Tibetan *PHD2*, an allele with loss of function properties

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Abstract

Tibetans have adapted to the chronic hypoxia of high altitude and display a distinctive suite of physiologic adaptations, including augmented hypoxic ventilatory response and resistance to pulmonary hypertension. Genome-wide studies have consistently identified compelling genetic signatures of natural selection in two genes of the Hypoxia Inducible Factor pathway, *PHD2* and *HIF2A*. The product of the former induces the degradation of the product of the latter. Key issues regarding Tibetan *PHD2* are whether it is a gain of function or loss of function allele, and how it might contribute to high-altitude adaptation. Tibetan PHD2 possesses two amino acid changes, D4E and C127S. We previously showed that in vitro, Tibetan PHD2 is defective in its interaction with p23, a cochaperone of the HSP90 pathway, and we proposed that Tibetan *PHD2* is a loss of function allele. Here, we report that additional PHD2 mutations at or near Asp-4 or Cys-127 impair interaction with p23 in vitro. We find that mice with the Tibetan *Phd2* allele display augmented hypoxic ventilatory response, supporting this loss of function proposal. This is phenocopied by mice with a mutation in *p23* that abrogates the PHD2:p23 interaction. *Hif2a* haploinsufficiency, but not the Tibetan *Phd2* allele, ameliorates hypoxia-induced increases in right ventricular systolic pressure. The Tibetan *Phd2* allele is not associated with hemoglobin levels in mice. We propose that Tibetans possess genetic alterations that both activate and inhibit selective outputs of the HIF pathway to facilitate successful adaptation to the chronic hypoxia of high altitude.

Significance

Tibetans have lived for thousands of years at an altitude where oxygen concentrations are low (hypoxia). They are known to have physiologic adaptations, including increased breathing responses to low oxygen and decreased blood pressure in the lungs. Tibetans also are known to have mutations in two genes, *PHD2* and *HIF2A*, that are central components of a molecular hypoxia response pathway. We provide evidence here that Tibetan PHD2 promotes increased breathing responses to hypoxia. The Tibetan *HIF2A* gene is likely to account for the decreased blood pressure in the lungs. Therefore, the hypoxia response pathway has been altered in Tibetans to facilitate adaptation to the hypoxia of high altitude.

Introduction

The Tibetan plateau has an average altitude of about 4,300 m (14,000 feet), an elevation at which the partial pressure of oxygen is only 60% of that at sea level. Yet Tibetans have occupied this plateau for at least 30,000 years, with some estimates as high as 40,000 years (1, 2). Studies have revealed a number of notable physiologic changes in the Tibetan population. In comparison to Andeans, another high-altitude adapted population, Tibetans display augmented hypoxic ventilatory response (HVR), resistance to pulmonary hypertension, and relatively low hemoglobin (Hb) levels (3, 4). These are considered to be adaptive. For example, the resistance to pulmonary hypertension lessens the risk of right heart failure, while the relatively low Hb levels (which approach that of lowlanders) may decrease the risk of thrombotic events associated with blood hyperviscosity. The augmented HVR can facilitate the intake of oxygen into the lungs.

Genome-wide studies have identified evidence of natural selection in Tibetans. The two genes that have consistently ranked the highest in these studies are the *PHD2* (also known as *EGLN1*) and *HIF2A* (also known as *EPAS1*) genes (4-7). The encoded proteins, Prolyl Hydroxylase Domain protein 2 (PHD2) and Hypoxia Inducible Factor-2α (HIF-2α), respectively, play central roles in the Hypoxia Inducible Factor (HIF) pathway, which in turn mediates the central transcriptional response to hypoxia in metazoans (8-10). In this pathway, PHD2-catalyzed, oxygen dependent, prolyl hydroxylation of HIF-α (of which HIF-1α and HIF-2α are the major paralogues) allows recognition by the von Hippel Lindau tumor suppressor protein, a component of an E3 ubiquitin ligase complex that targets HIF-α for degradation (Fig. 1A, left). Under hypoxia, this posttranslational modification is arrested, leading to the stabilization of HIF-α, its heterodimerization with Aryl Hydrocarbon Receptor Nuclear Translocator, and the activation of HIF target genes that promote adaptive responses to hypoxia (Fig.1A, right). HIF-1α and HIF-2α have overlapping and distinct gene targets (11). For example, HIF-1α is the

principal paralogue that upregulates glycolysis while HIF-2α is the main paralogue that activates the *ERYTHROPOIETIN* (*EPO*) gene, which allows oxygen-dependent regulation of red cell mass.

The Tibetan *HIF2A* allele arose through introgression from Denisovans, an archaic hominin species (12). The Tibetan *HIF2A* allele does not encode for amino acid changes in the HIF-2α protein. Rather, the SNPs under strongest selection, which number over 30, all reside within non-coding regions of the gene. In contrast, the Tibetan *PHD2* allele is not a result of introgression from Denisovans (13). Moreover, there are two SNPs, rs186996510 and rs12097901, in the *PHD2* gene which are tightly linked and enriched in Tibetans that encode for D4E and C127S amino acid substitutions, respectively (14, 15). Hence, the Tibetan PHD2 allele encodes for a D4E/C127S double amino acid substitution. A central question regarding this allele is whether it is a gain of function or loss of function allele. Some studies support a model in which Tibetan *PHD2* is a gain of function allele that derives from increased affinity for oxygen leading to enhanced hydroxylase activity towards HIF-α (15).

Other studies support a very different model. The affected amino acids flank a highly conserved MYND-type zinc finger at the N-terminus of PHD2 (Fig. 1B). The zinc finger binds to a Pro-Xaa-Leu-Glu (PXLE) motif found in p23 and FK506 Binding Protein 38 (FKBP38), two cochaperones of the HSP90 pathway (16). The principal function of the HSP90 pathway is to promote proper folding of client proteins (Fig. 1C). First, cochaperones that include select FKBPs such as FKBP38, FKBP51, and FKBP52, recruit partially folded client proteins to HSP90 (17, 18). These FKBPs employ a tetratricopeptide repeat domain to bind a Met-Glu-Glu-Val-Asp motif at the C-terminus of HSP90. ATP and p23 then bind. p23 binds to the N-terminal domain of HSP90 and inhibits ATP hydrolysis, allowing client protein maturation. Following ATP hydrolysis, the folded client protein and ADP are released. HIF-α is a client protein of HSP90 (19, 20). The PHD2:PXLE interaction therefore recruits PHD2 to a pathway that folds HIF-α,

and this interaction thereby facilitates HIF-α hydroxylation (Fig. 1D) (21). The D4E/C127S double amino acid substitution markedly impairs the interaction of PHD2 with p23 and, to a much lesser extent, FKBP38 (22). Importantly, this effect is not seen with either the D4E or the C127S substitution alone (22). Hence, the behavior of the D4E/C127S double amino acid substitution is very different from that of single amino acid substitutions. These studies therefore suggest that the Tibetan PHD2 allele is a hypomorphic loss of function allele that impairs PHD2-induced hydroxylation of HIF-α (22).

Here, we further characterize the Tibetan *PHD2* allele. We find that in vitro, additional PHD2 mutations at or in the vicinity of the Tibetan mutations impair interaction with p23. We generated a knockin mouse line with Tibetan *Phd2* to allow examination of respiratory parameters such as minute ventilation, respiratory frequency, tidal volume, and HVR, which can be measured by whole body plethysmography of mice. Tibetan *Phd2* mice display augmented HVR, supporting a model in which PHD2 is a hypomorphic loss of function allele. *Hif2a* haploinsufficiency in the mouse, which we have used to model the Tibetan *HIF2A* haplotype, protects against hypoxia-induced increases in right ventricular systolic pressure (RVSP) in the absence or presence of the Tibetan *Phd2* allele. We propose that the HIF pathway has been reconfigured in Tibetans to allow select adaptive phenotypes to emerge that promote successful adaption to hypoxia.

Results

Mutations in the vicinity of Asp-4 and Cys-127 of PHD2 impair interaction with p23 in vitro. The Tibetan D4E/C127S mutation in PHD2 decreases its interaction with p23 (22). We examined additional residues in the vicinity of Asp-4 and Cys-127 using coimmunoprecipitation assays. We first prepared mutations at or in the vicinity of Asp-4, an amino acid that is highly conserved across mammalian species (Fig. 1B). We find that, similar to the single D4E

substitution (22), N3A, N3S, D4A, D4N, and S5A substitutions all preserve the PHD2:p23 interaction (Fig. 1E). We took one of these substitutions, D4N, and combined it with the Tibetan C127S substitution, and we find that this combination (D4N/C127S) impairs binding (Fig. 1E, lane 22, top panel). Therefore, it behaves similarly to the Tibetan D4E/C127S double amino acid substitution (Fig. 1E, lane 4, top panel). We next examined mutations at or in the vicinity of Cys-127. Of note, Cys-127 is substituted for by serine in PHD2 orthologues from primates such as baboon and gorilla, and is not present at all in PHD2 orthologues from other species, such as mice or hamster (Fig. 1B). Similar to the C127S substitution (22), the S125A, P126A, and R128A substitutions all preserve the interaction (Fig. 1F). However, C127A, C127V, and C127P substitutions markedly diminish it (Fig. 1F, lanes 18, 20, and 24, top panel). It might be noted that amino acid 131 in bovine PHD2 (corresponding to amino acid 127 in human PHD2) is a proline (Fig. 1B). Whether the presence of Pro-131 in bovine PHD2 has an effect on its binding to p23 remains to be determined, since bovine PHD2 differs from human PHD2 at nearly 60 additional residues in the linker region between the zinc finger and the catalytic domain.

These in vitro studies show that in addition to the Tibetan D4E/C127S mutation, selective single or double amino acid mutations at the sites of the Tibetan substitutions also have the capacity to disrupt interaction with p23. We also examined whether the detrimental effect of the PHD2 D4E/C127S substitution on p23 binding depends on the exact spacing between residues 4 and 127 of PHD2. We generated two constructs bearing internal deletions of PHD2 that change the spacing by either 16 or 14 amino acids (∆76-91 and ∆97-110), respectively (Fig. 1G). Wild type versions maintain the interaction, but D4E/C127S versions abolish it (Fig. 1H). Together with the previous data, these results indicate that the PHD2:p23 interaction is unexpectedly sensitive to amino acid changes outside of the zinc finger itself. Furthermore, in all situations in which there was a change, it was a loss of function (i.e., decreased interaction).

Prior to generating a mouse model for Tibetan PHD2, we performed coimmunoprecipitation studies and confirmed that both human and mouse PHD2 can bind to p23, with the former binding somewhat more tightly than the latter (Fig. S1A, lanes 3 and 5, top panel). We inspected the amino acid sequences of the human and mouse PHD2 zinc fingers (residues 21-58), and noted that all are identical with the exception of amino acid 34, which is serine in human, glycine in mouse (Fig. S1B). A different protein, the transcriptional corepressor ETO, contains a MYND-type zinc finger homologous to that of PHD2, and mutation of the corresponding serine residue in ETO (Ser-675) to alanine weakens its interaction with its peptide ligand SMRT (1101-1113) (23). This suggests that the difference in affinity of human versus mouse PHD2 might be due at least in part to the amino acid residue at position 34. Indeed, mutation of Ser-34 in human PHD2 to Gly weakens the interaction (Fig. S1C, lanes 3 and 5, top panel), and conversely, mutation of Gly-34 in mouse Phd2 to Ser strengthens the interaction (Fig. S1D, lanes 3 and 5, top panel). Thus, there are some modest differences in affinity of human and mouse PHD2 for p23 due in part to amino acid 34. More important, however, is the observation that both bind p23.

The Tibetan *Phd2* allele is associated in augmented HVR in mice. Both Asp-4 and Cys-127, the residues affected by the Tibetan allele, reside within exon 1 of the *PHD2* gene, but only the former is conserved between human and mouse (Fig. 1B). We therefore replaced the coding sequence of murine *Phd2* exon 1 with that of wild type human or Tibetan *PHD2* exon 1 using homologous recombination in embryonic stem cells (Fig. 2A). The predicted chimeric protein is 99% identical at the amino acid level to the human protein, therefore effectively humanizing the murine *Phd2* gene (Fig. 2A and Fig. S2). Pilot transfection studies using murine Hepa1-6 (Fig. 2B) or N2a (Fig. S3) cells revealed that the D4E/C127S substitution in the predicted chimeric

PHD2 (hmPHD2) protein abolishes interaction with p23, showing that the defective binding is recapitulated in both a chimeric PHD2 protein context and in a murine cellular environment.

We proceeded to generate a mouse line with the Tibetan *PHD2* exon 1 substitution (*Phd2* ^{Tib}) (Fig. S4A). Sequencing confirmed the presence of the D4E and C127S substitutions (Fig. 2C). We have previously generated the corresponding control mouse line in which the coding sequence of murine *Phd2* exon 1 is replaced by that of wild type human *PHD2* exon 1 (hereafter referred to as *Phd2* ^{WT}) (24). There was no difference in litter sizes between the two genotypes [5.19 ± 1.38 (SD) for *Phd2* $^{WT/WT}$ x *Phd2* $^{WT/WT}$ crosses, vs. 5.00 ± 1.93 (SD) for *Phd2* $^{Tib/Tib}$ x *Phd2* $^{Tib/Tib}$ crosses; n = 16 litters for each; $P = 0.75$]. Protein expression of Phd2 ^{WT} and Phd2 ^{Tib} were comparable in heart, lung, liver, kidney, brainstem, and brain cortex (Fig. S5). We generated mouse embryonic fibroblasts from these mice and we immunoprecipitated endogenous Phd2 from these cells, confirming defective binding of Tibetan Phd2 to endogenous p23 (Fig. 2D, lanes 3 and 6).

We employed whole body plethysmography to measure ventilation under normoxia and after acute exposure to 12% O_2. Representative plethysmography traces are shown in Fig. S6. In these experiments, we included a low concentration (3%) of CO_2 under both normoxic and hypoxic conditions in order to blunt the fall in P_{CO2} caused by hypoxic hyperventilation, resulting in a more sustained HVR (25, 26). In control experiments, the *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice did not display any differences in ventilatory responses to hypercapnia alone (5% CO_2; Fig. S7). In comparing *Phd2* $^{Tib/Tib}$ with *Phd2* $^{WT/WT}$ mice (Fig. 3A) under normoxia, we did not observe any differences in baseline minute ventilation (Fig. 3B). We did, however, observe an increased HVR in the *Phd2* $^{Tib/Tib}$ as compared to *Phd2* $^{WT/WT}$ mice (Fig. 3C). There was an increased hypoxia-induced change in tidal volume, but not respiratory frequency, in the *Phd2* $^{Tib/Tib}$ as compared to *Phd2* $^{WT/WT}$ mice (Fig. 3D and Fig. 3E, respectively).

Chronic exposure to hypoxia can potentiate HVR, which has been termed ventilatory acclimatization to hypoxia (27). We exposed *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice to seven days of hypoxia (12% O_2) and then measured HVR. We observe that following this chronic hypoxia exposure, *Phd2* $^{Tib/Tib}$ mice display increased HVR as compared to *Phd2* $^{WT/WT}$ mice (Fig. S8). The *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice were maintained in C57BL/6 background. The C57BL/6J mouse strain is prone to exhibiting spontaneous respiratory pauses (28). We found no differences in the frequency of spontaneous pauses between *Phd2* $^{Tib/Tib}$ with *Phd2* $^{WT/WT}$ mice under either normoxic or hypoxic conditions (Fig. S9).

The zinc finger of PHD2 shows a defective interaction with p23 as well as a more modest decrease in binding to FKBP38 (22). Therefore, we sought to determine whether this respiratory phenotype could be recapitulated by reciprocal mutations in either partner protein (Fig. 4A). In vitro, PXLE > PXAA mutations in either p23 (L159A/E160A) or FKBP38 (L55A/E56A) abolish their interaction with PHD2 (Fig. 4B and Fig. 4C, respectively). We employed Crispr technology to knock in these mutations into the murine *p23* (also known as *Ptges3*) and *Fkbp38* (also known as *Fkbp8*) loci (Fig. 4D). We denote these alleles as *p23* ^{AA} and *Fkbp38* ^{AA}, respectively. As with the *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice, the *Fkbp38* $^{AA/AA}$ mice were generated in a C57BL/6 background. The *p23* $^{AA/AA}$ mice were generated in a mixed BL6/129 background because of difficulty generating them in a C57BL/6 background. Plethysmography studies showed that the PXLE > PXAA mutations have no effect on baseline minute ventilation in either mouse line (Fig. S10A and Fig. S10B). In addition, no differences were seen in HVR between *Fkbp38* $^{AA/AA}$ and *Fkbp38* $^{+/+}$ mice (Fig. S10C). In contrast, we did observe increased HVR with *p23* $^{AA/AA}$ mice as compared to *p23* $^{+/+}$ mice (Fig. 4E), as was previously observed in the *Phd2* $^{Tib/Tib}$ mice (Fig. 3C). It should be noted that we cannot rule out the possibility that the mixed background of the *p23* $^{AA/AA}$ and control *p23* $^{+/+}$ mice might influence the magnitude of this difference in HVR.

To extend these studies, we considered the possibility that the Tibetan respiratory phenotype would be maintained in a *Fkbp38* $^{AA/AA}$ background, given that the Tibetan mutation appears to predominantly affect interaction with p23. We therefore crossed the *Fkbp38* $^{AA/AA}$ mouse with *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice, and generated *Phd2* $^{WT/WT}$; *Fkbp38* $^{AA/AA}$ and *Phd2* $^{Tib/Tib}$; *Fkbp38* $^{AA/AA}$ mice in a C57BL/6 background. We did not observe differences in baseline minute ventilation under normoxia (Fig. S11). However, there was an increased HVR in the *Phd2* $^{Tib/Tib}$; *Fkbp38* $^{AA/AA}$ as compared to *Phd2* $^{WT/WT}$; *Fkbp38* $^{AA/AA}$ mice (Fig. 4F). It will be of interest in the future to compare *Phd2* $^{WT/WT}$; *p23* $^{AA/AA}$ and *Phd2* $^{Tib/Tib}$; *p23* $^{AA/AA}$ mice.

Previous studies examining *Phd2* loss of function due to either point mutations in or genetic deletion of the *Phd2* gene have consistently shown that *Phd2* loss of function is associated with increases in either HVR or hypoxia-induced tidal volume, respiratory frequency, or minute ventilation, or a combination of these (21, 26, 29). These findings therefore support the notion that the Tibetan *Phd2* allele is a loss of function allele, consistent with the biochemical studies demonstrating impaired interaction of Tibetan PHD2 with p23.

The Tibetan *Phd2* allele is not associated with either Hb or RVSP in mice. We examined whether the Tibetan *Phd2* allele might be associated with changes in Hb levels. We exposed *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice to three weeks of hypoxia (12% O_2) and then measured Hb and Hct. As shown in Fig. 5A and Fig. S12, respectively, we do not observe any differences in either. In this experiment, we also examined whether there might be an interaction between *Phd2* and *Hif2a* alleles. While the Tibetan *HIF2A* allele does not involve any coding sequence substitutions, and the causative SNP(s) has yet to be defined, the following lines of evidence suggest that Tibetan *HIF2A* allele may be a hypomorphic loss of function allele. (1) The Tibetan *HIF2A* allele is strongly correlated with low Hb concentration (30-33). (2) *HIF2A* mRNA levels from peripheral blood mononuclear cells, umbilical endothelial cells, and placentas from

Tibetans are lower than those from control Han Chinese (32, 34). (3) Tibetans who are homozygous for the Tibetan *HIF2A* allele have a blunted plasma EPO response to hypoxia in comparison to Tibetans who are homozygous for the lowland allele (34). Accordingly, we employed mice with heterozygous deficiency of *Hif2a* (*Hif2a* $^{+/-}$) to model this allele. In the presence of heterozygous loss of *Hif2a*, we do not observe differences in Hb or Hct between *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice (Fig. 5A and Fig. S12, respectively). Under normoxia, we similarly did not observe any differences in Hb or Hct levels between any of the four genotypes (Fig. S13A and Fig. S13B). In addition, there were no differences in red blood cell (RBC), white blood cell (WBC), platelet (Plt) counts, serum EPO levels, or renal *Vegfa* (a Hif gene target) mRNA levels (Fig. S14).

The lack of an effect of *Hif2a* haploinsufficiency on these parameters in this experiment is consistent with the lack of effect of *Hif2a* haploinsufficiency observed by others upon exposure to hypoxia (29, 35). Importantly, however, others have observed that acute global deletion of *Hif2a* reverses hypoxia-induced erythrocytosis (29), consistent with a critical role for Hif-2α in boosting red cell mass under hypoxic conditions. Evidence for a central role for HIF-2α in erythropoiesis is also provided by patients with protein stabilizing missense mutations in the *HIF2A* gene (36). We crossed the *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice to mice with one such *Hif2a* gain of function mutation (*Hif2a* $^{G536W/+}$) (37). As expected, the *Hif2a* $^{G536W/+}$ allele increases Hb or Hct in *Phd2* $^{WT/WT}$ mice (Fig. S15). However, no differences were observed in the Hb or Hct of *Phd2* $^{Tib/Tib}$; *Hif2a* $^{G536W/+}$ as compared to *Phd2* $^{WT/WT}$; *Hif2a* $^{G536W/+}$ mice. Furthermore, no differences were seen between *Phd2* $^{Tib/Tib}$; *Hif2a* $^{G536W/+}$ compared to *Phd2* $^{WT/WT}$; *Hif2a* $^{G536W/+}$ mice in terms of WBC, Plt counts, or serum EPO (Fig. S16).

We exposed *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice with or without *Hif2a* haploinsufficiency to three weeks of hypoxia (12% O_2) and measured RVSP. We did not observe any significant difference in RVSP between the *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice (Figure 5B). *Hif2a*

haploinsufficiency decreased RVSP in *Phd2* $^{WT/WT}$ mice (compare first and third columns), consistent with previous observations (32, 35). It also decreased RVSP in *Phd2* $^{Tib/Tib}$ mice (compare second and fourth columns). However, we did not observe differences between *Phd2* $^{WT/WT}$; *Hif2a* $^{+/-}$ and *Phd2* $^{Tib/Tib}$; *Hif2a* $^{+/-}$ mice. Under normoxic conditions, we did not observe any differences in RVSP between *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice, either in the absence or presence of *Hif2a* haploinsufficiency (Fig S17).

Discussion

We propose that the Tibetan *PHD2* allele is a hypomorphic loss of function allele that leads to an augmented HVR, while the Tibetan *HIF2A* allele is a loss of function allele that provides protection against pulmonary hypertension and erythrocytosis (Fig. 5C). Lowlanders have a robust HVR, but after long-term acclimatization to high altitude, their HVR declines (3, 27, 38). Tibetans have an augmented HVR that approaches the ancestral response of lowlanders (3, 38-40), which may allow them to maintain high oxygen delivery from the lungs. In contrast to Tibetans, Andeans exhibit a blunted HVR (41). Thus, this study provides evidence that Tibetans possess a distinct combination of *PHD2* and *HIF2A* alleles that reconfigures the HIF pathway in a manner that facilitates adaptation to the chronic hypoxia of high altitude.

The proposition that Tibetan PHD2 is hypomorphic might appear to be at odds with the observations that acute global knockout of *Phd2* leads to erythrocytosis and conditional knockout of *Phd2* in endothelial cells induces pulmonary hypertension (42-46). However, it must be emphasized that, in vitro, the Tibetan amino acid substitutions have a relatively selective detrimental effect on the interaction with the PXLE motif of p23 as opposed to the PXLE motif of other proteins such as FKBP38. Moreover, the zinc finger of PHD2 facilitates, but is not essential, for PHD2-induced hydroxylation of HIF-α (21). Hence, the Tibetan allele is a hypomorphic as opposed to a complete loss of function allele, and one would therefore not

expect it to behave in a similar manner to a complete loss of function. Indeed, we have previously reported that a partial loss of function *Phd2* $^{P294R/+}$ mouse line does not exhibit pulmonary hypertension (47). Furthermore, while patients with erythrocytosis-associated heterozygous *HIF2A* mutations are at risk for developing pulmonary hypertension (48), those with erythrocytosis-associated *PHD2* mutations do not appear to be. In addition, while some studies have shown an association of the Tibetan *PHD2* allele with lower Hb levels (14, 49), other studies have not (50). It may also be noted that in one study which specifically examined the D4E/C127S *PHD2* haplotype, there was no association with Hb level in Tibetan highlanders (when examined irrespective of *HIF2A* haplotype) (51). Finally, our experiments here demonstrate that the Tibetan *Phd2* allele in genetically engineered mice neither augments nor inhibits the erythrocytosis and RVSP increase induced by hypoxia (Fig. 5A and Fig. 5B).

We speculate that loss of PHD2 function in Tibetans is sufficient to cause an augmentation of HVR, but has not reached the threshold to produce changes in the hypoxia-induced levels of either Hb level or RVSP. Whether this is due to intrinsic threshold differences of these physiologic responses to changes in the PHD2:p23 interaction, tissue specific differences in the utilization of this interaction, or tissue specific differences in the utilization of other PXLE-containing proteins will require further investigation. For example, p23 may be important for proper PHD2 function in the carotid body, while FKBP38 may be important for some other PHD2 function in some other tissue. Alternatively, differential sensitivity of outputs to the degree of loss of function is also plausible, and indeed this idea is observed here in another context. Heterozygous loss of *Hif2a* is sufficient to protect against hypoxia-induced increases in RVSP (Fig. 5B). On the other hand, heterozygous loss of *Hif2a* is not sufficient to protect against hypoxia-induced erythrocytosis (Fig. 5A), at least in this experimental setting, as has been observed by others (29, 35). However, as has been mentioned above, acute global deletion of *Hif2a* is protective (29).

There is evidence that the Tibetan *HIF2A* allele was present in the Tibetan gene pool at an earlier time than the Tibetan *PHD2* allele (14). Thus, it is possible that the early emergence of the Tibetan *HIF2A* allele, which presumably dampens the HIF pathway and produces the proposed beneficial effects on Hb levels and pulmonary artery pressure, may have facilitated the appearance of a loss of function Tibetan *PHD2* allele which acts in the opposite direction on the HIF pathway and that otherwise might be unfavorable under certain circumstances. This could then allow the *PHD2*-dependent contribution of a beneficial respiratory phenotype. Such an epistatic relationship will require further investigation. Interestingly, in high altitude adapted deer mice, intramolecular epistasis has been observed in Hb, allowing the emergence of Hb variants with high affinity for oxygen (52). Alternatively, it is possible that the emergence of the Tibetan *PHD2* allele might have served to ameliorate potentially detrimental effects of a preexisting Tibetan *HIF2A* allele, i.e., it may represent an example of genetic compensation (53). A possible detrimental effect of a loss of function *HIF2A* allele is suppression of the hypoxic ventilatory response (29, 54). In this regard, an issue that remains to be resolved is whether the Tibetan *PHD2* allele-associated augmentation of HVR is HIF-1α or HIF-2α dependent. Evidence for the involvement of both HIF-α paralogues in respiratory control has been presented (29, 37, 54-56).

In summary, we propose that this particular configuration of the HIF pathway both activates and inhibits selective outputs of the pathway and provides the core framework for high-altitude adaptation in Tibetans. Tibetans are protected against chronic mountain sickness (also known as Monge's Disease), which is characterized by erythrocytosis, hypoventilation, and pulmonary hypertension (57), and our model may provide a mechanistic explanation for this. Additional genes, which also display signals of selection, may further contribute to this phenotype (5, 13, 33).

Materials and Methods

Mouse lines. A mouse line with the *Phd2* ^{Tib} allele was generated by homologous recombination in ES cells followed by injection into blastocysts. Mouse lines with the *p23* ^{AA} and *Fkbp38* ^{AA} alleles were generated by injection of Crispr reagents into fertilized oocytes. Full details of this as well as other Materials and Methods are provided in the SI Appendix.

Statistical analysis. One way analysis of variance with Tukey's post hoc test (GraphPad Prism) or unpaired Student's t tests were employed for statistical analysis, as appropriate. Differences were considered significant when $P < 0.05$. Unless otherwise specified, data are presented as mean ± standard error of the mean (SEM).

Data availability. All data are included in the manuscript and SI Appendix.

Acknowledgments

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Figure Legends

Fig. 1. Effect of mutations in vicinity of PHD2 Asp-4 and Cys-127 on the PHD2:p23 interaction. (*A*) The HIF pathway. See text for details. VHL, von Hippel Lindau protein. For simplicity, Aryl Hydrocarbon Nuclear Receptor Translocator has been omitted. (*B*) Diagram of PHD2, showing locations of zinc finger (ZF) domain, prolyl hydroxylase (PH) domain, Asp-4 (diamond), and Cys-127 (triangle). Sequence of residues 1-7 and 121-133 are shown at the bottom, with Asp-4 and Cys-127 are as indicated. (*C*) The HSP90 pathway. See text for details. (*D*) Model for zinc finger-dependent recruitment of PHD2 to the HSP90 pathway to facilitate HIF-α hydroxylation. PLXE motifs in p23 and FKBP38 are shown, as are Asp-4 and Cys-127 in PHD2. (*E, F,* and *H*) HEK293 FT cells were transfected with expression plasmids for the indicated proteins. The cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies, and then the immunoprecipitates and aliquots of lysate were examined by Western blotting as indicated. In (*H*), the D4E and C127S substitutions refer to the native amino acid sequence. (*G*) Diagram of internal deletion mutants of PHD2.

Fig. 2. *Phd2* ^{Tib} gene targeting strategy. (*A*) Numbers directly above exons denote exon number. Italicized numbers either above or below exons indicate % identity of amino acid sequence encoded by exon with that of the corresponding Tibetan exon. (*B*) Hepa 1-6 cells were transfected with expression plasmids for the indicated proteins. hmPHD2 denotes a chimeric protein consisting of human PHD2 (1-297) and mouse Phd2 (275-400). The amino acid sequence of this humanized WT Phd2 is provided in Fig. S2. The cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies, and then the immunoprecipitates and aliquots of lysate were examined by Western blotting as indicated. (*C*) DNA sequencing chromatograms of tail DNA confirming D4E and C127S substitutions. The sequence is from 3'

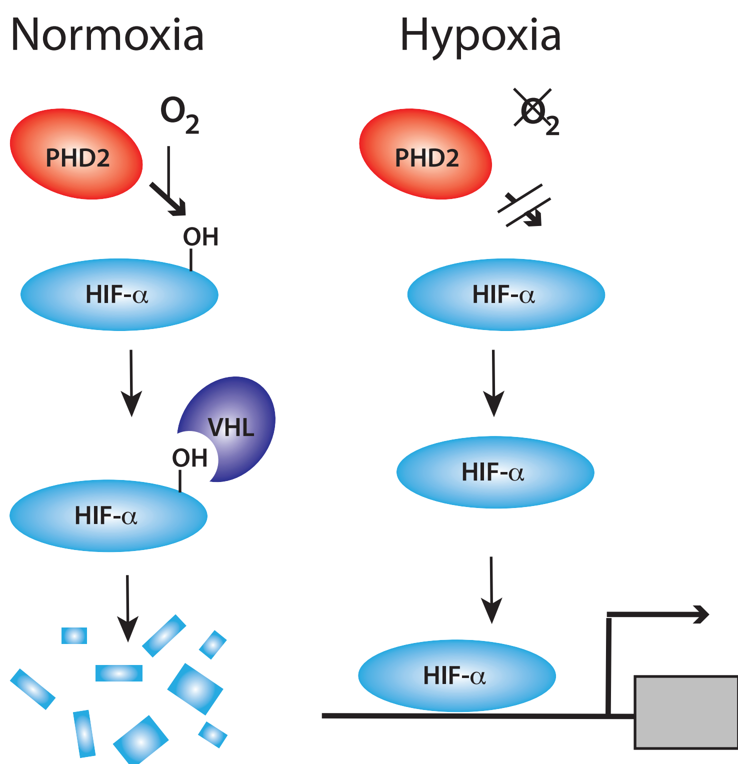
to 5' (reverse complement). Amino acids 4 and 127 are as indicated. (*D*) Immortalized mouse embryonic fibroblasts with the indicated genotypes were lysed, incubated with either control mAb or anti-PHD2 mAb 6.9, and immunoprecipitations were performed with protein G-agarose. The immunoprecipitates and aliquots of the lysate were then examined by Western blotting with anti-p23 antibodies.

Fig. 3. Increased HVR in Tibetan *Phd2* mice. (*A*) Model for Tibetan PHD2. Compared to wild type PHD2, Tibetan PHD2 has a defect in its interaction with p23. Hence, Tibetan PHD2 hydroxylates HIF-α less efficiently than wild type PHD2 (as indicated by difference in thickness of the arrow from PHD2 to HIF-α). (*B*) Minute ventilation under normoxia (21% O_2/3% CO_2) was compared between *Phd2* $^{WT/WT}$ and *Phd2* $^{Tib/Tib}$ mice. (*C*-*E*) Mice were acutely exposed to 12% O_2/3% CO_2, and HVR (*C*), tidal volume change (*D*), and respiratory frequency change (*E*) were measured. In (*B*-*E*), mice were 2-4 months of age, with n = 18-19 per group. * $P < 0.05$. *P* values were as follows: (*B*) 0.246, (*C*) 0.020, (*D*) 0.018, (*E*) 0.493.

Fig. 4. Increased HVR in *p23* $^{AA/AA}$ and *Phd2* $^{Tib/Tib}$;*Fkbp38* $^{AA/AA}$ mice. (*A*) The recruitment of PHD2 to HSP90 pathway (left) is impaired by PXLE > PXAA mutations in p23 or Fkbp38 (right). (*B* and *C*) HEK293 FT cells were transfected with expression plasmids for the indicated proteins. The cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies, and then the immunoprecipitates and aliquots of lysate were examined by Western blotting as indicated. (*D*) DNA sequencing chromatograms of tail DNA from *p23* $^{AA/AA}$ and *Fkbp38* $^{AA/AA}$ mice. The sequences are from (left*)* 5' to 3' and (right) 3' to 5'. Mutated amino acids are in bold. (*E* and *F*) Mice with the indicated genotypes were maintained in normoxia (21% O_2/3% CO_2) and then acutely exposed to 12% O_2/3% CO_2, and HVR were measured. In (*E*), mice were 2-3

months of age, with n = 14 per group. In (*F*), mice were 2-3 months of age, with n = 25-26 per group. * $P < 0.05$, ** $P < 0.01$. *P* values were as follows: (*E*) 0.007, (*F*) 0.030.

Fig. 5. Hypoxic Hb and RVSP responses in Tibetan *Phd2* mice with or without *Hif2a* haploinsufficiency. (*A* and *B*) Mice with indicated genotypes were exposed to three weeks of hypoxia (12% O_2). Hb (*A*) or RVSP (*B*) was then measured. In (*A*), mice were 3-9 months of age, with n = 9-10 per group. In (*B*), mice were 6-9 months of age, with n = 7-9 per group. * $P < 0.05$, ns = non-significant ($P > 0.05$). (*C*) Model for reconfiguration of the HIF pathway in Tibetans. Low altitude inhibitants at low altitude (normoxia) have high PHD2 activity and low HIF-2α protein levels. At high altitude (hypoxia), PHD2 activity decreases, resulting in high HIF-2α protein levels. Tibetans harbor a hypomorphic PHD2 allele that leads to augmented HVR. Tibetans also possess a hypomorphic HIF-2α allele that blunts selective aspects of the hypoxic response, including the right ventricular pressure and erythropoietic responses. Sizes of PHD2 and HIF-2α icons indicate their relative activity. *P* values were as follows: (*A*) *Phd2* $^{WT/WT}$ vs. *Phd2* $^{Tib/Tib}$ = 0.999. *Phd2* $^{WT/WT}$; *Hif2a* $^{+/-}$ vs. *Phd2* $^{Tib/Tib}$; *Hif2a* $^{+/-}$ = 0.932. (*B*) *Phd2* $^{WT/WT}$ vs. *Phd2* $^{Tib/Tib}$ = 0.949. *Phd2* $^{WT/WT}$; *Hif2a* $^{+/-}$ vs. *Phd2* $^{Tib/Tib}$; *Hif2a* $^{+/-}$ = 0.852. *Phd2* $^{WT/WT}$ vs. *Phd2* $^{WT/WT}$; *Hif2a* $^{+/-}$ = 0.010. *Phd2* $^{Tib/Tib}$ vs. *Phd2* $^{Tib/Tib}$; *Hif2a* $^{+/-}$ = 0.005.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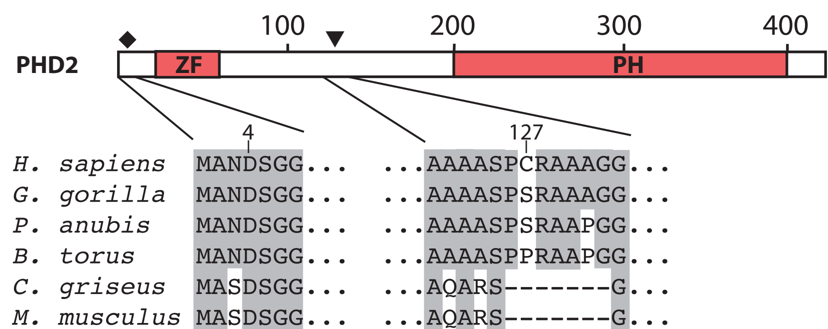
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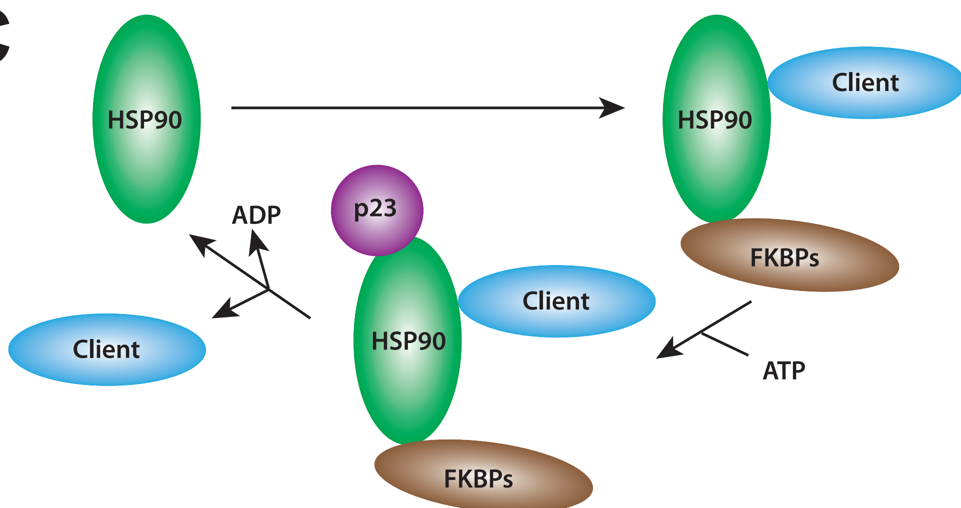
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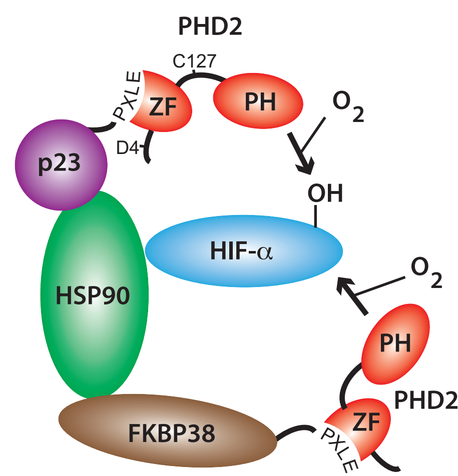
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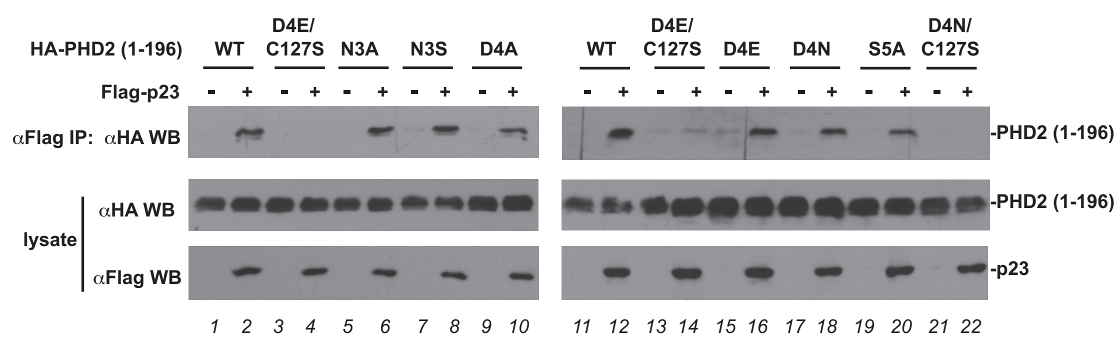
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D4E/ C127S
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D4N
S5A
D4N/ C127S
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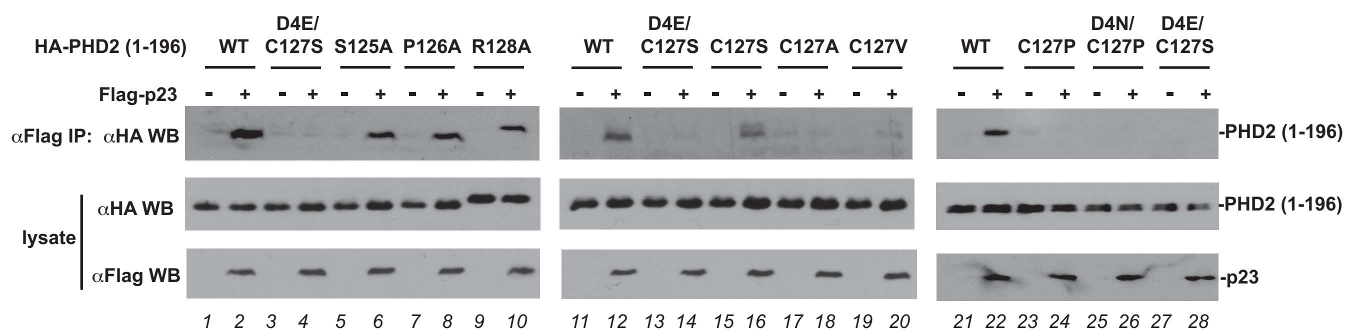
11 12 13 14 15 16 17 18 19 20 21 22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lysate
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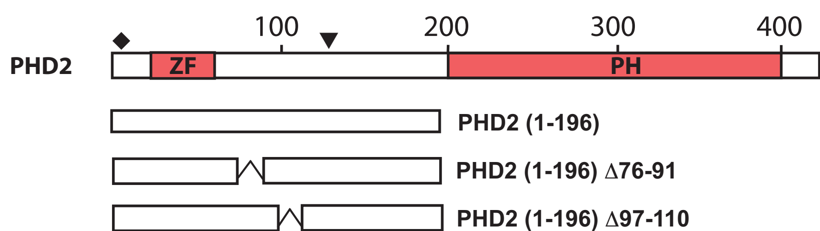
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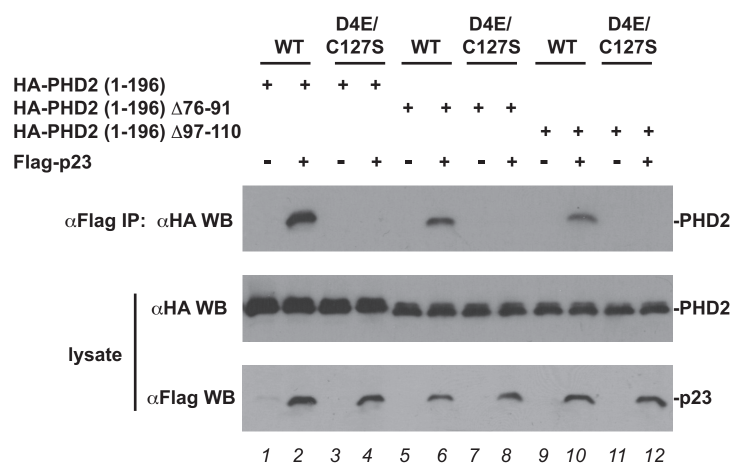
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HA-PHD2 (1-196) Δ97-110
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-PHD2
lysate
αHA WB
-PHD2
αFlag WB
-p23
1 2 3 4 5 6 7 8 9 10 11 12

A

50 kb

human *PHD2* gene 1 2 3 4 5

mouse *Phd2* gene 1 2 3 4 5

77% 100% 100% 100% 89%

targeting vector neo DTA

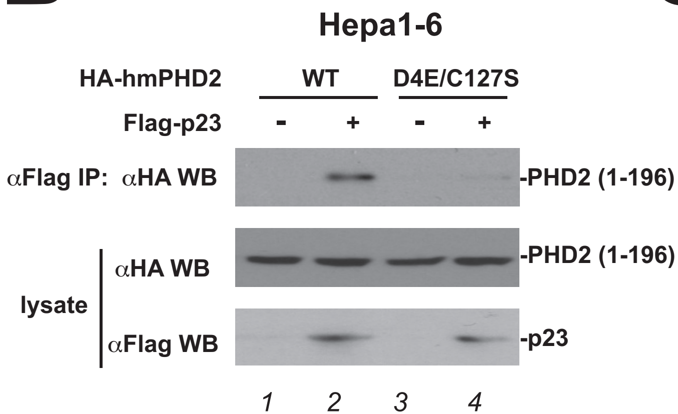
targeted *Phd2* allele neo

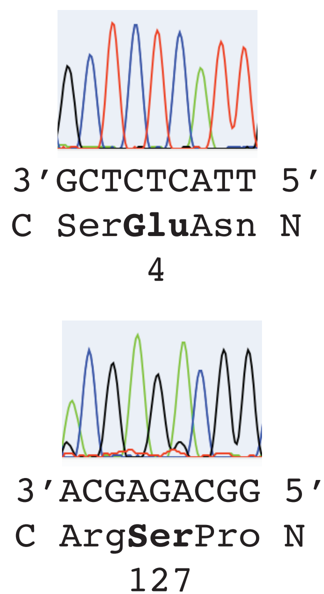
Cre

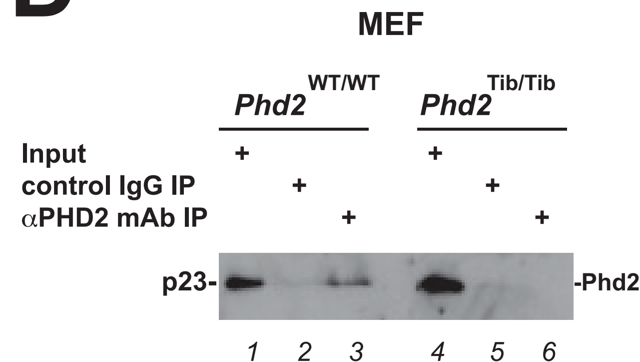
knockin *Phd2* allele
(aa seq 99% identical to human)

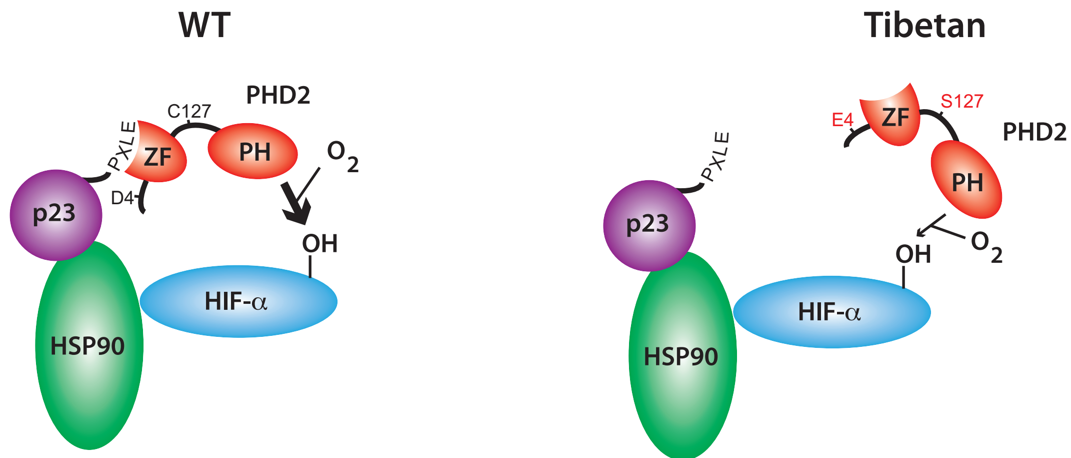
100% 100% 100% 100% 89%

* = Tibetan SNPs
= human exon
= mouse exon
= human UTR
= mouse UTR
= loxP
Cre = cre recombinase





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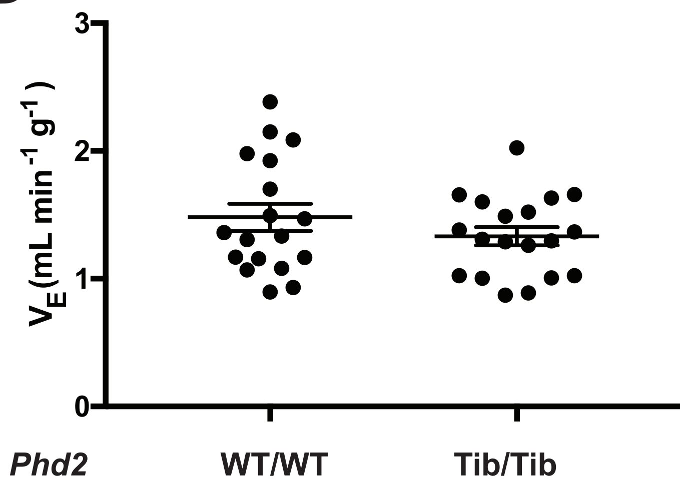
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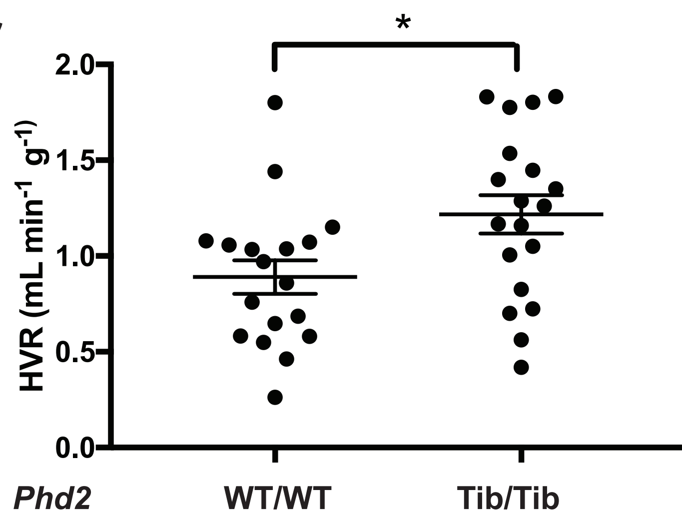
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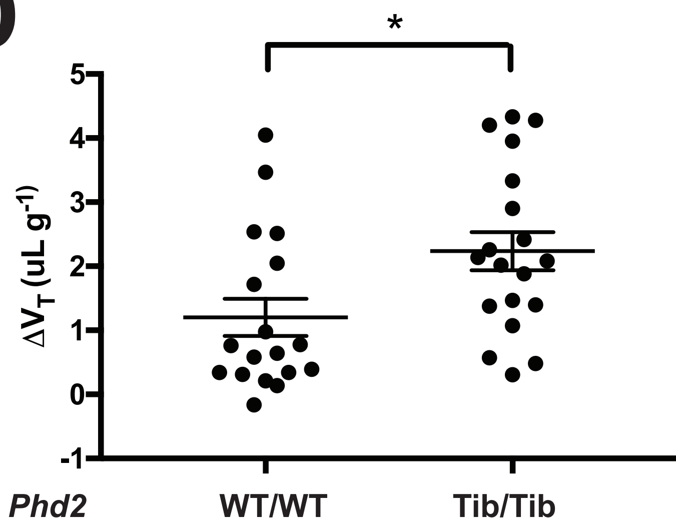
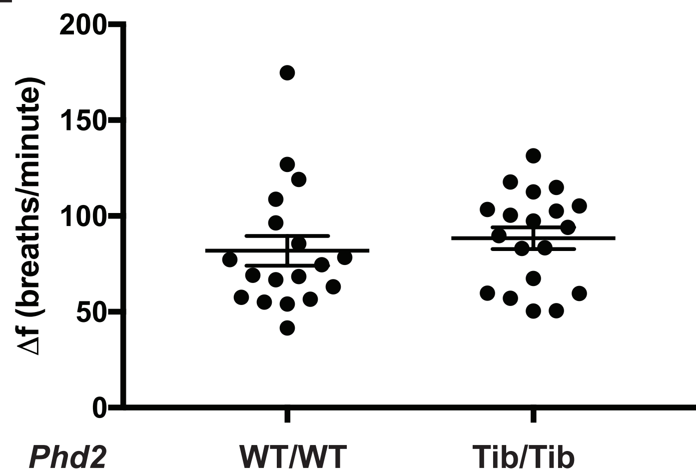
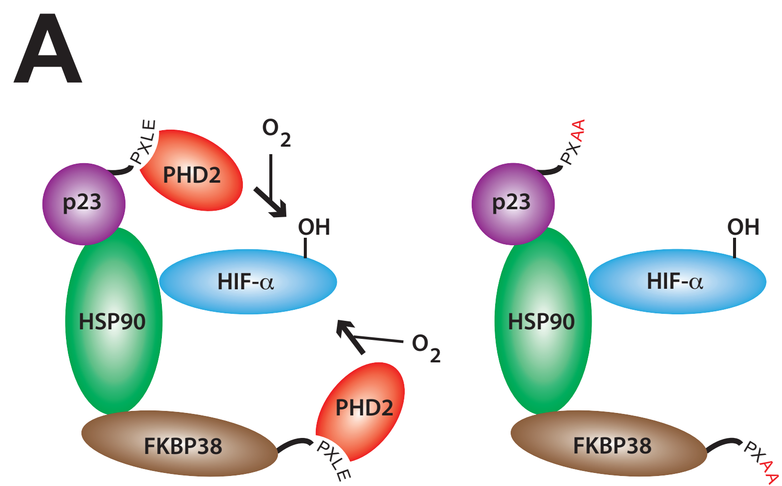
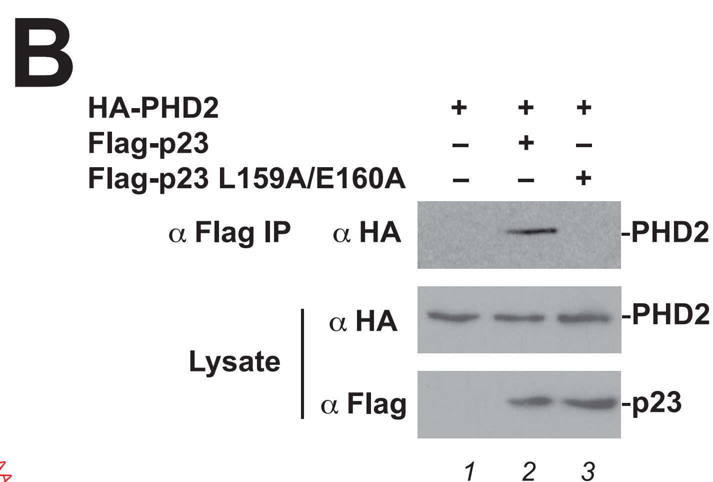
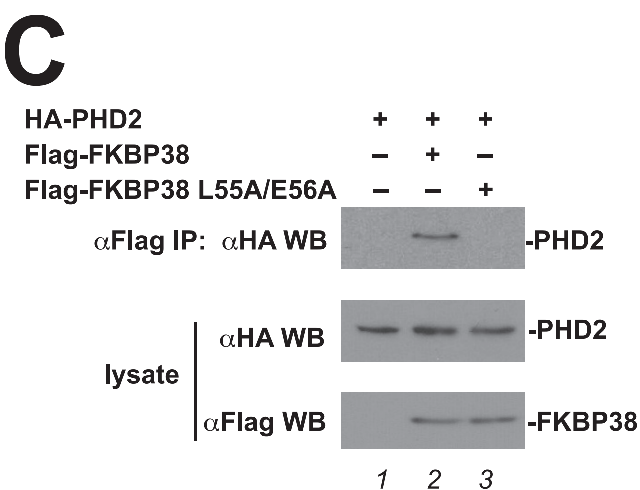
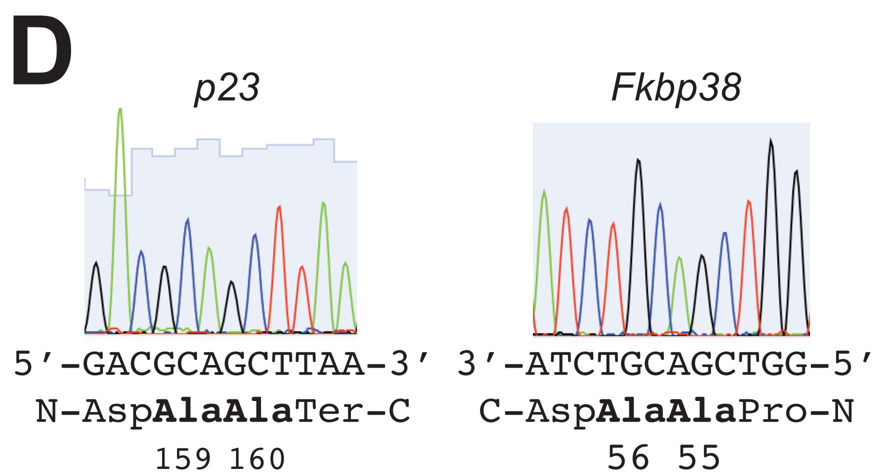

D
*
ΔV_T (uL g^{-1})
-1
0
1
2
3
4
5
Phd2
WT/WT
Tib/Tib

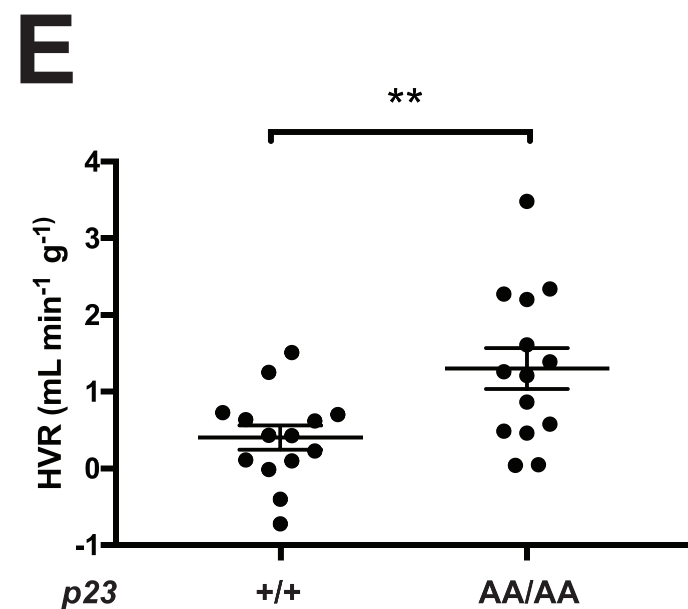

E
Δf (breaths/minute)
0
50
100
150
200
Phd2
WT/WT
Tib/Tib

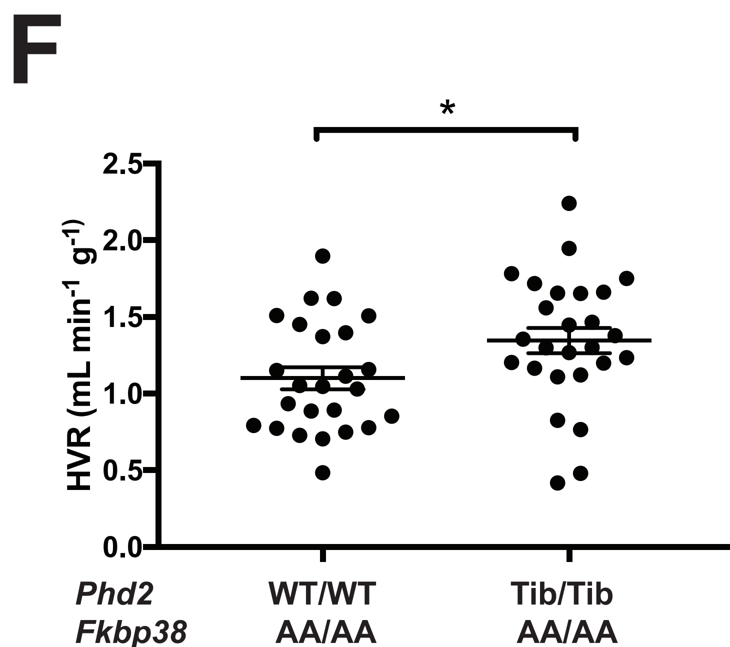

A
p23
PXLE
PHD2
O2
OH
HIF-α
HSP90
O2
PHD2
FKBP38
PXLE
p23
PXAA
OH
HIF-α
HSP90
FKBP38
PXAA

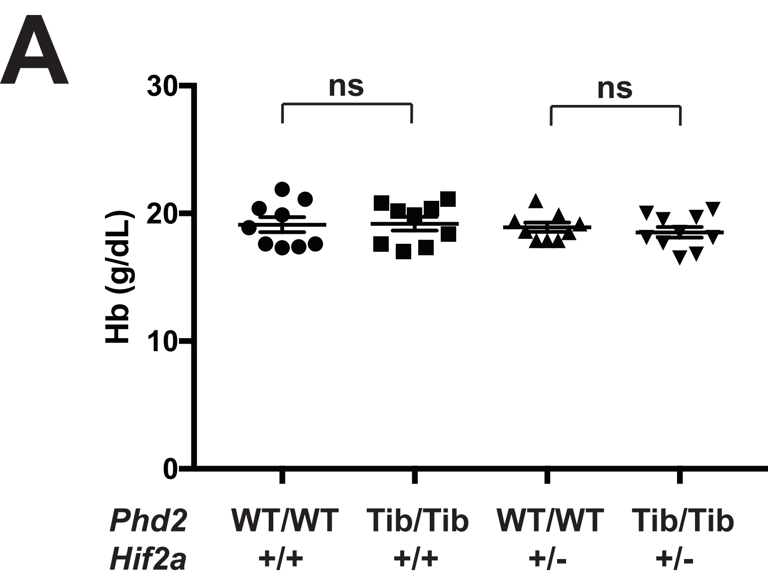

B
HA-PHD2 + + +
Flag-p23 – + –
Flag-p23 L159A/E160A – – +
α Flag IP α HA -PHD2
Lysate
α HA -PHD2
α Flag -p23
1 2 3

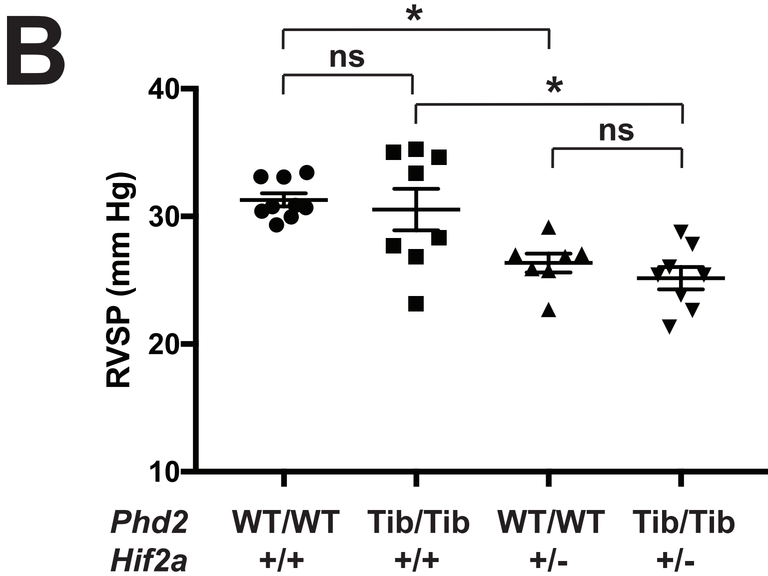

C
HA-PHD2 + + +
Flag-FKBP38 – + –
Flag-FKBP38 L55A/E56A – – +
αFlag IP: αHA WB -PHD2
lysate
αHA WB -PHD2
αFlag WB -FKBP38
1 2 3

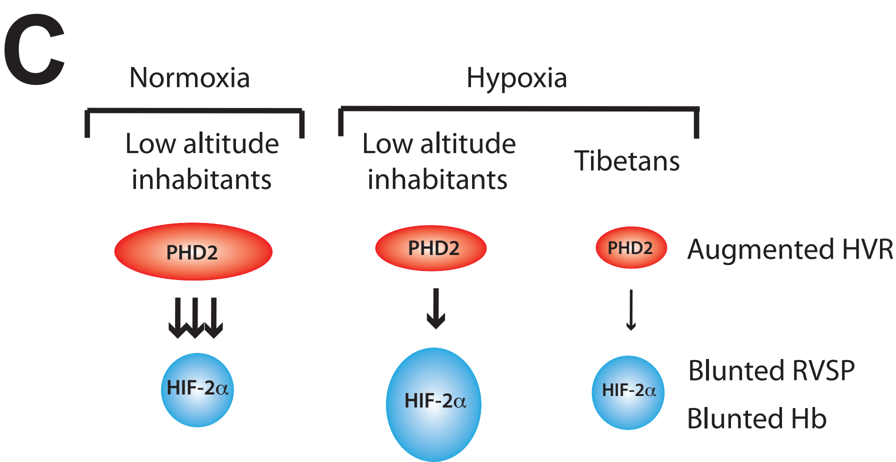

D
p23
Fkbp38
5′-GACGCAGCTTAA-3′
N-AspAlaAlaTer-C
159 160
3′-ATCTGCAGCTGG-5′
C-AspAlaAlaPro-N
56 55


E
**
HVR (mL min-1 g-1)
4
3
2
1
0
-1
p23
+/+
AA/AA


F
*
HVR (mL min-1 g-1)
2.5
2.0
1.5
1.0
0.5
0.0
Phd2
Fkbp38
WT/WT
AA/AA
Tib/Tib
AA/AA


A
ns
ns
Hb (g/dL)
30
20
10
0
Phd2
Hif2a
WT/WT
+/+
Tib/Tib
+/+
WT/WT
+/-
Tib/Tib
+/-


B
*
ns
*
ns
RVSP (mm Hg)
40
30
20
10
Phd2
Hif2a
WT/WT
+/+
Tib/Tib
+/+
WT/WT
+/-
Tib/Tib
+/-


C
Normoxia
Hypoxia
Low altitude inhabitants
Low altitude inhabitants
Tibetans
PHD2
PHD2
PHD2
Augmented HVR
HIF-2α
HIF-2α
HIF-2α
Blunted RVSP
Blunted Hb