**Prolyl Hydroxylase Domain Protein 2 (PHD2) Binds a Pro-Xaa-Leu-Glu Motif, Linking it to the Heat Shock Protein 90 Pathway**

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Running Title: *PHD2 is linked to the HSP90 pathway*

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**Background:** PHD2 is the central enzyme that controls Hypoxia Inducible Factor (HIF)-α protein levels.

**Results:** PHD2 binds a Pro-Xaa-Leu-Glu motif in two HSP90 co-chaperones, and knockdown of one of these, p23, augments hypoxia-induced HIF-1α protein levels.

**Conclusion:** PHD2 is linked to the HSP90 pathway, facilitating its hydroxylation of HIF-1α.

**Significance:** This uncovers a new model by which PHD2 controls HIF-1α.

**SUMMARY**

Prolyl Hydroxylase Domain protein 2 (PHD2, also known as Egg Laying Defective Nine homolog 1) is a key oxygen-sensing protein in metazoans. In an oxygen-dependent manner, PHD2 site-specifically prolyl hydroxylates the master transcription factor of the hypoxic response, Hypoxia Inducible Factor-α (HIF-α), thereby targeting HIF-α for degradation. In the present report, we show that the Heat Shock Protein 90 (HSP90) co-chaperones p23 and FKBP38 interact via a conserved Pro-Xaa-Leu-Glu motif (where Pro = Proline, Xaa = any amino acid, Leu = Leucine, Glu = Glutamic acid) in these proteins with the N-terminal Myeloid Nervy and DEAF-1 (MYND)-type zinc finger of PHD2. Knockdown of p23 augments hypoxia-induced HIF-1α protein levels and HIF target genes. We propose that p23 recruits PHD2 to the HSP90 machinery to facilitate HIF-1α hydroxylation. These findings identify a link between two ancient pathways—the PHD:HIF and the HSP90 pathways—and suggest that this link was established concurrent with the emergence of the PHD:HIF pathway in evolution.

The central pathway regulating the transcriptional response to hypoxia is the PHD:HIF:von Hippel Lindau protein (VHL) pathway (1-3). HIF is a heterodimeric complex consisting of an α and a β subunit. In mammals, there are two main α subunits, HIF-1α and HIF-2α, and one β subunit (HIF-1β, also known as the Aryl Hydrocarbon Nuclear Translocator). The protein stability of the α subunit is regulated in an oxygen-dependent manner. Under normoxic conditions, PHD constitutively hydroxylates HIF-1α on specific prolyl residues. In the case of HIF-1α, the primary site of hydroxylation is Pro-564 (4-6). This provides a binding platform for VHL, a component of an E3 ubiquitin ligase complex that selectively recognizes modified HIF-α and targets it for degradation by the ubiquitin-proteasome pathway (7). Under hypoxic conditions,
prolyl hydroxylation of HIF-α is attenuated, allowing the stabilization of HIF-α and activation of a broad range of HIF target genes (2). These genes promote glycolysis, glucose uptake, angiogenesis, and erythropoiesis (3,8).

Three mammalian PHD isoforms have been identified: PHD1, PHD2, and PHD3 (9,10). All three share a conserved prolyl hydroxylase domain. Among the three PHD isoforms, PHD2 is regarded as the central one mediating HIF-α turnover. Knockdown of PHD2 in most cell types is sufficient to induce HIF activity (11,12). PHD2 is also the most abundant isoform in most cell types (12). Knockout of the Phd2 gene in mice results in embryonic lethality, whereas Phd1-/- and Phd3-/- mice are viable (13). In humans, patients with germline PHD2 mutations display erythrocytosis due to dysregulation of the ERYTHROPOIETIN gene (14-17).

Moreover, PHD2 haplotypes are associated with high-altitude adaptation in Tibetans (18,19). PHD2 is distinctive among the three PHDs in that it harbors, at its N-terminus, a domain that is predicted to be a zinc finger of the Myeloid Nervy and DEAF-1 (MYND) type (20).

The PHD:HIF:VHL pathway shows broad conservation in animals (21,22), and experiments demonstrate that it is functional in the simplest metazoan, Trichoplax adhaerens (21). T. adhaerens contains single isoforms for PHD, HIF-α, and VHL, and intriguingly, its PHD contains a MYND-type zinc finger homologous to that seen in mammalian PHD2. These observations indicate that prolyl hydroxylation is an ancient mechanism to transduce changes in oxygen concentration to changes in cellular function, and furthermore suggest that mammalian PHD2 is the isoform most closely related to the single ancestral PHD.

The HSP90 pathway is an even more ancient pathway, showing conservation in bacteria (23). It is centered around the HSP90 chaperone protein. While it was originally identified as a pathway that is heat inducible and that promotes protein folding, it is clear that it plays a central role in the maturation of a multitude of client proteins, particularly ones involved in signal transduction and transcription. Indeed, HIF-1α is a client of the HSP90 pathway (24). A salient feature of the HSP90 pathway is that it employs a set of co-chaperones that regulate the activity of HSP90 and can interact with client proteins (25,26). These co-chaperones show varying degrees of conservation in evolution (27). As one example, the co-chaperone p23, which binds to HSP90 in an ATP-dependent manner and stabilizes a closed conformation of HSP90, shows extensive conservation. It is present, for example, in Saccharomyces cerevisiae. The conservation of various HSP90 co-chaperones raises interesting questions as to the selective pressures that have maintained them in evolution.

The PHDs site-specifically hydroxylate HIF-α on prolyl residues that occur in an LXXLAP motif (where L = Leu, X = any amino acid, A = Ala, and P = Pro). In vitro experiments demonstrate that HIF-1α peptides as short as 19 amino acids in length that contain this motif are sufficient to allow for site-specific hydroxylation and subsequent recognition by VHL (5,28,29). While the data imply that the core machinery of the pathway consists of PHD, HIF, and VHL, the extent to which it is integrated with other cellular pathways remains poorly understood. In the present report, we identify a direct interaction between the MYND-type zinc finger of PHD2 and a peptide motif in select co-chaperones of the HSP90 pathway, including p23. We provide evidence for the functional importance of this interaction in promoting efficient HIF-α degradation. Taken together, the data establish a link between oxygen-sensing and the HSP90 pathway, and suggest that this link emerged early in the evolution of metazoans.

**EXPERIMENTAL PROCEDURES**

Plasmids—pcDNA5/FRT/TO-FlagPHD2 was constructed by subcloning the 1.5 kb Hind III/Sph I fragment of pcDNA3-FlagPHD2 (30) into the Hind III/Sph I site of pcDNA5/FRT/TO. pcDNA3-HA-PHD2 was constructed by subcloning the 1.3 kb BamH
I/Xba I fragment of pcDNA3-FlagPHD2 into the BamH I/Xba I site of pcDNA3-HA. pcDNA3-HA-PHD2 (1-196) was constructed by subcloning the 0.6 kb BamH I/Xho I fragment of pcDNA3-FlagPHD2 (30) into the BamH I/Xho I site of pcDNA3-HA. pcDNA3-HA-PHD2 (130-426) was constructed by digesting pcDNA3-HA-PHD2 with Nhe I and Not I, blunting the ends with the Klenow fragment of E. coli DNA polymerase I, and then self-ligating. pcDNA3-HA-PHD2 (1-196) C36,42S was constructed by PCR-mediated mutagenesis. Then, the 0.7 kb Xho I/Xba I fragment of pcDNA3-FlagPHD2 was subcloned into the Xho I/Xba I site of pcDNA3-HA-PHD2 (1-196) C36,42S to yield pcDNA3-HA-PHD2 C36,42S.

pGEX-PHD2 (1-63) was constructed by subjecting pGEX-PHD2 (1-124) to digestion with Not I, partial digestion with Sfo I, blunting with the Klenow fragment of E. coli DNA polymerase I, and self ligation. pMAL-PHD2 (1-63) was constructed by subcloning the 0.2 kb BamH I/Not I fragment of pGEX-PHD2 (1-63) into the BamH I/Not I site of pMAL-5X-1.

pcDNA3-HA-PHD1 was constructed by subcloning the 1.8 kb BamH I/Xba I fragment of pcDNA3-FlagPHD1 (30) into the BamH I/Xba I site of pcDNA3-HA. pcDNA3-HA-PHD3 was constructed by subcloning the 0.8 kb BamH I (partial)/Xho I fragment of pcDNA3-FlagPHD3 (30) into the BamH I/Xho I site of pcDNA3-HA.

pcDNA5/FRT/TO-3xFlag-p23 was constructed by amplifying the coding sequence of pOTB7-p23 (IMAGE clone 2821965) by PCR using the following primers: 5’ GTA CGG ATC CAA ATG CAG CCT GCT TCT GCA AAG 3’ and 5’ GTA CCT CGA GCT CGC TCT AGA TTA GTT CCT GGC AGC GAT GAC CAC AGA GAG TGC CAC ACC CCCCAA G 3’. The 1.4 kb product was digested with BamH I and Not I, and subcloned into the BamH I/Not I site of pcDNA3-HA. pcDNA5/FRT/TO-3xFlag-FKBP38 was constructed by digesting pcDNA3-HA-FKBP38 with BamH I and Not I, and then subcloning the 1.2 kb coding sequence into the BamH I/Not I site of pcDNA5/FRT/TO-3xFlag.

pcDNA3-3xFlag-FKBP52 was constructed by amplifying the coding sequence of pOTB7-FKBP52 (IMAGE clone 3542330) with the following primers: 5’ GTA CCT TAA GAT GAC AGC CGA GGA GAT GAA G 3’ and 5’ GTA CCT CGA GCT ATG CTT CTG TCT CCA CCT G 3’. The 1.4 kb product was digested with Afl II and Xho I, and subcloned into the into Afl II/Xho I site of pcDNA3-3xFlag. pcDNA5/FRT/TO-3xFlag-FKBP51 was constructed by amplifying the coding sequence of pCMV-SPORT6-FKBP51 (IMAGE clone 4539929) with the following primers: 5’ GTA CGG ATC CAA ATG CAG CCT GCT TCT GCA AAG 3’ and 5’ GTA CCT CGA GCT CGC TCT AGA TTA GTT CCT GGC AGC GAT GAC CAC AGA GAG TGC CAC ACC CCCCAA G 3’. The 1.4 kb product was digested with Afl II and Xho I, and subcloned into the into Afl II/Xho I site of pcDNA3-3xFlag. pcDNA5/FRT/TO-3xFlag-p23 was constructed by amplifying the coding sequence of pOTB7-p23 using the following primers: 5’ GTA CGG ATC CAA ATG CAG CCT GCT TCT GCA AAG 3’ and 5’ GTA CCT CGA GCT CGC TCT AGA TTA GTT CCT GGC AGC GAT GAC CAC AGA GAG TGC CAC ACC CCCCAA G 3’.
pcDNA3-3xFlag-FKBP16 was constructed by amplifying the coding sequence of pcDNA3 (IMAGE clone 8143800) with the following primers: 5’ GTA CGA ATT CAG ATG GCG GAT ATC ATC GCA AG 3’ and 5’ GTA CCT CGA GTC AAT GGG AGA AGA TCC 3’. The 1.0 kb product was digested with EcoR I and Xho I, and then subcloned into the EcoR I/Xho I site of pcDNA3-3xFlag. pcDNA5/FRT/TO-3xFlag-PPP5C was constructed by amplifying the coding sequence of pCMV-SPORT-PPP5C (IMAGE clone 3459309) with the following primers: 5’ GTA CGG ATC CAG ATG GCG ATG GCG GAG GGC GAG 3’ and 5’ GTA CCT CGA GTC ACA TCA TTC CTA GCT GCA G 3’. The 1.5 kb product was digested with BamH I and Xho I, and then subcloned into the BamH I/Xho I site of pcDNA5/FRT/TO-3xFlag.

The authenticity of recombinant plasmids was confirmed by sequencing. The (eHRE)3-Luc plasmid, which contains three copies of the Hypoxia Response Element from the human EPO gene enhancer, and pGEX-HIF-1α (531-575) have been described (6,28). The plasmid pRL-TK, which expresses Renilla luciferase from a minimal thymidine kinase promoter, was obtained from Promega.

Cell lines—A cell line with doxycycline-inducible expression of FlagPHD2 was prepared using pcDNA5/FRT/TO-FlagPHD2, HEK293 Flp-In T-Rex cells, and Flp recombinase according the manufacturer’s instructions (Invitrogen). These cells are hereafter referred to as HEK293 Flp-In T-Rex/FlagPHD2. Stably transfected HEK293 Flp-In T-Rex/Flag-p23 cells were prepared in an analogous manner using pcDNA5/FRT/TO-3xFlag-p23. Hela and PC3 cells were obtained from ATCC. HEK293FT cells were obtained from Invitrogen. MCF7 and RCC4 cells were kind gifts from Dr. Mark Lemmon (Perelman School of Medicine, University of Pennsylvania) and Dr. Celeste Simon, respectively (Perelman School of Medicine, University of Pennsylvania). All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfections of plasmids and siRNA were performed using Lipofectamine 2000 and antibiotic-free media. Hypoxia experiments were performed in an In Vivo 200 Hypoxia Workstation (Ruskinn Technologies). Sf9 cells were maintained in SF-900 II serum free media. 17-AAG was obtained from Cayman Chemical. MG132 was from Sigma.

Mass spectrometry—HEK293 Flp-In T-Rex and HEK293 Flp-In T-Rex/FlagPHD2 cells were grown in 15 cm dishes, using light isotope-containing DMEM for the former, and heavy isotope (13C6 L-lysine and 13C6 15N4 L-arginine)-containing DMEM for the latter (SILAC kit from Pierce) (31). The cells were induced with 1 µg/ml of doxycycline for 18 hr. The cells were then gently washed with PBS, then lysed in 1 ml of buffer A (50 mM Hepes, pH 7.5, 0.5% Triton X-100) supplemented with mammalian protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation at 15,800 x g for 10 minutes at 4 °C. Equal protein amounts (as determined by the Bio-Rad DC Protein Assay) were then added to 15 µl aliquots of anti-Flag (M2) agarose (Sigma), and rocked for 1 hr at 4 °C. The resins were washed three times with buffer A, and then eluted by the addition of 40 µl of a 0.2 µg/µl solution of 3X Flag peptide. Aliquots of eluates were saved for later western analysis. Immunoprecipitations of heavy labeled Flag-p23 were performed in an analogous manner.

Eluates were mixed, and subjected to alkylation and trypsin digestion using Filter Aided Sample Preparation (FASP) and Microcon YM-30 filtration units (32), followed by desalting and concentration using C18 StageTips (33). Peptides were separated on 50 cm x 75 µM column packed with 4 µM C12 Jupiter Proteo beads (Phenomenex) that was heated to 50 °C (Phoenix S&T). Nanoflow chromatography was performed at 300 nL/min using a 90 minute gradient from 2% to 42% acetonitrile in 0.5% acetic acid. Eluents were electrosprayed for analysis at 2.7 kV with a Phoenix S&T µAutoNano spray.  

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source which was coupled to a Thermo Scientific LTQ-Orbitrap hybrid mass spectrometer. Mass spectra (MS) data was acquired in the Orbitrap, and MS/MS fragmentation was performed in the linear ion trap using a top-5 data-dependent MS/MS method. Data was analyzed using MaxQuant software v. 1.2.2.5 (34), and spectra were searched using MaxQuant’s included search engine, Andromeda, against the IPI human database v. 3.68. SILAC doublets for Arg 10 and Lys 6 were specified. Oxidation of methionine and proline, and acetylation of protein N-termini were employed as variable modifications, while carbamidomethylation of cysteine was employed as a fixed modification.

**Peptide binding assays**—Peptides corresponding to residues 151 to 160 of p23, and residues 47-56 of FKBP38 were synthesized by Genscript. p23 peptides were also prepared with the following amino acid substitutions: P157A/L159A/E160A, E160D, E160Q, L159A, L159I, L159V, D158A, D158P, D158L, P157A, P157S, M156A, M156L, M156V, M156F, M156E, K155A, E154A, D153A, D152A, and D152A/D153A/E154A. An FKBP38 peptide with a P53A/L55A/E56A substitution was prepared. All peptides possessed an N-terminal tyrosine residue to allow spectrophotometric quantitation using a molar absorptivity of 1,280 M⁻¹ cm⁻¹ at 280 nm (35), and all were biotinylated at the N-terminus.

Peptides (0.5 µg) were prebound to 15 µl aliquots of streptavidin-agarose (Sigma). The resins were incubated with SF9 lysates containing baculovirus-expressed (His)₉FlagPHD2 (30) for 1 hr with rocking at 4 °C in buffer B (20 mM Tris, pH 7.6, 150 mM NaCl, 25 mM β-glycerolphosphate, 2 mM pyrophosphate, 10% glycerol, 1% Triton X-100). The resins were washed four times with buffer A, eluted, and the eluates subjected to SDS-PAGE and western blotting using anti-Flag antibody-alkaline phosphatase conjugates (Sigma). In the case of the VHL capture assays, recombinant Flag-tagged VHL (in a complex with Elongin B and Elongin C) purified from SF9 cells (30) was incubated with the extracts for 1 hr prior to immunoprecipitation, and western blotting was conducted using anti-HIF-1α antibodies (Santa Cruz Biotechnology, H-206). For immunoprecipitation of endogenous PHD2, we employed anti-PHD2 monoclonal antibody 6.9 (36) and protein G-agarose (Invitrogen); for control antibody, we used monoclonal antibody G3A1 (Cell Signaling Technology).

**Western blotting**—Western blotting was performed essentially as described (28), except that the secondary antibodies were alkaline phosphatase conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology), and the substrate was CDP-Star (Roche). Rabbit polyclonal antibodies to PHD2 were prepared as follows. First, GST-PHD2 (1-63) and MBP-PHD2 (1-63) were purified from E. coli transformed with pGEX-PHD2 (1-63) and pMAL-PHD2 (1-63), respectively, using affinity chromatography on GST-HIF-1α (531-575) were purified from E. coli DH5α transformed with pGEX-5X-1 and pGEX-HIF-1α (531-575), respectively, using affinity chromatography on glutathione (GSH)-sepharose (GE Healthcare). GST or GST-HIF-1α (531-575) prebound to 15 µl of GSH-sepharose was incubated in buffer C (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5% Triton X-100) with rocking with cellular extracts for 1 hr at 4 °C. The resins were washed three times, and eluates subjected to SDS-PAGE and western blotting. **Immunoprecipitations**—Following a wash with PBS, cells were lysed in buffer C supplemented with mammalian protease inhibitor cocktail. The lysate was clarified by centrifugation at 15,800 x g for 10 minutes at 4 °C. These whole cell extracts were then added to 10 µl aliquots of anti-Flag (M2) agarose, and rocked for 1 hr at 4 °C. The resins were washed four times with buffer C, eluted, and the eluates subjected to SDS-PAGE and western blotting using anti-HA antibody-alkaline phosphatase conjugates (Sigma). In the case of the VHL capture assays, recombinant Flag-tagged VHL (in a complex with Elongin B and Elongin C) purified from SF9 cells (30) was incubated with the extracts for 1 hr prior to immunoprecipitation, and western blotting was conducted using anti-HIF-1α antibodies (Santa Cruz Biotechnology, H-206). For immunoprecipitation of endogenous PHD2, we employed anti-PHD2 monoclonal antibody 6.9 (36) and protein G-agarose (Invitrogen); for control antibody, we used monoclonal antibody G3A1 (Cell Signaling Technology).
GSH-sepharose and amylase-agarose (New England Biolabs), respectively. Then, polyclonal antibodies to MBP-PHD2 (1-63) were then raised in rabbits and affinity purified on GST-PHD2 (1-63) coupled to agarose by Covance Research Products. A rabbit monoclonal antibody to PHD2 was obtained from Epitomics (cat # 5859).

Anti-p23 (cat # 2731) and anti-FKB38 (cat # 5792) were from Epitomics. Anti-HSP90 (clone C45G5) and anti-HSP70 (cat # 4872) were from Cell Signaling. Anti-FKBP16/ARA9 (cat # GTX110665) was from GeneTex. Mouse monoclonal antibody against β-tubulin developed by Dr. Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the The University of Iowa, Department of Biology, Iowa City, IA 52242.

Real Time PCR—Total RNA was harvested from cells using TRIzol reagent (Invitrogen). Reverse transcription reactions were performed using High Capacity cDNA kits (ABI). Real Time PCR reactions were performed on 20 ng equivalents of cDNA using an ABI 7300 Real Time PCR machine and SYBR Green Master mixes. Relative quantification was performed using the ΔΔCt method and 18S RNA as the endogenous control. The following primer pairs were employed, and in all cases dissociation curve analysis revealed a single peak for each. P23, 5’ CCA AAT GAT TCC AAG CAT AAA AGA 3’ and 5’ GGC CAG ATT CTC CTT TTC GTA A 3’. PHD2, 5’ TGA AGC TGG CGC TCG AGT A 3’ and 5’ CAC ACA GAT GCC GTG CTT GT 3’. CA9, 5’ CGG AAG AAA ACA GTG CCT ATG A 3’ and 5’ CTT CCT CCG CGG CCC AGG ATC AG 3’. GLUT1, 5’-TGC TCA TGG GCT TCT CGA A 3’ and 5’-AAG CGG CCC AGG ATC AG 3’. HIF1α, 5’ TTT TAC CAT GCC CCA GAT TCA 3’ and 5’ AGT GCT TCC ATC GGA AGG ACT 3’. I8S, 5’ TCG GAA CTG AGG CCA TGA TT 3’ and 5’ TAG CGG CGC AAT ACG AAT G 3’.

siRNA—siRNAs to p23 [PTGES3_1 (SI02780911) and PTGES3_2 (SI02781170); the former was employed for most experiments] and FKBP38 [FKBP8_5 (SI03035977)] were obtained from Qiagen. siRNA to PHD2 (PHD2-A) has been described (37). The control Non-targeting siRNA #2 (D-001210-02) and Non-targeting siRNA #3 (D-001210-03) were from Dharmacon (the latter was employed for most experiments). Transfections were performed with siRNA concentrations of 20 nM. Lysates were prepared in buffer C supplemented with mammalian protease inhibitor cocktail, and clarified by centrifugation at 15,800 x g for 10 minutes at 4 °C. Protein concentrations were measured using a Bio-Rad DC Protein Assay, and equal amounts examined by western blotting.

Luciferase assays—Luciferase assays were performed on a Wallac LB9507 luminometer using a Dual Luciferase Reporter Assay System (Promega).

Statistical analysis—ANOVA (GraphPad Prism) was employed for statistical analysis. Differences were considered significant when p < 0.05.

RESULTS

PHD2 Interacts With Select HSP90 Co-chaperones—In many signal transduction pathways, key enzymes do not act in isolation, but rather interact with other proteins. We hypothesized that this might be the case with PHD2. To pursue this, we generated a Human Embryonic Kidney (HEK) 293 cell line expressing Flag-tagged PHD2. Using a Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) approach (31), we subjected this cell line (labeled with heavy isotope) as well as the parental cell line (labeled with light isotope) to immunoprecipitation with anti-Flag antibodies, and eluted with Flag peptide (Fig. 1A). The eluates were mixed, digested with trypsin, and analyzed by mass spectrometry. As expected, PHD2 displays a very high heavy isotope to light (H/L) isotope ratio (39.5). The group of proteins with the highest degree of enrichment were ones of the HSP90
pathway, including FKBP38 (H/L ratio of 22.5), p23 (19.0), FKBP51 (17.5), FKBP52 (6.3), and HSP90 itself (6.6 for the AA isoform, 6.4 for the AB isoform).

HSP90 can associate with a multitude of co-chaperones that perform a number of functions, including the recruitment of regulatory or client proteins to the HSP90 machinery (23,25,26). Only a few co-chaperones were identified in the SILAC experiment just described. To explore the possibility that select co-chaperones may interact with PHD2, we coexpressed PHD2 with various HSP90 co-chaperones and immunoprecipitated the latter. We find that PHD2 selectively coimmunoprecipitates with p23 and FKBP38 (Fig. 1B, top panel, lanes 2 and 5). In reciprocal immunoprecipitations, we confirmed the presence of p23, FKBP38, and HSP90 in PHD2 immunoprecipitates (Fig. 1C).

We next employed a monoclonal antibody that can specifically immunoprecipitate PHD2 but not PHD1 or PHD3 (36). We immunoprecipitated endogenous PHD2 from HEK293FT cells, and demonstrate the presence of endogenous p23 in these immunoprecipitates (Fig. 1D, top panel, lane 2). Exposure of cells to 1% O₂ did not affect the association (Fig. 1D, top panel, compare lanes 2 and 4). We also knocked down p23 using siRNA in these cells, immunoprecipitated endogenous PHD2, and examined the immunoprecipitates for the presence of HSP90. We find, as expected, that HSP90 coimmunoprecipitates with PHD2 (Fig. 1E, top panel, lane 2). Knockdown of p23 substantially diminishes this coimmunoprecipitation (Fig. 1E, top panel, compare lanes 2 and 3), suggesting that p23 is an important, though not necessarily the exclusive, means by which PHD2 interacts with HSP90.

Other proteins of the HSP90 pathway, such as HSP70, HOP (also known as STIP1), PPP5C, or FKBP16 (also known as AIP or XAP2), as well as others involved in protein folding, such as HSP60, GRP94, HSP73, or GRP75, were not enriched in PHD2 immunoprecipitates, as assessed by western blotting (Fig. 1B and C) or SILAC analysis (data not shown). The selective interaction of PHD2 with p23 and FKBP38 is consistent with their relatively high heavy to light isotope ratio in the SILAC experiment.

PHD2 Binds to a PXLE Motif—p23 is an evolutionarily conserved co-chaperone that binds to HSP90 in an ATP-dependent manner and plays a critical role in numerous HSP90-dependent processes (25,38). For certain client proteins, it enters late in the HSP90-dependent maturation cycle. FKBP38 appears to have emerged later in evolution and is a co-chaperone anchored to the endoplasmic reticulum (39). It also appears to have a more specialized function. For example, it has been implicated in the regulation of Bcl-2 (40). The interaction between PHD2 and FKBP38 has been reported previously and shown to depend on the interaction of PHD2 with an acidic region in FKBP38 comprising residues 37 to 56 (41). We confirmed this by deleting the N-terminal 57 amino acids of FKBP38 and observing that this abolishes interaction with PHD2 coimmunoprecipitation assays (data not shown). Inspection of the p23 amino sequence reveals that it possesses an acidic region at its C-terminus which shares a PXLE motif (Fig. 2A). This motif shows strong conservation in p23 through evolution (Fig. 2B).

We examined the importance of the PXLE motif in p23 by mutating the conserved Pro, Leu, and Glu simultaneously to Ala. Using coimmunoprecipitation assays with full length proteins, we observe that this mutation abolishes interaction with PHD2 (Fig. 2C, top panel, lanes 3 and 4). We also immobilized a peptide comprising p23 (151-160) on agarose beads (Fig. 3A), and find that this peptide is sufficient to confer interaction with PHD2 (Fig. 3B, top left panel, second lane). A parallel experiment with an FKBP38 peptide (residues 47-56) containing the PXLE motif shows that this peptide is sufficient to confer interaction with
PHD2, which again is abolished by mutation of this motif (Fig. 3B, bottom right panel, last two lanes).

In further experiments with p23, we find that mutation of E160 (to A, D, or Q), L159 (to A, I, or V), or P157 (to A or S) substantially reduces, if not abolishes PHD2 binding, highlighting the stringency of this amino acid motif. In contrast, amino acid substitutions at position 158 (to A, P, and L), which is not conserved between p23 and FKBP38, can be tolerated. Amino acid 156 is a hydrophobic residue (Met), and we find that substitutions to Leu and Val preserve the interaction; indeed, the Leu substitution has a somewhat increased binding. In contrast, substitutions to Ala, Phe, or Glu essentially abolish it, suggesting a preference for select hydrophobic amino acids. Mutations of each of the four amino acids preceding the MPDLE motif preserves the interaction, with the exception of D153A—which substantially diminishes it, as does a triple mutation of the three acidic residues (D152A/D153A/E154A). The importance of Asp at position 153 is likely to be specific to p23, as the corresponding residue in FKBP38 is Leu (Fig. 3A), but may nevertheless point to a necessity for having one or more acidic residues preceding the PXLE motif.

The MYND-type Zinc Finger of PHD2 Binds the PXLE Motif—PHD2 is one of three PHD isoforms that have the capacity to hydroxylate HIF-\(\alpha\) (9,10). We coexpressed Flag-p23 with HA-tagged versions of PHD1, PHD2, or PHD3, and find that only PHD2 interacts with p23 (Fig. 4A, lane 5). A similar result has been observed with FKBP38 (42). The three PHDs share a conserved C-terminal hydroxylase domain but differ at their N-termini, raising the possibility that the N-terminus of PHD2 mediates this interaction. Indeed, an N-terminal fragment of PHD2 interacts with p23, whereas a C-terminal fragment does not (Fig. 4B, compare lanes 5 and 7). The N-terminus of PHD2 is distinctive among the three PHDs in that it is predicted to harbor a MYND-type zinc finger (9,20) (Fig. 4C). Mutation of predicted zinc-chelating residues (C36,42S) in PHD2 abolishes its ability to coimmunoprecipitate with p23 (Fig. 4B, lane 9). This mutation also eliminates the ability of GST-PHD2 (1-124) to bind FKBP38 (data not shown). These results strongly suggest that the integrity of the MYND-type zinc finger is essential for interaction with the PXLE motif.

p23 is Functionally Important for HIF-1\(\alpha\) Regulation—We knocked down p23 or PHD2 using siRNA in Hela cells, and examined the levels of various proteins by western blotting. Under normoxic conditions, we find that p23 knockdown has, at best, a marginal effect on HIF-1\(\alpha\) levels (Fig. 5A, left upper panel, lane 2). We then examined these cells under moderate hypoxia (2% O\(_2\)), a situation in which PHD2 continues to have activity; indeed, PHD2 knockdown results in a substantial increase in HIF-1\(\alpha\) levels (Fig. 5A, left upper panel, compare lanes 4 and 6). Importantly, we find that p23 knockdown augments HIF-1\(\alpha\) levels to ones comparable to those seen following PHD2 knockdown (lane 5). Equally significant, this p23 knockdown augmentation of hypoxic HIF-1\(\alpha\) levels is seen in a range of other cell types, including HEK293, PC3, and MCF7 cells (compare lanes 4 and 5 in each set of panels). Augmentation of normoxic HIF-1\(\alpha\) levels following p23 knockdown is seen in PC3 cells, but not in HEK293 and MCF7 cells (compare lanes 1 and 2 in each set of panels). Western blotting indicates that the effects of p23 knockdown cannot be attributed to changes in PHD2 levels (Fig. 5A). Knockdown of p23 using an independent siRNA yields the same effect (Fig. 5B, lanes 11 and 12); in addition, under the conditions of this experiment, we find that FKBP38 knockdown does not have an effect on HIF-1\(\alpha\) levels (lanes 2 and 8).

We performed Real Time PCR on RNA extracted from Hela cells following p23 or PHD2 siRNA treatment. This analysis confirmed the efficacy of knockdown of p23 and PHD2 (Fig. 6A and 6B, respectively). Consistent with the previous results examining HIF-1\(\alpha\) protein levels (Fig. 5), knockdown of
PHD2 yields an increase in hypoxia-induced mRNA levels of the canonical HIF target genes, \textit{CA9} and \textit{GLUT1} (Fig. 6C and 6D, respectively). Importantly, we find that p23 knockdown also augments message levels of these same targets. For either p23 or PHD2 knockdown, this augmentation cannot be attributed to changes in \textit{HIF1A} mRNA levels (Fig. 6E).

The data support an important role for an interaction between p23 and the zinc finger of PHD2. In a different experiment to assess this, we transfected HEK293FT cells with a luciferase reporter gene with a hypoxia response element reporter gene and constructs for either wild-type or zinc finger-defective (C36,42S) PHD2. The cells were exposed to hypoxia, and luciferase activities measured. We find that hypoxia, as expected, robustly induces activity from this reporter gene (Fig. 6F). Under conditions where wild type PHD2 effectively inhibits activation of this reporter, zinc finger-defective PHD2 does not. This, therefore, provides further evidence for a functionally significant interaction between p23 and PHD2.

We next generated a HEK293 cell line expressing Flag-tagged p23, and then immunoprecipitated the p23 in a SILAC experiment analogous to the one that originally identified p23 as a PHD2-interacting protein. By mass spectrometry, we find that p23 displays the highest H/L ratio, 26.9, and that other components of the HSP90 pathway are also enriched, consistent with the well-established role of p23 in HSP90 dynamics. These proteins include FKBP51 (H/L ratio of 12.0), PPP5C (6.8), FKBP52 (4.8), HSP90AB1 (3.8) and HSP90AA1 (3.6). Among non-HSP90 pathway proteins, KIF1-binding protein displays the highest H/L ratio (21.6). Strikingly, PHD2 displays the next highest (13.3). Western blotting confirms the presence of endogenous PHD2 in p23 immunoprecipitates in this cell line (data not shown). Therefore, this unbiased SILAC experiment independently supports an interaction between p23 and PHD2.

\textbf{p23 is Functionally Important for HIF-1α Hydroxylation}—One possible explanation for the present results is that PHD2 itself is a client of the HSP90 pathway, i.e., the HSP90 pathway properly folds PHD2 into a catalytically active conformation. A number of observations argue against this. First, p23 knockdown does not affect PHD2 protein levels (Fig. 5). Second, mutation of the MYND-type zinc finger of PHD2 abolishes its interaction with p23 (Fig. 4B), indicating that at least for this particular domain of PHD2, it is actually the mature folded state—as opposed to an unfolded state—that is essential for interaction. To explore the possibility that p23 may be necessary for folding of the other domain of PHD2, i.e. its catalytic domain, we assessed the interaction of PHD2 with HIF-1α (530-575), which contains the hydroxylacceptor proline, Pro-564. We knocked down p23 in Hela cells, incubated cellular extracts with GST-HIF-1α (531-575), and assessed PHD2 binding by western blotting. As shown in Fig. 7A, we do not find any detectable effect of p23 knockdown on PHD2 binding to HIF-1α (530-575), suggesting that p23 is not essential for the folding of the PHD2 catalytic domain.

HSP90 inhibitors, such as geldanamycin and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), are known to produce a biphasic response on HIF-1α levels. At high (µM) concentrations, they diminish HIF-1α levels (24), while at low (nM) concentrations, they increase it (43). The former is consistent with HIF-1α being an HSP90 client protein. The latter, based on the present data, might conceivably be due to effects on the PHD2:p23 axis, since it is known that both HSP90 inhibitors and p23 bind to the N-terminus of HSP90 (38,44). To examine this, we treated PC3 cells with 17-AAG and then either maintained cells under normoxia or exposed them to hypoxia. Consistent with previous results, we find diminished hypoxia-induced HIF-1α levels at high (3 µM) 17-AAG concentrations (Fig. 7B, compare lanes 4 and 6). Importantly, we observe increased HIF-1α levels at low (6 µM)
nM) 17-AAG concentrations, particularly under hypoxic conditions (lane 5), a situation that mirrors p23 knockdown. We also examined the effect of 17-AAG on HIF-1α levels in RCC4 cells, which lack functional VHL and thereby dissociate prolyl hydroxylation from degradation. Again, consistent with previous results (24), high 17-AAG concentrations diminish HIF-1α levels (Fig. 7C, lanes 3 and 5). Low 17-AAG concentrations, in contrast, have no effect on HIF-1α levels in these cells under either normoxic or hypoxic conditions (lanes 2 and 4), thereby arguing that the effects of low 17-AAG concentrations seen in PC3 cells are not due to effects on HIF-1α translation.

We further employed the RCC4 cells to examine the hydroxylation state of HIF-1α. We treated cells with or without 6 nM 17-AAG in the absence or presence of hypoxia (2% O2) for twenty hours. Cells were lysed and mixed with recombinant Flag-tagged VHL (as a complex with Elongin B and Elongin C) to capture hydroxylated HIF-1α. The FlagVHL was then immunoprecipitated, and eluates then examined for the presence of HIF-1α by western blotting. Examination of the lysates indicates that neither low dose (6 nM) 17-AAG nor hypoxia has an appreciable effect on HIF-1α levels (Fig. 7D, second panel from bottom), consistent with results of Fig. 7C. In the absence of VHL capture probe, no HIF-1α is detectable in the anti-Flag immunoprecipitates while in its presence, HIF-1α is readily detectable (top panel, compare lanes 1 and 2), confirming the specificity of the capture probe (Fig. 7E, top panel, compare lanes 1 and 2). In control cells, hydroxylated HIF-1α is readily detected, indicating that prolyl hydroxylation occurs and is detectable under these modest hypoxic conditions (lane 2). Strikingly, we find that p23 knockdown results in markedly lower levels of HIF-1α hydroxylation (lane 4). Additional experiments using this same VHL capture approach reveal that the decrease in HIF-1α hydroxylation induced by p23 knockdown is comparable to that induced by PHD2 knockdown (Fig. 7F, top panel, lanes 2 and 3). We conclude that p23 serves to recruit PHD2 to HSP90 in order to promote optimal HIF-1α hydroxylation.

DISCUSSION

These data provide evidence for a critical link between PHD2 and the HSP90 pathway (Fig. 8). A model consistent with the data is that p23 recruits PHD2 to HSP90 in order to facilitate HIF-1α hydroxylation. This model is compatible with previous observations that HIF-1α is itself an HSP90 client protein (24), and also with previous work that indicates that p23 enters the HSP90 cycle at a late stage in the maturation cycle of HSP90 client proteins (23). The model would allow for efficient PHD2-mediated hydroxylation, since HIF-1α proceeds through an obligatory HSP90-dependent pathway. The more pronounced effect of p23 knockdown seen under hypoxic conditions would suggest...
that the coupling to the HSP90 pathway is relatively more important under these conditions.

HSP90 co-chaperones are diverse in terms of both structure and function (25,26). Many HSP90 co-chaperones serve to recruit HSP90 client proteins (23). Prominent examples include the recruitment of protein kinases by Cdc37 (45), and Nod-like receptors by Sgt1 (46,47). In addition, select co-chaperones have intrinsic catalytic activities. Examples of these include FKBP52, which has peptidyl prolyl isomerase activity, and PPP5C, which has protein phosphatase activity. Our model is different from these previously described uses of co-chaperones in that it proposes that a co-chaperone (p23) recruits an enzyme (PHD2) which posttranslationally modifies proteins. The enzymatic activity of PHD2 is clearly essential for its ability to regulate HIF-1α (9,10,30), and it will be of interest to determine if the proposed mechanism may also be used to promote the hydroxylation of non-HIF protein targets. In addition, it will be of interest to examine whether there might be hydroxylase-independent functions of PHD2. Interestingly, previous studies on PHD2 point to this possibility (48,49). It might be noted that the capacity of FKBP52 to serve as a co-chaperone in the glucocorticoid receptor maturation pathway is independent of its peptidyl prolyl isomerase activity (50).

The present studies also identify a functional role for the MYND-type zinc finger of PHD2, namely, interaction with a PXLE motif present in p23 and FKBP38. MYND domains serve as protein:protein interaction motifs in other proteins (51). For example, the MYND domain-containing transcriptional corepressor BS69 binds to a PXLXP motif present in E1A (52), whereas the MYND domain-containing AML1/ETO fusion protein binds to a PPPL1 motif in SMRT (53). These motifs share similarity to the PXLE motif to which the PHD2 MYND zinc finger binds. It is conceivable that MYND zinc fingers as a whole bind a core consensus sequence of PXL, with adjacent residues determined in a MYND domain-specific manner; additional studies on other MYND zinc fingers will be necessary to address this possibility. The SMRT PPPL1 motif binds in an extended conformation to AML1/ETO (53). The p23 PXLE motif is C-terminal, and studies indicate that it is unstructured (54,55), raising the possibility that it may also bind in an extended conformation to the PHD2 MYND-type zinc finger.

Taken together, these findings provide evidence for a direct link between two ancient pathways, the oxygen-sensing pathway and the HSP90 pathway. The significance of this link is supported by the strong conservation of the MYND domain of PHD2 through evolution (21,22). This domain, for example, is present in the single PHD isoform from the simplest animal, T. adhaerens, which possesses both a PXLE motif-containing p23 homologue (Fig. 2B) and a functional PHD:HIF pathway (21). This suggests that the coupling of the PHD:HIF pathway to the HSP90 machinery emerged early in evolution—indeed, perhaps concurrent with the appearance of the PHD:HIF pathway itself.
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FOOTNOTES

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2The abbreviations used are: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; FKBP, FK506 binding protein; HIF, Hypoxia Inducible Factor; HSP, Heat Shock Protein; PCR, polymerase chain reaction; PHD, Prolyl Hydroxylase Domain protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VHL, von Hippel Lindau protein.
FIGURE LEGENDS

FIGURE 1. Mass spectrometry identification of PHD2-interacting proteins. (A) Flp-In T-Rex HEK293 or Flp-In T-Rex HEK293/FlagPHD2 cells were grown in the presence of amino acids with light or heavy labeled lysine/arginine, respectively, subjected to immunoprecipitation (IP) with anti-Flag antibodies, and the eluates mixed, subjected to tryptic digest, and mass spectrometry. The square and hexagon depict proteins that bind specifically to PHD2, while the small circles depict proteins that bind non-specifically to the antibody (or resin). (B) HEK293FT cells were transfected with expression vectors for the indicated proteins, lysed, subjected to immunoprecipitation with anti-Flag antibodies, and western blots (WB) were then performed. The positions of HA-PHD2 or molecular weight markers are shown to the right. (C) Flp-In T-Rex HEK293 or Flp-In T-Rex HEK293/FlagPHD2 cells were induced with doxycycline, lysed, and subjected to immunoprecipitation with anti-Flag antibodies. The immunoprecipitates were eluted with 3X Flag peptide. Aliquots of lysate or eluate were then subjected to western blotting using antibodies against the indicated proteins. Anti-Flag antibodies were employed to detect Flag-PHD2. (D and E) HEK293FT cells were (D) maintained under normoxia or hypoxia (1% O₂ for 4 hr), or (E) treated with control or p23 siRNA. Cells were lysed, incubated with 10µg of either control or anti-PHD2 monoclonal antibody, immunoprecipitated with protein G-agarose, and western blots then performed.

FIGURE 2. A conserved PXLE motif in select HSP90 co-chaperones. (A) Schematic diagram of p23. Shading denotes the CHORD and Sgt1 (CS) domain, and numbers indicate amino acid number. The C-terminal 20 amino acids of p23 are shown and compared to the indicated residues of FKBP38. (B) Comparison p23 sequences from H. sapiens (Unigene Hs.50425), M. musculus (Mm.305816), X. laevis (Xl.14340), D. rario (Dr.77365), C. elegans (Cel.8134), and Trichoplax adherans (XP_002111186). Shading indicates residues conserved in all of the proteins shown. (C) HEK293FT were transfected and immunoprecipitated as in panel B. AAA denotes a P157A/L159A/E160A mutation in p23.

FIGURE 3. PHD2 binds to a PXLE motif. (A) Comparison of p23 residues 151-160 to FKBP38 residues 47-56. The numbers above the sequences correspond to p23 residues. Shading indicates conserved residues of the PXLE motif. (B) Sf9 lysates containing (His)₆-FlagPHD2 were incubated with p23 peptides immobilized on streptavidin-agarose, washed, and the eluates examined for the presence of PHD2 by western blotting using anti-Flag antibodies. Amino acid substitutions are indicated in each lane. PLE>AAA denotes P157A/L159A/E160A, while DDE>AAA denotes D152A/D153A/E154A. Experiments with FKBP38 peptides are shown in the last two lanes of the bottom right panel. FKBP38 AAA denotes P53A/L55A/E56A. In each panel, peptide was omitted in the first lane. The relative degree of binding for each peptide, normalized to that of wild type (WT) p23 peptide, is given below each panel.

FIGURE 4. The MYND-type zinc finger of PHD2 is required for its interaction with p23. (A) and (B) HEK293FT were transfected and immunoprecipitated as in Figure 1C. (C) Schematic diagram of PHD2. Dark shading denotes the N-terminal MYND-type zinc finger (ZF), light shading denotes the C-terminal Prolyl Hydroxylase (PH) domain, and numbers indicate amino acid number. Shown at the top are amino acids 21 to 58. Underlining indicates predicted zinc chelating residues. Inverted triangles indicate Cys-36 and Cys-42.

FIGURE 5. Knockdown of p23 augments HIF-1α protein levels. (A) The indicated cell lines were treated with the indicated siRNA, and then maintained under normoxia (NX) or subjected to...
Cell lysates were prepared, and equal protein amounts were then subjected to western blotting using antibodies to the indicated proteins. Con = control Non-targeting siRNA #3. The p23 siRNA was PTGES3_1. (B) Hela cells were treated with the indicated siRNA, and then examined as in (A). The p23-1 siRNA was PTGES3_1, while the p23-2 siRNA was PTGES3_2.

FIGURE 6. Knockdown of p23 augments HIF-1α target genes. (A-E) Hela cells were treated with the indicated siRNA for 72 hr, and then maintained under normoxia (NX) or subjected to 2% O₂ (HX) for 16 hr. mRNA levels for the indicated genes were measured by Real Time PCR and normalized to that of 18S rRNA. Shown are means ± SD, n = 3. *, p < 0.05 compared to the respective normoxic or hypoxic control; **, p < 0.01 compared to the respective normoxic or hypoxic control; ns, not significant compared to the respective normoxic or hypoxic control. (F) HEK293FT cells were transfected with 50 ng of (eHRE)3-Luc, 50 ng of pRL-TK, and either 5 or 15 ng of either pcDNA3-Flag-PHD2 or pcDNA3-HA-PHD2 (C36,42S). DNA doses were held constant by addition of pcDNA3. After 8 hr, cells were exposed to 1% O₂ (HX) for an additional 16 hr, or maintained under normoxia (NX). Cells were lysed, luciferase activity measured and normalized to that of Renilla luciferase. Shown are means ± standard deviations, n = 3. *, p < 0.05 compared to the respective normoxic or hypoxic control; **, p < 0.01 compared to the respective normoxic or hypoxic control; ns, not significant compared to the respective normoxic or hypoxic control.

FIGURE 7. The PHD2:p23 interaction facilitates prolyl hydroxylation of HIF-1α. (A) Hela cells were treated with control (Con) or p23 siRNA, and cellular extracts prepared. GST or GST-HIF-1α (531-575) prebound to GSH-agarose was incubated with the extracts, washed, and bound PHD2 then assessed by western blotting using the indicated antibodies. (B) PC3 cells were treated with the indicated concentrations of 17-AAG and then immediately subjected to normoxia (NX) or 2% O₂ (HX) for 4 hr. Cell lysates were prepared and equal protein amounts subjected to western blotting using antibodies to the indicated proteins. (C) RCC4 cells were treated with the indicated concentrations of 17-AAG and then immediately subjected to normoxia or 2% O₂ for 4 or 20 hr. Cell lysates were prepared and equal protein amounts subjected to western blotting using antibodies to the indicated proteins. (D) RCC4 cells were subjected to either 6 nM 17-AAG, 2% O₂ (HX), or both for 20 hr. The cells were lysed, equal protein amounts incubated with or without recombinant FlagVHL, the FlagVHL immunoprecipitated, and proteins captured by VHL analyzed by western blotting using antibodies to HIF-1α. Lysates were also analyzed by western blotting. (E) Hela cells were treated with the indicated siRNA and then subjected to 2% O₂ for 4 hr in the presence of 10 µM MG132. The cells were lysed, equal protein amounts incubated with or without recombinant FlagVHL, the FlagVHL immunoprecipitated, and proteins captured by VHL analyzed by western blotting using antibodies to HIF-1α. Lysates were also analyzed by western blotting. (F) Hela cells were treated with the indicated siRNA and then subjected to 2% O₂ for 4 hr in the presence of 10 µM MG132. The cells were lysed, equal protein amounts incubated with recombinant FlagVHL, the FlagVHL immunoprecipitated, and proteins captured by VHL analyzed by western blotting using antibodies to HIF-1α. Lysates were also analyzed by western blotting.

FIGURE 8. Model for interaction of PHD2 with HSP90 pathway. PHD2 associates with p23 via a PXLE motif in the latter (denoted by curved line). p23, in turn, is known to bind to the N-terminus of HSP90. The facilitates PHD2-catalyzed hydroxylation of proline in HIF-1α, which is known to be an HSP90 client protein. Prolyl hydroxylation, in turn, marks HIF-1α for degradation. The C-terminus of HSP90 is known to bind a subset of HSP90 co-chaperones that
includes FKBP38, FKBP51, and FKBP52. FKBP38 possesses a PXLE motif and therefore provides an independent means for recruiting PHD2 to the HSP90 machinery.
Fig 2

A

P23 (141-160): ...VDGADDSQDSDDKEKMPDLE
FKBP38 (37-56): ...EEEEEEEEEEDDLSELPPLE...

B

H. sapiens (145-160)  DDDSQDSDEDEKMPDLE
M. musculus (145-160)  DDDSQDSDEDEKMPDLE
X. laevis (145-160)  DDDSPDSDEDEKMPDLE
D. rario (144-159)  EEEESPDSDEDEKMPDLE
C. elegans (147-162)  GGLEDDEEDDDMPDLE
T. adhaerans (245-260)  DDDNDDSDDEDEKMPDLE

C

HA-PHD2
Flag-p23
Flag-p23 AAA

IP: α Flag  WB: α HA

Lysate

WB: α HA

WB: α Flag

1 2 3 4

HA-PHD2
Flag-p23

Downloaded from http://www.jbc.org/
A

P23 (151–160): SDDEKMPDLE
FKBP8 (47–56): DDLSELPPLE

B

|       | WT    | PLE-AAA | E160A | E160D | E160Q |
|-------|-------|---------|-------|-------|-------|
| SDDEKMPDLE | 1.00  | 0.08    | 0.06  | 0.10  | 0.09  |

|       | WT    | L159A  | L159I | L159V | D158A |
|-------|-------|--------|-------|-------|-------|
| DDLSELPPLE | 1.00  | 0.04   | 0.03  | 0.10  | 0.84  |

|       | WT    | D158P  | D158L | P157A | P157S |
|-------|-------|--------|-------|-------|-------|
| SDDEKMPDLE | 1.00  | 0.96   | 0.71  | 0.15  | 0.01  |

|       | WT    | M156A  | M156L | M156V | M156F |
|-------|-------|--------|-------|-------|-------|
| DDLSELPPLE | 1.00  | 0.03   | 2.61  | 0.92  | 0.02  |

|       | WT    | M156E  | K155A | E154A | D153A |
|-------|-------|--------|-------|-------|-------|
| SDDEKMPDLE | 1.00  | 0.11   | 1.69  | 1.31  | 0.08  |

|       | WT    | D152A  | DDE-AAA | FKBP8 WT | FKBP8 AAA |
|-------|-------|--------|----------|----------|----------|
| DDLSELPPLE | 1.00  | 1.10   | 0.06     | 3.61     | 0.07     |

Fig 3
A

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|----------|---|---|---|---|---|---|---|
| HA-PHD1  |   |   |   |   |   |   |   |
| HA-PHD2  | + | + | + | + | + | + | + |
| HA-PHD3  | + | + | + | + | + | + | + |
| Flag-p23 |   |   |   |   |   |   |   |

WB: α HA

Lysate

WB: α Flag

B

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------|---|---|---|---|---|---|---|---|---|
| HA-PHD2  | + | + | + | + | + | + | + | + | + |
| HA-PHD2 (1-196) |   |   |   |   |   |   |   |   |   |
| HA-PHD2 (130-426) |   |   |   |   |   |   |   |   |   |
| HA-PHD2 C36,42S |   |   |   |   |   |   |   |   |   |
| Flag-p23 | + | + | + | + | + | + | + | + | + |

WB: α HA

Lysate

WB: α Flag

C

PHD2 (21-58): CELCGKMNLLRCSRCSRSSFYCCkehQRQDWWKHKLVC

Fig 4
**Fig 6**

A. Relative mRNA levels of p23.

B. Relative mRNA levels of PHD2.

C. Relative mRNA levels of CA9.

D. Relative mRNA levels of GLUT1.

E. Relative mRNA levels of HIF1A.

F. Relative Luciferease Activity.
Fig 7

A

| siRNA | GST | Con | Con | p23 |
|-------|-----|-----|-----|-----|
| GST-HIF-1α (531-575) | pulldown | WB: PHD2 |

Lysate

| WB: β tubulin |

B

| NX | 2% O₂ |
|-----|-------|
| 17-AAG (nM) | 0 | 6 | 3000 | 0 | 6 | 3000 |

| HIF-1α |

| β-tubulin |

C

| 17-AAG (nM) | 0 | 6 | 3000 | 6 | 3000 |
|-------------|-----|-----|-------|-----|-----|

| HIF-1α |

| β-tubulin |

D

| NX | HX |
|-----|-----|
| 17-AAG (6 nM) | - | - | + | + |

| VHL IP |

| HIF-1α |

| lysate |

| β-tubulin |

E

| siRNA | Control | p23 |
|-------|---------|-----|
| VHL IP | - | + | - | + |

| HIF-1α |

| lysate |

| β-tubulin |

F

| siRNA | Con | p23 | PHD2 |
|-------|-----|-----|------|
| VHL IP | HIF-1α |

| lysate |

| β-tubulin |

Fig 7
Fig 8
Prolyl Hydroxylase Domain Protein 2 (PHD2) Binds a Pro-Xaa-Leu-Glu Motif, Linking it to the Heat Shock Protein 90 Pathway
Daisheng Song, Lin-Sheng Li, Katherine J. Heaton-Johnson, Patrick R. Arsenault, Stephen R. Master and Frank S. Lee

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