1α-mut–ECFP with PHD2Δ6–20–EYFP also low amounts of HIF-1α-mut–ECFP localised in cell nuclei, where interaction with PHD2Δ6–20–EYFP lead to nuclear FRET signal (Fig. 4A). These data confirm interaction between PHD2 and HIF-1α to both compartments. However, the intracellular localisation of PHD2 is important for its role as oxygen sensor in the HIF-1 pathway.

Overexpression of PHD2 in the nucleus inhibits HIF-1α protein expression

The impact of PHD2 intracellular localisation on HIF-1α protein level was investigated by western blot analysis. For this reason, U2OS cells were transiently transfected with HIF-1α

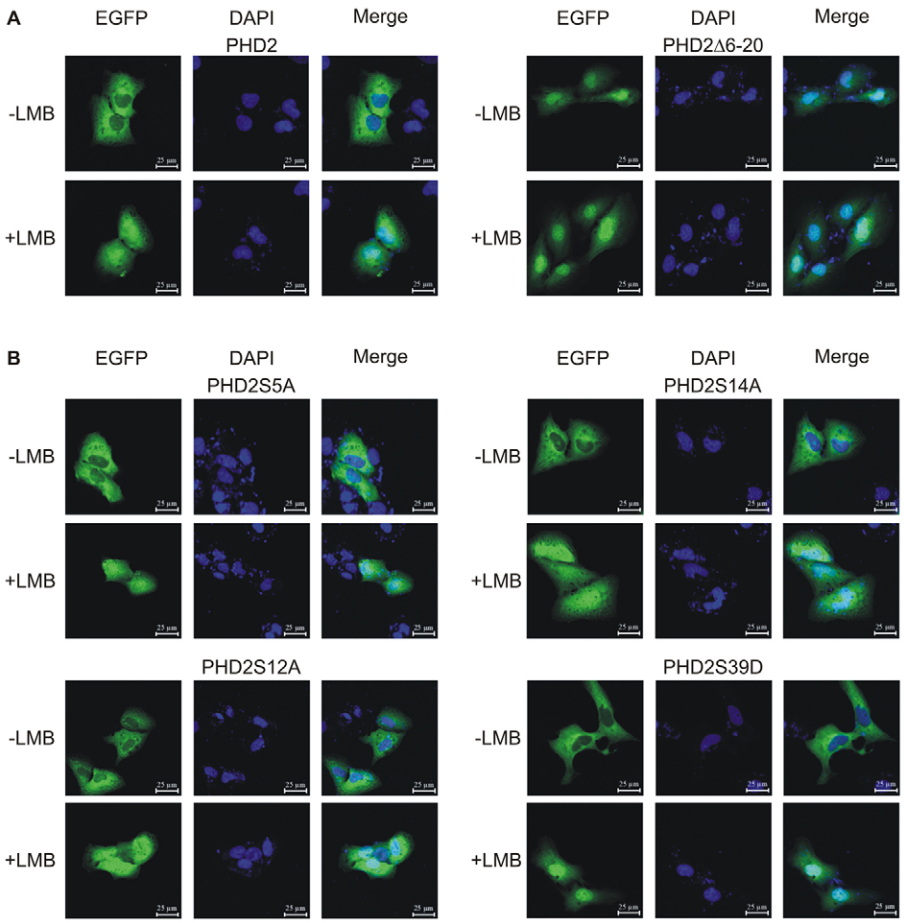

Fig. 2. Characterisation of the nuclear export of PHD2. U2OS cells were transiently transfected with (**A**) EGFP-tagged wild-type PHD2, the deletion mutant PHD2Δ6–20, or (**B**) the point mutants PHD2S5A, PHD2S12A, PHD2S14A and PHD2S39D. Deletion of the NES (PHD2Δ6–20) causes nuclear PHD2 accumulation with and without LMB treatment, whereas mutation of crucial serine residues does not affect subcellular PHD2 localisation. Cells were treated without (upper panel) and with (lower panel) LMB. Representative microscopic images (Leica TCS SP5, Leica, Germany) are shown, taken at a final magnification of 1000 fold. Scale bars: 25 µm.

and the EGFP-tagged PHD2 constructs. Overexpression of the PHD2 mutant restricted to the cytoplasm, PHD2Δ196–205, HIF-1α protein expression levels were significantly higher than in cells overexpressing wild-type PHD2 or PHD2Δ6–20 (Fig. 5) indicating that PHD2Δ196–205 shows less enzymatic activity for HIF-1α hydroxylation. These data demonstrate that HIF-1α protein levels are affected by intracellular localisation of PHD2.

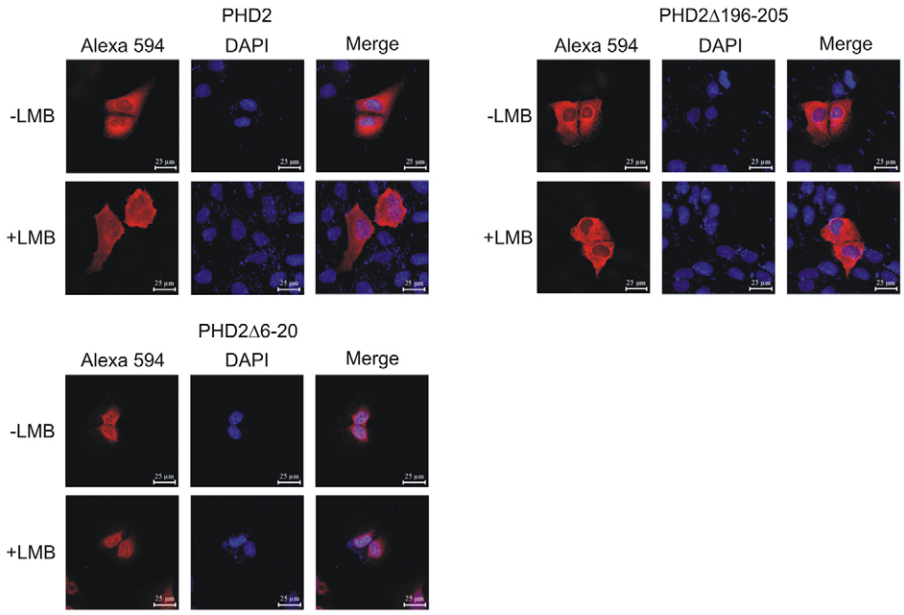

Fig. 3. Intracellular PHD2 localisation is not influenced by expression of the EGFP tag. U2OS cells were transiently transfected with wild-type PHD2 or the deletion mutants PHD2Δ196–205 and PHD2Δ6–20. Deletion of a putative NLS (PHD2Δ196–205) results in cytoplasmic PHD2 retention with and without treatment with LMB. Deletion of the NES (PHD2Δ6–20) causes nuclear PHD2 accumulation with and without the addition of LMB. PHD2 was detected by immunofluorescence analysis. Cells were treated without (upper panel) and with (lower panel) LMB. Representative microscopic images (Leica TCS SP5, Leica, Germany) are shown, taken at a final magnification of 1000 fold. Scale bars: 25 µm.

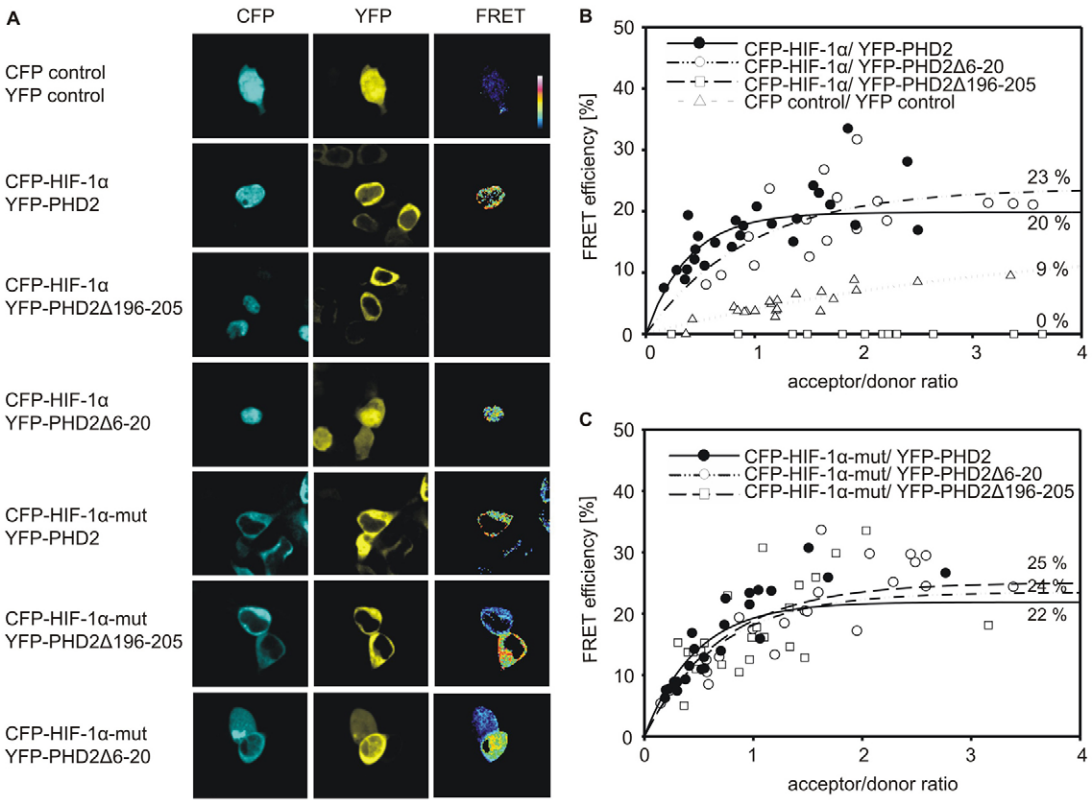

Fig. 4. NLS deletion inhibits the interaction between ECFP–HIF-1α and PHD2–EYFP. U2OS cells were transiently co-transfected with ECFP-tagged HIF-1α or HIF-1α-mut (HIF-1α K719AK753A) and EYFP-tagged wild-type PHD2 (PHD2) or the deletion mutants PHD2Δ6–20 and PHD2Δ196–205. Cells co-transfected with ECFP or EYFP empty vectors served as controls. (**A**) Representative microscopic images of the respective co-transfected cells observed in the CFP channel (cyan colour), YFP channel (yellow colour) and FRET channel (false colours indicate signal intensity), taken at a magnification of 400 fold. Because nuclear-localised HIF-1α and cytoplasmic-localised PHD2Δ196–205 were not colocalised in cells, FRET could not be observed. (**B**) FRET efficiency (%) was calculated for cells co-transfected with ECFP-tagged HIF-1α and EYFP-tagged PHD2 constructs. The maximum FRET efficiency of co-transfected cells was 23% for HIF1α and PHD22Δ6–20, and 20% for HIF1α and PHD2 whereas the cells transfected with ECFP and EYFP empty vector showed a maximum FRET efficiency of 8.9%. FRET efficiency of HIF-1α and PHD2Δ196–205 was 0% because of the lack of colocalisation. (**C**) FRET efficiency (%) was calculated for cells co-transfected with ECFP-tagged HIF-1α-mut (HIF-1α K719AK753A) and EYFP-tagged PHD2 constructs. HIF-1α-mut–ECFP was predominantly localised in the cytoplasm where it colocalised with all of the PHD2 variants. The maximum FRET efficiency of co-transfected cells was 25% (PHD2Δ196–205), 24% (PHD2Δ6–20) and 22% (PHD2).

Nuclear PHD2 localisation inhibits HIF-dependent transcriptional activity

We next sought to investigate the functional relevance of intracellular localisation of PHD2 on HIF transactivation. Therefore, HIF-dependent reporter gene assays were performed for the evaluation of the prolyl hydroxylase activity of wild-type and mutant PHD2 which is represented by a reduction of HIF reporter activity. U2OS cells were transiently transfected with a HIF-dependent firefly luciferase gene, a *Renilla* luciferase reporter vector for internal normalisation and the indicated EGFP tagged PHD2 constructs (wild-type, Δ196–205, Δ6–20) (Fig. 6). In line with earlier studies HIF-dependent firefly responded to hypoxia by more than 5-fold higher transcriptional activity than in normoxia (data not shown). The hypoxic luciferase activity was significantly reduced by overexpression of EGFP-tagged wild-type PHD2 compared to control transfections with the empty vector control (pEGFP-N1). Transfection with PHD2Δ6–20 caused a likewise significant reduction of reporter activity (Fig. 6) despite differential patterns of intracellular distribution. However, wild-type PHD2 and PHD2Δ6–20 have in common that both are expressed in the cytoplasm and can be imported into the nucleus. In transient transfected cells PHD2Δ196–205 protein localised exclusively in the cytoplasm and had no influence on HIF-dependent transcriptional regulation compared to the empty vector control (Fig. 6). These data show that the intracellular localisation of PHD2 strongly influences the activity of PHD2 for HIF-1α degradation and that induction of PHD2 activity is predominantly mediated in the nucleus.

The level of hypoxia induced HIF-1α target gene expression is inhibited by nuclear PHD2

To evaluate the role of PHD2 intracellular localisation on the transcriptional regulation of HIF-1 target genes, total RNA was isolated from U2OS cells transiently transfected with the empty control vector pEGFP-N1 or EGFP-tagged PHD2 constructs (wild-type, Δ196–205, Δ6–20). By qRT-PCR, mRNA levels of HIF-1 target genes VEGF, GLUT1 and MCT4 were analysed under normoxic and hypoxic conditions (Fig. 7). Hypoxia induced the expression of HIF-1 target genes (data not shown). LMB treatment lasting over more than 4 hours resulted in high cell toxicity preventing the analysis of hypoxic HIF-1 target gene expression in LMB treated cells. Under hypoxia, overexpression of wild-type

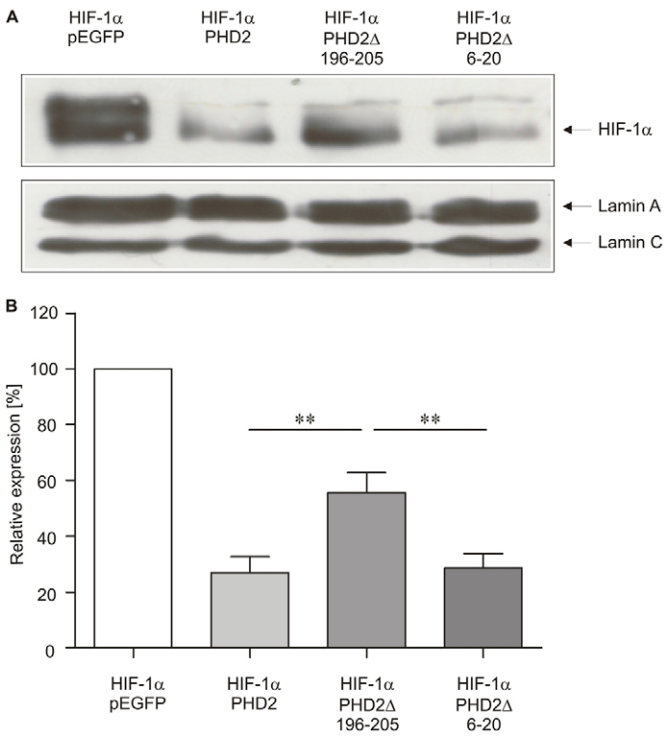

Fig. 5. HIF-1α protein expression is inhibited by nuclear PHD2. HIF-1α and the indicated EGFP-tagged PHD2 constructs (wild-type, Δ196–205 and Δ6–20) were co-transfected in U2OS cells. (**A**,**B**) Whole-cell lysates were analysed for HIF-1α protein expression with a monoclonal anti-HIF-1α antibody by western blotting. The anti-lamin-A/C antibody serves as control for equal protein loading and transfer. The immunoblot shown is representative of at least five independent experiments. Densitometry analysis was performed using ImageJ software and relative protein expression was calculated after normalisation to lamin A/C. Data are the mean ± s.e.m. (n=6). **P<0.01.

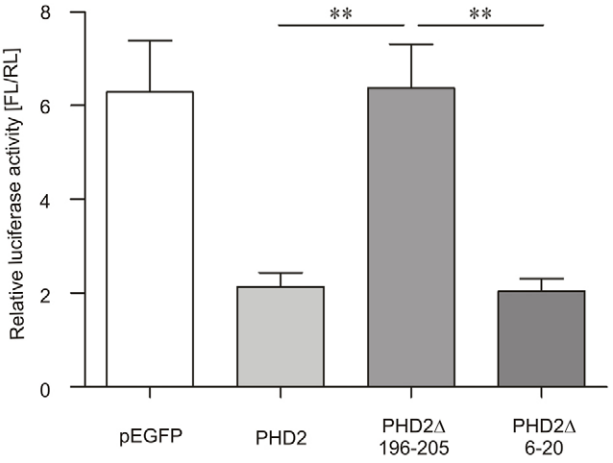

Fig. 6. Nuclear PHD2 localisation inhibits HIF-dependent transcriptional activity. A HIF-dependent firefly luciferase gene and the indicated EGFP-tagged PHD2 constructs (wild-type, Δ196–205 and Δ6–20) were co-transfected in U2OS cells. Cells were exposed to hypoxic conditions (3%) for 16 hours. The prolyl hydroxylase activity of wild-type and mutant PHD2 is represented by a reduction of HIF reporter activity. Firefly luciferase (FL) activities were normalised to *Renilla* luciferase (RL) activities. Data are the mean ± s.e.m. for triplicate determinations (n=6). **P<0.01.

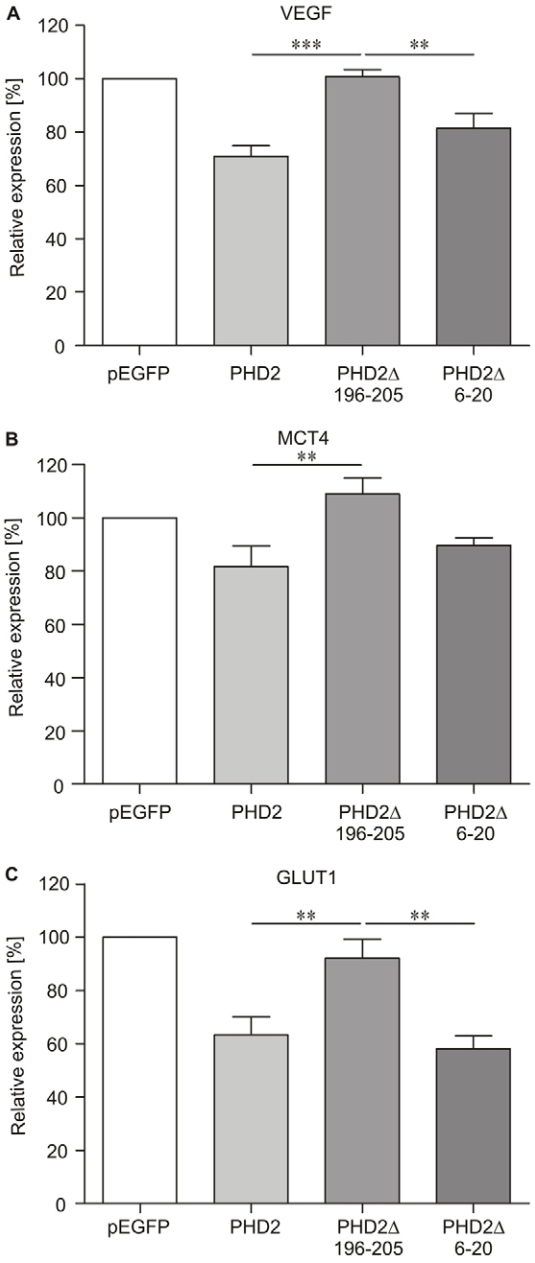

Fig. 7. Level of HIF-1α target gene expression is inhibited by nuclear PHD2. U2OS cells were transiently transfected with EGFP-tagged wild-type PHD2, PHD2Δ196–205 or PHD2Δ6–20. After exposure to hypoxic conditions (3%) for 16 hours, the total mRNA from the cells was reverse-transcribed and analysed by qRT-PCR. The normalised ratios for mRNA encoding VEGF(**A**), MCT4 (**B**) and GLUT1 (**C**) to that of mRNA encoding β-actin are shown. Data are the mean ± s.e.m. for triplicate determinations (n=6). *P<0.05, **P<0.01, ***P<0.001.

PHD2 and PHD2Δ6–20, but not of PHD2Δ196–205 reduced mRNA levels of VEGF, GLUT1 and MCT4 compared to control transfections (Fig. 7).

Discussion

The prolyl hydroxylase PHD2 acts as the oxygen sensor in oxygen-dependent regulation of HIF-1α stability. In normoxia, PHD2 hydroxylates the HIF-1α subunit at specific prolyl residues

initiating the degradation of the transcription factor subunit. When oxygen-dependent PHD2 activity is reduced in hypoxia, HIF-1α evades degradation and can accumulate. A line of data indicates that PHD2 hydroxylase-dependent activity plays a crucial role in cell survival, proliferation, cell growth and tumorigenesis. Accordingly, Takeda and colleagues investigated the contribution of PHD isoforms to mouse development showing that PHD2 is a major regulator of vascular growth in adult mice, while mice homozygous for targeted disruptions in *phd1* or *phd3* genes had no apparent defects and were viable (Takeda et al., 2008).

The overall expression of PHD2 has been studied at protein and mRNA levels demonstrating that PHD2 is widely expressed, but is most abundant in adipose tissue and the heart (Lieb et al., 2002). Studies on subcellular expression patterns indicated a mainly cytoplasmic localisation of PHD2 (Metzen et al., 2003). Recently, we were able to show that inhibition of nuclear export causes nuclear accumulation of PHD2, indicating intracellular shuttling of PHD2 between nucleus and cytoplasm (Steinhoff et al., 2009). By interaction studies, we demonstrated that nuclear PHD2 import is not mediated via the classical nuclear import pathway involving receptors importin α and β (Steinhoff et al., 2009).

Herein, we now investigated the presence of molecular determinants for nuclear localisation of PHD2. Many types of NLSs with little sequence or structural similarities make the prediction of NLS sequences in candidate import cargos very difficult (Marfori et al., 2011). Although the search for specific consensus patterns in the primary PHD2 amino acid sequence using different databases (PredictNLS, PSORT II Prediction) failed, we could show that effective import of PHD2 clearly depends on an intact motif in the primary sequence between residues E196 and G205 (EYIVPCMNKHG) (Fig. 1). The identification of an atypical putative NLS, together with the finding that PHD2 does not bind to the classical importin α and importin β receptors, suggests that import of PHD2 is mediated by one of the other known importin-β-like nuclear transport receptors. Unfortunately, preliminary data so far failed to prove specific physical interaction between PHD2 and β-like nuclear transport receptors importin 4 and importin 7 (unpublished data). Thus, future characterisation of the PHD2 nuclear import process is of particular interest.

On the other hand nuclear export is the other major step regulating nuclear accumulation. Previously, we showed that nuclear export of PHD2 depends on the major exportin CRM1 (Steinhoff et al., 2009). Interestingly, we could not confirm a predicted NES between Leu188 and Iso198 (NetNES), but identified a specific motif in the N-terminal part of PHD2 enabling nuclear export of the protein. By immunofluorescence analysis, we demonstrated that nuclear export of PHD2 clearly depends on an intact motif between amino acids G6 and Y20 (GGPGGPSPSERDRQY). However, this part of the primary sequence does not contain the classical leucine-rich NES consensus motif (Fig. 2A). We identified four serine residues within or in close vicinity to the NES of PHD2 suggesting protein phosphorylation as another step for regulation of nucleocytoplasmic trafficking. It is well-established that phosphorylation can stimulate or inhibit nuclear transport in different ways (Nardozzi et al., 2010). Consistently, Mylonis et al. (Mylonis et al., 2006) showed that phosphorylation of HIF-1α serine residues by MAPK promotes nuclear accumulation and transcriptional activity by blocking its export via CRM1. However, the four point mutations, S5A, S12A, S14A and S39D had no influence on intracellular PHD2 localisation, indicating that phosphorylation of serine residues in the N-terminal part is not linked to nuclear export of PHD2 (Fig. 2B).

Varying cofactors provided in different subcellular compartments may influence PHD activity. Our identification of a nuclear localisation signal in the PHD2 sequence for the first time enables us to analyse the activity of PHD2 in a specific cellular environment, e.g. with PHD2Δ196–205 activity restricted to the cytoplasm.

HIF-1 transcriptional activity, HIF-1α protein levels and HIF-1 target gene expression were decreased by overexpression of wild-type PHD2 and nucleus located mutant PHD2Δ6–20 but not by overexpression of cytoplasmic PHD2Δ196–205, indicating that nuclear PHD2 localisation is important for HIF-1α hydroxylation (Figs 5, 6, 7). Consistently, FRET measurements revealed interaction of HIF-1α with PHD2Δ6–20 in the nucleus but not with PHD2Δ196–205 in the cytoplasm. Since catalytic activity of PHD2 has been shown to depend on His-313, Asp-315 and His-374 (McDonough et al., 2006), deletion of amino acids 196–205 does not directly disturb the protein's active site, pointing to the fact that a crucial role of intracellular localisation for PHD2 hydroxylase activity exists. This data suggest a specific HIF-1α hydroxylase activity of PHD2 that depends on the intracellular localisation and thus the subcellular microenvironment of the nucleus in particular. In agreement with our results, Berchner-Pfannschmidt et al. (Berchner-Pfannschmidt et al., 2008) demonstrated by nuclear fractionation and subsequent PHD activity assays that nuclear extracts exhibit more PHD2 activity than the respective cytoplasmic extracts. This difference in subcellular activity became even more prominent when nuclear PHD2 abundance was increased by hypoxia or nitric oxide because higher abundance was associated with a substantial induction of PHD2 activity in the nucleus (Berchner-Pfannschmidt et al., 2008).

Several lines of evidence indicate that ubiquitylation and degradation of nuclear proteins depends on subcellular localisation of both the substrate protein and the components involved in degradation processes. In our model, the induction of PHD2 hydroxylase activity which is required for HIF-α ubiquitylation by VHL occurs predominantly in the nucleus. Consistently, Groulx and Lee suggested that nuclear–cytoplasmic shuttling of von-Hippel–Lindau tumour suppressor protein (VHL) is essential for oxygen-dependent ubiquitylation and degradation of the HIF-α subunits (Groulx and Lee, 2002). Moreover, they showed that oxygen-dependent nuclear ubiquitylation of HIF-α can be prevented by inhibition of the HIF-specific prolyl hydroxylases indicating that nuclear ubiquitylation of HIF-α requires nuclear prolyl hydroxylation by the PHD proteins (Groulx and Lee, 2002).

Earlier studies using different cancer cell lines have demonstrated that nuclear PHD2 localisation promotes a more malignant cancer phenotype, suggesting a role for PHD2 in the regulation of cellular proliferation and differentiation (Couvelard et al., 2008; Jokilehto and Jaakkola, 2010). In head and neck squamous cell carcinoma, nuclear translocation of PHD2 is associated with less differentiating and strongly proliferating tumours and predicts radiation resistance (Jokilehto et al., 2006; Luukkaa et al., 2009). Moreover, Couvelard et al. (Couvelard et al., 2008) showed that nuclear expression of PHD2 is increased in aggressive pancreatic endocrine tumours with higher grade and

stage. In accordance with these data, nuclear localisation of PHD2 enhances anchorage-independent cell growth in colon carcinomas while cytoplasmic PHD2 less efficiently downregulates HIF expression. Nevertheless, two hydroxylase-activity-depleting point mutations revealed only minor effects on anchorage-independent growth, indicating that the effect of PHD2 is independent of the HIF hydroxylation activity (Jokilehto and Jaakkola, 2010). Our results in osteosarcoma cells cannot unveil why nuclear PHD2 expression might promote tumour growth but are fully consistent with Jokilehto et al. (Jokilehto and Jaakkola, 2010) implying a central role of nuclear PHD2 localisation for its function. Finally, the outcome of PHD2 in cancer strongly depends on hydroxylase-independent activity, non-HIF targets such as the tumour suppressor ING4 or members of the NF-κB signalling pathway and a cell- and cancer-type-specific expression (Chan et al., 2009; Jokilehto and Jaakkola, 2010; Ozer and Bruick, 2005). It might be well worth considering that inhibition of nuclear translocation of the oxygen sensor PHD2 could provide therapeutic applicability to interfere with the regulation of HIF-1 activity and the oxygen sensor pathway in tumours.

Materials and Methods

Cell culture and DNA transfection

U2OS (human osteosarcoma cell line) cells and HEK293 cells (human embryonic kidney) were grown in DMEM culture medium containing 10% fetal calf serum (Gibco, Darmstadt, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Cölbe, Germany) at 37℃ in a 5% CO_2 incubator. 60% confluent cells were transiently transfected using X-tremeGENE HP (Roche, Mannheim, Germany) or GeneJuice (Merck, Darmstadt, Germany) for 24 hours as described by the manufacturer. Cells were exposed to a humidified atmosphere containing 3% O_2, 92% N_2 and 5% CO_2 for another 16 hours for hypoxic incubation. All transfections were, as a minimum, performed in triplicate.

For FRET experiments HEK293 cells were grown on glass-bottomed cell culture dishes (WillcoWells BV, Amsterdam, The Netherlands). Cells were transiently transfected with Fugene6 (Roche, Mannheim, Germany) according to the manufacturer's protocol for 24 hours. Medium was renewed for another 24 hours and PHD2 activity was inhibited by addition of 2 mM DMOG. Again, medium was renewed 1 hour before starting the experiment.

Plasmids and DNA modification

Standard procedures, as described by Ausubel et al. (Ausubel et al., 1993) were used for DNA isolation and transformation of *E. coli*. Vector constructs encoding a N-terminal EGFP-fused version of PHD2 were kindly provided by E. Metzen (Metzen et al., 2003). For FRET analysis plasmids encoding HIF-1α fused to ECFP (Clontech, Heidelberg, Germany) and PHD2 fused to EYFP were used. The PCR based Lightning Mutagenesis® kit (Stratagene, Waldbronn, Germany) was used to produce site-directed deletions within PHD2. Corresponding primer sequences are available upon request to the authors. Mutations were verified by DNA sequencing (GATC, Konstanz, Germany). Primers were purchased from Invitrogen (Darmstadt, Germany).

Fluorescent microscopy and fluorescence resonance energy transfer (FRET) analysis

U2OS cells were grown on coverslips in 24 well plates to 60% confluence. For localisation studies using EGFP-tagged fusion proteins, cells were transiently transfected with the indicated EGFP-fused versions of PHD2. After 24 hours of incubation, cells were washed with PBS and fixed with 250 µl of 3.7% formaldehyde in PBS for 15 minutes. For immunofluorescence analyses cells were transiently transfected with the indicated PHD2 constructs. After 24 hours of incubation, cells were washed with PBS, fixed with 250 µl of 3.7% formaldehyde in PBS for 15 minutes, permeabilised with 0.1% Triton-X 100 and blocked with 0.2% gelatine from cold water fish skin in PBS. Endogenous as well as transfected PHD2 was detected using a monoclonal rabbit anti-PHD2 antibody (Novus, Littleton, CO, USA) overnight at 4℃ and a secondary Alexa 594-conjugated goat anti-rabbit antibody for 1 hour at 4℃ (Invitrogen, Darmstadt, Germany). To block CRM1-mediated nuclear export, 10 ng/ml Leptomycin B (LMB, Merck, Darmstadt, Germany) was applied for 4 hours. Samples were mounted on glass slides in ProLong® Gold antifade reagent with DAPI (Invitrogen, Darmstadt, Germany) and analysed the following day with a laser scanning fluorescence microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Heidelberg, Germany; objective lenses HCX PL APO CS 20.0×0.70 IMM UV; Leica LAS AF Lite software, Leica Microsystems CMS GmbH, Heidelberg, Germany).

For FRET analysis a standard inverted confocal microscope with object lenses plan Apochromat 40×(Nikon, Duesseldorf, Germany) was used with two band pass emission filters 480/40 and 564/40 nm (AHF, Tuebingen, Germany). To determine protein–protein interaction HEK 293 cells were transfected with ECFP–HIF-1α or HIF-1α-mut–ECFP (HIF-1α K719AK753A) (donor) and EYFP–PHD2 constructs (acceptor) as described above. PHD2 activity was inhibited by addition of 2 mM DMOG for 24 hours resulting in stabilisation of ECFP–HIF-1α or ECFP–HIF-1α-mut. 48 hours after transfection FRET was monitored as described previously and FRET signals were analysed according to the sensitised FRET method using *FRET* software (Wotzlaw et al., 2010).

Luciferase reporter gene assay

U2OS cells were used to monitor HIF-1 activity in reporter gene studies. Cells (1.5×10^4 cells/well) were allowed to attach overnight. The following day, cells were co-transfected with a hypoxia responsive luciferase plasmid (200 ng/well) containing six copies of HIF-1 binding sites from the transferrin 3′ enhancer and a *Renilla* luciferase plasmid (2 ng/well) for standardisation. Additionally, cells were transfected with plasmids encoding EGFP tagged wild-type PHD2 and PHD2 deletion mutants (100 ng/well), PHD2Δ196–205 (PHD2ΔNLS) or PHD2Δ6–20 (PHD2ΔNES), resulting in a compartment-specific localisation. Empty pGL4 (Promega, Mannheim, Germany) vector and empty pEGFP-N1 (Clontech, Heidelberg, Germany) were used as controls. After 24 hours transfection the medium was replaced and cells were incubated in normoxic (20% O_2) or hypoxic (3% O_2) conditions for 16 hours. After incubation, cells were lysed with 1×passive luciferase buffer (Promega). Luminescence was measured using the luciferase assay system (Promega) with a MicroLumat LB 96P (Berthold Technologies). Firefly luciferase (FL) activities were normalised to *Renilla* luciferase (RL) activities. All experiments were carried out in triplicate.

Quantitative real-time PCR

For the quantification of mRNA, cells were washed with PBS and lysed with 500 µl 1×Nucleic acid purification lysis solution (Applied Biosystems). Total RNA was extracted using the 6100 Nucleic Prepstation (Applied Biosystems) following the manufacturer's protocol. 9 µl of total RNA were used for cDNA synthesis with the Cloned AMV First Strand Synthesis Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's specifications. RT–qPCR was accomplished with Platinum SYBR Green qPCR Supermix (Invitrogen, Darmstadt, Germany) and 2 µl cDNA in a volume of 25 µl on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Expression values were normalised to β-actin expression values. The following primers were used for detection of specific cDNA: human β-actin forward, 5′-AAGATCATTGCTCCTCCTGAGC-3′; human β-actin reverse, 5′-CATACTCCTGCTTGCTGATCCA-3′; human GLUT1 forward, 5′-GGCCTT-TTCGTTAACCGCTT-3′; human GLUT1 reverse: 5′-AGCATCTCAAAGGAC-TTGCCC-3′; human MCT4 forward, 5′-CGCCCGATCAGTGTTTTGA-3′; human MCT4 reverse, 5′-AGGATGCCTTGTAACCTTGCG-3′; human VEGF forward, 5′-CGAGGCAGCTTGAGTTAAACG-3′; human VEGF reverse, 5′-AGATCTGG-TTCCCGAAACCCT-3′.

Protein extraction and immunoblot analysis

Whole cell extracts were prepared in order to analyse protein expression levels of HIF-1α. Cells were transiently transfected with HIF-1α and one of the EGFP-tagged PHD2 constructs. Following incubation for 24 hours, cells were collected, washed with ice-cold PBS and extracted with UREA lysis buffer containing 10 mM Tris HCl (pH 6.8), 6.7 M Urea, 10 M Glycerin, 1% SDS and 5 mM DTT. All protein extracts were supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined by the Bradford method using BSA as standard. Per lane 20 µg were subjected to 7.5% SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare, Freiburg, Germany) by semidry blotting. Membranes were stained with Ponceau S (Sigma, Munich, Germany) in order to monitor protein transfer. Blocking of the membranes was carried out at 4℃ in 5% nonfat dry milk powder in PBS for at least 1 hour. Membranes were incubated with a monoclonal mouse anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) with cautious shaking overnight at 4℃. We used a polyclonal goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Hamburg, Germany). A polyclonal goat anti-lamin-A/C antibody (Santa Cruz Biotechnology, Heidelberg, Germany) followed by a polyclonal rabbit anti-goat antibody (Dako, Hamburg, Germany) served as control for equal protein loading and transfer. Chemiluminescence detection of immunoreactive proteins was performed by incubation of the membrane with ECL detection reagents (Amersham ECL Western Blotting Detection Reagents, GE Healthcare, Freiburg, Germany) for 1 minute, followed by exposure to X-ray films (Amersham Hyperfilm MP, GE Healthcare, Freiburg, Germany).

Statistical analysis

If not otherwise indicated, experiments were performed in triplicate and results are presented as mean ± standard error of the mean of at least $n=4$ independent experiments. Statistical differences of means between treated and control groups

were assessed using one-way ANOVA and Bonferroni's test. Significance was accepted at $P<0.05$ (*$P<0.05$; **$P<0.01$; ***$P<0.001$). All statistics were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

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