Tumor and Stem Cell Biology

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Prolyl Hydroxylases 2 and 3 Act in Gliomas as Protective Negative Feedback Regulators of Hypoxia-Inducible Factors

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Abstract

Adaptive responses to hypoxia in tumors are transcriptionally regulated by the hypoxia inducible factors (HIF-1α/HIF-2α), which are tightly controlled by the HIF-prolyl hydroxylases (PHD). Hypoxia induces expression of the PHD2 and PHD3 proteins in tumors but the pathobiological significance of these events is uncertain. Here, we show that PHD2 and PHD3 induction acts within a negative feedback loop to limit the hypoxic HIF response. In glioblastomas, PHD2 and PHD3 are hypoxia-inducible *in vitro* and expressed in hypoxic areas of tumors *in vivo*. Comparison with other PHDs revealed distinct cytoplasmatic and nuclear localization patterns of PHD2 and PHD3. Gain and loss of function experiments defined PHD2 and PHD3 as HIF target genes that remained operative even at low oxygen concentrations. We found that increased PHD levels could compensate for reduced oxygen availability to regulate the HIF response. This negative feedback loop protected tumor cells against hypoxia-induced cell death, functionally implicating this pathway in the control of the tumor-suppressive components of the HIF system in glioblastoma. Moreover, PHD inhibition facilitated cell death induction by staurosporine or tumor necrosis factor–related apoptosis-inducing ligand, hinting at a more general protective role of PHD in the regulation of cell viability. In summary, our findings recognize the PHD/HIF regulatory axis as a novel therapeutic target to disable a tumor's ability to adjust to hypoxic conditions and control cell survival, helping to potentially overcome therapeutic cell death resistance in glioblastomas. *Cancer Res; 70(1); 357–66. ©2010 AACR.*

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Introduction

Regions of low oxygen tension are common findings in malignant tumors, being associated with increased frequency of tumor invasion and metastasis and a poor therapy outcome (1). Consequently, the ability to initiate homeostatic responses to adapt to hypoxia represents an important and crucial aspect in solid tumor growth. Hypoxia-inducible factor (HIF) has been identified as a key transcriptional system that regulates these adaptive responses in tumors (2). The HIF complex exists as a heterodimer composed of the constitutively expressed HIF-β (aryl hydrocarbon receptor nuclear translocator) and the oxygen-regulated HIF-1α or HIF-2α subunits, respectively (3). Although there are multiple mechanisms regulating HIF activity, a critical step is the oxygen-dependent regulation of HIF-α stability. In normoxia, HIF-α is destabilized by the enzymatic activity of oxygen-sensing enzymes termed prolyl 4-hydroxylase domain (PHD). To date, three PHD paralogues (PHD1–3) and a related PHD isoform (PH4; PHD4) have been identified (4–6). PHDs hydroxylate HIF-1/2α at two specific prolyl residues in the oxygen-dependent degradation domain allowing capture by the von Hippel-Lindau tumor suppressor protein (pVHL), ubiquitylation and subsequent proteasomal degradation (7).

The relevance of the HIF system for tumor growth and progression is highlighted by the variety of mechanisms regulated by HIF target genes ranging from angiogenesis (to increase tissue oxygenation) over glycolysis and pH regulation (allowing for energy generation when oxygen is scarce) to cell proliferation and survival pathways. Although these mechanisms are commonly believed to promote tumor growth, the HIF pathway is also known to exert proapoptotic activities suggesting that HIF function in tumors may be more complex. Indeed, several studies have revealed conflicting data with regard to the effect of HIF manipulation on tumor growth. Some reported impaired tumor growth and angiogenesis following HIF-1α loss of function (8, 9). In contrast, others provided evidence for the tumor-suppressive function of HIF (10, 11). We recently showed that HIF-1α and HIF-2α suppress tumor growth in part by increasing tumor cell death (12). These studies suggest a dual role of HIF in tumor biology, implicating HIF-α levels and activity in the regulation of the tumor-promoting and tumor-suppressing functions (3, 13).

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PHDs as key regulators of HIF activity may critically shape these functions. Although PHDs share a highly conserved domain which allows binding of 2-OG and ferrous iron, the four PHDs partly differ with respect to tissue expression, cellular localization, substrate specificity, and regulation (3). Thus, the precise role of the various PHD isoforms in hypoxia signaling remains to be further elucidated. Recent evidence has indicated that PHD2 and PHD3 are transcriptionally regulated by HIF to promote an accelerated HIF degradation following reoxygenation (14, 15). *In vitro* studies also suggest that PHDs retain some activity under hypoxic conditions (16). We were interested to see whether HIF-dependent regulation of PHDs may be part of a negative feedback loop limiting the extent of the hypoxic tumor response. We show here that PHD2 and PHD3 are themselves target genes of HIF-1α and HIF-1/2α, respectively, helping to downregulate HIF levels and HIF function under prolonged hypoxia in human glioblastomas. Thus, PHD2 and PHD3 remain operative even in low oxygen concentrations. Our gain and loss of function experiments indicate that one of the biological functions of PHDs in glioblastoma is the control of hypoxia-induced cell death via the regulation of HIF. Thus, HIF-dependent regulation of PHD may be viewed as a preventative and self-limiting adaptive response within a self-regulatory loop to control and adjust HIF function in tumor growth and progression.

Materials and Methods

Reagents. hPHD1–4 open reading frames were cloned into pcDNA3.1-TOPO-V5 using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). hHIF-1α and hHIF-2α expression constructs were kindly provided by E. Huang (Department of Neurosurgery, University of Utah, Salt Lake City, UT) and W.G. Kaelin (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). Dipyridyl, desferrioxamine, cobaltous ions, or dimethyloxaloylglycine (DMOG) were purchased from (Sigma).

Cell culture, transfection. Glioblastoma cell lines G55, G120, G123, G141, and G142 were kindly provided by M. Westphal and K. Lamszus (Department of Neurosurgery, University Hospital Hamburg-Eppendorf, Hamburg, Germany; ref. 17), GBM by H. Weich (Departments of Gene Regulation and Differentiation, Helmholtz Centre for Infection Research, Braunschweig, Germany), U343 by M. Nistér (Department of Oncology-Pathology, Karolinska University Hospital, Stockholm, Sweden; ref. 18), LN-229, U87, SNB19, and U118 at American Type Culture Collection (CRL-2611, HTB-14, CRL-2219, and HTB-15, respectively), and U251 at RIKEN cell bank (no. RCB0461). Cell lines were cultured in DMEM (Invitrogen), containing 10% fetal bovine serum (PAN Systems). For hypoxic treatment, cells were incubated in the Invivo$_2$ 400 (Ruskinn Technology). For overexpression studies, cells were transfected with SuperFect (Qiagen). Short interfering RNAs (siRNA) were purchased from Eurogentec. Alternatively, PHD siRNAs were synthesized with the Silencer siRNA Construction Kit (Ambion). siRNA transfections were performed twice at 24-h intervals with OligofectAMINE reagent (Invitrogen).

Luciferase reporter assay. Cells were transiently transfected with a vascular endothelial growth factor (VEGF) promoter (19) firefly and a SV40 Renilla luciferase construct for normalization of transfection efficiency and assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

Real-time reverse transcription-PCR. Quantitative PCR (qPCR) was carried out in an iCycler iQ-Systems (Bio-Rad) as previously described (12). For primer sequences, see Supplementary Table.

Western blotting. Western blotting was carried out as described (12) using the following antibodies: HIF-1α (BD Transduction Laboratories), HIF-2α, PHD1, PHD2, PHD3, PHD4 (Novus Biologicals), V5 (Invitrogen), and tubulin as a loading control (The Jackson Laboratory). *In vitro* transcription of PHD lysates was performed using the TNT Coupled Reticulocyte Lysate System (Promega).

***Immunohistochemistry/*in situ hybridization.** Immunohistochemistry and *in situ* hybridization was carried out as described (20). Sections were stained with PHD1–4 antibodies (Novus Biologicals). Hybridization was carried out with a digoxigenin-labeled (Boehringer Mannheim) cRNA generated by *in vitro* transcription using PHD2 and PHD3 cDNA templates.

Cell death. Cell death (propidium iodide uptake) was determined by flow cytometry following staining of tumor cells with 10 μg/mL of propidium iodide ± addition of 100 nmol/L of staurosporine (Sigma) or Killer TRAIL (50 ng/mL; Alexis Biochemicals).

Statistical analysis. Results are presented as mean ± SEM. Statistical comparisons of values were made using Student's *t* test. All experiments were repeated at least thrice with similar results in indicated replicates (n). Statistical significance was defined as $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

PHD2 and PHD3 are hypoxia-inducible in human glioblastomas. mRNA expression levels of PHD1–4 varied across a panel of human glioblastoma cell lines (Fig. 1*A*; Supplementary Fig. S1*A*) demonstrating moderate variations of up to 10-fold for PHD1, PHD2, and PHD4 and high variations of up to 1,000-fold for PHD3 (note logarithmic scale). Interestingly, PHD2 (8 of 12 cell lines) and PHD3 (11 of 12 cell lines) increased markedly under hypoxia, with PHD3 showing a particularly strong induction of up to two log units. Correlating with the respective mRNA profile, hypoxia treatment induced PHD2 and most markedly PHD3 proteins (Fig. 1*B*) using validated PHD antibodies (Supplementary Fig. S1*B*). Similarly, treatment with the hypoxia mimetics dipyridyl, desferroxamine, and cobaltous ions induced PHD2 as well as PHD3 (Fig. 1*C*). To study whether PHD protein expression is posttranslationally regulated by oxygen tension similarly to HIF-α, we analyzed PHD protein levels at different time points following re-oxygenation (Fig. 1*D*). Hypoxia-induced HIF-1α and HIF-2α protein levels returned to or even decreased below normoxic levels. In contrast, PHD2 and PHD3 protein

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concentrations remained at increased hypoxic levels. Taken together, these results show that the PHD isoforms differ in their relative expression levels and identify PHD2 and PHD3 as hypoxia-regulated isoforms in human glioblastoma.

PHD2 and PHD3 are differentially regulated by HIF-1α and HIF-2α in human glioblastomas. To provide evidence for a HIF-dependent regulation of PHD2 and PHD3 in different cellular contexts, we performed loss- and gain-of-function studies in a battery of six glioblastoma cell lines. The hypoxia-mediated increase in PHD2 expression (five of six cell lines) was reduced by HIF-1α knockdown but remained unaffected by HIF-2α knockdown (Fig. 2*A*). Hypoxia-induced PHD3 was lowered by HIF-1α siRNA (five of six cell lines) as well as by HIF-2α siRNA (two of six cell lines) with an additive suppression by combined HIF-1/2α knockdown (four of six cell lines). To examine potential cell-specific differences in PHD regulation, we confirmed these results on the protein level with glioblastoma cell lines that show differential hypoxic induction of HIF-1α and HIF-2α. Under hypoxia, G142 strongly induces HIF-1α, whereas G141 preferentially activates HIF-2α (Fig. 1*B* and *D*). The hypoxia-mediated increase of PHD3 in G141 was diminished by HIF-1α and HIF-2α siRNA with an additive suppression achieved by combined HIF-1/2α knockdown (Fig. 2*B*). In contrast, in G142 cells, the hypoxia-mediated increase of PHD3 was reduced by HIF-1α siRNA alone and was not affected by HIF-2α silencing. In line with the silencing experiments, PHD2 protein levels were elevated by transient overexpression of HIF-1α, but remained unaffected by HIF-2α overexpression (Fig. 2*C*). In contrast, PHD3 protein levels increased under hypoxic conditions following HIF-1α as well as HIF-2α overexpression. Thus, loss- and gain-of-function studies unequivocally identify PHD2 as a HIF-1α and PHD3 as a HIF-1α and, cell type–specifically, also as a HIF-2α target gene.

PHD2 and PHD3 are efficient regulators of HIF under hypoxia. Given the HIF-dependent regulation of PHD2/3, the PHD system may function in a negative feedback loop that serves to prevent HIF levels from reaching excessive concentrations during chronic hypoxia. Indeed, during prolonged hypoxia, HIF-1α and, slightly later, HIF-2α protein gradually decreased at 24 and 48 hours (Fig. 3*A, left*), respectively, whereas mRNA levels were not significantly altered (HIF-1α) or even increased (HIF-2α; Fig. 3*A, right*). The decrease in HIF-1/2α protein was accompanied by increased PHD2/3 expression. PHD inhibition by DMOG treatment 6 hours prior to cell harvesting prevented this gradual decrease and resulted in re-accumulation of HIF-1α and HIF-2α (Fig. 3*A*). Similarly, PHD3 silencing was sufficient to decrease HIF-1α degradation, whereas combined PHD2/3 silencing was necessary to rescue HIF-2α accumulation at 24 hours (Fig. 3*B*; Supplementary Fig. S2). These results underline the importance of PHD2 and PHD3 as regulators of HIF-α in hypoxia-adapted cells. Such a functional feedback loop implies that PHDs retain their enzymatic activity even under conditions of reduced oxygen availability. Therefore, we next assessed the ability of PHDs to mediate the degradation of HIF-1α and HIF-2α in hypoxic glioblastoma cells. Cotransfection of PHD1–4 effectively lowered ectopic HIF-1/2α protein under normoxia but also under hypoxia (Fig. 3*C, top*). In particular, PHD2 and PHD3 reduced HIF-1/2α expression to the highest extent, whereas PHD1 and PHD4 showed weaker activity. HIF-1α or HIF-2α transactivation activity as measured in a VEGF promoter luciferase assay was equally suppressed by PHDs (Fig. 3*C, bottom*). PHD2 and PHD3 most efficiently reduced HIF-1/2α–induced luciferase activity under both normoxic and hypoxic conditions in tumor cells. We next analyzed whether the gradual increase in cellular PHD protein abundance observed in hypoxic conditions could compensate for reduced oxygen availability. A stepwise increase in cotransfected PHD levels progressively decreased hypoxia-induced VEGF promoter activity (Fig. 3*D*). Similarly, VEGF promoter activity induced by the iron chelators desferrioxamine or dipyridyl was reduced by PHD overexpression (Supplementary Fig. S3). These results indicate that increased PHD concentrations can counterweigh decreased oxygen and iron availability. Taken together, the PHD system, in particular PHD2 and PHD3, efficiently suppresses HIF-1/2α function and maintains activity even under low oxygen and iron availability. PHD2 and PHD3 could therefore act as potent feedback regulators of both HIF-α subunits within the complete physiologic and pathologic range of the hypoxia response.

PHDs protect against hypoxia-induced cell death in glioblastoma. We have recently shown that HIF activation can inhibit tumor growth, in part by induction of tumor cell death (12). Because PHDs are the main regulators of HIF, we investigated a possible causal role for PHD in regulating glioblastoma cell survival. PHD inhibition by hypoxia and/or DMOG significantly reduced tumor cell survival in our panel of glioblastoma cell lines as measured by quantification of propidium iodide uptake (Fig. 4*A*), clonal growth, free oligonucleosomes, or terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells (Supplementary Fig. S4*A–D*). PHD inhibition by hypoxia and/or DMOG treatment resulted in increased cell death. In line with the clonal survival results, DMOG treatment under hypoxia enhanced tumor cell death to a higher extent than hypoxia alone, clearly demonstrating that PHD function is necessary for cell survival under normoxic and hypoxic conditions. Of note, PHD inhibition by DMOG under hypoxia (1% O_2) increased HIF-1α and HIF-2α to levels comparable to or slightly lower than physiologic HIF levels detected under 0.5% or 0.1% O_2 (Supplementary Fig. S4*E*)—ruling out that DMOG treatment induced HIF to excessive or supraphysiologic levels that might nonspecifically affect tumor cell behavior. Combined knockdown of HIF-1/2α abrogated the DMOG-induced cell death under hypoxia (Fig. 4*B*) demonstrating that the PHD-mediated control of HIF activity is indeed crucial for the control of hypoxic cell survival. In support of this notion, PHD inhibition by hypoxia and DMOG induced the expression of the known HIF-dependent cell death regulators BNIP, NIX, and RTP801 (Supplementary Fig. S5). To further confirm the role of PHD2 and PHD3 in hypoxic cell survival, we measured propidium iodide uptake following silencing of PHD2/3 (Fig. 4*C*). Combined silencing of PHD2/3 increased hypoxia-induced cell death supporting the protective

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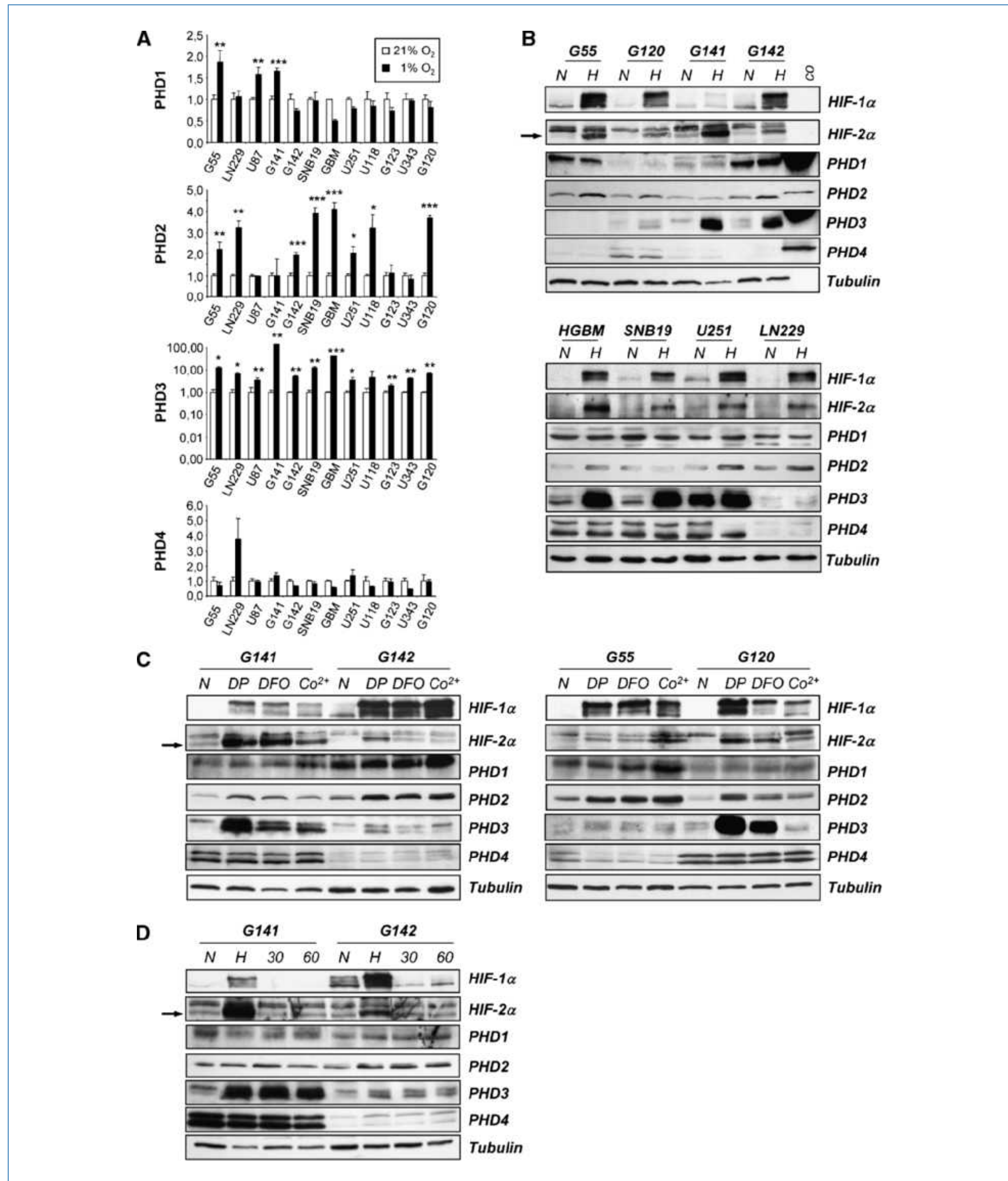

Figure 1. Differential PHD regulation by oxygen tension. *A,* PHD1–4 mRNA expression varies across different glioblastoma cell lines. PHD mRNA quantification by qPCR of glioblastoma cells exposed to 21% or 1% O_2 for 18 h. Values are normalized to HPRT housekeeping gene and expressed as fold induction relative to normoxic control (n = 3). Note logarithmic scale for PHD3. *B,* PHD2/PHD3 protein levels are hypoxia-inducible. Western blot of glioblastoma cells exposed to 21% (*N*) or 1% O_2 (*H*) for 18 h. *In vitro* transcribed PHD1–4 TNT proteins served as positive controls (*co*). *C,* hypoxia mimetics induce PHD2/3. Western blot of glioblastoma cells incubated in the absence (*N*) or presence of 100 μmol/L of dipyridyl (*DP*), 100 μmol/L of desferrioxamine (*DFO*), or 100 μmol/L of cobaltous ions (Co^{2+}) for 18 h. *D,* re-oxygenation does not alter PHD protein levels. Western blot of glioblastoma cells exposed to 21% (*N*) or 1% O_2 (*H*) for 18 h or re-exposed to 21% oxygen for the indicated time points (min) prior to harvesting. *Arrows,* specific HIF-2α protein bands (*B, C,* and *D*).

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feedback role of both isoforms. In contrast, elevating cellular PHD2/3 abundance by transient overexpression functionally compensated for reduced oxygen availability and abrogated hypoxia-induced tumor cell death (Supplementary Fig. S4*F*). To expand on the protective role of PHD function, we challenged tumor cells with the pro–cell death stimuli staurosporine and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; Fig. 4*D*). Many tumor cells remain resistant to the induction of cell death by TRAIL, often as a result of the aberrant activation of survival pathways (21). Interestingly, PHD inhibition by hypoxia or DMOG not only potentiated the cell death induction by staurosporine or TRAIL, but even sensitized glioblastoma cells to TRAIL-induced cell death. Taken together, these results provide evidence for PHDs as a feedback survival mechanism that protects tumor cells against hypoxia-induced cell death through the control of HIF-α levels. Moreover, our findings hint at a general protective role of PHD under conditions of cell stress.

PHD2 and PHD3 are expressed in perinecrotic areas in glioblastoma biopsies. In line with a negative regulatory feedback function of PHD2 and PHD3 *in vivo*, we observed enhanced expression of PHD2 and PHD3 transcripts in pseudopalisading cells in the perinecrotic/hypoxic areas in a panel of five human glioblastoma specimens by *in situ* hybridization (Fig. 5; for sense control Supplementary Fig. S6*A*). Analysis of PHD2 and PHD3 protein expression in human glioblastoma specimens by immunohistochemistry confirmed these results (Fig. 5; for antibody control Supplementary Fig. S6*B*). Strong PHD2 and PHD3 immunoreactivity was predominantly seen in tumor cells located in perinecrotic regions. Similar to the subcellular localization of PHD2 and PHD3 in cultured tumor cells (Supplementary Fig. S7), PHD2 was localized in the nucleus and cytoplasm, whereas PHD3 was predominantly seen in the cytoplasm of tumor cells (Fig. 5, *higher magnification*). Thus, PHD2 and PHD3 mRNA and protein are strongly upregulated in perinecrotic tumor areas, regions with reduced oxygen availability, indicating that *in vivo* hypoxia is also a strong inducer of PHD2 and PHD3 expression. In contrast, PHD1 and PHD4 protein expression was not upregulated in perinecrotic tumor areas, but was evenly expressed in tumor cells throughout the tumor (Supplementary Fig. S6*C*).

Discussion

The hypoxia inducibility of PHD2 and PHD3 observed throughout various cell types and animal models has raised the question about the biological role of increased PHD levels during hypoxia in physiologic and pathologic settings. In this study, we provide evidence that the hypoxic PHD accumulation in glioblastoma acts within a negative feedback loop to limit the HIF response during conditions of reduced oxygen availability. Our experiments show that this loop protects tumor cells against hypoxia-induced cell death via the control of HIF, indicating that the PHD-mediated control of HIF response is essential for the survival of tumor cells in low oxygen conditions. Finally, we present evidence that PHD inhibition sensitizes cells to cell death induction by cell stress activators, indicating that PHDs may represent an attractive antitumor therapy target to overcome cell death resistance in glioblastoma.

Although the HIF system receives various regulatory inputs, control of the degradation rate and transcriptional activity by hydroxylation represents the dominant route for regulating HIF activity (reviewed in ref. 3). As HIF-α prolyl hydroxylation is a nonequilibrium reaction, the abundance of each PHD isoform in the cell will critically influence the regulatory range of HIF activation. Interestingly, the relative PHD abundance is affected by various stimuli. PHD1 is estrogen-inducible in breast cancer cell lines (22), whereas PHD2 and

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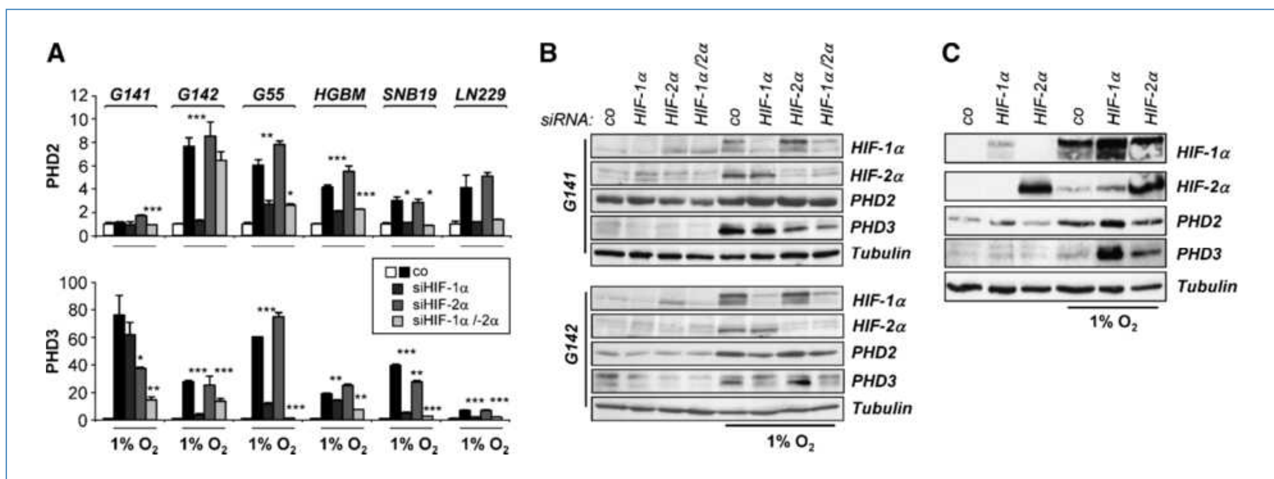

Figure 2. PHD2 and PHD3 are hypoxia-inducible HIF target genes. *A* and *B,* PHD2 is suppressed by HIF-1α, whereas PHD3 is suppressed by HIF-1α and HIF-2α knockdown. *A,* glioblastoma cells were transiently transfected with HIF-1α, HIF-2α, or control SIMA (*co*) siRNA and exposed to 21% or 1% O_2 for 18 h. PHD2/3 mRNA were quantified by qPCR, normalized to HPRT, and expressed as fold induction relative to normoxic control (*n* = 3). *B,* Western blot of glioblastoma cells treated as in *A*. *C,* HIF-1α induces PHD2 and PHD3, HIF-2α induces only PHD3. Western blot of G55 cells transiently transfected with HIF-1α, HIF-2α, or empty vector (*co*), and exposed to 21% (*N*) or 1% O_2 (*H*) for 18 h.

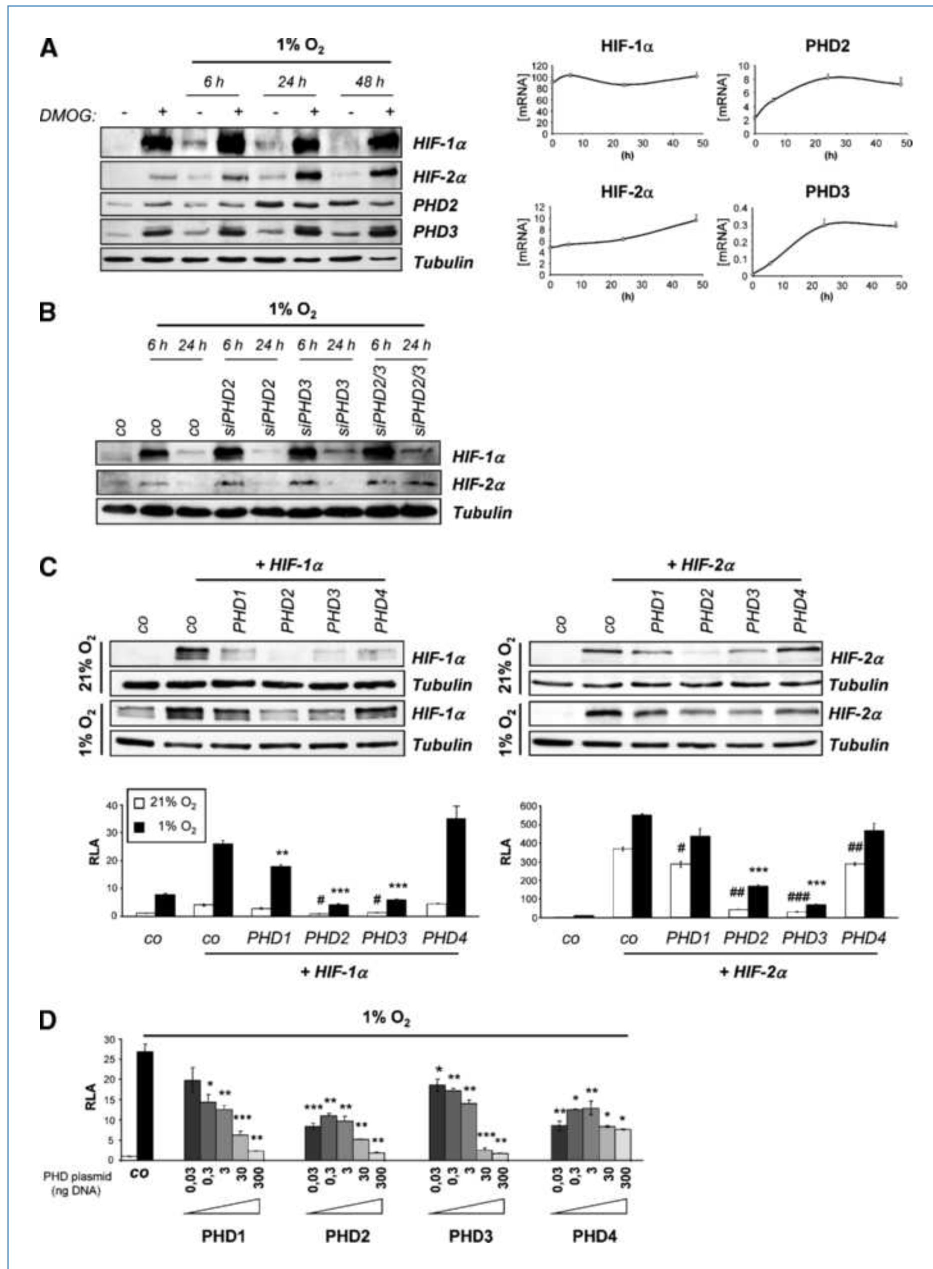

Figure 3. PHDs control HIF activity under normoxic and hypoxic conditions. *A,* HIF-1/2α proteins decrease during chronic hypoxia in parallel with increased PHD2/3 levels and reaccumulate following PHD inhibition. *Left,* Western blot of glioblastoma cells exposed to 21% or 1% O_2 for the indicated times ± PHD inhibitor DMOG 6 h prior to cell harvesting. *Right,* glioblastoma cells were exposed to 21% or 1% O_2 for the indicated times. mRNA levels were quantified by qPCR and normalized to HPRT (n = 3). *B,* PHD2/3 control HIF-1/2α protein levels during chronic hypoxia. Western blot of G55 cells transiently transfected with PHD2, PHD3, or control SIMA (*co*) siRNA and exposed to 21% or 1% O_2 for the indicated times (h). *C,* PHD1–4 reduce HIF-1/2α proteins and transactivation activity under normoxia and hypoxia. *Top,* Western blot of G55 cells transiently transfected with HIF-1α, HIF-2α, or empty vector (*co*) together with the indicated PHDs and exposed to 21% or 1% O_2 for 18 h. *Bottom,* G55 cells were cotransfected with HIF-1α (*left*) or HIF-2α (*right*), the indicated PHDs or empty vector (*co*), VEGF promoter firefly luciferase reporter and pSV40-renilla luciferase to control for transfection efficiency and exposed to 21% or 1% O_2 for 18 h. Relative luciferase activity (*RLA*) is expressed as fold induction relative to normoxic control (n = 3; #, versus normoxic control; *, versus hypoxic control). *D,* increased PHD levels can compensate for reduced oxygen availability. G55 cells were cotransfected with the indicated amounts of PHDs or empty vector, VEGF promoter firefly luciferase reporter and pSV40-Renilla luciferase. Relative luciferase activity was determined as in *C*.

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PHD3 are induced by growth factors (23). PHD3 is additionally upregulated in response to nerve growth factor withdrawal (24) following smooth muscle differentiation (25) or p53 activation (26). Our analysis of PHD1–4 expression in a panel of glioblastoma cell lines revealed the hypoxia-inducibility of PHD2 and PHD3, confirming previous findings with other cell types (4, 14, 15, 27–29). Accordingly, HIF binding sites have been identified in the promoters of PHD2 (30) and PHD3 (31). Using gain- and loss-of-function experiments, we show that in glioblastomas, PHD2 is under the control of HIF-1α, whereas PHD3 is a transcriptional target of HIF-1α as well as HIF-2α. These results are in agreement with recently published data showing differential regulation of PHD2 and PHD3 by the two HIF isoforms also in hepatocellular carcinoma (27). It is interesting to note that a relative selectivity has also been reported for the hydroxylation of the two HIF-α isoforms by PHD2 versus PHD3 (28). PHD2 was found to preferentially regulate HIF-1α, whereas PHD3 more substantially affected HIF-2α hydroxylation. We observed a similar preference of PHD2 and PHD3 for HIF-1α and HIF-2α, respectively (Fig. 3*C*). Moreover, we identified PHD2 and PHD3 as the most potent isoforms among the four PHDs to reduce HIF-1/2α protein expression and HIF-1/2α transactivation activity, in line with previous results (28, 32).

It has been suggested that PHD accumulation during hypoxia operates within a negative feedback loop to accelerate HIF degradation following re-oxygenation, thus efficiently and quickly shutting off the hypoxia response when oxygen becomes available again (14, 15, 29). Our data in glioblastomas, and a recent study on the physiologic role of PHDs in hypoxic mice (33), indicate that the reciprocal control of HIF levels and HIF transactivation activity during prolonged hypoxia is a second function of increased hypoxic PHD levels. This would imply that PHDs retain activity at low oxygen

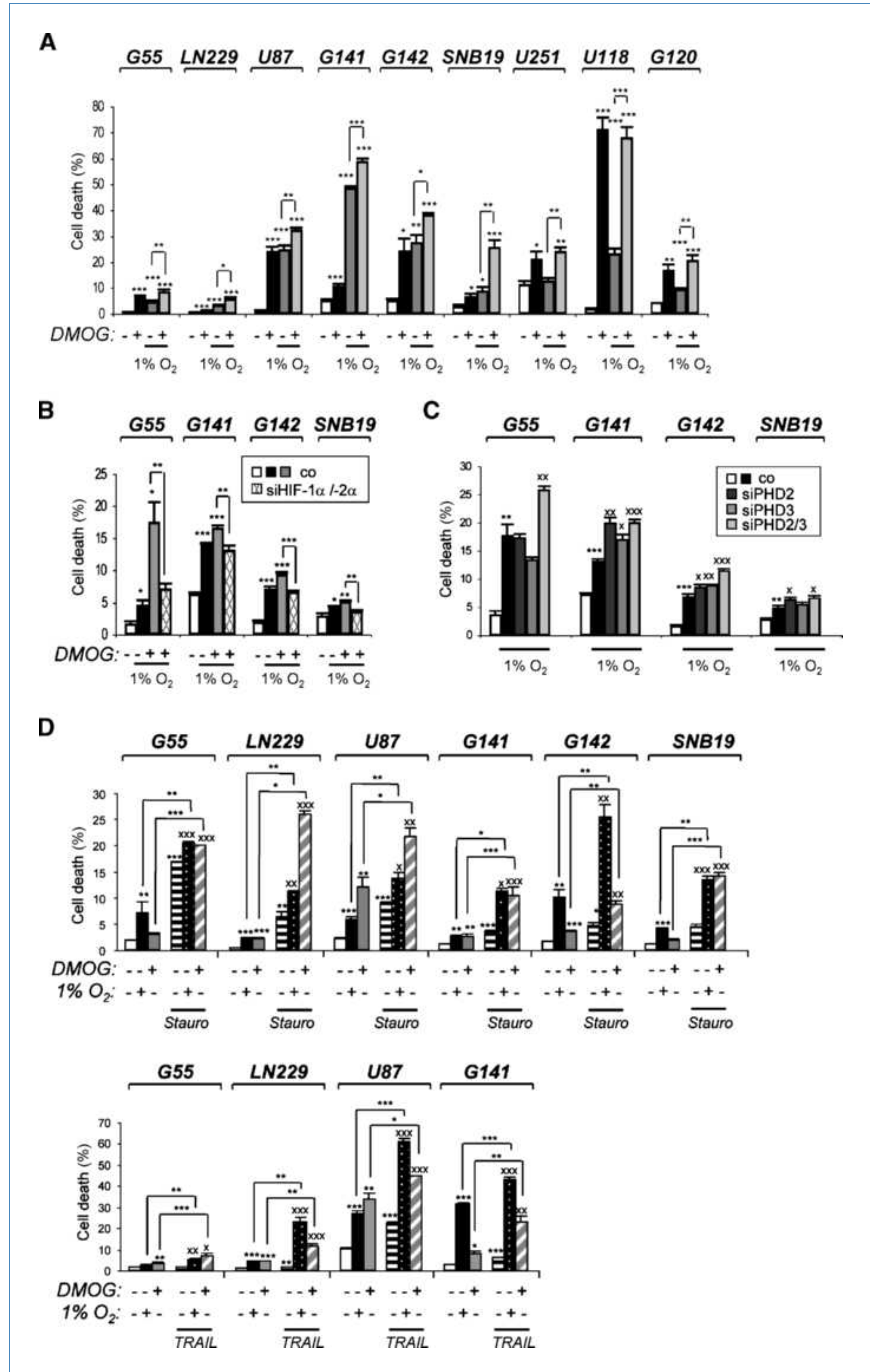

Figure 4. PHDs inhibit tumor cell death in glioblastoma. *A,* PHD inhibition induces tumor cell death. Quantification of tumor cell death (propidium iodide permeability) by flow cytometry after exposure to 21% or 1% O_2 ± PHD inhibitor DMOG for 72 h ($n = 3$). *B,* HIF-1/2α silencing protects from cell death following PHD inhibition. Tumor cells were transfected with HIF-1α, HIF-2α, or control (*co*) SIMA siRNA and treated and analyzed as in *A*. *C,* PHD2 and PHD3 protect against hypoxia-induced cell death. Tumor cells were transiently transfected with PHD2, PHD3, or control (*co*) SIMA siRNA. Cells were treated and analyzed as described in *A*. *D,* PHD inhibition sensitizes tumor cells to cell death inducers. Cells were treated and analyzed as described in *A* with or without (+/–) treatment with the pro–cell death reagents staurosporine (100 nmol/L; *top*) or killer TRAIL (50–100 ng/mL; *bottom*; $n = 3$; *, versus normoxic control; $^{\times}$, versus normoxic control + staurosporine/TRAIL).

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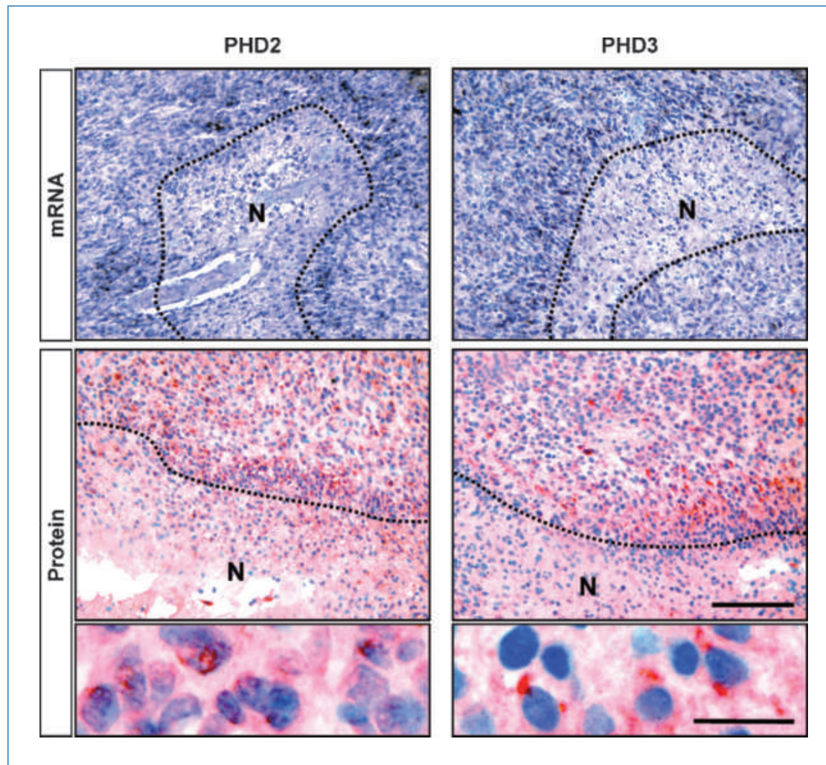

Figure 5. PHD2 and PHD3 are expressed in pseudopalisading cells in glioblastoma biopsies. Nonradioactive *in situ* hybridization and immunohistochemistry reveals perinecrotic upregulation of PHD2/3 mRNA transcripts (*black*) and protein (*red*), respectively, in pseudopalisading cells in glioblastoma (*bar,* 200 μm). PHD2 is localized to the nucleus and cytoplasm, PHD3 is seen in the cytoplasm predominantly in tumor cell processes (*bottom,* higher magnification; *bar,* 25 μm).

concentrations. Indeed, we show that PHD1–4 efficiently decrease HIF protein levels and HIF transactivation activity even at a pO_2 as low as 1%. Although PHDs have a high K_m for O_2 *in vitro* (16), the actual K_m under physiologic conditions could be markedly modulated by the intracellular concentration of cofactors (e.g., Fe^{2+}, 2-OG) or the redox state of the cells. Indeed, several reports have shown persistent hydroxylation activity of PHDs at low oxygen tension using HIF-α peptides (16, 34), recombinant HIF-α proteins (35), HIF-1α oxygen-dependent degradation domain hybrid reporter constructs (33), or hydroxylation-specific antibodies (36). Thus, PHDs operate over a wide range of oxygen concentrations encountered in physiologic and pathologic conditions. Our data suggest that the hypoxia-induced increase in PHD abundance can at least partially compensate for the reduced PHD activity under hypoxic conditions. This is supported by our observation that a stepwise increase in PHD levels translated into a gradual decrease in hypoxia-induced transcriptional activity. Indeed, enhanced total HIF hydroxylase capacity after prolonged hypoxia has been reported (4, 14). In keeping with this, PHD inhibition by DMOG treatment in glioblastoma cells reduced the steady decrease in HIF levels during prolonged hypoxia, confirming previous reports (28, 33). Additionally, chronic hypoxia induces PHD activity by increasing O_2 availability as a consequence of HIF-mediated inhibition of mitochondrial respiration (37).

What is the biological role of an oxygen-dependent and HIF-dependent regulation of PHD abundance in tumor physiology? From a mechanistic point of view, increasing the relative PHD abundance would allow the cell to adjust the threshold of HIF activation, extending the regulatory range of the system to lower oxygen levels. We propose that tumor cells exploit this physiologic feedback mechanism to adapt to the heterogeneous oxygen distribution within the tumor. Due to the high metabolic demand and the changing blood flow within a chaotic tumor vasculature, oxygen distribution in tumors varies spatially and temporally. Thus, tumor cells frequently experience periods of acute hypoxia when, e.g., tumor blood vessels become occluded. Modulating PHD levels would allow the tumor cell to reset its threshold of HIF activation in response to the surrounding pO_2. Tumor cells adapted to a chronically low pO_2 would therefore be able to mount a hypoxia response in a similar manner as tumor cells located in the vicinity of blood vessels adapted to higher pO_2. This mechanism will enable all cells of a tumor to individually respond to acute changes in oxygen tension.

In addition, this pathway may help the cell to limit the extent of its adaptive response during hypoxia. We and others have shown that HIF activation can inhibit tumor growth, in part by induction of tumor cell death (10–12). As PHDs are the main regulators of HIF function, we reasoned that PHD induction might help to balance these tumor-suppressive components. Indeed, we show here that PHD inhibition increased whereas PHD activation protected against hypoxic cell death as well as cell death induced by cell stress activators such as TRAIL or staurosporine, arguing for a more general protective role of PHDs in glioblastoma. It is tempting to speculate that the perinecrotic upregulation of PHD2 and PHD3 in glioblastoma biopsies may represent such an adaptive response. We believe that

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these results are of high importance for cell death therapies of glioblastoma, as resistance to cell death is the predominant cause for treatment failure in glioblastoma. From a clinical perspective, our findings of a synergistic action of PHD inhibition with chemotherapeutic agents argue for further investigation of PHD inhibitors in combination with chemotherapeutics such as TRAIL to overcome cell death resistance in glioblastomas. However, as with HIF function, the role of PHD in determining cell survival may be more complex and cell type–specific. A proapoptotic function of PHD3 in neuronal cells and other cells has been reported (24, 38, 39). Similarly, increasing PHD1 expression reduced colon carcinoma growth (40). In contrast, in head and neck carcinoma or pancreatic endocrine cancer, a positive correlation between PHD expression and tumor progression has been established (41, 42).

In conclusion, we show here that the HIF-dependent increase in PHD2 and PHD3 expression serves within a negative feedback during hypoxia to regulate the cell-specific oxygen threshold for HIF activation. We propose that this feedback loop enables the cells to adapt to the heterogeneous intratumoral oxygen distribution and regulate the extent and quality of the hypoxia response. Thus, PHDs belong to a growing list of negative feedback factors that help to attenuate the hypoxic response during chronic hypoxia such as antisense HIF-1α or CITED2 (43–45). Future studies will be necessary to determine the therapeutic utility of interfering with this loop. Given the large differences in tissue-specific expression patterns of PHDs, the qualitative role of the PHD system may also vary between tumor entities, underscoring the need for a detailed and tumor type–specific analysis of this crucial oxygen sensor pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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