Materials and methods

2.1 Materials

$NiSO_4$, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $FeSO_4$, L-ascorbic acid, and 2-oxopentanedioic acid (2-OG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-PHD3 was obtained from Abcam (Cambridge, MA), and anti-PHD2 was from Cell Signaling Technology (Beverly, MA). The HIF-1α peptide containing residues 556 to 575 denoted as F-P564 (fluorescein isothio-cyanate (FITC)-AminoCaproic Acid (ACA)-DLDLEALAPYIPADDDFQLR) was synthesized by AnyGen (KwangJu, Korea). All other chemicals were of the highest grade commercially available.

2.2 Site- directed mutagenesis of PHD3

PCR primers were synthesized by Bioneer (Deajeon, Korea). Site-directed mutagenesis of Cys, Glu, Asp, Arg or His residues of PHD3 was conducted using the quikchange mutagenesis protocol provided by Stratagene, USA. All mutations were confirmed by DNA sequencing.

2.3 Preparation of recombinant proteins

Proteins were prepared as previously described (Cho et al, 2005). Briefly, plasmids for human VHL (amino acids 54–213) and human Elongin B (amino acids 1–118) in pGEX-4T-1 and for human Elongin C (amino acids 17–112) in pET29b were co-expressed in *Escherichia coli* (*E. coli*) BL21(DE3). Full-length human PHD2, truncated human PHD2 (amino acids 184–418) denoted as PHD2F and PHD2T, respectively, and full-length human PHD3 were subcloned into pET-28a (Novagen), pET-21b (Novagen), and pET-22b (Novagen), respectively and expressed in *E. coli* BL21(DE3). After induction with 0.5 mM isopropyl β-D-thiogalactoside at 18 °C for 15 hours, GST-VBC was purified using glutathione-Sepharose

(Amersham Biosciences) and PHD2F, PHD2T, and PHD3 were lysed by sonication in the buffer containing 50 mM sodium phosphate (pH = 8.0), 500 mM NaCl, 14 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and 10 mM imidazole. Triton X-100 was added into the lysate to final concentration of 1% and the lysate was incubated for 10-30 minutes at 4 °C. Proteins were purified using Ni-NTA agarose (Qiagen). The purified recombinant proteins were confirmed by SDS-PAGE and quantified by Bradford assay (Bio-Rad).

2.4 Fluorescence polarization (FP)-based activity assays

For determination of inhibitory effects of divalent metal ions on PHDs, 1 μM of F-P564 was incubated with 1 μg PHD2F, 0.25 μg PHD2T or 1 μg PHD3 in hydroxylation buffer containing 200 μM ascorbic acid, 200 μM 2-OG, 5 mM KCl, and 1.5 mM $MgCl_2$ in the absence and presence of increasing concentrations of Zn(II), Ni(II), Mn(II), and Co(II) at 30 °C. To analyze effects of Zn(II) on PHDs' activities, concentrations of Zn(II) were varied from 0 to 160 μM. For competition with Fe(II), varying concentrations of Zn(II) and Fe(II) were used. For Zn(II) pretreatment, PHD3 was incubated with 40 or 80 μM of Zn(II) for 10 minutes at room temperature, followed by assaying in the presence of 0.5 mM DTT or 1 mM TCEP, respectively. After 2 hours of incubation at 30 °C, the hydroxylation reaction was stopped by heating for 1 minute at 95 °C. VBC (200 nM) was then added to the reaction solution and diluted to a final peptide concentration of 100 nM in EBC buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40). The hydroxylation yields were analyzed by FP measurements using an Appliskan multimode microplate reader (Thermo Scientific, Waltham, MA, USA) with an excitation/emission filter set of 485/535 nm. Assays were performed in triplicate. IC_{50} values for compounds were determined by nonlinear regression with sigmoidal dose-response curves using Sigmaplot software.

2.5 Western blot analysis of the PHDs' oligomers

PHDs (1 μg) were incubated in the absence and presence of 80 μM Zn(II) and/or 1 mM TCEP at 30 °C for 10 minutes. For immunoblotting, reaction mixtures were prepared in the SDS sample buffer with or without 2-mercaptoethanol, boiled for 2 minutes, and run on 4-15% linear gradient Tris-HCl ready gels (Bio-Rad). After electrophoresis, the protein was electroblotted onto poly(1,1-difluoroethylene) (PVDF) membranes (Millipore) by tank-electroblotting (Bio-Rad), and the membranes were blocked with 5% BSA for 1 hour. Then the membranes were incubated with anti-PHD2 (Cell signaling catalog #: 3293S) or anti-PHD3 (Abcam catalog #: ab30782) as the primary antibody and anti-rabbit IgG as the secondary antibody. Blots were developed with the ECL Plus Western Blotting Detection System (Thermo Scientific).

Table S1 Primer list used in this study

PHD3 Mutants	Primer name	Primer sequence (5′→ 3′)
E37A	E37A F	GGA CAA CTT CCT GGG CGC CGT GGT GGG CGA CTG C
	E37A R	GCA GTC GCC CAC CAC GGC GCC CAG GAA GTT GTC C
C42A	C42A F	CGA GGT GGT GGG CGA CGC CGT CCT GGA GCG CGT CA
	C42A R	TGA CGC GCT CCA GGA CGG CGT CGC CCA CCA CCT CG
E45A	E45A F	GCG ACT GCG TCC TGG CCC GCG TCA AGC AGC TG
	E45A R	CAG CTG CTT GAC GCG GGC CAG GAC GCA GTC GC
C52A	C52A F	GCG TCA AGC AGC TGC ACG CCA CCG GGG CCC TGC
	C52A R	GCA GGG CCC CGG TGG CGT GCA GCT GCT TGA CGC
R57A	R57A F	CGG GGC CCT GGC GGA CGG CCA G
	R57A R	CTG GCC GTC CGC CAG GGC CCC G
R71A	R71A F	CGC CGG CGT CTC CAA GGC ACA CCT GCG GGG CGA
	R71A R	TCG CCC CGC AGG TGT GCC TTG GAG ACG CCG GCG
H72A	H72A F	CCG GCG TCT CCA AGC GAG CCC TGC GGG GCG ACC A
	H72A R	TGG TCG CCC CGC AGG GCT CGC TTG GAG ACG CCG G
E85A	E85A F	GGG GGC AAC GCG GAG GGC TGC
	E85A R	GCA GCC CTC CGC GTT GCC CCC
E86A	E86A F	GGC AAC GAG GCG GGC TGC GAG
	E86A R	CTC GCA GCC CGC CTC GTT GCC
C88A	C88A F	GGC AAC GAG GAG GGC GCC GAG GCC ATC AGC TTC C
	C88A R	GGA AGC TGA TGG CCT CGG CGC CCT CCT CGT TGC C
E89A	E89A F	ACG AGG AGG GCT GCG CCG CCA TCA GCT TCC TC
	E89A R	GAG GAA GCT GAT GGC GGC GCA GCC CTC CTC GT
C124A	C124A F	AAG GCA ATG GTG GCT GCC TAT CCG GGA AAT GG
	C124A R	CCA TTT CCC GGA TAG GCA GCC ACC ATT GCC TT
D183A	D183A F	GTG GAG CCC ATT TTT GCC AGA CTC CTG TTC TTC
	D183A R	GAA GAA CAG GAG TCT GGC AAA AAT GGG CTC CAC
R71AH72A	R71AH72A F	CGC CGG CGT CTC CAA GGC AGC CCT GCG GGG CGA CCA
	R71AH72A R	TGG TCG CCC CGC AGG GCT GCC TTG GAG ACG CCG GCG
E85AE86A	E85AE86A F	CGG GGG CAA CGC GGC GGG CTG CGA G
	E85AE86A R	CTC GCA GCC CGC CGC GTT GCC CCC G