Loss of PHD3 allows tumours to overcome hypoxic growth inhibition and sustain proliferation through EGFR

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Solid tumours are exposed to microenvironmental factors such as hypoxia that normally inhibit cell growth. However, tumour cells are capable of counteracting these signals through mechanisms that are largely unknown. Here we show that the prolyl hydroxylase PHD3 restrains tumour growth in response to microenvironmental cues through the control of EGFR. PHD3 silencing in human gliomas or genetic deletion in a murine high-grade astrocytoma model markedly promotes tumour growth and the ability of tumours to continue growing under unfavourable conditions. The growth-suppressive function of PHD3 is independent of the established PHD3 targets HIF and NF-κB and its hydroxylase activity. Instead, loss of PHD3 results in hyperphosphorylation of epidermal growth factor receptor (EGFR). Importantly, epigenetic/genetic silencing of PHD3 preferentially occurs in gliomas without EGFR amplification. Our findings reveal that PHD3 inactivation provides an alternative route of EGFR activation through which tumour cells sustain proliferative signalling even under conditions of limited oxygen availability.

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Growing tumours frequently exist within a hypoxic tumour microenvironment because of insufficient blood supply. Hypoxia initiates a wide array of adaptive cellular responses that ultimately promote a more aggressive tumour phenotype. In nonmalignant tissues hypoxia induces a process termed oxygen conformance that is associated with decreased proliferation and enables cell survival under conditions when energy becomes scarce. Little is known, however, about how tumour cells are able to overcome and counteract the growth inhibitory effects of hypoxia to sustain their aberrant growth. The cellular response to hypoxia is primarily mediated by the hypoxia-inducible factors (HIFs). HIF abundance is tightly regulated by the prolyl hydroxylase domain proteins 1–3 (PHDs); also called EglN3,4, which hydroxylate prolyl residues within the oxygen-dependent degradation domain of HIFs. In addition, PHD substrates other than HIF, and PHD functions independent of its enzymatic activity, are being increasingly identified. Mechanistic insight into the function of PHDs in tumourigenesis remains limited. Both pro- and antitumourigenic functions have been attributed to PHD1 (refs 12,13) and PHD2 (refs 6,14,15), while recent work suggests a role for PHD3 in suppressing the growth of diverse tumour types. Apart from hypoxia, which is a strong stimulus for PHD3 expression, PHD3 abundance is regulated by other stress-related mechanisms such as growth factor deprivation. These features may allow PHD3 to act as a key sensor of stress signals within the tumour microenvironment. We therefore investigated by which mechanisms inactivation of PHD3 may enable tumours to sustain their growth and overcome growth inhibitory signals within the hypoxic microenvironment.

Results

PHD3 is silenced in glioma progression. We first examined PHD3 mRNA expression levels during glioma progression in a panel of 76 WHO (World Health Organization) grade II–IV glioma patients. Despite a strong induction of the hypoxia marker CAIX in primary and secondary glioblastomas, mRNA levels of PHD3, which can be highly upregulated by hypoxia, remained unchanged or were even significantly lower, respectively, compared with low-grade gliomas (WHO grade II; Fig. 1a,b). These results suggested that PHD3 expression levels are attenuated in glioma progression and, importantly, are kept low even though tumours activate the hypoxic response. We therefore examined whether PHD3 was genetically or epigenetically inactivated in gliomas. Copy number analysis revealed that the PHD3 genomic region was within a large region of deletion in over 20% of all gliomas from different cohorts (Supplementary Fig. 1a–c). Furthermore, PHD3 genetic loss was associated with downregulation of PHD3 expression (Supplementary Fig. 1d), suggesting that single-copy loss of PHD3 may contribute in part to clonal selection of cells carrying this broad deletion. We next assessed whether PHD3 may also be epigenetically silenced in gliomas by promoter hypermethylation, as has been recently reported in multiple myeloma. Methylation-specific PCR (Supplementary Fig. 1e) revealed that PHD3 CpG sites were methylated in more than 80% of all patients with low-grade and anaplastic astrocytomas as well as secondary glioblastomas (Fig. 1c) and to a lower degree in primary glioblastoma patients. Notably and in line with an attenuation of PHD3 expression by promoter methylation, gliomas with increased PHD3 CpG methylation exhibited significantly lower PHD3 levels (Fig. 1d). These findings were corroborated with the TCGA glioblastoma cohort (Fig. 1e, Supplementary Fig. 1f). Importantly, treatment with the DNA methyltransferase inhibitor 5-Azacytidine (5-AzaC) and the histone deacetylase inhibitor trichostatin A (TSA) significantly upregulated PHD3 expression in glioma cell lines with a methylated promoter (Fig. 1f, Supplementary Fig. 1g), supporting the role of promoter methylation in the control of PHD3 expression. Taken together, these results show that PHD3 expression is frequently downregulated by both genetic deletion and promoter hypermethylation.

Loss of PHD3 increases tumour growth. To gain insight into the role of PHD3 downregulation in tumour growth, we generated lentiviral knockdown G55 glioblastoma cells for PHD3 (Fig. 2a, Supplementary Fig. 2a). Lentiviral PHD3 silencing elevated HIF-2α levels during acute hypoxia (Fig. 2a) and allowed HIF-1α and HIF-2α accumulation during chronic hypoxia (Supplementary Fig. 2b), confirming the functionality of the knockdown. PHD3-deficient G55 cells revealed a strikingly accelerated tumour growth and decreased survival in an intracranial xenograft model (Fig. 2b–d). Within 2 weeks, shPHD3 tumours grew to a fivefold larger size than control tumours. This effect was specific for the PHD3 isomorph, as PHD2 knockdown did not alter tumour growth (Fig. 2c–e). We corroborated our results in the primary glioblastoma cell line GBM046x, isolated from a patient biopsy, in which PHD3 silencing significantly promoted intracranial tumour growth (Supplementary Fig. 3a–c). To confirm our findings of a tumour growth-suppressive role of PHD3 in a PHD3 null background, we genetically inactivated PHD3 in a mouse glioma model. We generated murine high-grade astrocytomas lacking PHD3 by Cre-recombination in immortalized and transformed astrocytes isolated from PHD3flox/flox mice (Fig. 2e, Supplementary Fig. 4). Intracranial injection of PHD3−/− astrocytomas yielded tumours with a highly increased growth and over fourfold larger volume than control tumours (Fig. 2f,g).

PHD3 regulates tumour cell apoptosis and proliferation. Next we sought to understand how PHD3 disruption promotes tumour growth. The acquisition of a vascular supply is rate-limiting for tumour growth and is centrally regulated by the PHD/HIF system. Therefore, we investigated whether PHD3 deficiency affects tumour angiogenesis. Analysis of tumour blood vessels did not reveal a significant difference in vessel density or vessel morphology between control and PHD3-deficient tumours (Supplementary Fig. 4) indicating that the tumour growth-promoting effects of PHD3 loss are independent of the regulation of tumour vascularization. Consistently, vascular endothelial growth factor (VEGF) mRNA levels were not increased following PHD3 silencing (Supplementary Fig. 2a). Instead, the increased growth of PHD3-silenced G55 tumours could be attributed to a combination of decreased tumour cell apoptosis as assessed with TdT-mediated dUTP nick end labeling (TUNEL) staining (Fig. 2h,i) and increased cell proliferation as determined by the proliferation marker phospho-histone H3 (Fig. 2j,k). These results were corroborated in tumours originating from the PHD3-silenced primary glioblastoma cell line GBM046x (Supplementary Fig. 5a–d) and from the PHD3−/− astrocytomas (Supplementary Fig. 5e–h). These data suggest that PHD3 loss promotes resistance to apoptosis induction and increases cell proliferation.

We next set up an in vitro assay to mechanistically dissect the PHD3-dependent control of tumour cell growth. We used a three-dimensional (3D) tumour spheroid culture system under defined serum-independent conditions that more closely replicates the growth characteristics of tumours in vivo and sensitively responds to changes in growth factor concentration. The number of spheres as a parameter of clonal cell growth was significantly increased by PHD3, but not by PHD2 inactivation following
EGF/FGF stimulation (Fig. 3a,b, Supplementary Fig. 6a). The capacity to sustain proliferative signalling is one of the hallmarks of malignancy\cite{14} and can be tested in vitro by the ability of tumour cells to grow under growth factor-deprived conditions. While sphere formation was drastically reduced upon growth factor withdrawal in control tumour cells, PHD3 inactivation allowed tumour cells to continue growing in the absence of exogenous growth factors (Fig. 3a,b). Correspondingly and in line with the in vivo results, PHD3 loss almost completely protected cells against cell death induction under starving conditions (Fig. 3c,d) and enhanced cell proliferation both in the presence and in the absence of growth factors (Fig. 3e,f). The effect of PHD3 inactivation on cell growth was confirmed in PHD3\textsuperscript{−/−} astrocytomas (Fig. 3g), two additional glioblastoma cell lines (G141 and HGBM), as well as the primary line GMB046x (Supplementary Fig. 6b–d). Taken together, these data establish that PHD3 is a critical regulator of cell growth and survival and that PHD3 deficiency in gliomas confers a pronounced growth advantage in vitro and in vivo.

**PHD3 mediates growth inhibition.** Given the importance of PHD3 in growth control, we wanted to test the hypothesis that changes in PHD3 levels may allow cells to direct the cell growth response. Except for hypoxia, little is known about additional stimuli that regulate PHD3 expression. We therefore first determined whether PHD3 levels may be flexibly altered by growth-stimulating and -inhibiting conditions. Notably, hypoxic treatment, induction of hypoglycaemia with 2-deoxyglucose or incubation with the cytokine tumour-necrosis factor (TNF)-α (Fig. 4a,b, Supplementary Fig. 7a,b) strikingly increased PHD3 levels, whereas growth stimulation with EGF reduced PHD3 levels (Fig. 4c, Supplementary Fig. 7b), indicating that growth inhibitory conditions stimulate PHD3 expression. We next addressed whether increased PHD3 levels could relay the growth inhibitory signals. While hypoxia strikingly reduced total cell number and sphere-forming capacity in the glioma cell line G141, which robustly induces PHD3 under hypoxia, PHD3 loss allowed for a continued sphere formation and cell accumulation under hypoxia (Fig. 4d, Supplementary Fig. 7a–d). To functionally corroborate this role of PHD3 in tumour growth suppression we used G55 cells with increased PHD3 expression levels, and control G55 cells expressing green fluorescent protein (GFP). Increased PHD3 expression significantly reduced intracranial tumour growth (Fig. 4e,f), confirming the growth-suppressive function of PHD3. Similarly, PHD3 decreased sphere formation even in the presence of growth factors (Fig. 4g), concomitantly with an increase in cell death and a decrease in proliferation (Fig. 4h,k). Collectively, these results demonstrate that tumour growth responses are highly sensitive to PHD3 expression levels, indicating that PHD3 is a key regulator of tumour growth control in response to microenvironmental cues.

**PHD3-mediated growth control is hydroxylase-independent.** We next addressed the mechanisms by which PHD3 disruption...
Figure 2 | Loss of PHD3 increases tumour growth. (a) Immunoblot of G55 glioblastoma cells stably transduced with control (co), PHD2 or PHD3 shRNA following exposure to 21% (N) or 1% O₂ (H) for 24 h. (b,c) PHD3, but not PHD2, loss promotes intracranial glioma growth. Tumour xenografts of polyclonal G55 pools expressing control, PHD2, PHD3 or PHD2/PHD3 shRNA were stained with haematoxylin and eosin (HE; N), or immunostained for Ki-67 (C) or β-catenin (D). (e,f) Genetic inactivation of PHD3 increases mouse astrocytoma growth (n = 9–10). (g,h) Kaplan–Meier survival curves of nude mice intracranially injected with polyclonal G55 pools expressing control or PHD3 shRNA. (i,j) PHD3 silencing reduces tumour cell apoptosis in xenografts of polyclonal G55 pools (n = 8) assessed by quantifying the number of TUNEL-positive cells per tumour area. (k,l) Silencing of PHD3 increases cell proliferation in xenografts of polyclonal G55 pools (n = 8) quantified as the number of phospho-histone 3-positive cells per tumour area. Western blots images (a,e) have been cropped for presentation. Full size images are presented in Supplementary Fig. 10. All values are means ± s.e.m. *P<0.05; **P<0.01; ***P<0.001. Scale bars, 1 mm (b,f), 50 μm (h,j).
tyrosine phosphorylation of epidermal growth factor receptor (EGFR) was selectively increased following PHD3 loss under basal conditions (Supplementary Fig. 9a) as well as after stimulation with EGF (Supplementary Fig. 9b). In agreement with a specific effect on EGFR phosphorylation, PHD3 loss did not result in an overall increase in tyrosine phosphorylation (Supplementary Fig. 9b). On the contrary, phosphorylation of other RTKs including PDGFRα, VEGFR1 and EphB2 was even reduced (Supplementary Fig. 9a). The apparent increased phosphorylation of Axl was not confirmed when using specific antibodies for this receptor (data not shown). We next confirmed that PHD3 alters EGFR phosphorylation using two different phospho-specific antibodies for this receptor (data not shown). We next confirmed that the growth-promoting effects of PHD3 inactivation are functionally dependent on the increased EGFR activity. Taken together, these data demonstrate that the growth advantage conferred by PHD3 loss is because of hyperactivation of EGFR signalling, suggesting a molecular crosstalk between PHD3 and EGFR signalling.

**Discussion**

Carcinogenesis is a multistep process that endows tumour cells with a set of hallmarks including sustained proliferative

**Figure 3** | Loss of PHD3 promotes tumour cell survival and proliferation. (a, b) PHD3 silencing increases clonal cell growth in a 3D spheroid culture system. G55 cells expressing control or PHD3 shRNA were cultured as spheroids in B27-supplemented serum-free medium ± EGF/FGF and the number of spheroids was quantified after 3 days (n = 6). (c, d) PHD3 loss protects against cell death induction following growth factor withdrawal. Apoptosis was assessed by quantifying the number of TUNEL-positive cells after 48 h of incubation in B27-supplemented serum-free medium ± EGF/FGF (n = 15). (e, f) PHD3 loss increases cell proliferation. Cell proliferation was assessed by quantifying the number of 5-bromodeoxyuridine-positive cells after 48 h of incubation in B27-supplemented serum-free medium ± EGF/FGF (n = 15). (g) PHD3 disruption by genetic inactivation confers a growth advantage in additional glioma cell systems. Clonal cell growth was quantified in b in the presence or absence of EGF/FGF in PHD3−/− astrocytoma cells (n = 6). All values are means ± s.e.m., **P < 0.01; ***P < 0.001. Scale bars, 50 μm.

**PHD3 is lost in glioblastomas without EGFR amplification.** As our findings show that the tumor growth-promoting effect of PHD3 silencing is exerted through activation of EGFR, we next sought to determine how PHD3 inactivation relates to other mechanisms that enhance EGFR signalling, for example, genetic EGFR amplification, which is a characteristic feature of primary glioblastoma. We found that, while a high level of EGFR amplification was observed in around half of the glioblastoma patients from the TCGA cohort, PHD3 suppression through deletion and/or promoter methylation was much more common in the tumors without EGFR amplification: over 70% of the patients with PHD3 deletion and/or promoter methylation did not have an EGFR amplification, while over 80% of the patients with an EGFR amplification did not harbour a PHD3 deletion and/or promoter methylation (Fig. 6d,e). These findings suggest that PHD3 depletion plays a complementary role as an alternative EGFR-activating mechanism and may be particularly relevant for tumours without EGFR amplification.
Figure 4 | PHD3 is a mediator of hypoxic growth inhibition. (a–c) Growth inhibitory signals induce PHD3 expression. Immunoblot of G141 and HGBM tumour cells following exposure to 21% (N) or 1% O2 (H) for 24h, in combination with ± hypoglycaemia (treatment with 6 mM 2-deoxyglucose) (a), incubation with TNFα (10 ng ml−1) (b) or EGF (20 ng ml−1) (c). (d) PHD3 loss protects against hypoxic growth inhibition. Clonal spheroid growth (n = 6) of G141 tumour cells expressing control or PHD3 shRNA was quantified following exposure to 21% (N) or 1% O2 (H) for 3 days. (e,f) PHD3 inhibits intracranial glioma growth. Tumour xenografts of polyclonal G55 pools expressing PHD3 or GFP control were stained with HE (e) and the tumour volume was quantified (f) (n = 9–10). (g) PHD3 decreases clonal cell growth in a 3D spheroid culture system. G55 cells expressing PHD3 or GFP control were cultured as spheroids in B27-supplemented serum-free medium (phospho-) and the number of spheroids was quantified after 3 days (n = 6). (h,i) PHD3 expression enhances apoptosis. Cell apoptosis was assessed in G55 pools expressing PHD3 or GFP control by quantifying the number of TUNEL-positive cells after 48h of incubation in B27-supplemented serum-free medium ± EGF/FGF and the number of spheroids was quantified after 3 days (n = 6). (j,k) PHD3 inhibits proliferation. Clonal spheroid growth (n = 18). (j,k) PHD3 inhibits proliferation. Cell proliferation was assessed in G55 pools expressing PHD3 or GFP control by quantifying the number of BrdU-positive cells after 48h of incubation in B27-supplemented serum-free medium ± EGF/FGF (n = 15). Western blot images (a–c) have been cropped for presentation. Full size images are presented in Supplementary Fig. 10. All values are means ± s.e.m., *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars, 1 mm (e), 50 μm (h,j).
The upregulation of PHD3 together with PHD2 can be part of a negative feedback loop that restrains the pro-apoptotic functions of HIF. Notably, the HIF-dependent effects of PHD3 on apoptosis under these conditions are only consistently manifested if PHD2 is co-silenced. Our present work reveals that the silencing of PHD3, which occurs during glioma progression, primarily affects tumor growth by different mechanisms that do not depend on HIF, but centre around the control of EGFR activity, resulting in a net stimulation of cell growth coupled to reduced apoptosis. Thus, our data indicate

**Figure 5** | PHD3 function in tumour growth is hydroxylase-independent. (a,b) The growth-promoting effect of PHD3 loss is HIF-1/2α-independent. G55 cells expressing control or PHD3 shRNA in combination with control, HIF-1α or HIF-2α (a) or double HIF-1α/2α (b) shRNA were cultured as spheroids in B27-supplemented serum-free medium ± EGF/FGF and the number of spheroids was quantified after 3-5 days (n = 6). (c,d) NF-κB signalling is not altered following PHD3 loss. G55 cells expressing control or PHD3 shRNA were cultured as spheroids ± treatment with TNF-α (10 ng ml⁻¹, 30 min). Immunofluorescence with anti-p65 antibodies shows that basal and TNF-α-stimulated translocation of p65 to the nucleus is not affected by PHD3 loss (c). PHD3 loss does not activate NF-κB signalling. G55 cells expressing control, PHD3 or PHD2 (positive control) shRNA were transfected with an NF-κB luciferase reporter construct ± treatment with TNF-α (10 ng ml⁻¹, 6 h) and EGF (20 ng ml⁻¹, 6 h; n = 3) (d). (e) The hydroxylase function of PHD3 is not required for the regulation of tumour cell growth. G55 cells expressing PHD3 or control shRNA were transfected with either wild-type PHD3, the hydroxylase mutant PHD3-H196A or empty vector control, cultured as spheroids in B27-supplemented serum-free medium ± EGF/FGF and the number of spheroids was quantified after 3 days (n = 6). (f) PHD3 wild-type and hydroxylase mutant decrease the PHD3-loss-mediated increase in intracranial tumour growth (n = 9-10). All values are means ± s.e.m., *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars, 10 μm.
that PHD3 suppression represents an important step in the transition of tumour cells from a dormant to a proliferative state under hypoxia or other growth-inhibiting conditions.

Two features may allow PHD3 to act as a key regulator of growth control: its robust induction by growth inhibitory signals and its hydroxylase-independent control of tumour growth. PHD3 is most potently upregulated under hypoxic conditions compared with other PHD isoforms and is also induced through growth factor deprivation in neuronal cells. Importantly, we show here that in glioblastoma cells hypoxia cooperates with other growth-inhibiting signals, such as growth factor/nutrient deprivation and treatment with inhibitory cytokines, to robustly upregulate PHD3 expression.

Furthermore, the dispensability of PHD3 hydroxylase activity for its HIF-independent tumour-suppressive role in glioblastoma uncouples PHD3 function from the requirement for cofactors (Fe^{2+}, ascorbate) and co-substrates (O_2, 2-oxoglutarate). PHD3 is thus able to control cell proliferation over a broad physiological range, since its influence on cellular processes is not constrained by the availability of these rate-limiting factors within the tumour microenvironment. This implies that the control of PHD3 expression levels is critical for tumour growth and progression in particular under hypoxic and nutrient-deprived conditions. Such extrinsic cues dynamically regulate PHD3 levels and could contribute to its downregulation in tumour progression. In addition, genetic and epigenetic mechanisms can also lead to PHD3 depletion to promote tumour growth. Our clinical data show that PHD3 expression is attenuated during glioma progression even though high-grade gliomas are highly hypoxic. The majority of gliomas in our cohort had a methylated PHD3 promoter and this was associated with a marked decrease in PHD3 expression. The general relevance of this phenomenon is supported by recent reports that have linked PHD3 promoter methylation to impaired expression in B-cell neoplasias, colorectal cancer and various cancer cell lines. Genetic deletion of PHD3. It is nevertheless feasible that the loss of additional genes located within this region may also contribute to the selection of cell clones in which it is deleted. Additional functions of PHD3, for example, related to the control of the hypoxic response, are likely to further contribute to the selection of tumour cells with PHD3 loss, as suggested by the fact that epigenetic PHD3 silencing was also observed in low-grade gliomas, where EGFR signalling is not thought to play a major role.
Although the regulation of HIF stability by hydroxylation is the best studied function of the PHD protein family so far, a growing number of studies demonstrate that PHDs also possess important HIF-independent functions. Interestingly, there is particularly abundant evidence of HIF-independent interaction partners and functions for PHD3 (refs 10,11,45–48 including the regulation of the candidate tumour suppressor KIF1Bβ15), as well as the stress response protein and transcription factor ATF4 (ref. 8). Our own results show that the growth advantage conferred by silencing of PHD3 was not affected by suppression of HIF proteins, arguing against a role for HIF in this process. Similarly, PHD3 depletion did not affect the activity of the NF-κB pathway, which has also been proposed as a PHD target11,12,15. Several reports indicate the necessity for functional PHD3 enzyme even for targets different from HIF1α,20,25,35. At the same time, however, there is also evidence that PHD3 can act in a hydroxylation-independent manner. For example, the tumour-suppressor function of PHD3 in colorectal cancer, which is mediated through an IKK-B- dependent pathway, does not require the enzymatic activity of PHD3 (ref. 3). Our data show that the hydroxylase function of PHD3 is not dispensable for sustaining tumour growth, as demonstrated by our findings that two PHD3 mutants incapable of hydroxylation phenocopied the growth inhibitory functions of the wild-type protein. Instead, we have discovered that PHD3 loss sustains proliferative signalling in glioblastomas through the upregulation of EGFR phosphorylation. As shown in the accompanying paper19, the increased EGFR phosphorylation is a result of a deregulated EGFR endocytosis. The accompanying study19 unravels a novel scaffold function for PHD3 that regulates the internalization and signalling of EGFR by controlling the recruitment of the endocytic adaptor machinery to EGFR. Thus, our findings uncover the PHD3-dependent regulation of EGFR as a novel mechanism, extending the range of PHD3 targets to a new signalling pathway with a key role in tumour progression.

In summary, both studies together (see ref. 49) support a model whereby PHD3 levels in tumour cells are flexibly regulated by various growth-promoting and growth inhibitory signals from the tumour microenvironment through the regulation of EGFR signalling. Within this context, genetic deletion and epigenetic silencing would contribute to a downregulation of PHD3 in tumour progression, thereby decreasing the sensitivity to growth inhibitory signals and shifting the overall balance towards growth promotion. Taken together, our results demonstrate that PHD3 downregulation can indeed provide a common mechanism through which tumours overcome and counteract the growth inhibitory effect of hypoxia, allowing aberrant tumour growth even at low oxygen tension.

Methods

Cell culture. The glioblastoma cell lines G5T1 and G414 were kindly provided by M. Weissthal and K. Lamszus (Hamburg, Germany), HGBM by H. Weich (Braunschweig, Germany). Glioblastoma cell lines were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (PAN Systems). For propagating cells under neurosphere conditions, cell culture dishes, plates or flasks were coated with 10 mg ml−1 pHEMA and dried. Neurosphere medium (Invitrogen) was supplemented with 1 pHEMA and 2 blasticidin (Invitrogen, pGIPZ lentiviral shRNAmir vectors for HIF-1α; B pathway, which has also been proposed as a PHD target11,12,15. Several reports indicate the necessity for functional PHD3 enzyme even for targets different from HIF1α,20,25,35. At the same time, however, there is also evidence that PHD3 can act in a hydroxylation-independent manner. For example, the tumour-suppressor function of PHD3 in colorectal cancer, which is mediated through an IKK-B- dependent pathway, does not require the enzymatic activity of PHD3 (ref. 3). Our data show that the hydroxylase function of PHD3 is not dispensable for sustaining tumour growth, as demonstrated by our findings that two PHD3 mutants incapable of hydroxylation phenocopied the growth inhibitory functions of the wild-type protein. Instead, we have discovered that PHD3 loss sustains proliferative signalling in glioblastomas through the upregulation of EGFR phosphorylation. As shown in the accompanying paper19, the increased EGFR phosphorylation is a result of a deregulated EGFR endocytosis. The accompanying study19 unravels a novel scaffold function for PHD3 that regulates the internalization and signalling of EGFR by controlling the recruitment of the endocytic adaptor machinery to EGFR. Thus, our findings uncover the PHD3-dependent regulation of EGFR as a novel mechanism, extending the range of PHD3 targets to a new signalling pathway with a key role in tumour progression.

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GW/EmGFP-miR vectors (Invitrogen) expressing the target (PHD3 and PHD2) micro RNAs (short hairpin RNA, shRNAs) according to the manufacturer’s instructions. The pLenti6/V5-GW/EmGFP-miR vector (Invitrogen) was used as control. The Rapid BP/LR recombination reaction (Block-it Lentiviral Pol II miRNA Expression System; Invitrogen) between pDONR 221, pCDNA6.2-GW/EmGFP-miR and pLent6/V5-DEST was performed to generate the pLent6/V5-GW/EmGFP-miR expression construct.

Transfection, virus production and infection. G55TL cells were stably transfected with the pTet-Off regulator plasmid (Clontech) and the pTRE2hyg/pur-PHD3 or -GFP-inducible expression plasmids, selected with 200 µg/ml puromycin and screened for low transgene background and high transgene induction by western blot and qPCR analyses.

Lentiviral particles using the pLIPGZ vectors were produced according to the manufacturer’s instructions. Proteins were transduced using the Lentiviral shRNA Bulk Packaging System (Open Biosystems). Cells were selected with 2 µg/ml puromycin to obtain resistant polyclonal cell pools. Lentiviral particles using the Block-it system were produced in HEK293T cells using the ViraPower Lentiviral Expression System (Invitrogen) and Lipofectamine 2000 according to the manufacturer’s instructions or the pCI-VSVG and psPAX2 packaging plasmids and calcium phosphate transfection. G55TL and HGBM were selected with 6 µg/ml G418 with the pTet-Off regulator plasmid (Clontech) and the pTRE2hyg/pur-PHD3 or -GFP-inducible expression plasmids, selected with 200 µg/ml puromycin to obtain resistant polyclonal cell pools. Gene expression analysis was performed using western blot and qPCR analyses.

Tumour xenographs and tumour volume measurement. Xenograft transplantations were performed in a nonblinded manner in nonrandomized 6- to 7-week-old female athymic NMRI nu/nu mice according to the institutional guidelines and permission and the animal experiments, obtained from the regional authorities of the State of Hessen. In all, 5,000 cells (all G55TL-derived transgenic cell pools), 25,000 cells (PHD3/- and control murine astrocytomas), 20,000 cells (PHD3 WT and PHD3 hydroxylysyl-defective mutant astrocytoma rescue experiments) in a volume of 1 µl, or 200,000 cells (GM4064-α-laxata pools) in a volume of 2 µl, were stereotactically implanted intracranially into the left striatum. Group size was chosen based on previous empirical experience with analogous animal tumour models23,24. After the onset of neurological symptoms all mice were killed at the same time point. For drug treatment experiments, mice that developed premature morbidity were killed and excluded from the experiment. Mice were anaesthetized with Ketamine and Xylazine and sacrificed. The brain was removed and the vasculature was perfused with 0.9% NaCl solution for 2 min and fixative (4% paraformaldehyde, PFA) for 6 min. Brains were removed and additionally fixed overnight in 4% dehydrated in 30% sucrose and rapidly frozen on dry ice for sectioning with a cryotome. The sections were stained with haematoxylin and eosin and tumour volume was determined using stereological quantification of series of 20 µm cross-sections (240 µm intervals, for G55 tumours) or every twelfth 40 µm sections (480 µm intervals for mouse astrocytoma and GM4064-α-laxata tumours) throughout the brain. The tumour area was traced with a semiautomatic stereology system (MicroBrightField) or measured on light micrographs using ImageJ.

For immunohistochemical experiments, 100 µl PBS were injected subcutaneously into the flanks of 6- to 7-week-old female nude (NMRI nu/nu) mice. Mice were given 100 mg/kg -1 Erolitinib (10% dimethylsulphoxide (DMSO)) in saline or 10% DMSO in saline solution orally every day. The tumour size was measured every 2 days with a caliper and the tumour volume was calculated using the formula: tumour volume = 0.52 x D² x D (where D is the short tumour diameter and D is the long tumour diameter).

Vessel density, cell apoptosis and proliferation in vivo. For immunohistochemical detection of vessel density, free-floating intracranial tumour sections were washed in PBS. Endogenous peroxidase was neutralized with 0.6% H₂O₂ in PBS for 30 min. After washing again in PBS, sections were mounted on microscope slides and dried for 3 h at room temperature (RT). Antigen retrieval was performed for 5 min in Tris-EDTA buffer, pH 8.0, in a steamer. Sections were blocked with 20% normal goat serum (NGS)/0.01% Triton in PBS for 2 h. Sections were then treated overnight at 4°C with CD34 primary antibody (1:100, BD Pharmingen) in 10% NGS/0.01% Triton in PBS, respectively. After washing in PBS, sections were incubated with a secondary peroxidase-conjugated goat-anti-rat IgG antibody, and diluted at 1:20 in Clarity Western ECL (Bio-Rad). Protein (25 µg) lysates were subjected to SDS–PAGE and western blot analysis was performed using antibodies specific for HIF–1α (BD Transduction Laboratories; no. 610958), HIF-2α, PHD1, PHD2, PHD3 and PHD4 (BD Transduction Laboratories, no. 610958).

Immunofluorescence. In all, 200,000 cells were plated on PHEMA-coated 6-cm dishes and cultivated as tumour cultures. For p65 staining, cells were stimulated for 30 min with 10 ng/ml TNF-α. Phalloidin was visualized by incubating with Alexa Fluor 594 conjugated Phalloidin (Invitrogen). The collection of series of 20 µm cross-sections (80 µm intervals, for G55 tumours) or every twelfth 40 µm sections (80 µm intervals for mouse astrocytoma and GM4064-α-laxata tumours) throughout the brain. The tumour area was traced with a semiautomatic stereology system (MicroBrightField) or measured on light micrographs using ImageJ.

Cell apoptosis and proliferation in vitro. To detect cell apoptosis, cells were seeded at 400,000 cells per 10-cm dish and incubated for 24 h in F12 medium ± EGF/FGF. In all, 250,000 cells were transferred to slides using a Cyto-Tek Centrifuge (Sakura Finetek) after dissociation with accutase. TUNEL staining was performed using the ApoTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon), Apoptosis was quantified as the percentage of TUNEL–positive cells in five randomly selected optical fields (×20) per condition.

Luciferase reporter assay. Cells were transiently transfected with an NF-xB-responsive promoter firefly and a SV-40 Renilla luciferase construct (Promega) for normalization of transfection efficiency, grown for 48 h under sphere conditions and assayed for luciferase activity with the Dual-Luciferase Reporter-Assay System (Promega).

Real-time qRT–PCR. RNA was extracted with the RNeasy Mini Kit (Qiagen), and reverse-transcribed using standard protocols (Superscript II Reverse Transciptase, Life Technologies or RevertAid H Minus M-MuLV Reverse Transcriptase, Fermentas). cDNA was amplified with either the Absolute QPCR SYBR Green Mix or the Absolute PCR Mix (Agene) using specific primer sequences. Genes were selected based on their expression profiles in PHD3+/+ and PHD3−/− cells. Compensations were performed in three independent experiments using the ΔΔCt method. In addition, the presence of specific primer/probe sequences was confirmed by melting curve analysis using qRT–PCR.
Phospho-RTK array analysis. The phosphorylation level of 42 human RTKs was assessed using the Phospho-RTK Array Kit (R&D Systems). 350,000 cells were plated and cultivated for 48 h in Neurosphere medium without EGF/FGF. Cells were lysed in NP-40 Buffer (1% NP-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). 150 μg of total protein were incubated with RTK array membranes. Detection of proteins was accomplished using mouse anti-phospho-tyrosine antibody conjugated to HRP and enhanced chemiluminescence, according to the manufacturer’s instructions.

PHD3 promoter methylation analyses. Cells were incubated from the second day on for 72 h with 5-AzaC (20 μM), which was changed every day (for demethylation). The last 24 h, TSA (1 μM) was additionally added to hypoxic treatment at 1% O2. DNA isolated from the cells using commercial kits (DNAeasy Blood&Tissue Kit no. 69506 and QIAamp DNA FFPE Tissue Kit no. 56404, Qiagen). CpG-Universal Methylation DNA (Chemicon/Millipore no. S 7821) served as a positive control, DNA isolated from blood with the Blood & Cell Culture DNA Spin Kit (Genomed no. 440250) was used as a negative control. DNA was then subjected to sodium bisulphite modification using the EZ DNA Methylation kit (EZ DNA Methylation-Gold Kit no. D5006 Zymo Research). To assess the methylation status of the PHD3-associated 5′ CG island, methylation-specific PCR was carried out. Two sets of primers (primer sequences) detecting either methylated or unmethylated sequences were used with slight modifications (see Supplementary Methods: Primer and shRNA sequences). Methylation-specific PCR was performed using Qiagen HotStar DNA Polymerase in a reaction volume of 25 μl. The PCR conditions were as described in ref. 16. The PCR reaction mixture was loaded on a 2% agarose gel, stained with ethidium bromide and assessed the methylation status of the PHD3-associated 5′ CpG island. DNA was then subjected to sodium bisulphite modification using the EZ DNA Methylation kit (EZ DNA Methylation-Gold Kit no. D5006 Zymo Research). To assess the methylation status of the PHD3-associated 5′ CG island, methylation-specific PCR was carried out. Two sets of primers (primer sequences) detecting either methylated or unmethylated sequences were used with slight modifications (see Supplementary Methods: Primer and shRNA sequences). Methylation-specific PCR was performed using Qiagen HotStar DNA Polymerase in a reaction volume of 25 μl. The PCR conditions were as described in ref. 16. The PCR reaction mixture was loaded on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

Bioinformatic analysis. PHD3 gene expression data (Agilent 244 K Custom Gene Expression G4402A-07 array, log 2 tumour/norm ratio, total of 424 samples with available expression data) and PHD3 promoter methylation data ( Illumina Infinium Human DNA Methylation 27 array, probe cg05769169, total of 251 samples with available methylation data) for the glioblastoma cohort of The Cancer Genome Atlas (TCGA) were downloaded from the TCGA data portal (http://tcga-portal.nci.nih.gov/tcga-portal/Anomaly/Anomaly.jsp) on 7 May 2012. EGRF and PHD3 promoter methylation data (CNA) data for the TCGA cohort, determined using the GISTIC algorithm (total of 501 samples with available CNA data) were downloaded using the WebAPI of the cBio portal (http://www.cbioportal.org/) on 9 September 2012. For PHD3 promoter methylation analysis, tumours with a beta-value ≤ 0.1 were considered as methylated. CNAs along chromosome 14 were determined using GISTIC analysis at the TCGA Copy Number Portal (http://www.broadinstitute.org/cgi/home) and the Integrative Genomics Viewer14.

Statistical analysis. Results are presented as mean ± s.e.m. Statistical analysis was performed using the Student’s t-test or, in the case of multiple comparisons in the glioma patient cohort, the ANOVA test followed by a post test for linear trend. The Kruskall test was used to eliminate outliers for the expression of CAIX and PHD3 and for in vitro tumour growth experiments. Statistical significance was defined as P < 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001).

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Author contributions
A.-T.H. generated and biochemically and functionally characterized knockdown and overexpression cell pools, analysed xenograft experiments, performed immunostainings and qPCR. B.K.G. collected data and performed bioinformatic analysis. S.S. performed transplantsations and analysed tumour volume, carried out in vivo and in vitro proliferation and apoptosis assays. A.M.C. performed in vivo tumour experiments and drug treatment. M.R. and A.F. generated stable cell lines. F.F. performed immunofluorescence and biochemistry. H.D. and C.L.E. performed biochemistry. P.H.M. and P.C. generated the conditional PHD3/-. A.M.C. provided and characterized the grade II–IV glioma collection. T.A., A.A.-P. and B.K.G. designed experiments, analysed the data, prepared the figures and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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