A genetic mechanism for Tibetan high-altitude adaptation

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Abstract

Tibetans do not exhibit increased hemoglobin concentration at high altitude. We describe a high-frequency missense mutation in the *EGLN1* gene, which encodes prolyl hydroxylase 2 (PHD2), that contributes to this adaptive response. We show that a variant in *EGLN1*, c.[12C>G; 380G>C], contributes functionally to the Tibetan high-altitude phenotype. PHD2 triggers the degradation of hypoxia-inducible factors (HIFs), which mediate many physiological responses to hypoxia, including erythropoiesis. The PHD2 p.[Asp4Glu; Cys127Ser] variant exhibits a lower K_m value for oxygen, suggesting that it promotes increased HIF degradation under hypoxic conditions. Whereas hypoxia stimulates the proliferation of wild-type erythroid progenitors, the proliferation of progenitors with the c.[12C>G; 380G>C] mutation in *EGLN1* is significantly impaired under hypoxic culture conditions. We show that the c.[12C>G; 380G>C] mutation originated ~8,000 years ago on the same haplotype previously associated with adaptation to high altitude. The c.[12C>G; 380G>C] mutation abrogates hypoxia-induced and HIF-mediated augmentation of erythropoiesis, which provides a molecular mechanism for the observed protection of Tibetans from polycythemia at high altitude.

High-altitude populations have developed genetic adaptations that permit their survival in extremely hypoxic environments. In most non-adapted individuals, exposure to high-altitude hypoxia leads to an elevation of hematocrit levels and an increased number of erythrocytes (polycythemia). In contrast, the majority of Tibetan highlanders maintain hematocrit levels comparable to those for populations living at sea level[1]. Although increased hemoglobin concentration might be considered to be a beneficial adaptation to hypoxia, excessive erythrocytosis results in high blood viscosity, which impairs tissue blood flow and oxygen delivery[2,3]. Several studies have reported evidence for positive natural selection at the *EGLN1* and *EPAS1* loci, both of which are associated with the HIF pathway[4–6]. *EGLN1* encodes HIF prolyl 4-hydroxylase 2 (PHD2), and *EPAS1* encodes the HIF-2α subunit. Mutations or dysregulation of these genes or their products has been reported to be associated with anemia and polycythemia[2,3]. In addition, variation at the *EGLN1* locus is associated with protection against polycythemia in Tibetan highlanders[4,7,8], and SNPs at the *EPAS1* locus are associated with hemoglobin levels in Tibetans[5,6].

HIFs are transcription factors that function as master regulators of oxygen homeostasis. They are composed of a hypoxia-inducible α subunit and a constitutively expressed β subunit. The α subunit has three different isoforms; HIF-1α, HIF-2α (encoded by *EPAS1*) and HIF-3α (refs. 8–10). The HIF-1α subunit is ubiquitously expressed, whereas HIF-2α and HIF-3α have tissue-specific patterns of expression. HIF-1 was discovered as a result of studies on erythropoietin (EPO)[11], the key hormone that stimulates erythroid progenitors and regulates the production of erythrocytes; however, it was later found that EPO production is primarily regulated by HIF-2α (refs. 12,13). In the presence of oxygen, HIF α

subunits are hydroxylated by PHD2, which generates a binding site for the von Hippel–Lindau (VHL) protein and results in their polyubiquitination and proteasomal degradation[10]. Thus, PHD2 and VHL are the major oxygen-dependent negative regulators of HIFs. HIFs can also augment erythropoiesis by EPO-independent mechanism(s) that are yet to be elucidated[13,14] and can regulate the uptake of iron that is required for hemoglobin synthesis[13].

We report a missense variant of the *EGLN1* gene, c.12C>G, that is in almost complete linkage disequilibrium (LD) with a previously reported missense variant in this gene, c.380G>C. Both c.12C>G and c.380G>C are observed almost exclusively on a haplotype we previously identified as a strong candidate for conferring Tibetan high-altitude adaptation on the basis of tests of extended haplotype homozygosity (iHS and XPEHH) and association with protection from polycythemia[4]. Kinetic studies of recombinant p.[Asp4Glu; Cys127Ser] PHD2 and functional assessment of native erythroid progenitors homozygous for the variant encoding p.[Asp4Glu; Cys127Ser] PHD2 demonstrate that the variant protein has increased hydroxylase activity under hypoxic conditions. We demonstrate that this variant contributes to the molecular and cellular basis of Tibetan adaptation to high altitude by blunting the erythropoietic response to hypoxia.

RESULTS

Genomic screening for adaptive mutations in Tibetans

We first screened for sequence variants in candidate genes. Twenty-six Tibetans living in Virginia and Utah (designated hereafter as US Tibetans) were recruited for our studies. The ancestral composition of the local Tibetans is depicted in Table 1, and that of 121 Asian (Chinese, Japanese, Korean, Mongolian and Filipino) and European control subjects is shown in Supplementary Table 1. In earlier studies, variant alleles of *EGLN1* (NCBI RefSeq NG_015865), *EPAS1* (NCBI RefSeq NG_016000) and *PPARA* (NCBI RefSeq NG_012204) were significantly associated with lower hemoglobin concentrations in Tibetan highlanders[4–6]. Genomic DNA sequencing of the exons, exon-intron boundaries and promoter region of *PPARA*[15] did not identify any sequence variants in four Tibetans. No sequence variants in *EPAS1* were identified in the 16 exons and exon-intron boundaries of genomic DNA from 2 Tibetans with the *EPAS1* haplotype previously reported to be under positive selection. Initially, exon sequencing of *EGLN1* in Tibetan genomic DNA identified 2 missense mutations in *cis* in exon 1: 22 genomic DNA samples had a new c.12C>G variant (NM_022051), and all 26 samples (at the time of the original evaluation)[7] had a known but unvalidated SNP, rs12097901 (c.380G>C; NM_022051). The minor allele frequency for the c.380G>C variant was reported to be 20% in the 1000 Genomes Project Phase 1 sample in 2011.

Frequency of the *EGLN1* variants

We then determined the frequency of the c.12C>G and c.380G>C *EGLN1* alleles in Tibetans and non-Tibetans (Table 1 and Supplementary Table 1). The c.380G>C variant was found in all 26 US Tibetan DNA samples and occurred in non-Tibetan controls at a frequency of ~20%, which is consistent with the 1000 Genomes Project estimate. By

comparison, the frequency of the c.12C>G variant was 85% in local Tibetans (20 homozygotes, 2 heterozygotes and 4 wild type) but only 0.8% in 242 non-Tibetans (occurring in 1 Mongolian subject and 1 Japanese subject, both of whom were heterozygous). This variant was submitted to the NCBI dbSNP database as ss538786686/ rs186996510. More recently, we studied an additional 26 Tibetans living in Himachal Pradesh in northern India. We also collaborated with the Research Center for High-Altitude Medicine of Qinghai University in China, and the following subjects living on the Qinghai-Tibetan Plateau agreed to participate in our studies: 13 subjects from the provincial capital of Xining and 26 subjects from Huashixia township (Zorgenrawa in Tibetan), Madoi county in Qinghai province. In subjects from these expanded Tibetan cohorts ($n = 65$) and US Tibetans ($n = 26$), the frequency of the c.12C>G variant was 88% ($n = 91$; 46 homozygotes, 34 heterozygotes and 11 wild type; Supplementary Tables 2 and 3). With the exception of one apparent intragenic crossover (Supplementary Table 2, subject MG08), the c.12C>G allele was always observed on a haplotype containing the c.380G>C allele. Although we are not able to determine the level of population structure and/or admixture in each Tibetan cohort examined, we suspect that these factors underlie the differences observed between local and other Tibetans with respect to the frequency of c.12C>G homozygosity. Our recent analyses of regional Tibetan populations identify clustering of geographically separate groups within Tibet in addition to distinct differences in the amount of genetic admixture[16].

Positive selection of the *EGLN1* variants

To search for genomic evidence of positive selection for the c.[12C>G; 380G>C] compound allele of *EGLN1*, we genotyped >900,000 SNPs in 17 US Tibetan samples using the Genome-Wide Human SNP Array 6.0 (Affymetrix). We identified two pairs of Tibetans as close relatives (TU02 and TU04; TU05 and TU08) and excluded one of the related individuals from each pair from all subsequent analyses (Online Methods). Principal-components and ADMIXTURE analyses indicated that the ancestries of the current Tibetan samples were similar to those of the Tibetan population in which the selection signal at the *EGLN1* locus was identified (Supplementary Fig. 1). Next, we estimated the population branch statistic (PBS), which compares pairwise estimates of population differentiation (F_{ST}) among three populations to detect sites that are highly divergent in the population of interest[6]. When comparing Europeans, Mongolians and Tibetans, the PBS for the c.12C>G variant of *EGLN1* was 1.02, which was the fifth most extreme value that we observed (empirical P value = 9×10^{-6}) (Fig. 1). The four SNPs with more extreme PBS values (rs12063614, rs12563076, rs11122250 and rs6541261) were also located at the *EGLN1* locus (Fig. 1) but did not cause amino acid changes in PHD2. Using population-based phasing on 16 US Tibetans, we estimated that the major allele for each of these 4 SNPs was present on 25 of the 26 chromosome copies (of a total of 32) that carried the c.12C>G variant. Thus, the elevated PBS values of these SNPs are likely the result of genetic hitchhiking with the c.12C>G mutation.

Age of the *EGLN1* variant

On the basis of high-density SNP data from 16 unrelated US Tibetans, we estimated the age of the c.12C>G mutation in *EGLN1* by measuring the decay of LD surrounding the mutation using a previously described approach[17]. The maximum-likelihood estimate for the age of

the c.12C>G mutation was 8,000 years, on the basis of a 25-year generation time (95% confidence interval (CI) = 5,100–11,900 years; Supplementary Table 4). Because the c. 380G>C allele was present at intermediate frequency in several non-Tibetan populations, the more recent c.12C>G mutation probably arose on a chromosome that contained the c. 380G>C mutation. The relatively young estimated age and high frequency of this Tibetan-specific variant are consistent with a rapid increase in allele frequency due to strong positive natural selection.

Kinetic analyses and *in cellulo* experiments

To characterize the functional properties of the selected Tibetan p.[Asp4Glu; Cys127Ser] PHD2 double mutant and the p.Asp4Glu and p.Cys127Ser single mutants in the context of high-altitude adaptation, we performed kinetic analyses of recombinant proteins under normoxic and hypoxic conditions. The p.Asp4Glu and p.Cys127Ser single-mutant and p. [Asp4Glu; Cys127Ser] double-mutant enzymes were expressed as recombinant proteins in insect cells and were affinity purified. The mutant enzymes migrated similarly to wild-type PHD2 on SDS-PAGE and showed no signs of degradation (Supplementary Fig. 2). The K_m and V_{max} values for DLD19, a 19-residue synthetic peptide encompassing the HIF-1α C-terminal hydroxylation site, were identical in all mutants to the values for the wild-type enzyme (Table 2). The K_m value was also assessed for a more natural substrate, the HIF-2α O_2-dependent degradation domain (ODDD), a 250-aminoacid recombinant polypeptide encompassing both the N- and C-terminal prolyl hydroxylation sites of HIF-2α. For p. [Asp4Glu; Cys127Ser] PHD2, the K_m was 100 nM, which is slightly higher than the 70 nM seen for wild-type PHD2 ($P < 0.05$; Table 2). However, the V_{max} of p.[Asp4Glu; Cys127Ser] PHD2 for the HIF-2α ODDD was 30% greater than that for wild-type PHD2 ($P < 0.005$; Table 2), most likely reflecting compensation for a possible minor difference in hydroxylation due to the slightly higher K_m value of the mutant.

Next, we performed kinetic analyses of the two other cosubstrates of PHD2, 2-oxoglutarate (α-ketoglutarate) and oxygen[18]. The K_m and V_{max} values of p.[Asp4Glu; Cys127Ser] PHD2 for 2-oxoglutarate were determined using DLD19 as a substrate and were identical to those for the wild-type enzyme (Table 2). The K_m and V_{max} values of the p.Asp4Glu and p.Cys127Ser PHD2 single mutants for O_2 were determined using the HIF-2α ODDD as a substrate and were identical to those of the wild-type enzyme (Table 2). However, the K_m value of p.[Asp4Glu; Cys127Ser] PHD2 for O_2 was 110 μM, which is ~30% lower than the value of 150 μM for wild-type PHD2 ($P < 0.05$; Fig. 2a and Table 2), whereas the V_{max} value was identical to that for the wild-type enzyme (Table 2). The K_m values of the p.Asp4Glu and p.Cys127Ser single mutants for O_2 were also significantly higher than that for the p.[Asp4Glu; Cys127Ser] double mutant ($P = 0.05$ and $P < 0.05$, respectively; Table 2), suggesting an epistatic interaction between the two mutations. Thus, the p.[Asp4Glu; Cys127Ser] protein is capable of more efficiently hydroxylating HIF α subunits at lower oxygen tensions than wild-type PHD2.

We then asked whether the lower K_m of the p.[Asp4Glu; Cys127Ser] variant for oxygen resulted in alterations in HIF-2α stabilization. Hep3B hepatoma cells were infected with a lentivirus expressing a Venus fluorescent protein as a negative control, wild-type PHD2 or

the PHD2 p.[Asp4Glu; Cys127Ser] variant. To directly compare wild-type PHD2 and the p.[Asp4Glu; Cys127Ser] mutant, endogenous PHD2 was knocked down using a short hairpin RNA (shRNA) targeting the 3′ UTR of *EGLN1*, which was lacking in the cDNAs used to express exogenous PHD2. After antibiotic selection for stably transduced cells, we cultured the cells in 21% or 1% O_2 for 12 h and quantified HIF-2α levels by protein blotting. Cells expressing the p.[Asp4Glu; Cys127Ser] variant had decreased HIF-2α protein levels under hypoxic conditions when compared to cells expressing wild-type PHD2 (Fig. 2b). A similar trend was noted for HIF-1α, but the effect was less consistent. Knockdown of endogenous *EGLN1* and equal transgene expression were confirmed to rule out differences due to dissimilar PHD2 expression levels (Fig. 2c). These data suggest that the p.[Asp4Glu; Cys127Ser] variant is more active in downregulating HIF α subunits under hypoxic conditions.

In vitro analysis of erythropoiesis by BFU-E assay

HIFs directly stimulate erythropoiesis[3,19], in addition to and independently of the augmentation of circulating EPO. Peripheral blood contains early erythroid progenitors, which form erythroid colonies *in vitro* (burst-forming units–erythroid, BFU-Es) when cultured in methylcellulose in the presence of EPO. BFU-Es were isolated from the peripheral blood of three US Tibetans and ten control subjects. Progenitors from two Tibetans who were homozygous for the variant encoding p.[Asp4Glu; Cys127Ser] PHD2 were hypersensitive to EPO in comparison to progenitors from the ten controls, whereas progenitor cells from the US Tibetan who was heterozygous for the variant encoding p.[Asp4Glu; Cys127Ser] PHD2 had decreased EPO sensitivity in comparison to BFU-Es homozygous for the variant encoding p.[Asp4Glu; Cys127Ser] PHD2 (Fig. 3a). Hypersensitivity to EPO is a feature found in inherited disorders associated with increased HIF activity[3,19]. Indeed, HIF-regulated gene expression was increased in normoxic cultures of erythroid colonies from Tibetans homozygous for the variant encoding p.[Asp4Glu; Cys127Ser] (Supplementary Fig. 3). However, analysis of erythropoiesis at ambient oxygen concentration does not reflect physiological hypoxia in the bone marrow, which is further accentuated by hypoxia at high altitudes. Thus, erythropoiesis was then reevaluated *in vitro* in a controlled hypoxic environment.

Normal erythroid progenitors had exaggerated growth, increased hemoglobinization and increased sensitivity to EPO when incubated under hypoxic conditions (1% or 5% O_2; Fig. 3b–d), as previously reported[20,21]. However, concomitantly tested erythroid progenitors from six Tibetans (five subjects homozygous for the variant encoding p.[Asp4Glu; Cys127Ser] and one subject heterozygous for the variant encoding p.Asp4Glu; cells homozygous for the variant encoding p.Cys127Ser did not grow at all at 1% O_2 (Fig. 4) and, at 5% O_2, their proliferation and hypersensitivity to EPO were decreased (Fig. 3b,c). Additionally, the mean size of the BFU-Es from controls increased under hypoxic conditions, whereas the mean size ($P = 0.0017$) and hemoglobinization of the BFU-Es from homozygotes for the variant encoding p.[Asp4Glu; Cys127Ser] were decreased (Figs. 3d and 4).

DISCUSSION

We report a new missense mutation in *EGLN1*, c.12C>G, that coexists in *cis* in c.[12C>G; 380G>C] with the previously reported c.380G>C *EGLN1* variant and is present in the majority of Tibetans (SNP ss538786686/rs186996510)[7]. The c.12C>G mutation originated ~8,000 years ago, most likely on a haplotype with the c.380G>C mutation. The c.12C>G variant was not reported in an analysis of 50 Tibetan exomes[6], likely owing to the high GC content in this region. Similarly, this region of exon 1 of *EGLN1* could not be successfully sequenced in 54 high-coverage whole genomes from the Complete Genomics Diversity Panel[22]. Therefore, this region appears to have particularly poor coverage in next-generation sequence data, which explains why the c.12C>G variant escaped detection in previous studies.

The association of SNPs at the *EGLN1* locus with high-altitude adaptation (absence of polycythemia) was reported previously in Tibetans[4], as well as in Andeans[23], and loss-of-function *EGLN1* mutations are associated with dominantly inherited congenital polycythemia[24–28]. Furthermore, two SNPs in the first intron of *EGLN1*, rs479200 and rs480902, differ substantially in frequency between samples from highland and lowland populations in India and are associated with increased risk of high-altitude pulmonary edema in the latter[29].

The catalytic domain of PHD2 starts at residue 181 and extends through the end of the 426-residue polypeptide. Several missense mutations affecting the catalytic domain and mutations that lead to a truncated protein lacking the catalytic domain have been reported previously to be linked to familial polycythemia[22–28]. The high-altitude Tibetan variant c.[12C>G; 380G>C] in *EGLN1* enhances the catalytic activity of PHD2 under hypoxic conditions; however, it does not affect the catalytic domain. The currently available three-dimensional structure of PHD2 only contains the catalytic domain; therefore, modeling of the p.Asp4Glu and p.Cys127Ser alterations is not feasible. However, our data suggest that the Tibetan c.[12C>G; 380G>C] variant alters oxygen binding and perhaps also HIF-2α substrate binding to PHD2 and, under hypoxia, is a gain-of-function mutation of PHD2, the principal negative regulator of HIFs.

When we demonstrated that the *EGLN1* haplotype in Tibetans is associated with protection from polycythemia[4], we hypothesized that this haplotype might be associated with an *EGLN1* gain-of-function mutation resulting in decreased HIF activity and, possibly, decreased EPO levels and blunted erythropoiesis. However, our initial functional assessment of erythroid progenitors bearing the homozygous haplotype found that, under normoxia, it is associated with augmented rather than decreased erythropoiesis (Fig. 3a). This feature is found in inherited disorders associated with increased HIF levels, as first reported for Chuvash polycythemia, which is caused by a missense mutation in *VHL* encoding a p.Arg200Trp alteration that reduces binding to hydroxylated HIF α subunits and thus increases HIF-1 and HIF-2 levels[30]. Missense mutations in *EGLN1* (ref. 31) and *EPAS1* (ref. 32) interfere with the hydroxylation and degradation of HIF-2α by PHD2 and are also associated with polycythemia in humans. Similarly, a study of erythropoiesis in *Hif1a*^{−/−} mice demonstrated that HIF-1 stimulates erythropoiesis through a mechanism that is

independent of EPO[14]. The molecular basis for the normoxic augmentation of erythropoiesis that we observed in individuals who express the c.[12C>G; 380G>C] variant remains to be determined and might be explained by co-selected haplotypes at other loci, such as *EPAS1*.

The erythroid progenitor cells from six Tibetans homozygous for c.[12C>G; 380G>C] in *EGLN1* had decreased proliferation, EPO sensitivity, colony size and hemoglobinization when incubated at 5% O_2 and did not grow at all at 1% O_2. In striking contrast, control erythroid progenitors had exaggerated growth and increased EPO sensitivity, colony size and hemoglobinization under hypoxic conditions (1% or 5% O_2; Figs. 3a–d and 4), as previously reported[20,21].

Tibetan high-altitude adaptation is not determined by a single gene but by multiple evolved genetic adaptations acting in concert[33]. These mutations may have different effects on the regulation of erythropoiesis, as well as on metabolism, which is another major target of HIF regulation[34]. In aggregate, the data we present here support the key role of the c.[12C>G; 380G>C] variant in *EGLN1* as a major molecular mechanism by which Tibetans are protected from developing polycythemia at high altitude. Further studies are warranted to determine whether these alleles also alter other pathological responses to high-altitude hypoxia, such as pulmonary hypertension. A more complete understanding of the genetic basis for adaptation to high altitude may lead to new approaches for the treatment of common causes of mortality, such as ischemic cardiovascular disease, in which hypoxia has a major pathophysiological role.

ONLINE METHODS

Subjects

Twenty-six Tibetans residing in Virginia and Utah (referred to as US Tibetans) were recruited for the study. Institutional review board approval was obtained from the University of Utah Regulatory Knowledge and Clinical Research Ethics Committee in the United States, from the Sheri-Kashmir Institute of Medical Science Ethics Committee in India and from the Quinghai University Institutional Ethics Committee in China; informed consent was obtained from all participants. Of the 26 US Tibetans, 11 originate in U-Tsang, 8 originated in Khamba, 1 originated in Amdo and 6 were of mixed Tibetan ancestry; none of the 26 subjects had any known chronic illness or history of smoking. DNA from healthy Salt Lake City volunteers was complemented by anonymous DNA from the Sorensen Molecular Genealogy Foundation (SMGF) and the Baylor College of Medicine Department of Genetics (BCMDG) population database, serving as controls. These controls constituted DNA from 74 European Americans, 14 subjects of mixed Asian ancestry (Korean, Japanese and Filipino) from Salt Lake City, 11 anonymous Mongolians[4,35], 6 Japanese and 16 Han Chinese (SMGF and BCMDG). Additional information on subject recruitment is given in the Supplementary Note.

Sample preparation

Peripheral blood was collected using acid citrate buffer (ACD) tubes, and plasma, mononuclear cells, granulocytes and platelets were separated and archived for future use with the Histopaque density gradient method (Sigma-Aldrich). Nucleic acids were extracted

from granulocytes and mononuclear cells using TRI Reagent (Molecular Research Center), and, for some samples only, genomic DNA was isolated using the PureGene DNA purification kit (Qiagen). Mononuclear cells from subjects were used for evaluation of the sensitivity of erythroid progenitors to EPO (BFU-E assays; see below), and five BFU-E colonies were pooled from each individual separately and used for RNA isolation.

Genomic analyses

In general, for the initial screening of the *PPARA* and *EGLN1* genes, four representative samples were used and two representative samples were used for *EPAS1*. The sequences for the promoter region of the *PPARA* gene[15] and the exons and intron-exon boundaries of the *PPARA*, *EPAS1* and *EGLN1* genes were obtained from NCBI GenBank via the NCBI website; the corresponding sequences were amplified using the primers and conditions listed in Supplementary Table 5 and were sequenced in both directions. PCR was carried out using Phusion High-Fidelity Master Mix (New England Biolabs) according to the manufacturer's recommended protocol.

Analysis of *EGLN1* exon 1

Exon 1 of *EGLN1* spans 4,047 bp, and the distal 891-nt region encodes the N terminus of the PHD2 protein. This region of exon 1 is GC rich (70%) with multiple repeats, and amplification was performed using forward primers flanking the coding region of exon 1 (Supplementary Table 5) with PreMix G from the FailSafe PCR System (Epicentre). The 1,025-bp amplicon generated by PCR was then purified using the QIAquick Gel Extraction kit (Qiagen) and sequenced in both directions.

SNP genotyping

We genotyped 16 local Tibetans using the Affymetrix 6.0 SNP array (>900,000 SNPs) and performed genotype calling using the Affymetrix Genotyping Console 3.1 (Affymetrix). The genotypes used for analyses included all autosomes (no sex chromosomes or mitochondrial DNA). We augmented these samples with the HapMap CHB and JPT (Chinese and Japanese) populations (International HapMap Consortium, 2007) and with 31 Tibetan individuals and 25 Buryat Mongolian individuals who we previously genotyped[4,35]. The sample of 25 Mongolian individuals included the 11 individuals genotyped for the c.12C>G and c.380G>C variants in *EGLN1*. We performed principal-components and AMIXTURE analyses (Supplementary Fig. 1) as previously described[36], excluding related individuals. Using the tool Estimation of Recent Shared Ancestry (ERSA)[37], we determined that 2 of the 18 local Tibetans were close relatives and excluded 1 of the related individuals from all subsequent analyses. We calculated PBS in the 16 local Tibetans and 11 Mongolians and 14 Europeans for c.12C>G and c.380G>C in *EGLN1* and for all Affymetrix SNPs with a call rate of at least 95% in each sample. We used the Beagle software package[38] to estimate phase in the 16 local Tibetans. We then estimated the age of the c.12C>G mutation from the decay of LD in the local Tibetans, according to the methods described[17,39].

Reverse-transcription and real-time PCR

A portion (1 μg) of RNA was treated with DNase I to eliminate genomic DNA contamination. The integrity of the RNA was verified by agarose gel electrophoresis and by an optical density at 260/280 nm of 2.0 ± 0.2 as measure by NanoDrop 2000 (Thermo Fisher Scientific). RNA was then reverse transcribed using SuperScript VILO (Life Technologies) according to the manufacturer's instructions. Hydrolysis probes were acquired from Applied Biosystems (Life Technologies) and are listed in Supplementary Table 5. The amount of each target mRNA was normalized using *HPRT1* and *GAPDH* mRNA for granulocytes, whereas *RPLP0* and *TFRC* mRNA were used for normalization of the erythroid progenitor colonies, on the basis of the eight reference genes used to validate normalization for blood and erythroid progenitors in using geNorm Plus (Biogazelle) (data not shown). Amplification reactions were carried out using the 7500 Real-Time PCR System (Applied Biosystems, Life Technologies) with 45 cycles of 95 °C for 15 s followed by 60 °C for 1 min. To assess reaction specificity, analyses were also carried out without reverse transcriptase or template, with both serving as negative controls.

Quantitative RT-PCR data and statistical analysis

The Livak method with REST 2009 software (Qiagen) was employed to analyze the generated data set using at least two reference genes. The data presented represent the mean and s.d. of at least two separate experiments performed in duplicate. Differences between control and Tibetan samples were analyzed using the Student's *t* test, and a *P* value of less than 0.05 was considered statistically significant.

Expression, purification and kinetic analyses of p.Asp4Glu, p.Cys127Ser and p.[Asp4Glu;Cys127Ser] PHD2

The mutations encoding p.Asp4Glu and p.Cys127Ser were introduced into human *EGLN1* cDNA in a baculovirus vector[40] with a sequence encoding a C-terminal Flag-His tag using the primer pairs listed in Supplementary Table 5 and the QuikChange site- directed mutagenesis kit (Stratagene). The resulting recombinant vectors were sequenced for the correct mutations. The vectors were cotransfected into *Spodoptera frugiperda* (Sf9) cells (Invitrogen) with BaculoGold DNA (Pharmingen) by calcium phosphate transfection, and the recombinant baculoviruses generated were amplified. Mutant and wild-type PHD2 proteins were expressed in H5 insect cells (Invitrogen) cultured in suspension in Sf900IISFM serum-free medium (Invitrogen) and were purified to near homogeneity with anti-Flag M2 affinity material, as described[40]. Kinetic analyses of the PHD2 mutants in comparison to wild-type enzyme were performed by an assay measuring the hydroxylation-coupled stoichiometric release of ^{14}C-labeled CO_2 from 2-oxo[1-^{14}C]glutarate in a reaction volume of 0.250 ml (ref. 18). Equal amounts of freshly purified wild-type and mutant enzyme were used as catalysts. The K_m and V_{max} values for the mutant enzymes relative to wild-type enzyme for a synthetic peptide DLDLEMPAYIPMDDDFQL (DLD19) and HIF-2αODDD produced and purified in *Escherichia coli*[41] were determined by varying the concentration of the substrate while keeping the concentrations of the cosubstrates constant. The K_m and V_{max} values for oxygen were determined by varying the oxygen concentration in a hypoxia workstation (InVivo$_2$ 400, Ruskinn Technology) and keeping the concentration

of the substrate (HIF-2αODDD) and those of the other cosubstrates constant. The K_m values for 2-oxoglutarate were determined under normoxia by varying the concentration of 2-oxoglutarate while keeping those of the substrate (DLD19) and the other cosubstrates constant. Statistical analyses comparing the K_m and V_{max} values for each mutant PHD2 with the values for wild-type enzyme were performed using the Student's two-tailed t test.

EGLN1 shRNA knockdown and rescue assays

EGLN1 cDNA (wild-type and variant) was subcloned into pLenti-Ubcp-Flag-HA-gateway-PGK-HYG expression vector using Gateway technology (Life Technologies), and constructs were confirmed by DNA sequencing. Hep3B cells (American Type Culture Collection; tested negative for mycoplasma) were infected with lentiviruses encoding Venus fluorescent protein or the PHD2 constructs. After selection in 200 μg/ml hygromycin, cells were infected with lentivirus expressing control shRNA or EGLN1 shRNA (TRCN0000001042, Broad Institute TRC shRNA library) and were selected in 2 μg/ml puromycin. Cells were maintained in DMEM containing 10% FBS, 200 μg/ml hygromycin, 2 μg/ml puromycin and 1% penicillin-streptomycin in the presence of 21% O_2 and 5% CO_2 at 37 °C. For hypoxia experiments, cells were grown instead in 1% O_2 and 5% CO_2 at 37 °C. Cell extracts were prepared on ice with 1× lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP-40) supplemented with a protease and phosphatase inhibitor cocktails (Roche Applied Science), resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBST with 5% nonfat milk and probed with monoclonal antibody to HIF-2α (1:1,000 dilution; generated in house), monoclonal antibody to PHD2 (1:1,000 dilution; D31E11, Cell Signaling Technology) and monoclonal antibody to tubulin (1:10,000 dilution; T5168, Sigma-Aldrich). Bound proteins were detected with HRP-conjugated secondary antibody (Pierce) and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Total (endogenous and transgene-expressed) EGLN1 transcript levels were quantified using the primers described in Supplementary Table 5.

Analysis of erythroid progenitors: BFU-E sensitivity assays

Erythroid colonies (BFU-Es) were grown from peripheral blood mononuclear cells for 14 d in the presence of different concentrations of EPO (Stemcell Technologies)[42]. Briefly, mononuclear cells from peripheral blood were isolated with Histopaque 1077 (Sigma-Aldrich) and cultured at a final density of 6×10^5 cells/ml in Methocult H-4531 medium (Stemcell Technologies) in 35-mm Petri dishes in duplicate. Cultures were maintained at 37 °C with 5% CO_2 and 21% O_2 for normoxic conditions. Culture under reduced oxygen tension was achieved using a modular incubator chamber (Billups Rothenberg), which was flushed for 10 min with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 or 1% O_2, 5% CO_2 and 94% N_2, sealed and placed at 37 °C in a conventional cell incubator. After 14 d of culture, colonies from plates with different EPO concentrations were counted and compared with the controls, as previously described[42]. In addition, each colony was imaged in an identical setting using a Stereomicroscope Stemi 2000-C (Carl Zeiss Microscopy) connected to an Olympus DP Digital camera (Olympus Imaging America) at a fixed magnification (40×). Digital images of every colony were analyzed using a pixel count algorithm provided in ImageScope software (Aperio) to calculate the area occupied by each individual colony (pixel count) as a measure of colony size. Colonies were individually collected, and RNA

was extracted for expression studies. For colony growth in 15 mU and 3,000 mU concentrations of EPO, data were analyzed using repeated-measures ANOVA to compare colony counts by group at different oxygen concentrations and assess interaction effects (oxygen-by-group interactions). The PROC MIXED procedure (a component of the SAS System) was used to generate these mixed models. All statistical analyses were performed using SAS software, Version 9.3 of the SAS System (2012 SAS Institute).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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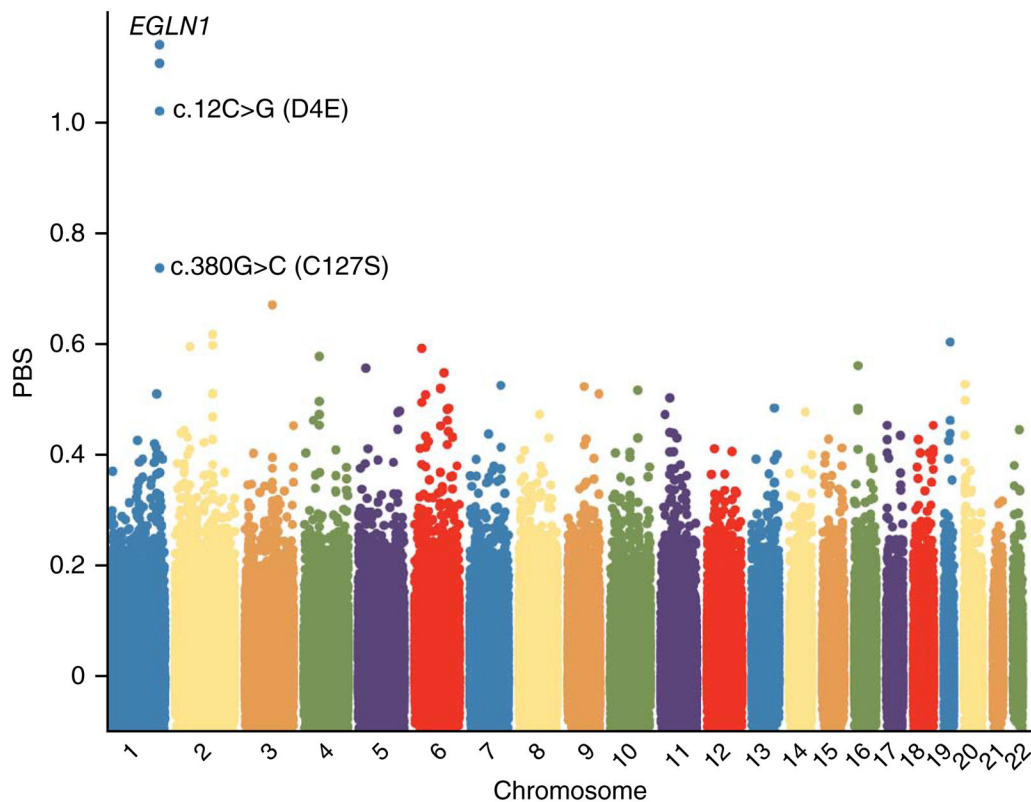
Figure 1.
Genome-wide allele frequency differentiation between Tibetans, Mongolians and Europeans. To measure genome-wide differentiation in allele frequency between Tibetans and Mongolians, we calculated the PBS for the c.12C>G and c.380G>C variants in *EGLN1* for each Affymetrix SNP. We included all unrelated Tibetan, Mongolian and European individuals in the analysis for whom we had Affymetrix 6.0, c.12C>G and c.380G>C genotype data. The final set included 16 Tibetans, 11 Mongolians and 14 Europeans. The PBS for c.12C>G was 1.02 (empirical *P* value = 9×10^{-6}). The four SNPs with PBS greater than 1.02 were all located in the *EGLN1* region.
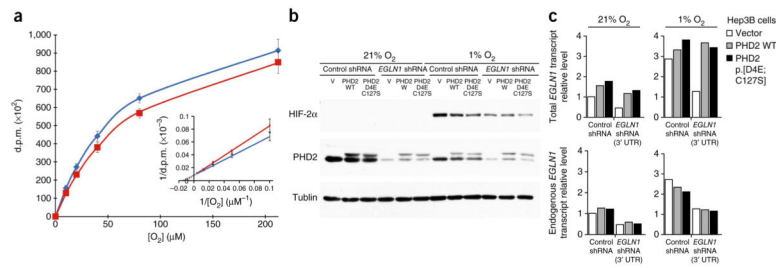
Figure 2.
The p.[Asp4Glu; Cys127Ser] PHD2 mutant shows gain of function under hypoxia. (**a**) The K_m value for O_2 for the p.[Asp4Glu; Cys127Ser] PHD2 mutant is lower than that for wild-type PHD2. Shown is the effect of O_2 concentration on the reaction velocity of wild-type PHD2 (red line) and the p.[Asp4Glu; Cys127Ser] mutant (blue line), using the HIF-2α ODDD as a substrate. The inset shows the corresponding double-reciprocal plot. The data presented are the average ± s.e.m. of eight independent assays comparing wild-type PHD2 and the p.[Asp4Glu; Cys127Ser] mutant in the same assay. Data from independent assays were normalized relative to the disintegrations per minute (d.p.m.) obtained with wild-type PHD2 at an O_2 concentration of 212 μM. (**b**) Decreased HIF-2α accumulation in hypoxic cells expressing p.[Asp4Glu; Cys127Ser] PHD2 in knockdown-rescue experiments. Shown is a representative immunoblot analysis of Hep3B cells expressing Venus fluorescent protein (V), wild-type (WT) PHD2 or p.[Asp4Glu; Cys127Ser] PHD2. Cells were stably coinfected with an shRNA targeting the 3′ UTR of endogenous *EGLN1* (not present in the rescue constructs) or a negative control shRNA and were grown in the presence of 21% or 1% O_2 for 12 h. Decreased HIF-2α levels in cells expressing the p.[Asp4Glu; Cys127Ser] variant were observed in three independent experiments. The electrophoretic mobility of the exogenous PHD2 protein was slightly decreased owing to the presence of the Flag epitope. Tubulin was used as a loading control. (**c**) RT-PCR demonstrates knockdown of endogenous *EGLN1* in Hep3B cells by the *EGLN1*-targeted shRNA and equal levels of transgene transcripts in cells expressing the transgenes for wild-type and p.[Asp4Glu; Cys127Ser] PHD2. Shown are representative data from three independent experiments.
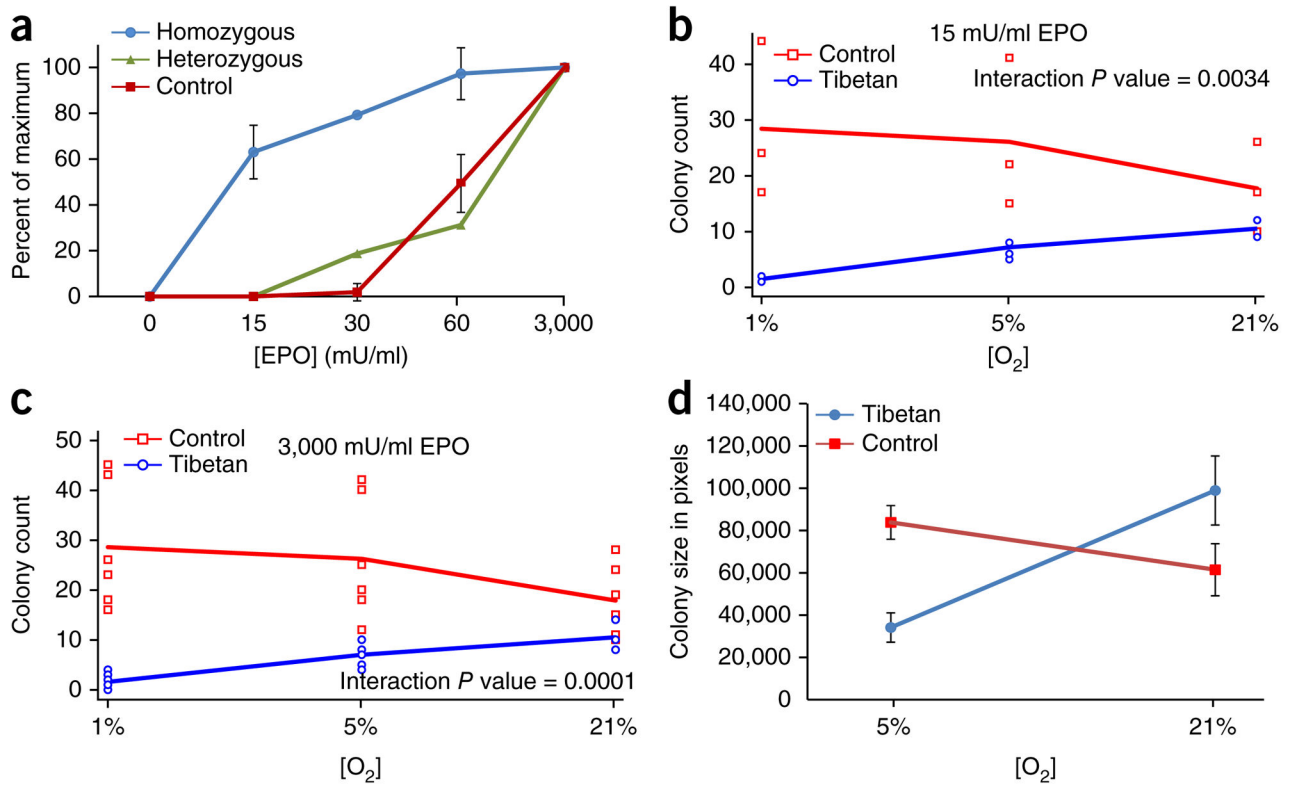
Figure 3.

Erythroid colony (BFU-E) assays. Erythroid colonies were enumerated after stimulation with various concentrations of EPO. (**a**) BFU-Es homozygous for the c.[12C>G; 380G>C] variant in *EGLN1* are hypersensitive to EPO under normoxia. BFU-E colony assays in Tibetans homozygous ($n = 2$ biological replicates) or heterozygous ($n = 1$) for the c.[12C>G; 380G>C] variant in comparison to normal controls ($n = 10$) at different EPO concentrations. Values are normalized to the number of BFU-Es grown at ambient oxygen tension at 3,000 mU/ml EPO, which was expressed as 100%. Designated error bars in controls and for cells homozygous for c.[12C>G; 380G>C] represent ±s.d. (**b**) Sensitivity of BFU-Es to low EPO concentration (15 mU/ml). Hypersensitive BFU-Es from Tibetans with the c.[12C>G; 380G>C] variant ($n = 6$) lose their hypersensitivity to EPO as the oxygen tension decreases to 5% and fail to form colonies at 1% O_2, whereas control colonies ($n = 3$) become hypersensitive to EPO with increasing hypoxia. (**c**) Colony growth of progenitor cells from a Tibetan with the c.[12C>G; 380G>C] mutation and controls at 3,000 mU/ml EPO. Tibetan BFU-E colonies with the c.[12C>G; 380G>C] mutation have decreased colony formation with ambient O_2 levels and 5% O_2, and they fail to grow at 1% O_2. In contrast, BFU-Es from control subjects ($n = 3$) form more colonies as the oxygen tension decreases. (**d**) Size of erythroid colonies increases in BFU-Es from controls but decreases for progenitor cells from a Tibetan with the c.[12C>G; 380G>C] mutation. Average BFU-E colony size represented in pixels (*y* axis) is greater in BFU-E colonies from the control subject under hypoxia (5% O_2), whereas, in the Tibetan subject with the c.[12C>G; 380G>C] mutation, hypoxia decreases BFU-E colony size. Error bars in Tibetans and controls represent ±s.d. (Fig. 3a is an independent experiment performed with different individuals and at a different
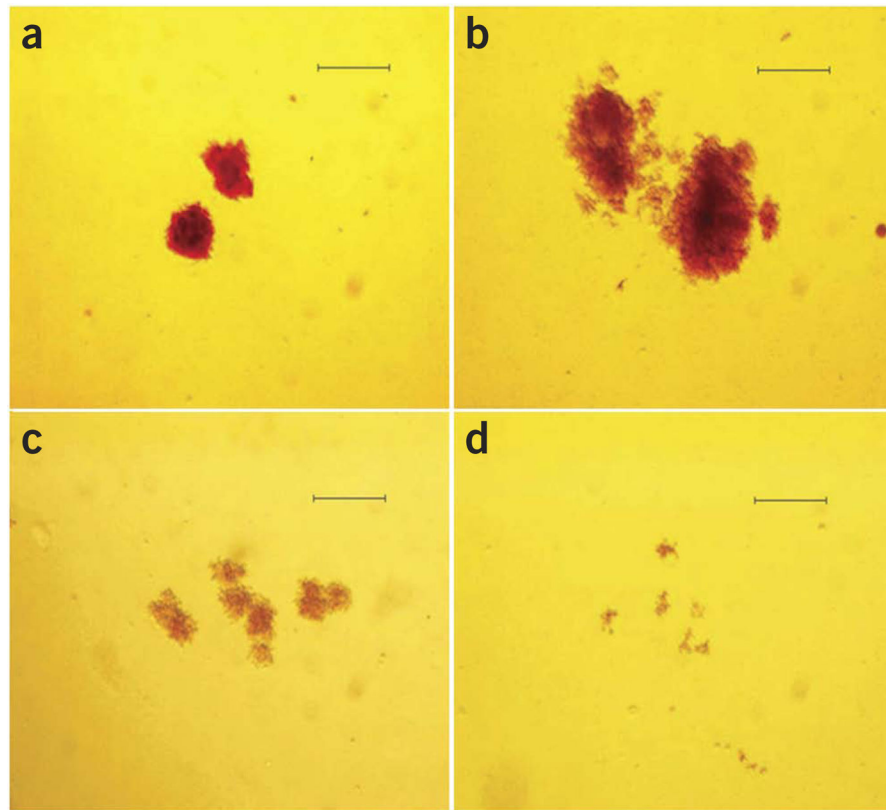
time than the experiments in Fig. 3b–d, which consist of 6 biological replicates (5 Tibetans homozygous and 1 Tibetan heterozygous for c.[12C>G; 380G>C]) together with 3 controls.)

Figure 4.
Hypoxia increases the proliferation of control BFU-E colonies but decreases that of colonies with the c.[12C>G; 380G>C] mutation in *EGLN1*. BFU-Es were grown in 3,000 mU/ml EPO, and all images were acquired at 40× magnification (scale bars, 1 mm). (**a,b**) Representative colonies from a control (wild type) subject and (**c,d**) a Tibetan subject homozygous for the c.[12C>G; 380G>C] mutation. Note the larger colony sizes for the control BFU-E colonies under hypoxia (5% O_2) (**b**) relative to ambient oxygen tension (**a**). BFU-Es with the c.[12C>G; 380G>C] mutation exhibit smaller colony sizes under normoxia (**c**) in comparison to control cells (**a**), and the colonies are paler than the controls, reflecting the decreased hemoglobinization with the c.[12C>G; 380G>C] erythroid progenitors in comparison to the controls. (**d**) The decrease in colony size and hemoglobinization for Tibetan BFU-Es with the c.[12C>G; 380G>C] mutation is even more pronounced under 5% O_2 (in comparison to **b**).

Table 1

Genotyping results for *EGLN1* exon 1 mutations in 26 Us tibetans

Sample	Sex	Age (years)	Region	p.Asp4Glu	p.Cys127Ser
TU01	F	35	U-Tsang	Homozygous	Homozygous
TU02	F	36	Mixed	Homozygous	Homozygous
TU03	M	31	Kham	Heterozygous	Heterozygous
TU04	M	61	Mixed	Homozygous	Homozygous
TU05	M	44	Kham	Homozygous	Homozygous
TU06	M	34	U-Tsang	Homozygous	Homozygous
TU07	M	38	U-Tsang	Homozygous	Homozygous
TU08	M	ND	U-Tsang	Homozygous	Homozygous
TU09	M	ND	Mixed	Wild type	Heterozygous
TU10	F	ND	Mixed	Heterozygous	Heterozygous
TU11	F	46	Kham	Homozygous	Homozygous
TU12	M	46	Mixed	Wild type	Heterozygous
TU13	M	46	Mixed	Homozygous	Homozygous
TU14	M	38	Kham	Homozygous	Homozygous
TU15	M	53	Kham	Homozygous	Homozygous
TU16	F	24	U-Tsang	Homozygous	Homozygous
TU17	M	30	Kham	Homozygous	Homozygous
TU18	M	36	U-Tsang	Homozygous	Homozygous
TU19	F	43	U-Tsang	Wild type	Homozygous
TU20	M	39	U-Tsang	Homozygous	Homozygous
TU21	M	41	Kham	Homozygous	Homozygous
TU22	M	40	U-Tsang	Homozygous	Homozygous
TU23	F	41	U-Tsang	Wild type	Heterozygous
TU24	M	41	Kham	Homozygous	Homozygous
TU25	F	36	U-Tsang	Homozygous	Homozygous
TU26	F	38	U-Tsang	Homozygous	Homozygous

Individuals of Tibetan ancestry are generally divided into three known major subgroups: U-Tsang, Kham and Amdo. M, male; F, female; ND, no data.

Table 2

Kinetic values for the PHD2 p.Asp4Glu and p.cys127ser mutants

Enzyme	DLD19			HIF-2α ODDD			2-oxoglutarate			O₂		
	K_m (µM)	V_{max} (%)	n	K_m (nM)	V_{max} (%)	n	K_m (µM)	V_{max} (%)	n	K_m (µM)	V_{max} (%)	n
Wild-type PHD2	13 ± 3	100	3	70 ± 20	100	5	1.3 ± 0.3	100	4	150 ± 50	100	8
p.Asp4Glu PHD2	11 ± 2	90	3	ND	ND		ND	ND		150 ± 30	100	4
p.Cys127Ser PHD2	14 ± 3	105	3	ND	ND		ND	ND		160 ± 50	110	4
p.[Asp4Glu; Cys127Ser] PHD2	14 ± 1	100	3	100 ± 40*	130**	5	1.4 ± 0.5	80	4	110 ± 30*	100	8

Values are mean ± s.d. V_{max} values are expressed relative to the V_{max} value obtained for wild-type PHD2.

*$P < 0.05$,

**$P < 0.005$, Student's two-tailed t test. n is the number of assays conducted. ND, not determined.