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Supplemental Information

The mTOR and PP2A Pathways Regulate PHD2 Phosphorylation to Fine-Tune HIF1α Levels and Colorectal Cancer Cell Survival under Hypoxia

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Supplemental Information

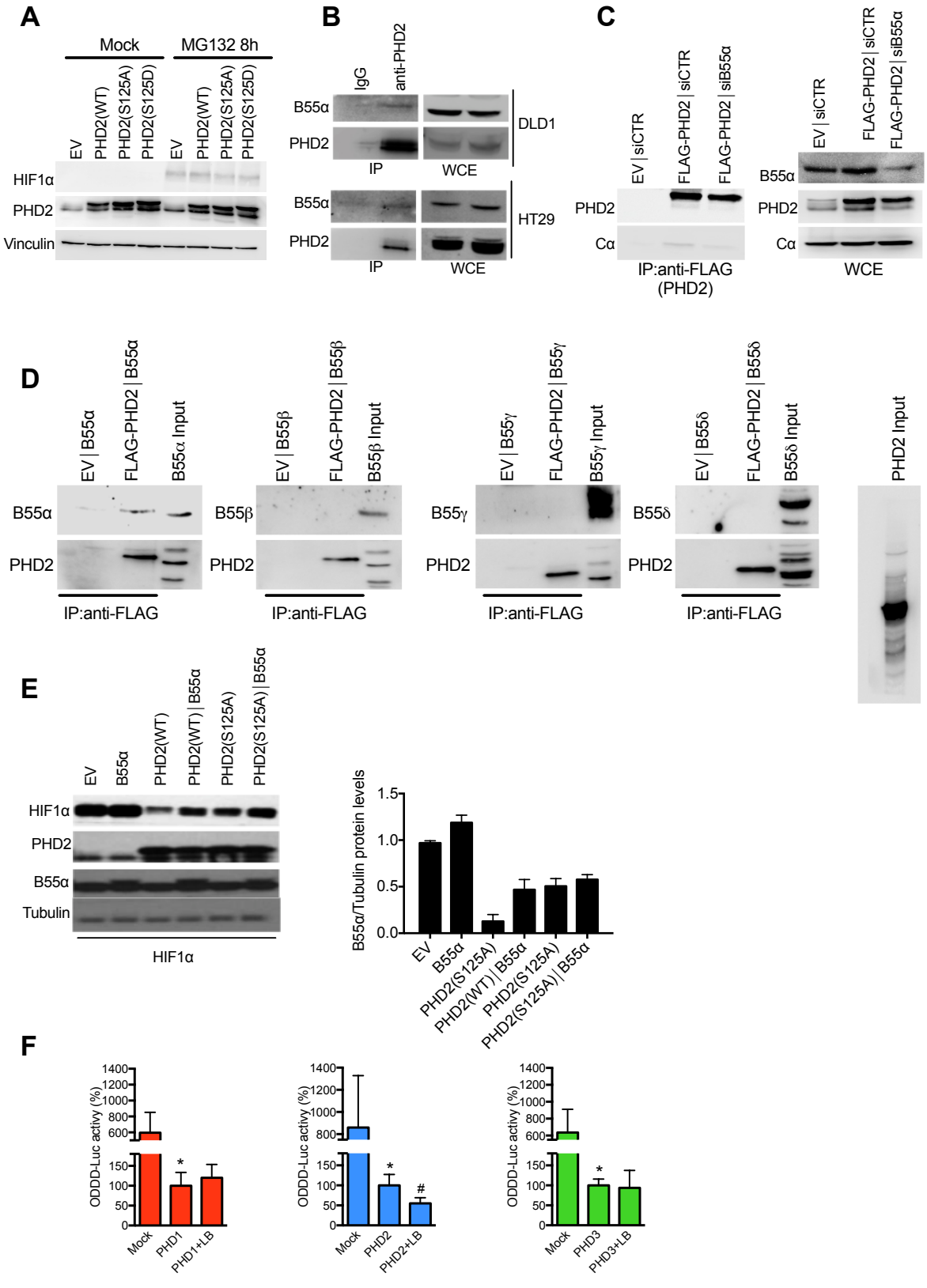
A
Mock
MG132 8h
EV
PHD2(WT)
PHD2(S125A)
PHD2(S125D)
HIF1α
PHD2
Vinculin
B
IgG
anti-PHD2
B55α
PHD2
IP
WCE
DLD1
HT29
C
EV | siCTR
FLAG-PHD2 | siCTR
FLAG-PHD2 | siB55α
Cα
IP:anti-FLAG
(PHD2)
WCE
D
EV | B55α
FLAG-PHD2 | B55α
B55α Input
EV | B55β
FLAG-PHD2 | B55β
B55β Input
EV | B55γ
FLAG-PHD2 | B55γ
B55γ Input
EV | B55δ
FLAG-PHD2 | B55δ
B55δ Input
PHD2 Input
B55β
B55γ
B55δ
IP:anti-FLAG
E
EV
B55α
PHD2(WT)
PHD2(WT) | B55α
PHD2(S125A)
PHD2(S125A) | B55α
Tubulin
HIF1α
B55α/Tubulin protein levels
1.5
1.0
0.5
0.0
F
ODDD-Luc activy (%)
1400
1200
1000
800
600
150
100
50
0
Mock
PHD1
PHD1+LB
PHD2
PHD2+LB
PHD3
PHD3+LB
*
#

Figure S1, related to Figure 1. PP2A/B55α specifically binds and dephosphorylates PHD2 in S125.
(A) HT29 cells were transfected with an empty vector (EV) or with PHD2 (WT, S125A or S125D). After 16 hours (h), cells were treated with the proteasome inhibitor MG132 (10μM) for 8h, lysed and analyzed by WB. (B) WCEs from DLD1 or HT29 cells were collected and immunoprecipitated with an anti-PHD2 antibody or with anti-IgG as control. Immunocomplexes with endogenous B55α were detected by WB analysis. (C) HEK293T cells transiently silenced for control (siCTR) or B55α (siB55α) were transfected with pcDNA3/FLAG (EV) or pcDNA3/FLAG-PHD2 (FLAG-PHD2). After 24h, whole cell extracts (WCEs) were immunoprecipitated with anti-FLAG M2 beads and immunocomplexes were analyzed by Western blot (WB) for the indicated proteins. (D) In vitro translated pcDNA3/FLAG-PHD2 (FLAG-PHD2) or pcDNA3/FLAG (EV) were immunoprecipitated with anti-FLAG M2 beads after incubation with in vitro translated B55α, B55β, B55γ or B55δ and immunocomplexes were detected by WB analysis. (E) WB analysis and quantification of HEK293T cells transiently transfected with HIF1α alone (EV) or in combination with plasmids carrying B55α and/or PHD2 (WT and S125A). (F) HEK293T cells were transfected with a luciferase fused to the oxygen-dependent degradation domain (ODDD-Luc) and either with PHD1, PHD2 or PHD3. After 8h the PP2A inhibitor LB100 (LB; 1μm) was added where indicated for an overnight. Cells were lysed and read at the luminometer. *P<0.05 versus Mock, #P<0.05 versus PHD2; N=3. Graph show mean ± SEM.

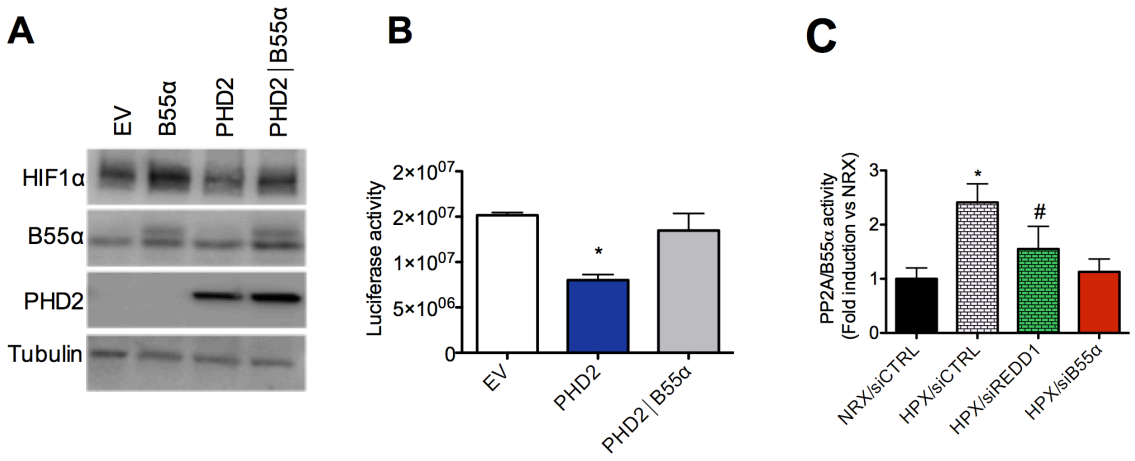

Figure S2, related to Figure 2 and 3. B55α negatively regulates PHD2 function resulting in increased HIF1α protein levels and activity.

(A) WB analysis from HT29 cells transiently transfected with pcDNA3/FLAG (EV), pcDNA3/HA-B55α, pcDNA3/FLAG-PHD2 or both and exposed to hypoxia for 16h.

(B) HEK293T cells stably overexpressing CMV-Luc-ODDD were transiently transfected with pcDNA3/FLAG (EV), pcDNA3/FLAG-PHD2 or pcDNA3/HA-B55α alone and in combination. After 8h the cells were exposed to hypoxia. After 24h cells were lysed and luciferase activity was measured and normalized for protein concentration.

(C) Measurement of the enzymatic activity of PP2A/B55α from WCE of HEK293T cells incubated in normoxia and hypoxia for 16h, as such or after silencing of REDD1. Silencing of B55α (siB55α) was used as negative control. *P<0.05 versus all the other conditions in B and C; # P<0.05 versus HPX16h in C. The graph shows mean ± SEM.

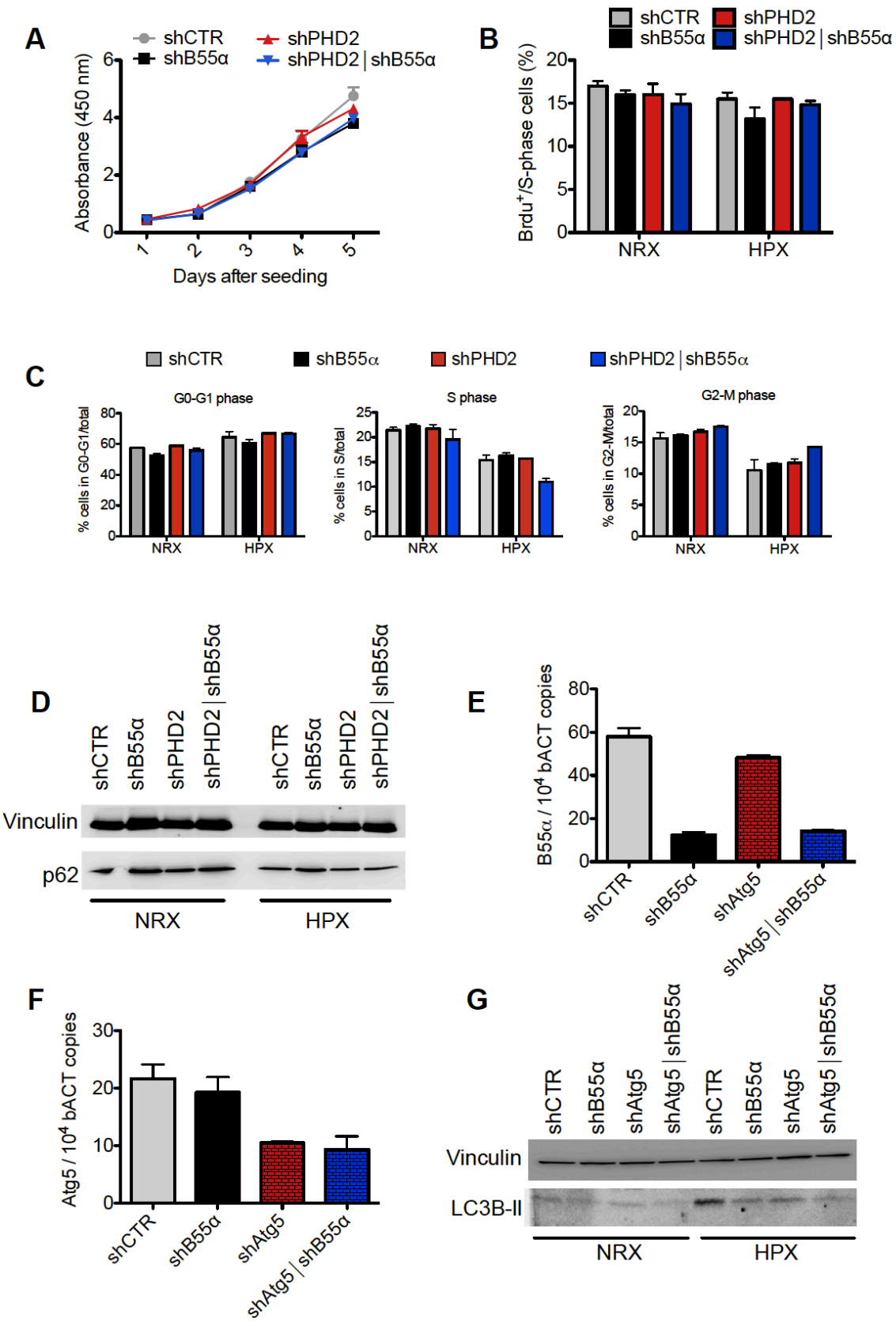
A
shCTR
shB55α
shPHD2
shPHD2 | shB55α
Absorbance (450 nm)
Days after seeding
B
Brdu+/S-phase cells (%)
NRX
HPX
C
G0-G1 phase
S phase
G2-M phase
% cells in G0-G1/total
% cells in S/total
% cells in G2-M/total
D
Vinculin
p62
E
B55α / 10^4 bACT copies
shAtg5
shAtg5 | shB55α
F
Atg5 / 10^4 bACT copies
G
LC3B-II

Figure S3, related to Figure 4. The B55α/PHD2 axis does not modify cell proliferation, but it promotes autophagy in response to hypoxia.

(A) Proliferation curve of DLD1 cells stably silenced for control (shCTR), B55α (shB55α), PHD2 (shPHD2), or both (shPHD2 | shB55α).

(B) BrdU and propidium iodide double staining was performed on DLD1 cells stably silenced for control (shCTR), B55α (shB55α), PHD2 (shPHD2), or both (shPHD2 | shB55α) following 24h in either normoxia (NRX; 21% O_2) or hypoxia (HPX; 0.2% O_2). After 1h incubation with Brdu, cells were fixed and analysed by FACS.

(C and D) DLD1 cells stably silenced for control (shCTR), B55α (shB55α), PHD2 (shPHD2), or both (shPHD2 | shB55α) were exposed to normoxia (NRX; 21% O_2) or hypoxia (HPX; 1% O_2) for 24h. Then cells were stained with propidium iodide and analysed by FACS (C). Same cells as in C were exposed to either normoxia or hypoxia for 48h. Then, WCE were analysed by WB (D).

(E and F) qRT-PCR for B55α (E) and Atg5 (F) in DLD1 cells stably silenced for control (shCTR), B55α (shB55α), Atg5 (shAtg5) or both (shAtg5 | shB55α).

(G) DLD1 stably silenced for control (shCTR), B55α (shB55α), Atg5 (shAtg5) or both (shAtg5 | shB55α) were exposed to normoxia (NRX; 21% O_2) or hypoxia (HPX; 1% O_2) for 48h and WCEs were analysed by WB. All graphs show mean ± SEM.

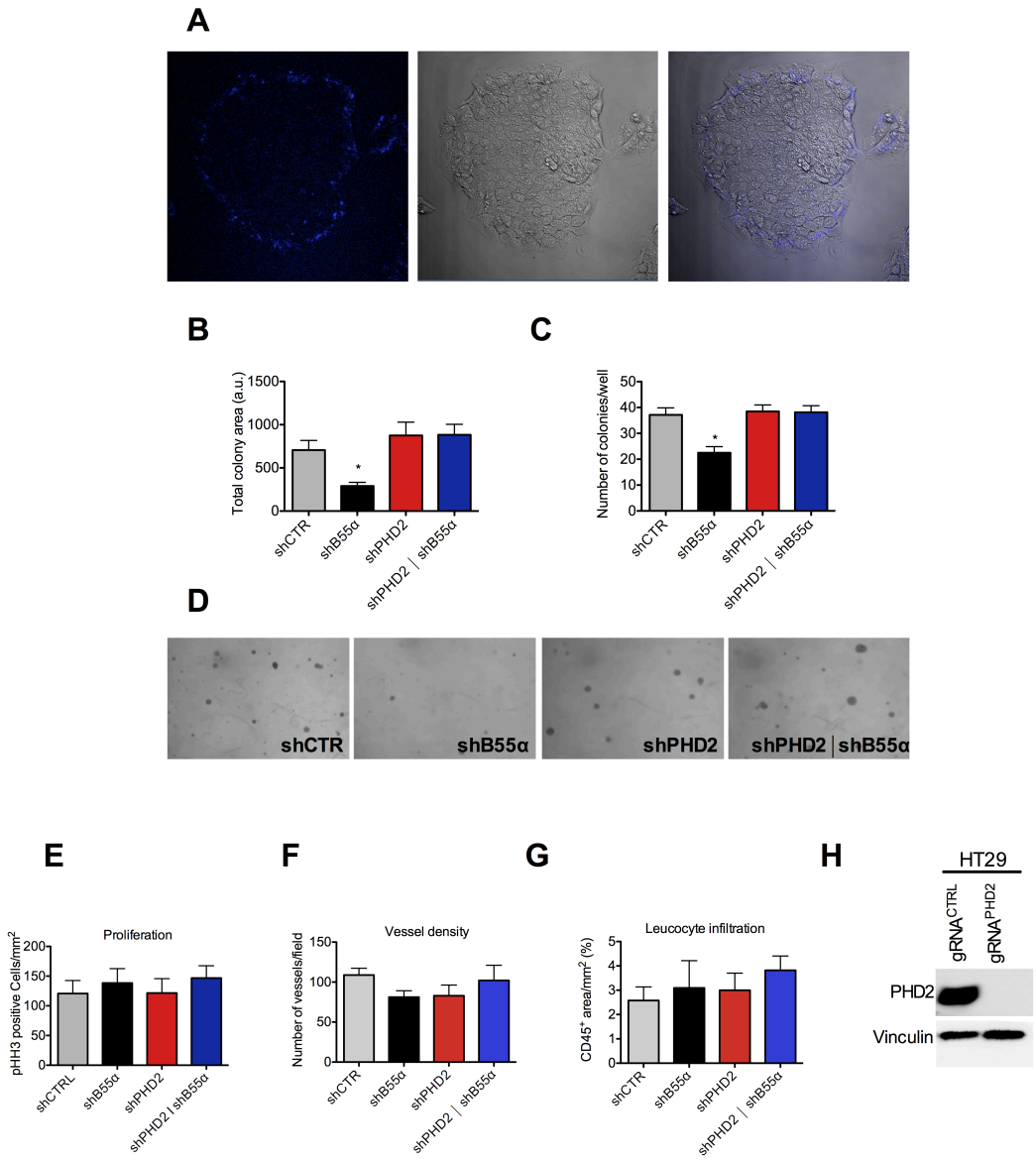

Figure S4, related to Figure 5. PP2A/B55α promotes colorectal cancer growth in a PHD2-dependent manner.

(A) Confocal images of a DLD1-derived foci after 4h incubation with the oxygen-specific probe $NaNO_2$, which labels in blue the oxygenated margin of the colony.

(B-D) Representative pictures and quantification of a soft agar assay with DLD1 cells stably silenced for control (shCTR), B55α (shB55α), PHD2 (shPHD2) or both (shPHD2 | shB55α).

(E) Morphometric quantification of DLD1 tumor sections stained for phosphoHistoneH3, showing proliferation.

(F and G) Morphometric quantification of HT29 tumour sections stained for CD31 showing vessel density (F) and for CD45 showing leucocyte infiltration (G).

(H) WB analysis for PHD2 in HT29 cells transduced with a CRISPR/Cas9 lentiviral construct harbouring a gRNA targeting PHD2 ($gRNA^{PHD2}$) or a non-targeting control ($gRNA^{CTRL}$). *P<0.05 versus all the other conditions. All graphs show mean ± SEM.

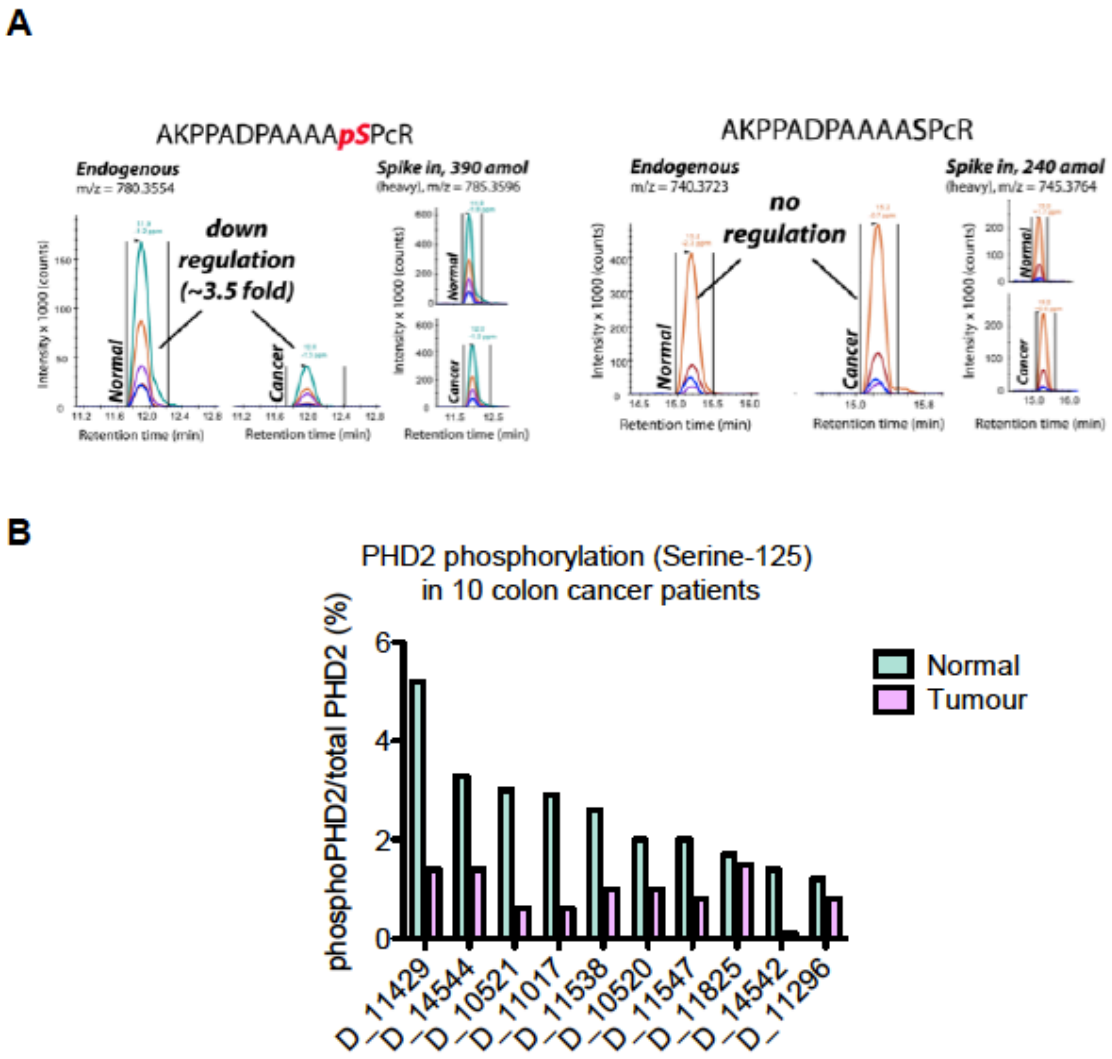

Figure S5, related to Figure 6. In human CRC, phosphorylation of PHD2 in Serine125 is abrogated in tumour samples compared to the healthy tissues.

(A) Targeted PRM to determine PHD2 S-125 phosphorylation stoichiometry in colon cancer and healthy tissue of 10 different patients. For quantification we spiked known amounts of stable-isotopic labelled (SIL) reference peptides to trypsin-digested protein. For quantification of phosphorylated PHD2, we spiked 390 amol of a SIL-peptide in 16 µg of a tryptic digest followed by phosphopeptide enrichment. For the unphosphorylated peptide we used 240 amol of the SIL-peptide in 1.2 µg of tryptic digest. PRM was performed using the 4 or 5 best transitions of each peptide.

(B) Phosphorylation stoichiometry of S-125 PHD2 reveals an average phosphorylation level of 2.5 % in healthy tissue and 0.9 % in cancerous tissue. The phosphorylation stoichiometry is given as percentage of phospho-PHD2 over total PHD2.

Fig 1C								
HIF1α /Vinculin	EV 2.36 ± 0.03	PHD2 0.52 ± 0.04	S12A 0.70 ± 0.05	S14A 0.69 ± 0.06	S125A 1.39 ± 0.061			
Fig 1D								
HIF1α/ Vinculin	EV 0.45 ± 0.05	WT 0.09 ± 0.02	S125A 0.26 ± 0.01	S125D 0.05 ± 0.05				
Fig 1E								
HIF1α/ Tubulin	EV 1.6 ± 0.04	WT 1.05± 0.03	S125A 1.5± 0.01	S125D 0.9± 0.02				
Fig 1F	MG132				HPX			
HIF1α/ Vinculin	EV 2.0±0.05	WT 1.2±0.06	S125A 1.9±0.05	S125D 1.1±0.06	EV 0.36±0.01	WT 0.21±0.04	S125A 0.29±0.01	S125D 0.19±0.04
Fig 1J								
p-PHD2/ total PHD2	Mock 1.35 ± 0.01	Rapamycin 0.61 ± 0.08	Torin2 0.40 ± 0.05	PD169316 1.38 ± 0.06	PD98059 0.95 ± 0.01			

Table S1, related to Figure 1.
Densitometric analysis performed on Western blots run from 3 independent experiments, represented in the indicated panel in Figure 1. All values show mean ± SEM.

			MS human PHD2		MS murine PHD2	
Accession	**Gene name**	**Protein name**	**Log2(L/H)**	**Peptides with valid L/H ratios**	**Log2(L/H)**	**Peptides with valid L/H ratios**
Q91YE3	Egln1	EGL nine homolog 1 (C. elegans)			0,00	26
P52480	PKM2	pyruvate kinase, muscle	1,21	30	1,30	22
Q9GZT9	EGLN1	egl nine homolog 1 (C. elegans)	0,00	15		
Q8BTM8	FLNA	filamin A, alpha			1,18	18
O35465	FKBP8	FK506 binding protein 8, 38kDa			0,01	2
Q6P1F6	**PPP2R2A**	**protein phosphatase 2, regulatory subunit B, alpha (B55α)**	**1,12**	**4**	**1,10**	**3**

Table S2, related to Figure 2. AP-MS data on PHD2 and its interactors.
A SILAC-based strategy was used to identify PHD2 interactors upon PHD2 affinity-based isolation. Log2 (L/H) ratio values are shown, together with the number of identified peptides for which L/H ratio values could be determined. Given the experimental setup, the ratio value for PHD2 was set to zero, and the ratio values for the indicated proteins were corrected. The table shows that known PHD2 interactors and B55α have similar ratio values, hinting to the fact that B55α is a PHD2 interactor.

Fig 2F				
HIF1α/ Vinculin	siCTR/ HIF1α 0.48 ± 0.06	siB55α/ HIF1α 0.13 ± 0.01	siCTR/ HIF1α–PP 0.78 ± 0.03	siB55α/ HIF1α–PP 0.91 ± 0.01
Fig 2G				
p-PHD2/ total PHD2	EV 0.00	PHD2 3.40 ± 0.02	PHD2/B55α 0.92 ± 0.04	S125A 0.00	S125A/B55α 0.00
Fig 2J				
p-PHD2/ total PHD2	EV 0.81 ± 0.03	B55α 0.18 ± 0.001		
Fig 2L				
PHD2/ Vinculin	siCTR 1.10	siCTR 1.24	siCTR 0.92	siB55α 0.78	siB55α 0.96	siB55α 1.05	siP70S6K 1.1	siP70S6K 0.97	siP70S6K 0.93

Table S3, related to Figure 2.
Densitometric analysis performed on Western blots run from 2 or 3 independent experiments, represented in the indicated panel in Figure 2. All values show mean ± SEM.

Fig 3A	HPX			
HIF1α/Tubulin	siCTR 0.89 ± 0.05	siB55α 0.39 ± 0.03	siPHD2 1.30 ± 0.01	siB55α/siPHD2 1.26 ± 0.03
Fig 3B	HPX			
HIF1α/Tubulin	siCTR 1.06 ± 0.02	siB55α 0.481 ± 0.02	siPHD2 1.76 ± 0.01	siB55α/siPHD2 1.93 ± 0.05
Fig 3G	HPX			
HIF1α/Vinculin	siCTR 1.21 ± 0.05	siB55α 0.91 ± 0.01	siREDD1 0.63 ± 0.02	siB55α/siREDD1 0.41 ± 0.05
Fig 3I				
HIF1α/Vinculin	siCTR 1.3 ± 0.02	siB55α 0.7 ± 0.02	siP70S6K 1.9 ± 0.05	siB55α/siP70S6K 2 ± 0.01

Table S4, related to Figure 3.
Densitometric analysis performed on Western blots run from 3 independent experiments, represented in the indicated panel in Figure 3. All values show mean ± SEM.

Fig 4B	NRX				HPX			
CleavPARP/Vinculin	shCTR 0.01±0.02	shB55α 0.15±0.07	shPHD2 0.05±0.04	shPHD2/shB55α 0.12±0.03	shCTR 0.21±0.05	shB55α 0.89±0.01	shPHD2 0.04±0.06	shPHD2/ shB55α 0.23±0.04
Fig 4E	NRX				HPX			
LC3BII / Vinculin	shCTR 0.46±0.01	shB55a 0.4± 0.007	shPHD2 0.4±0.003	shPHD2/ shB55a 0.35±0.03	shCTR 2.3± 0.01	shB55α 1.4± 0.001	shPHD2 2.04±0.05	shPHD2/ shB55α 1.85±0.009
Fig 4G								
HIF1α/ Vinculin	shCTR 1.21±0.04		shB55α 0.78±0.001		HIF1α 2.42±0.01		HIF1α/shB55α 2.9±0.05	
Fig 4I								
LC3BII / Vinculin	shCTR 0.55±0.05		shB55α 0.3±0.006		HIF1α 0.57±0.05		HIF1α/shB55α 0.52±0.032	

Table S5, related to Figure 4
Densitometric analysis performed on Western blots run from 3 independent experiments, represented in the indicated panel in Figure 4. All values show mean ± SEM

Supplemental Experimental Procedures

Plasmids, siRNA and lentiviral vectors. In the overexpression experiments the following plasmids were used: pcDNA3-PHD2-FLAG, pcDNA3 empty vector, pLA-B55α-FLAG, pcDNA3-B55α-HA, pcDNA3-PHD2(S12A), pcDNA3-PHD2(S14A), pcDNA3-PHD2(S125A), pcDNA3-PHD2(S125D), pcDNA3-HIF1α, pDONR223 B55β, pDONR223 B55γ, pDONR223 B55δ. Commercially available siRNAs were purchased from Invitrogen and their sequences or assay IDs are listed below: For B55α: PPP2R2AHSS108371, PPP2R2AHSS108372, PPP2R2AHSS108372 (used alone or in combination); For PHD2: EGLN1HSS123076, EGLN1HSS182577 (used in combination); For scramble: Stealth RNAi™ siRNA Negative Control Lo GC, 12935-200. To generate stable knockdown cell lines, mir-155 miRNA/microRNA lentiviral vectors (shPHD2 and respective scramble (SIMA)) carrying the following shRNA were used: for PHD2: TGCTGTCAACATGACGTACATAACCCGTTTTGGCCACTGACTGACGGGTTATGCGT-CATGTTGA; for the SIMA: TGCTGCATGAATATCTCTGTCTCCTTGTTTTGGCCACTGACTGACAAGGAGACAGATATTCATG Alternatively, PLKO lentiviral vectors (from SIGMA Aldrich) carrying a shB55α, shAtg5 or a scramble sequence as control, were used: for B55α: CCGGAGAAACACAAAGCGAGACATACTCGAGTATGTCTCGCTTTGTGTTTCTTTTTT; for Atg5: CCGGGATTCATGGAATTGAGCCAATCTCGAGATTGGCTCAATTCCATGAATCTTTTTTG; for the scramble: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT. For the overexpression of HIF1 and control the following lentiviral vectors were used: pLenti/UbC/V5-Dest-hHIF1a-deltaProline-V5 pLenti/UbC/LacZ control. Selection with Blasticidin (10 μg/ml) or puromycin (4 μg/ml) allowed the generation of a homogenous population of silenced or overexpressed (and scramble control) cells.

Western Blot analysis. Protein extraction was performed using RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Lysates were incubated on ice for 30 minutes before centrifuging 15 minutes at 4°C to remove cellular debris. Supernatants were subsequently collected. Protein concentration of cell extracts was determined by using bicinchoninic acid (BCA) reagent (Pierce) according to the manufacturer's instructions. Protein samples were denaturated by adding loading buffer 6X (β-mercaptoetanolo 0,6 M; SDS 8%; Tris-HCl 0,25 M pH 6,8; glycerol 40%; Bromophenol Blue 0,2%), incubated at 95°C for 5 minutes. After electrophoresis, proteins were transferred onto a nitrocellulose membrane using the iBlot® Dry Blotting System (Invitrogen) according to manufacturer's instructions. The membranes were blocked for non-specific binding in 5% non-fatty dry milk in Tris Buffered Saline-Tween 0.1 % (50 mM Tris HCl ph 7.6, 150 mM NaCl, 0.1% Tween; TBS-T) for 1h at room temperature (RT) and incubated with primary antibody for 2h at RT or overnight (ON) at 4°C. The following antibodies were used: PPP2R2A/B55α (Clone 2G9, Cell Signaling); EGLN1 (human PHD2) (NB-100-137, Novus Biological); Vinculin (Monoclonal anti-Vinculin, V9131, SIGMA Aldrich); Tubulin (HRP-conjugated anti-beta-tubulin, Abcam); HIF1α (human HIF1α, Cayman Chemical); Hydroxy-HIF1α (Clone D43B5, Cell Signaling); FLAG (Monoclonal anti-FLAG, SIGMA Aldrich); PP2A Ca (Clone 46, BD Bioscience); Cleaved PARP (rabbit, Cell Signaling); LC3B (Clone D11, Cell Signaling); SQSTM1 (p62) (Clone 2C11, Abnova), phosphoP70S6K (Thr389) (Cell Signaling); P70S6K (Clone 49D7, Cell Signaling); phospho-S125PHD2 (MABC1612 clone 4, Merck).
After incubation with the indicated primary antibodies, the membranes were washed for 15 minutes in TBS-T and incubated with secondary antibody (1/5000 in 5% non fatty dry milk in TBS-T) for 50 minutes at RT. The following secondary antibodies were used: goat anti-mouse and goat anti-rabbit (Santa Cruz biotechnology). The signal was visualized with Enhanced Chemiluminescent Reagents (ECL; Invitrogen) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) with a digital imager (ImageQuant LAS 4000, GE Health Care Life Science Technologies).

Immunoprecipitation. Cells were lysed using Extraction Buffer (EB; 1% triton, 20 mM Tris, 10% glycerol, 5 mM EDTA, 150 mM NaCl, pH 7.4). Protein extraction and determination of protein concentration were performed as described. For anti-FLAG immunoprecipitations, 1 or 2 mg of total protein was incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich®) followed by elution with FLAG-peptide, according to manufacturer's instructions. For endogenous co-immunoprecipitations, antibody anti-PHD2 (Novus Biological) was incubated for 2h at 4°C with magnetic Dynabeads Protein G (Life Technology) in PBS Tween 0.05% and afterwards the beads-antibody complexes were incubated 30' at 4°C with BS_3 (Sulfo-DSS, Thermo Scientific), a water soluble cross-linker in Conjugation buffer (pH 7-9) 20 mM Sodium Phosphate, 0.15 M NaCl (pH 7-9). Two mg of protein extracts from DLD1 or HT29 were incubated overnight with beads-Antibody conjugated. Immunoprecipitates were washed 3 times with EB Buffer and 3 times with TBS-Tween 0.1%. For the bead elution, NuPage LDS sample buffer (Novex, without 2-Mercaptoethanol) was added to the immunocomplexes and incubate for 10 min at 99 ºC. Supernatant was collected and denaturated by adding DTT 20mM for 10' at 99°C. Electrophoresis and Western blot were performed as described. For in vitro co-immunoprecipitation, in vitro translated FLAG-PHD2 or HA-B55a were produced by using TNT coupled wheat germ extract system/T7 promoter (Promega) according to manufacturer's instructions. 20 ml of the 50 ml reaction proteins were incubated with each other for 2 hours at 4°C in EB lysis buffer. Therefore, anti-FLAG IP was performed as previously described.

Luciferase assay. HEK293T or DLD1 cells were stably transfected with a plasmid carrying CMV-Luc-ODD (O_2-dependent degradation domain). Medium was changed every two days with culture medium containing 100 mg/ml G418 for 15 days until a homogenous population of ODDD-overexpressing cells was obtained. Subsequently, selected cells were transiently transfected with the indicated plasmids or siRNA. Alternatively DLD1 cells stably silenced for PHD2 or B55A or both, were transiently transfected with a construct encoding CMV-HRE-Luc. Cells were lysed in luciferase lysis buffer (PBS, 0.2% Triton X-100, 0.5% DTT) and luminescence was measured by using a luminometer (Microplate Luminometer LB 96 V), in presence of a luciferase Assay Reagent (CoA 500 mM, Luciferin 500 mM, ATP 1000 mM and luciferase assay buffer (20 mM, ($MgCO_3$) $4Mg(HO)_2.5H_2O$ 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM in H_2O). Luciferase activity was normalized for protein content, as determined by BCA.

Liquid Chromatography-Mass Spectrometry (LC-MS) to identify PHD2 phosphorylation sites. To identify PHD2 phosphorylation sites, overexpression of FLAG-tagged PHD2 has been performed in HEK293T cells. Cells were harvested, lysed in extraction buffer and 3 mg of total protein extracts was used for immunoprecipitation using anti-FLAG M2 affinity beads, as previously described. Immunoprecipitated proteins were separated by SDS-PAGE and stained using Coomassie. Gel bands of interest were excised, washed several times with water and acetonitrile, and completely dried in a SpeedVac. Subsequently, an in-gel trypsin digest was performed using sequence-grade modified trypsin, porcine (Promega, Madison, Wi USA) and samples were incubated overnight at 37°C. The supernatants containing the peptides were then isolated, transferred to MS-compatible vials and acidified with trifluoroacetic acid (TFA) (pH < 3). The obtained peptide mixtures were introduced into an LC-MS/MS system; the Ultimate 3000 RSLC nano (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) for analysis. Peptides were first loaded on a trapping column (made in-house, 100 mm internal diameter (I.D.) x 20 mm, 5 mm C18 Reprosil-HD beads, Dr. Maisch, Ammerbuch-Entringen, Germany). After back-flushing from the trapping column, the sample was loaded on a reverse-phase column (made in-house, 75 µm I.D. x 150 mm, 3 µm C18 Reprosil-HD beads, Dr. Maisch). Peptides were loaded with solvent A (0.1% TFA, 2% acetonitrile) and separated with a linear gradient from 98% solvent A' (0.1% formic acid in water) to 55% solvent B' (0.1% TFA, 80% ACN) at a flow rate of 300 nl/min followed by a wash reaching 100% solvent B'. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the ten most abundant peaks in a given MS spectrum. In the LTQ-Orbitrap Velos, full scan MS spectra were acquired in the Orbitrap at a target value of 1E6 with a resolution of 60,000. The ten most intense ions were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 40 s. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. From the MS/MS data in each LC run, Mascot Generic Files were created using Distiller software (version 2.4.3.3, Matrix Science, www.matrixscience.com/Distiller). While generating these peak lists, grouping of spectra was allowed in Distiller with a maximum intermediate retention time of 30 s and a maximum intermediate scan count of 5

was used where possible. Grouping was done with 0.005 Da precursor tolerance. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks. There was no deisotoping and the relative signal to noise limit was set at 2. These peak lists were then searched with Mascot search engine (MatrixScience) using the Mascot Daemon interface (version 2.3.01, Matrix Science). Spectra were searched against the Swiss-Prot database restricted to *Homo sapiens* (SwissProt 2012_04, 20.324 protein sequences). Variable modifications were set to pyro-glutamate formation of amino-terminal glutamine, acetylation of the protein N-terminus, oxidation of methionine, hydroxylation of proline, propionamidation of cysteine and phosphorylation on serine, threonine and tyrosine. Tolerance on precursor ions was set to ± 10 ppm (with Mascot's C13 option set to 1) and on fragment ions to ± 0.5 Da. The peptide charge was set to 2+, 3+, and the instrument setting was put on ESI-TRAP. Enzyme was set to trypsin, allowing for one missed cleavage, also cleavage was allowed when arginine or lysine is followed by proline. Only peptides that were ranked one and scored above the threshold score, set at 99% confidence, were withheld. All data management was done by ms_lims (PMID: 20058248).

Targeted LC-MS to determine PHD2 S125 phosphorylation levels. Targeted LC-MS to determine PHD2 S125 phosphorylation levels. To determine the levels of phosphorylated and unphosphorylated PHD2 (serine 125) in cell culture and patient samples, we performed bottom-up proteomics and phosphoproteomics. The purified protein fraction of each sample was digested using trypsin followed by spike-in of stable-isotope-labelled (SIL-) reference peptides of known concentrations. The known concentrations of the SIL-peptides enabled an LC-MS-based quantification of the peptides derived from endogenous PHD2 upon digestion.

For targeting the more abundant unphosphorylated peptide in patient samples, non-fractionated tryptic digests were analyzed by LC-MS. For targeting the unphosphorylated peptide in cell culture samples and the phosphorylated peptide in all samples, we introduced an additional purification step by HPLC: Using the corresponding SIL peptides, we determined the retention times (I) of the unphosphorylated peptide in high-pH-reversed phase separation and (II) the retention time of the phosphorylated peptide in electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) (Alpert, 2008; Loroch et al, 2015). Afterwards, samples were separated using the same conditions to collect a fraction at the specific retention times to yield a crude enrichment of the target peptides. Subsequent LC-MS analysis was done in parallel reaction monitoring (PRM) mode.

Experimental details are given in the following sections:

i) Synthesis and amino acid analysis of stable-isotope-labelled (SIL-) reference peptides:

Unphosphorylated and phosphorylated fully tryptic reference peptides (AKPPAD**P**AAAASPC**R)** containing PHD2 S125 were synthesized in-house using a Syro I synthesis unit (MultiSynTech, Witten, Germany) and Fmoc chemistry**.** A heavy-labelled arginine was incorporated at the C-terminus resulting in a mass shift of +10.0083 Da. Peptides were purified by preparative RP-LC coupled via a 1:20 split to an MS-Q (Thermo Fisher, Bremen, Germany) mass spectrometer for targeted fractionation. After lyophilization, peptides were solubilized in 0.1 % TFA and the peptide concentration was determined using amino acid analysis according to Cohen et al. (Cohen & Michaud, 1993). Quantification was conducted against a five-point calibration curve of derivatized amino acids in the range from (5 -25 pmol/µL).

ii) Sample processing: Samples were lysed using 1% SDS containing 1 tablet of PhosSTOP and 1 tablet Complete mini (Roche, Mannheim, Germany) per 10 mL. Samples were lysed by 2 cycles of ultrasonication on ice. Debris was removed by centrifugation at 16,000 g for 20 min at 4°C. Proteins were precipitated by adding 9 volumes of ice-cold ethanol, followed by incubation at -40° C for 1 h. Precipitates were spun down at 20,000 g for 40 minutes at 4° C and pellets were washed using 50 µL of ice-cold acetone. Protein pellets were solubilized in 6 M guanidinium-HCl followed by protein concentration determination using a BCA kit (Pierce by Thermo Scientific, Bremen, Germany). Afterwards, samples were adjusted to equal protein concentrations using 6 M guanidinium-HCl. Prior to proteolytic digestion, samples were diluted 20-fold using 50 mM ammonium bicarbonate, 1.5 mM $CaCl_2$. Trypsin (Sequencing grade, Promega, Mannheim, Germany) was added in a ratio of 1:10 (Dickhut et al, 2014) (trypsin : protein) and samples were incubated for 15 h at 37° C with slight agitation (for cell culture experiments, 100 µg of protein and for patient samples 70 µg were digested). The digestion was stopped by adding FA to a final concentration of 1% and peptides were purified using SPEC-C18 AR cartridges (Agilent, Boeblingen, Germany). Digests were quality controlled using a monolithic column HPLC separation, as described previously (Burkhart et al, 2012).

iii) Purification of the unphosphorylated peptide using HPLC-based high-pH-RP: High-pH-RP was done with an U3000 HPLC (Thermo Fisher, Bremen, Germany) equipped with a Biobasic-C18 column, 0.5 mm x 15 cm, 5 µm (Thermo Scientific). The binary gradient (12.5 µL/min) consisting of solvent A: 10 mM ammonium formate, pH 8 and solvent B: 84% (v/v) in 10 mM NH_4HCO_2, pH 8.0 was as follows: equilibration for 20 min with 3% B, followed by 3% B for 7 min, 3% to 31.5% B (linear) in 21.5 min, and 31.5% to 95% B in 15 min, 95% B for 2.5 min before returning to 3% B. A linear gradient from 3 to 95% B was used to wash the column between each of the runs.

We determined the retention time of the target peptide using 2 pmol of the SIL peptide (minute 17.5). Afterwards 12 µg of each sample including 600 amol of the SIL-peptide were injected in 15 µL of buffer A and a 2.5-minute fraction was collected at the specific retention time (± 1.25 min). All fractions were dried in a vacuum centrifuge and re-dissolved in 0.1% TFA for LC-MS analysis.

iv) Purification of the phosphorylated peptide using ERLIC: ERLIC was done with an Ultimate HPLC (Thermo Fisher, Bremen, Germany) equipped with a PolyWAX (weak anion exchange) column (2.1 mm × 200 mm, 5 µm, 300 Å, Poly LC, Columbia). The binary gradient (200 µL/min) consisting of solvent A': 20 mM sodium methylphosphonic acid, 70% acetonitrile (ACN), pH 2 and solvent B': 200 mM triethylammonium phosphate, 60% ACN, pH 2 was as follows: equilibration for 25 min with 100% A', followed by 100% A' for 10 min, 0% to 100% B' (linear) in 5 min, 100% B' for 10 min, followed by 100% A' for re-equilibration. A linear gradient from 0 to 100 % B was used to wash the column between each of the runs.

We determined the retention time of the target phosphopeptide using 400 pmol of the SIL-phosphopeptide. Afterwards, we spiked 2 fmol of the SIL-phosphopeptide into 30 µg (cell culture experiments) or 790 amol in 32 µg (patient tissue) of the tryptic digest. Samples were injected to ERLIC in 20 µL of buffer A' and a 4-minute fraction was collected at the specific retention time (± 2 min). Fractions were dried under vacuum, reconstituted in 0.1% trifluoroacetic acid (TFA) and desalted using Hypersep C18-RP 10-200 µL cartridges (Thermo Fisher). Peptides were eluted from the cartridge with 30% ACN. Samples were dried under vacuum and reconstituted in 0.1 % TFA.

v) Targeted LC-MS analysis: Targeted LC-MS analysis was done in parallel reaction monitoring (PRM) mode to absolutely quantify endogenous levels of the phosphorylated and unphosphorylated peptides in each sample using the known concentrations of the SIL peptides. PRM was performed using a Q-Exactive HF mass spectrometer online coupled to a U3000 RSLC nanoHPLC equipped with an Acclaim PepMap trap-column (100 µm x 2 cm, 5 µm particles, 100 Å pores) and an Acclaim PepMap main column (75 µm x 50 cm, 3 µm particles, 100 Å pores). Peptides were injected onto the trap column in 0.1% TFA using a flow rate of 10 µL/min. After 5 min the trap column was switched in-line and peptides were separated using a 1 h gradient ranging from 2.5 to 40 % ACN in 0.1% FA at a flow rate of 250 nL/min. For quantification of the unphosphorylated peptide in patients samples, 240 amol of the unphosphorylated SIL-peptide were spiked in 1,2 µg of tryptic digest prior to LC-MS. For quantification of the unphosphorylated peptide in cell culture samples, 50% of each high-pH-RP fraction was analyzed (corresponding to 6 µg digest + 300 amol of SIL peptide). PRM was conducted using the m/z of the +2 and +3 charged ions of both the endogenous (m/z: 740.3723 and 493.9173) and the SIL-peptide (m/z: 745.3764 and 497.2534).

Fragmentation was done using a 0.4 m/z isolation window and a normalized collision energy of 27. Fragment ion spectra were acquired at a resolution of 30,000; automatic gain control (AGC) was set to 2 x 10^5 with a maximum injection time of 500 ms.

For quantification of the phosphopeptide 50% of each ERLIC-sample were used (corresponding to 15 µg digest + 1 fmol SIL-phosphopeptide) and PRM was conducted using the m/z of the +2 and +3 charged ions of both the endogenous (m/z: 780.3554 and 520.5727) and the SIL-phosphopeptide (m/z: 785.3596 and 523.9088) and the same parameters as for the unphosphorylated peptide but with an AGC target value of 10^5.

vi) Data analysis: Raw-files were imported into Skyline v3.1 (MacLean et al, 2010) and the 3 to 5 best transitions of each peptide were manually selected and used for quantification. Obtained peptide ratios were exported to Excel and the amount of each peptide per µg of tryptic digest was calculated (in amol*µg digest^{-1}). Phosphorylation stoichiometry was determined using the following equation:

$$\%\,phosphorylation = \frac{amol_{phosphorylated}}{\mu g_{digest}} \cdot \frac{\mu g_{digest}}{\mathrm{amol}_{\mathrm{phosphorylated}} + \mathrm{amol}_{\mathrm{unphosphorylated}}} \cdot 100\%$$

In Vivo Labeling with [^{32}P]Orthophosphate and Immunoprecipitation. DLD1 cells were cultured in a humidified incubator at 37° C and 5% CO_2. The next day, the cells were washed with phosphate-free DMEM (Gibco) and incubated with the same medium containing 1 mCi of [^{32}P]orthophosphoric acid (NEN)/ml (PerkinElmer, Groningen, Netherlands) under hypoxic conditions (37°C, 5% CO_2 and 1% O_2). At indicated time points, cells were washed twice with ice-cold PBS and scraped in lysis buffer (50 mM Tris/HCL, pH 7.5, 150 nM NaCl, 1% Titon X-100, 2 mM EDTA, 2 mM EGTD, 1 mM PMSF and proteasome inhibitor cocktail tablet (Roche)). To recover endogenous PHD2, equal amounts of total protein (500 µg) were incubated with 4 µg of PHD2 antibody for 2 h at 4˚C before Sepharose beads (30 µl per reaction) were added overnight at 4˚C. Thereafter, the beads were washed five times with lysis buffer, recovered and pellets were dissolved in 2 X Laemmli buffer, boiled at 95˚C for 7 min and separated by 10% SDS PAGE. Afterwards, the gel was blotted onto a nitrocellulose membrane and the membrane was exposed to a KODAK phosphor imaging screen overnight. Autoradiography was detected with MolecularImager FX using the Quantity One software (all BioRad, Helsinki, Finland). Thereafter, the membrane was probed with an antibody against PHD2.

Propidium Iodide Staining. Supernatants derived from DLD1 and HT29 cells cultured in normoxia or hypoxia was collected in order to keep dead cells. Adherent cells were subsequently trypsinized, added to the supernatant previously collected and centrifuged at 300g for 5 minutes. After one wash with PBS, cells were fixed with 1 ml of 70% ethanol. Cells were incubated 2h or overnight at 4°C, prior to another centrifugation at 300g for 5 minutes. Supernatant was removed and the pellet was resuspended in 200 µl of PBS, containing 500 µg of RNase (10 mg/ml). 200 ml of Propidium Iodide (0,1mg/ml) was added to a final volume of 400 µl. Samples were incubated for 1-2 h at 37°C and subsequently analyzed by Fluorescence-Activated Cell Sorting by using FACS Canto II (BD Bioscience).

Immunocytochemistry. 2x10^5 DLD1 cells transfected with FLAG-PHD2WT, S125A and S125D were seeded on top of sterilized, gelatin treated coverslips in 6-well dishes. Cells were rinsed three times with PBS and fixed in methanol for 20 minutes on ice. Cells were washed two times with PBS and permeabilized and blocked for 1 hour in PBS 0.1% Triton 5% BSA at room temperature (RT). Coverslips were incubated with primary antibody (FLAG, 1:100, SIGMA Aldrich) in blocking solution (PBS, 5% BSA) for 2 hours. Cells were washed with PBS three times for 10 minutes, rinsed with blocking solution, and incubated for 1 hour with 1/500 donkey anti-rabbit-Alexa 568 in blocking solution. Cells were washed once with blocking solution and three times for 10 minutes with PBS before mounting coverslips with prolong gold with DAPI (Life Technologies).

Proliferation assays. For cell proliferation, 10 µM of BrdU was added to the cells previously incubated in normoxia or hypoxia. After 1 hour, cell proliferation was quantified on single cell suspensions with the FITC BrdU Flow Kit (BD Bioscience) according to manufacturer's protocol. Alternatively, cells were seeded in triplicate in 5 96-well plate. Proliferating cells were detected every 24 hours for 5 days by using Cell Proliferation Reagent WST1 (Roche Diagnostics), according to manufacturer's protocol.

Immunocytochemistry. 2x10^5 DLD1 cells were seeded on top of sterilized, gelatin treated coverslips in 6-well dishes and subsequently incubated in normoxia or hypoxia (0.2% O_2) for 48 hours. Cells were rinsed three times with PBS and fixed in methanol for 20 minutes on ice. Cells were washed two times with PBS and permeabilized and blocked for 1 hour in PBS 0.1% Triton 5% BSA at room temperature (RT). Immunostaining was performed on coverslips by using ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore) according to manufacturer's instructions. Prolong gold with DAPI (Life Technologies) was used to detect cell nuclei.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR). To assess gene expression, RNA from DLD1 or HT29 seeded in triplicate in 12-well plates was extracted with a RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Reverse transcription to cDNA was performed by using Quantitect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. cDNA, primer/probe mix and TaqMan Fast Universal PCR Master Mix were prepared in a volume of 10 ml according to manufacturer's instructions (Applied Biosystems). qRT-PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR system. Pre-made assays were purchased and their assay IDs are listed below:
PPP2R2A (B55a): Hs.PT.58.25465949 from IDT

bActin: Hs.PT.39a.22214847 from IDT
BNIP3: Hs.PT.56a.404067 from IDT
BNIP3L: Hs.PT.56a.1103799 from IDT
EGLN1 (PHD2): Hs00254393_m1 from Applied Biosystem
bActin: Hs99999903_m1 from Applied Biosystem.

Hypoxia assessment. Tumor hypoxia was detected by injection of 60 mg/kg pimonidazole hydrochloride into tumor-bearing mice 1 hour before tumor harvesting. To detect the formation of pimonidazole adducts, tumor cryosections were immunostained with Hypoxyprobe-1-Mab1 (Hypoxyprobe kit, Chemicon) following the manufacturer's instructions.

Histology and immunostainings. To obtain serial 7-μm-thick sections, tissue samples were immediately frozen in OCT compound or fixed in 2% PFA overnight at 4°C, dehydrated and embedded in paraffin. Paraffin slides were first rehydrated to further proceed with antigen retrieval in citrate solution (DAKO). Cryo-sections were thawed in water and fixed in 100% methanol. If necessary, 0.3% H_2O_2 was added to methanol to block endogenous peroxidases. The sections were blocked with the appropriate serum (DAKO) and incubated overnight with the antibody rabbit anti-LC3B (Cell Signaling; clone D11) 1:200 and Hypoxyprobe-1-Mab1. Appropriate secondary antibodies were used: Alexa488-or Alexa568-conjugated secondary antibodies (Molecular Probes) 1:200. When necessary, Tyramide Signaling Amplification (Perkin Elmer, Life Sciences) was performed according to the manufacturer's instructions. Whenever sections were stained in fluorescence, ProLong Gold mounting medium with DAPI (Invitrogen) was used. Otherwise, 3,3'-diaminobenzidine was used as detection method followed by Harris' haematoxilin counterstaining, dehydration and mounting with DPX. Apoptotic cells were detected by the TUNEL method, using the ApopTag peroxidase in situ apoptosis detection kit (Millipore) according to the manufacturer's instructions. Microscopic analysis was done with an Olympus BX41 microscope and CellSense imaging software. The analysis was performed by acquiring 4-6 fields per sections on 5 independent sections from the same biological tissue sample. The values in the graphs represent the average of the means of, at least, 5 samples and the standard error indicates the variability among the different samples.

Dephosphorylation assay. FLAG-PHD2 isolated from HEK293T cells was incubated alone or with immune-purified FLAG-B55α in dephosphorylation buffer (100 mM Tris-Cl, pH 7.4, 100 mM KCl, 20 mM $MgCl_2$, 30 mM EGTA) for 30' at 30°C.

Generation of CRISPR/Cas9 constructs. The lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). The gRNA targeting PHD2 exon 1 (CGGACAGCAGATCGGCGACG) was generated using Rule Set 2 (Doench et al, 2016). PHD2 targeting gRNA and non-targeting control gRNA (GAACAGTCGCGTTTGCGACT) were then cloned in lentiCRISPR v2 as described previously (Sanjana et al, 2014). A silent mutation was introduced within the PHD2 coding sequences ($PHD2^{wt}$, $PHD2^{S125A}$ and $PHD2^{S125D}$) in order to remove the protospacer adjacent motif (PAM) sequence that is absolutely necessary for CRISPR/Cas9 target binding. The cDNAs were then PCR amplified and cloned into a lentiviral vector after restriction with AgeI/SpeI.

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