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## Physiological Genomics of Adaptation to High-Altitude Hypoxia

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### Abstract

Population genomic studies of humans and other animals at high altitude have generated many hypotheses about the genes and pathways that may have contributed to hypoxia adaptation. Future advances require experimental tests of such hypotheses to identify causal mechanisms. Studies to date illustrate the challenge of moving from lists of candidate genes to the identification of phenotypic targets of selection, as it can be difficult to determine whether observed genotype–phenotype associations reflect causal effects or secondary consequences of changes in other traits that are linked via homeostatic regulation. Recent work on high-altitude models such as deer mice has revealed both plastic and evolved changes in respiratory, cardiovascular, and metabolic traits that contribute to aerobic performance capacity in hypoxia, and analyses of tissue-specific transcriptomes have identified changes in regulatory networks that mediate adaptive changes in physiological phenotype. Here we synthesize recent results and discuss lessons learned from studies of high-altitude adaptation that lie at the intersection of genomics and physiology.

### Keywords

adaptation; altitude; hypoxia; oxygen; maladaptive plasticity; phenotypic plasticity

## INTRODUCTION

The study of high-altitude adaptation has benefitted from the application of population genomic approaches, as illustrated by studies of highland human populations that have revealed evidence for positive selection on genes involved in the physiological response to hypoxia (1-14). The identified candidate genes include key components of the hypoxia inducible factor (HIF) signaling pathway, the central pathway that orchestrates the transcriptional response to hypoxia by transducing changes in cellular O<sub>2</sub> levels to changes in gene expression (15-20). In genomic studies of indigenous high-altitude people from the Andean altiplano, the Tibetan Plateau, and the Ethiopian highlands, some of the identified candidate genes show evidence of population-specific selection, whereas others appear to represent repeated targets of selection (21-27). HIF genes that exhibit strong evidence for

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selection in multiple highland human populations include *EPAS1* (*endothelial PAS domain containing protein 1*) and *EGLN1* (*egl-9 family hypoxia inducible factor*). *EPAS1* encodes HIF2 $\alpha$ , a member of the HIF family of transcription factors, and *EGLN1* encodes PHD2, a prolyl hydroxylase that induces degradation of HIF in an O<sub>2</sub>-dependent manner. Population genomic studies of wild and domesticated mammals and birds living at high altitudes have also identified many candidate genes for hypoxia adaptation, including some of the same components of the HIF pathway that were identified in humans (27-30). Such studies have generated hypotheses about the genes and pathways that may have contributed to hypoxia adaptation, but physiological experiments are required to test such hypotheses and to identify and characterize underlying mechanisms.

Integrated analyses of physiological phenotypes and genetic/genomic variation in high-altitude natives have yielded several mechanistic insights in evolutionary context. For example, targeted functional studies in high-altitude humans and other vertebrates have revealed evidence for putatively adaptive modifications of cardiac and skeletal muscle metabolism and mitochondrial oxidative capacity (26, 31-44). In high-altitude humans, evidence suggests that changes in muscle metabolism may have been mediated by genetic changes in *PPAR $\alpha$*  (*peroxisome proliferator-activated receptor  $\alpha$* ), a transcription factor that plays a key role in regulating lipid oxidation. In Tibetans, variants in *PPAR $\alpha$*  are associated with a reduced capacity for fatty acid oxidation, indicating a shift toward glucose as a preferred metabolic fuel (35, 39), and the gene exhibits signatures of positive selection in both Tibetans (3) and Amharas from the Ethiopian highlands (8). In numerous high-altitude birds and nonhuman mammals, targeted functional studies and reverse genetics experiments have revealed changes in the oxygenation properties of hemoglobin (Hb), and in many cases, population genetic analyses of sequence variation in the underlying globin genes provided corroborative evidence that the observed functional changes have adaptive significance (45-60).

Documenting genotype–phenotype associations represents the first step in any investigation of adaptive mechanisms, and ecologically relevant measures of whole-organism performance represent a logical point of focus (61, 62). Because the reduced partial pressure of O<sub>2</sub> ( $P_{O_2}$ ) at high altitude limits aerobic metabolism, there are good reasons to expect that measures of aerobic performance capacities in hypoxia are relevant to fitness in highland natives (63-66). At high altitude, both the capacity for sustained exercise and the ability to be active in the cold are highly relevant measures of performance and are directly related to aerobic metabolism. The upper limit of aerobic metabolism is defined by the maximal rate of O<sub>2</sub> consumption,  $\dot{V}O_{2\max}$  [typically measured in units of mL(min/kg)], a performance measure that reflects the integrated functioning of the respiratory, cardiovascular, and metabolic systems (67). Brutsaert (68) and McClelland & Scott (65) discuss other relevant measures of performance and aerobic metabolism at high altitude.

Much of what we know about the effects of hypoxia on aerobic performance capacities comes from studies of humans and rodents that are lowland natives (69). In humans, this includes a vast literature related to mountaineering and mountain medicine (70). Studies of hypoxia acclimation and acclimatization in lowland species have enriched our understanding of mechanisms of physiological plasticity and suggest testable hypotheses about evolved

mechanisms of adaptation in high-altitude species. Several recent studies involving indigenous high-altitude humans (71-73) and rodents (32-34, 36, 37, 40, 74-77) have investigated aerobic performance capacities in hypoxia to characterize genetic or physiological mechanisms of adaptation. These studies involved some or most of the following steps: (a) identification of fitness-relevant differences in physiological performance between high- and low-altitude natives, (b) experimental assessment of how such performance differences stem from changes in subordinate traits (respiratory, cardiovascular, or metabolic), (c) tests of genotype–phenotype associations, and (d) analysis of genome-wide transcriptional profiles to determine how changes in the measured phenotypes are brought about by hypoxia-induced changes in gene expression. Statistical associations between phenotypes and tissue-specific gene expression profiles can guide the design of follow-up experiments to identify causal mechanisms of acclimatization or genetic adaptation. By elucidating causal connections, it may be possible to determine why the up- or downregulation of particular genes and pathways is advantageous under cold, hypoxic conditions at high altitude. In addition to studies of aerobic performance capacities in hypoxia, several pioneering studies in high-altitude humans have conducted genetic association studies of pregnancy outcomes and offspring survival (78, 79).

Functional genomic approaches can reveal the transcriptional basis of plasticity in physiological phenotypes and can elucidate how changes in regulatory networks mediate acclimatization and adaptation to hypoxia. Population genomic approaches (followed by experiments in forward or reverse genetics) can potentially shed light on the genetic basis of evolved traits involved in hypoxia adaptation and can also provide insights into sources of adaptive genetic variation and the history of locus-specific selection. Here we review insights into mechanisms of high-altitude adaptation derived from both forms of genomic analysis, and we highlight case studies that demonstrate the value of integrating genomic analyses with experimental physiology. We start by reviewing the acclimatization response to hypoxia, and we then discuss the challenge of deciphering causes of observed genotypic associations with hypoxia-responsive phenotypes.

## THE ROLE OF PHENOTYPIC PLASTICTY IN HIGH-ALTITUDE ADAPTATION

HIF genes like *EPAS1* and *EGLN1* mediate plastic responses to hypoxia because they exert O<sub>2</sub>-dependent control over the expression of other genes. To varying degrees, the HIF pathway regulates every major component of hypoxia acclimatization, including respiration, blood flow, vascular remodeling, and intermediary metabolism (15, 17-20).

The benefit of phenotypic plasticity is the ability to make adjustments in response to environmental change. It is easy to envision how such benefits might accrue to migratory species like the bar-headed goose (*Anser indicus*), a species that spends most of the year at low to moderate altitudes but that undertakes trans-Himalayan migratory flights that reach altitudes of >7,200 m (80-82). Even in nonmigratory species that do not experience the same vicissitudes of O<sub>2</sub> availability on a daily or seasonal basis, there is always variation in metabolic O<sub>2</sub> demand (e.g., increased thermoregulatory demands associated with daily or seasonal changes in temperature).

There are also good theoretical reasons to expect that plasticity plays an important role in adaptation to environmental heterogeneity in species that are continuously distributed across steep altitudinal gradients. Among mammals, North American deer mice (*Peromyscus maniculatus*) are distributed from sea level to 4,300 m (66), and Andean yellow-rumped leaf-eared mice (*Phyllotis xanthopygus*) are distributed from sea level to altitudes >6,700 m (83). In such species, individual mice might spend their lives within a narrowly defined elevational zone, but the broader distribution of the species as a whole can produce a spatial averaging of selection pressures across generations. When average dispersal distances exceed the spatial scale of variation in relevant environmental factors, different generations will often experience different conditions. Under such circumstances, theory predicts that the capacity to express different phenotypes in response to prevailing conditions will be favored over a strategy of genotypic specialization (84, 85).

### Plasticity in Whole-Animal Performance in Hypoxia

Aerobic exercise capacity of lowland species is always reduced during exposure to hypoxia relative to the sea-level baseline (65, 68-70, 86) (Figure 1). However, in highland and lowland natives alike, acclimatization to hypoxia can help minimize the inevitable decline in maximal and submaximal performance at high altitude. In some high-altitude natives, the hypoxia-related decline in aerobic performance is partially compensated by the combined effects of evolved changes and environmentally induced changes in numerous subordinate traits (64-66, 87-91).

### Plasticity in Subordinate Traits

Aerobic performance depends on the flux capacity of the O<sub>2</sub> transport pathway, which consists of interrelated physiological processes arranged as a linear series of diffusive and convective steps (ventilation, pulmonary O<sub>2</sub> diffusion, circulatory O<sub>2</sub> delivery, and tissue O<sub>2</sub> diffusion), culminating in O<sub>2</sub> utilization in the tissue mitochondria. A different (but partly interrelated) pathway exists for the transport and utilization of metabolic substrates such as lipids and carbohydrates (65, 92, 93). Hypoxia-induced changes in the flux of O<sub>2</sub> and metabolic substrates through the relevant pathways could be caused by plasticity in the organs and tissues governing each step. In mammals and birds, acclimatization to chronic hypoxia involves a characteristic suite of plastic changes in respiratory, circulatory, and metabolic phenotypes (64-66, 87-91, 94). However, the various physiological and anatomical support systems for the transport of respiratory gases and metabolic substrates are not all equally labile and responsive to environmental stimuli. The process of acclimatization likely involves coordinated adjustments in serially integrated traits with varying degrees of plasticity that exert control over different steps in the same pathway (74, 75, 95-101). In the case of the O<sub>2</sub> transport pathway, for example, the benefits of increasing convective O<sub>2</sub> transport via plastic changes in cardiac output or red cell mass (the fraction of total blood volume consisting of red blood cells) are influenced by limits imposed by tissue diffusion capacity. Thus, a mechanistic understanding of physiological acclimatization and adaptation to hypoxia requires insights into the mosaic nature of plasticity at the level of individual traits and the functional interactions among such traits (69, 74, 75, 95).

## ADAPTIVE PLASTICITY AND ITS TRANSCRIPTIONAL UNDERPINNINGS

Plasticity in many physiological traits is mediated by regulatory changes in gene expression in response to environmental stimuli. In comparisons between high- and low-altitude populations that exhibit differences in gene expression, common-garden acclimation experiments can be used to separate genetic and environmental components of variation. Studies of thermogenic performance in high-altitude deer mice provide an example of this experimental approach.

In small mammals like deer mice that have high surface area-to-volume ratios, increased rates of heat loss must be compensated by higher rates of metabolic heat production, so a capacity for sustained aerobic thermogenesis may often be critical for survival during periods of extreme cold. When challenged with the combined stressors of cold and hypoxia, individuals with a higher thermogenic capacity (measured as cold-induced  $\dot{V}O_2\text{max}$ ) can maintain constant body temperature at lower ambient temperatures and can therefore remain active in the cold for a longer period of time (102). In placental mammals, whole-organism thermogenic capacity is determined by the joint capacities for both shivering and nonshivering thermogenesis (NST). In the wild, high-altitude deer mice exhibit greater thermogenic capacities in hypoxia than their lowland counterparts, and these population differences persist in lab-reared offspring at low altitude, suggesting that observed performance differences are at least partly genetically based (32, 34, 36, 74, 75, 103). Hypoxia acclimation improves thermogenic capacity of highland and lowland mice alike, but the magnitude of the performance enhancement is significantly greater in highlanders (74, 75). The superior performance of highland mice in hypoxia is attributable to accentuated plasticity in some subordinate physiological traits in conjunction with evolved changes in the mean values of other traits (36, 74, 75). These physiological changes, in turn, are associated with a combination of plastic and constitutive changes in tissue-specific gene expression that influence multiple steps in the transport pathways for  $O_2$  and metabolic substrates (32, 34, 36, 37, 40, 76, 77).

### Regulatory Changes in Gene Expression Are Associated with Adaptive Enhancements of Thermogenic Capacity

In deer mice, NST may account for more than 60% of total thermogenic capacity and is responsive to cold acclimation in adulthood (76, 104). As with whole-organism thermogenic capacity, wild-caught highland mice exhibit significantly higher NST capacities under hypoxia than wild-caught lowland mice. However, these population differences do not persist in lab-born progeny reared under normoxic conditions. In both highlanders and lowlanders, the hypoxia-induced enhancement of NST capacity is associated with changes in the expression of several sets of coregulated genes (transcriptional modules) in brown adipose tissue that influence brown adipocyte proliferation, innervation, and vascularization;  $\beta_3$  adrenergic receptor signaling; and mitochondrial uncoupling (76, 77). In lowland natives, changes in gene expression were associated with an increase in whole-organism NST that enabled them to achieve the same capacity as acclimated highlanders.

In contrast to the high degree of plasticity in NST, the capacity for shivering thermogenesis is less responsive to cold acclimation during adulthood (104). This is consistent with evidence for genetic differences in skeletal muscle phenotype between highland and lowland natives that may underlie population differences in the capacity for shivering thermogenesis (32, 34, 36, 37). For example, when subjected to hypoxia and cold in combination, highland mice exhibit an increased capacity for oxidizing lipids as a primary fuel source during intense shivering. Common-garden experiments revealed that the enhanced capacity for lipid catabolism in the skeletal muscle of highland mice is associated with muscle-specific increases in the activities of key enzymes that influence flux through fatty acid oxidation and oxidative phosphorylation pathways in conjunction with the concerted upregulation of numerous genes in these same pathways (32, 34).

Population differences in thermogenic performance are also associated with changes in skeletal muscle phenotype that may influence oxidative capacity during shivering thermogenesis. Relative to lowlanders, the skeletal muscle of highland deer mice contains a higher capillary surface density, higher total and subsarcolemmal mitochondrial volume density, and a higher density of slow (Type 1) oxidative fibers, and common-garden experiments indicate that these population differences in muscle phenotype are largely genetically based (36, 37, 41-44, 74). These differences in muscle phenotype between highland and lowland mice are associated with persistent expression differences in nearly 70 genes that participate in a variety of processes, including energy metabolism, muscle plasticity, vascular development, and cell stress response (37). One notable example involves peroxisome proliferator-activated receptor PPAR $\gamma$  (*Pparg*), which is a regulator of insulin sensitivity, mitochondrial biogenesis, and capillarity, all of which can contribute to the oxidative phenotype of skeletal muscle (105, 106). Highland deer mice exhibit higher *Pparg* transcript abundance and protein expression compared with lowlanders (36) (Figure 2), and these persistent expression differences may contribute to the observed population differences in oxidative muscle phenotype. Whereas the regulatory networks that mediate responses to cellular hypoxia have received a great deal of experimental attention (16), the deer mouse studies mentioned above provide some of the first glimpses of the regulatory changes in gene expression that contribute to adaptive variation in aerobic performance in high-altitude natives.

### Genetic Assimilation of Acclimatization Responses to Hypoxia

Plastic changes in a given trait that are beneficial as a response to acute exposure to a particular stressor may also be beneficial as a constitutive response to chronic exposure. In such cases, an environmentally induced response can become canalized (genetically fixed) by selection, a phenomenon known as genetic assimilation. A recent study documented an example of genetic assimilation in the hypoxia adaptation of Tibetan antelope, *Pantholops hodgsonii*, a highly athletic species that is endemic to the Tibetan Plateau and lives at altitudes of 3,600–5,500 m (60).

When exposed to hypoxia, adult bovid mammals like goats and sheep upregulate a juvenile Hb isoform (with relatively high O<sub>2</sub> affinity) at the expense of the normal adult isoform (with relatively low O<sub>2</sub> affinity). This isoform switch produces an increase in blood-O<sub>2</sub>

affinity that should help safeguard arterial O<sub>2</sub> saturation at low inspired *PO*<sub>2</sub>, thereby improving circulatory O<sub>2</sub> delivery in hypoxia (55, 107). An integrated genomic and biochemical analysis revealed that Tibetan antelope evolved a derived increase in blood-O<sub>2</sub> affinity by truncating the ancestral ontogeny of globin gene expression such that the high-affinity juvenile Hb isoform completely supplanted the lower-affinity adult isoform that is expressed in the adult red blood cells of other bovids. This juvenilization of blood properties in Tibetan antelope represents a novel mode of biochemical adaptation and illustrates how alteration of existing developmental channels can provide a ready mechanism of evolutionary change. Comparative genomic analysis revealed that the developmental switch in Hb isoform expression became canalized via deletion of a ~45-kb chromosomal region that spans the 3' end of the Tibetan antelope β-globin gene cluster (Figure 3). This deletion eliminated the adult-expressed β-globin gene while leaving the linked juvenile β-globin gene intact, with the result that the high-affinity juvenile Hb isoform became the sole expressed isoform in adult red cells. The chromosomal deletion produced a drastic regulatory switch in Hb isoform expression such that a reversible acclimatization response to acute hypoxia—as observed in goats and sheep—became genetically assimilated as an irreversible adaptation to chronic hypoxia in the Tibetan antelope (60).

## NONADAPTIVE PLASTICITY AND REGULATORY MECHANISMS OF GENETIC COMPENSATION

The examples discussed above represent cases in which acclimatization responses of specific traits appear to contribute to adaptive improvements in performance under hypoxia. In such cases, plasticity presumably evolved via selection that favored the capacity to express different phenotypes in response to changes in tissue O<sub>2</sub> supply or metabolic demand. It is less obvious how we should account for the occurrence of maladaptive or nonadaptive plasticity, but it may often result from evolutionary lag, whereby the response induced by a particular stimulus was beneficial in a species' ancestral environment but is not well-matched to recently changed conditions. Upon colonization of a new environment, a plastic response to a given stimulus may be miscued and misdirected if the stimulus had a different underlying cause in the past. For example, in lowland species, tissue hypoxia typically stems from transient limitations of circulatory O<sub>2</sub> transport rather than reductions in the *PO*<sub>2</sub> of inspired air (environmental hypoxia). When individuals of a lowland species colonize a high-altitude environment, the hypoxic stimulus may induce a miscued response that does not remedy the root cause of the problem (because environmental hypoxia was not a problem that the species' ancestors ever had to solve). If the induced phenotype is maladaptive, selection will favor an attenuation of the plastic response (genetic compensation) (87, 91, 108, 109).

### Hematological Responses to Hypoxia and Associations with *EPAS1* Polymorphism

In Tibetan highlanders, phenotypic associations with derived SNP alleles at *EPAS1* bring questions about maladaptive plasticity and genetic compensation into especially sharp focus (23, 24, 87, 91, 110). *EPAS1* encodes a subunit of HIF2, a transcription factor with numerous downstream target genes, so allelic variants may affect numerous physiological phenotypes (21, 23, 111-113). Although *EPAS1* has been identified repeatedly as a strong

candidate gene for hypoxia adaptation in highland humans and other vertebrates, the phenotypic target of selection remains unclear.

One of the most consistent features of high-altitude acclimatization is the increase in hematocrit (Hct), the ratio of the volume of red blood cells to total blood volume (which is positively correlated with blood Hb concentration). After several weeks at high altitude, increased Hct is sustained by renal synthesis and release of erythropoietin (EPO), a hormone that increases red blood cell production by stimulating the proliferation and differentiation of erythroid precursor cells in the bone marrow. As a result of the hypoxia-induced increase in circulating EPO, acclimatized people at high altitude, including Andean natives, tend to have elevated Hcts compared with people living at sea level. This was traditionally interpreted as an adaptive acclimatization response to hypoxia because increasing Hct results in a corresponding increase in the O<sub>2</sub> content of arterial blood, and, all else being equal, this should translate into an enhancement of tissue O<sub>2</sub> delivery. In contrast to the Andean pattern, Tibetans living at altitudes of 4,000 m or higher typically have Hb concentrations in the range expected for people living at sea level (114–118). The adaptive significance of this counterintuitive pattern received renewed attention when population genomic studies revealed striking evidence for positive selection on *EPAS1* and demonstrated that the derived SNP alleles that are present at especially high frequency in Tibetans are associated with low (i.e., nonelevated) Hb concentration at high altitude (1, 3, 4, 14, 119). The fact that the most hypoxia-tolerant high-altitude mammal and bird species do not exhibit elevated Hb concentrations lends credence to the idea that the Tibetan phenotype is adaptive (87, 91).

The Tibetan pattern is paradoxical because—if all steps in the O<sub>2</sub>-transport pathway remain constant—a Hct-related increase in arterial O<sub>2</sub> content would increase total circulatory convection of O<sub>2</sub> to working muscles, which, in turn, should translate into a higher aerobic exercise capacity. This is the rationale for blood doping in endurance sports. Similarly, mammals with high-endurance performance, like horses and dogs, use their own natural form of blood doping, as Hct can be rapidly increased from ~40% at rest to ~60% during exercise via release of stored red blood cells from the spleen (splenic contraction) (120–123). This reversible form of autotransfusion may increase  $\dot{V}O_{2\max}$  by up to 30% under normoxic conditions (124). However, experiments using thoroughbred horses suggest that the associated expansion of blood volume contributes more to the observed performance enhancement than the increased Hct per se (125). Likewise, among elite cyclists, evidence suggests that the performance-enhancing effects of EPO blood doping may be largely attributable to changes in cardiac performance, red cell properties, and pulmonary/tissue diffusion capacities that are largely independent of Hct (126).

Total circulatory O<sub>2</sub> transport is the product of arterial O<sub>2</sub> content and cardiac output (the total volume of blood pumped by the heart). Beyond a certain point, increasing Hct can be counterproductive because it increases blood viscosity, which can compromise cardiac output and microcirculatory blood flow, thereby limiting O<sub>2</sub> delivery to working muscle (127, 128). This in turn compromises aerobic exercise capacity. Optimal Hcts for exercise performance have been estimated in ex vivo experiments involving canine skeletal muscle (129) and in vivo experiments involving wild-type and transgenic mice with pharmacologically modulated Hcts (130). The traditional interpretation is that performance



is limited by arterial O<sub>2</sub> content at Hct levels below the optimum and is limited by hemodynamic impairments of cardiac output at Hct levels above the optimum. In mice, optimal Hcts for endurance exercise performance are higher than normal resting levels and are higher for animals subject to chronic erythrocytosis relative to those subject to acute treatments (Figure 4), which likely reflects compensatory cardiovascular adjustments to increased blood viscosity (130). In hypoxia, the optimal Hct at rest appears to be close to the normal value at sea level (131). It would be of interest to know whether optimal Hcts for endurance performance differ in normoxia and hypoxia, especially given that the effect of Hct on systemic hemodynamics can change with hypoxia acclimatization (132, 133).

In Tibetan highlanders tested at 4,200 m,  $\dot{V}O_{2\max}$  is negatively correlated with blood Hb concentration and is positively correlated with cardiac output and muscle diffusion capacity (69), although cause–effect relationships among these variables are not clear. Within the normal physiological range of variation, evidence suggests that the negative effect of increasing Hct on  $\dot{V}O_{2\max}$  is not primarily attributable to hemodynamic impairments of cardiac output (71, 134). Instead, increasing Hct may negatively affect  $\dot{V}O_{2\max}$  by limiting diffusive conductance of O<sub>2</sub> across the alveolar–capillary barrier (thereby reducing the  $PO_2$  of arterial blood) and across the capillary–cell barrier (thereby reducing tissue O<sub>2</sub> extraction) (71, 96). Because pulmonary O<sub>2</sub> diffusion is typically not limiting under normoxic conditions, the diffusion-limitation hypothesis predicts that increasing Hct may have beneficial effects at sea level that are not manifest at high altitude.

In summary, studies of high-altitude exercise performance in acclimatized lowlanders and native highlanders indicate that hypoxia-induced increases in Hct are nonadaptive at best, and excessive erythrocytosis is certainly maladaptive, as evidenced by its role in the pathogenesis of chronic mountain sickness (135). This seems consistent with the idea that selection on *EPAS1* contributed to a blunting of the normal hypoxia-induced increase in Hct—a potential example of genetic compensation. This interpretation may be mostly correct in broad outline, but the originally proposed mechanism is incorrect, and there are reasons to doubt that Hct represents the direct phenotypic target of selection. With regard to physiological mechanism, the nonelevated Hct of Tibetan highlanders was originally interpreted as a blunted erythropoietic response to chronic hypoxia. We now know that total red cell mass is actually increased in Tibetans living at high altitude and their nonelevated Hct is attributable to an associated expansion of plasma volume (73). Thus, Tibetans living at high altitude benefit from the augmentation of blood O<sub>2</sub>-carrying capacity afforded by the increased red cell mass, but the associated increase in plasma volume prevents an increase in Hct, thereby avoiding viscosity-related impairments of cardiac function and blood flow. With regard to the inferred target of selection, Hct is regulated by erythropoiesis and water balance via a feedback loop based on renal tissue  $PO_2$  (136). Regardless of the relative contributions of regulatory changes in erythropoiesis and plasma volume, attenuation of the hypoxia-induced increase in Hct could be an indirect consequence of changes at any step in the O<sub>2</sub>-transport pathway, and any such changes could potentially be mediated by selection on variation in HIF genes like *EPAS1* (23, 71, 87, 91, 137).

## ***EPAS1*, Hematocrit, and the Challenges of Mammalian Pregnancy at High Altitude**

In addition to effects on aerobic exercise performance, maternal Hct is also strongly associated with pregnancy outcomes and offspring survival in humans at high altitude (79, 138, 139). During human pregnancy at high altitude, the increase in blood viscosity associated with increased Hct is thought to reduce uteroplacental blood flow, which contributes to an increased risk of stillbirth, preterm birth, and reduced birth weight (139-142). It therefore seems plausible that the nonelevated Hct of Tibetan highlanders could have an accentuated impact on the fertility component of fitness. To explore this possibility, Jeong et al. (79) performed genome-wide association analyses using hematological measurements and extensive data on the reproductive histories of more than 1,000 indigenous Tibetan women living in high Himalayan valleys in Nepal (3,000–4,000 m). Integrating data on hematological traits and reproductive outcomes revealed that low (nonelevated) Hb concentration is positively associated with a higher proportion of live births among pregnancies and with lower proportions and lower absolute numbers of stillbirths and miscarriages (79, 138). The genome-wide association study of Tibetan women revealed that derived alleles at eight single-nucleotide polymorphisms (SNPs) in an intron of *EPAS1* were associated with low Hb concentration and were even more strongly associated with a composite phenotype, oxyHb, defined as the product of Hb concentration and arterial oxygen saturation. The aggregate effect size of the *EPAS1* variants on Hb concentration was consistent with results of other association studies based on independent population samples (1, 4). The association between Hb concentration and female reproductive success predicts that derived variants associated with low Hb concentration should exhibit increased frequencies in Tibetan highlanders relative to lowland population samples. Accordingly, in comparison with a reference panel of control SNPs, the *EPAS1* SNP alleles associated with low Hb concentration exhibited significantly higher frequencies in Tibetans than in Han Chinese and other lowland populations. However, the *EPAS1* SNPs explained only 2.7% of the variation in Hb concentration within the sample of Tibetan women and appeared to explain approximately half of the population difference in Hb concentration between Tibetans and Han Chinese. Moreover, outside of *EPAS1*, none of the other SNP alleles associated with low Hb concentration exhibited significant, population-specific increases in frequency relative to lowland populations (79), suggesting that the observed association between *EPAS1* and Hb concentration could reflect a correlated response to selective changes in a different (unmeasured) trait that affects one or more steps of the O<sub>2</sub>-transport pathway. This interpretation is also supported by the fact that *EPAS1* was more strongly correlated with the oxyHb phenotype than Hb concentration itself.

## **Phenotypic Associations with *EPAS1* Polymorphism in Nonhuman Mammals**

*EPAS1* has also been identified as a candidate gene for hypoxia adaptation in genomic analyses of several high-altitude animals (27), including wild species such as deer mice (30) and Tibetan wolves (28), but experimental validation and follow-up testing are lacking in the majority of cases. As discussed below, two studies reported genotype–phenotype associations and experimental results that suggest hypotheses about possible mechanisms of physiological adaptation.

### ***EPAS1* polymorphism in North American deer mice.**

A population genomic analysis of deer mice revealed a striking altitudinal pattern of variation in *EPAS1* across western North America, and tests that controlled for demographic history provided strong evidence that the observed allele frequency variation was shaped by a history of altitude-related selection (30). In contrast to the pattern of nucleotide variation in Tibetan humans, which is limited to noncoding sites, a nonsynonymous polymorphism in deer mouse *EPAS1* exhibits the largest altitudinal change in allele frequency. The derived amino acid variant at this site exhibits a steep altitudinal cline in frequency from the Great Plains to the crest of the Front Range of the Southern Rocky Mountains. Using segregating amino acid variation in an alpine population of deer mice living at 4,350 m, Schweizer et al. (30) tested for associations with a broad range of respiratory and cardiovascular phenotypes. Although high-altitude deer mice exhibit a less-pronounced increase in Hb concentration than lowland conspecifics in response to chronic hypoxia (36, 91)—similar to the case with Tibetan humans—the highland *EPAS1* variant exhibited no association with either Hb concentration or Hct (30). Likewise, no associations were detected with capillary density or oxidative capacity of skeletal muscle, O<sub>2</sub> consumption, hypoxia-induced depression of body temperature, or pulmonary O<sub>2</sub> extraction. However, there was a significant association with resting heart rate under hypoxia, a trait that was already known to exhibit genetically based differences between highland and lowland deer mice (143). An increase in heart rate (if not offset by corresponding reductions in stroke volume) increases cardiac output and should therefore increase circulatory O<sub>2</sub> delivery. Unlike lowland humans and rats (69, 144), deer mice increase cardiac output at  $\dot{V}O_2$ max during acclimatization to hypoxia, a plastic response that makes a significant contribution to aerobic capacity at high altitude (74, 75). It is therefore possible that heart rate represents the direct phenotypic target of selection, but it is probably more likely that the observed change represents a secondary consequence of selected changes in other components of the cardiovascular system that are regulated by *EPAS1*.

In addition to the association between *EPAS1* and cardiac function, mice with alternative *EPAS1* genotypes exhibited subtle but statistically significant differences in genome-wide gene expression in two tissues that determine cardiac function in hypoxia: the adrenal gland (which regulates vasoconstrictive responses via secretion of catecholamines) and the left ventricle of the heart. In the adrenal gland, *EPAS1* genotype was associated with a downregulation of genes involved in catecholamine synthesis, and in the left ventricle of the heart, *EPAS1* genotype was associated with an upregulation of HIF target genes.

In summary, the pattern of allele frequency variation in *EPAS1* and the associations with cardiovascular function and pathway-specific gene regulation suggest that the observed variants have contributed to hypoxia adaptation in high-altitude deer mice (30). As in the case with the studies of *EPAS1* in Tibetan humans, the evidence for selection is clear, but insights into causal mechanisms of physiological adaptation will require much additional experimental work.

### ***EPAS1* polymorphism in Tibetan horses.**

To test for evidence of altitude-related selection in native horse breeds of China, Liu et al. (29) sequenced whole genomes of 138 horses representing 17 Chinese native breeds over a broad range of altitudes, from the lowlands of northeast China to the highland Qinghai–Tibet Plateau. The population genomic data provided insights into the relationships and histories of different horse breeds and revealed that China was originally populated by a single lineage of modern domestic horses in the early Bronze Age (~3,700 years ago). This founding lineage subsequently gave rise to the geographically distinct breeds that are recognized today.

Genome scans of allelic differentiation identified *EPAS1* as an extreme outlier in comparisons between high-altitude horses from the Qinghai–Tibet Plateau and closely related lowland breeds. Subsequent genotyping in a panel of 908 horses revealed two nonsynonymous polymorphisms in the highly conserved PAS domains of the *EPAS1*-encoded protein (HIF2 $\alpha$ ), and frequencies of the derived alleles at both sites exhibited significant, positive associations with altitude of residence. Seemingly consistent with reported results of human studies, one of the two *EPAS1* SNPs was associated with numerous hematological changes, including a reduced Hb concentration at high altitude. However, these results do not lend themselves to straightforward interpretation because the set of phenotyped animals were sampled and measured at different altitudes (Hequ horses from 3,500 m and Guanzhong horses from sea level), so altitude-related differences in the measured hematological traits undoubtedly reflect differences in acclimatization history. It is therefore all the more surprising that horses living at 3,500 m were reported to have lower Hb concentrations than those living at sea level, as data from other mammals lead us to expect exactly the opposite. Reverse genetics experiments of the horse *EPAS1* variants also suggest that the observed missense mutations could have phenotypic consequences. Co-immunoprecipitation experiments revealed that one of the two mutations in the PAS domain increased heterodimerization affinity between HIF2 $\alpha$  (product of *EPAS1*) and its dimerization partner, HIF1 $\beta$  (product of the *aryl hydrocarbon receptor nuclear translocator, ARNT*), which conceivably enhances stability of the dimeric protein. Moreover, experimental measures of gene expression in transfected cells revealed that the same amino acid replacement is associated with an upregulation of downstream target genes in the HIF pathway. Results of this study of Tibetan horses provide compelling evidence for positive selection on *EPAS1*, but—similar to the studies of Tibetan humans—there is no conclusive evidence that allelic variation in this gene contributed to a response to direct selection on erythropoiesis.

In summary, multiple population genomic studies indicate that *EPAS1* has contributed to a response to local selection in high-altitude populations, but the phenotypic target of selection still remains unclear. Even when genotype–phenotype associations have been documented, it is difficult to rule out the possibility that changes in the implicated trait represent secondary consequences of selectively mediated changes in other physiological traits involved in the sensing, transport, or utilization of O<sub>2</sub>. Given that *EPAS1* and other components of the HIF pathway mediate the transcriptional regulation of diverse pathways in different tissues and

organs, it is also possible that the same gene has contributed to a response to selection on different traits in different species.

### Hypoxic Pulmonary Hypertension and Cardiac Hypertrophy

In response to localized hypoxia in the lung (e.g., owing to inflammation), the constriction of pulmonary arterioles shunts blood flow to better-ventilated, nonhypoxic regions, thereby enhancing O<sub>2</sub> uptake by matching ventilation and perfusion. At high altitude, by contrast, environmental hypoxia affects the entirety of the lung, and the constriction of pulmonary arteries is pervasive rather than localized. This global vasoconstriction response leads to a series of detrimental changes in the pulmonary vasculature (hypoxic pulmonary hypertension) that impairs O<sub>2</sub> uptake and overburdens the heart, causing right-ventricle hypertrophy and, in severe cases, pulmonary edema (145, 146). This maladaptive pulmonary vasoconstrictive response to environmental hypoxia is attenuated in Tibetan highlanders and several other high-altitude mammals and birds (147-151), potentially representing independently evolved mechanisms of genetic compensation (87, 91). For example, high-altitude deer mice show no change in relative right-ventricle mass following 20 weeks of laboratory acclimation to hypobaric hypoxia when compared with normobaric controls. By contrast, a closely related lowland congener, the white-footed mouse (*Peromyscus leucopus*), exhibits increases in relative right-ventricle mass of nearly 50% upon exposure to the same level of hypoxia (151). Right-ventricle hypertrophy in white-footed mice is associated with upregulation of two transcriptional modules that are enriched for genes in the interferon regulatory factor (*IRF*) pathway that mediate inflammatory responses. The expression of genes in the *IRF* pathway is associated with pulmonary hypertension, and misregulation of *IRF* genes is associated with several cardiovascular disease states in both rodents and humans. Neither the hypertrophy-associated transcriptional modules nor the individual *IRF*-signaling genes (*IRF1*, *IRF7*, and *IRF9*) are differentially expressed following acclimation in highland deer mice (Figure 5). Further experimental work is needed to unravel the cause-and-effect relationships between *IRF* signaling, hypoxic pulmonary vasoconstriction, and right-ventricle hypertrophy, but high-altitude mice appear to be a useful model to examine the naturally evolved attenuation of hypoxic pulmonary hypertension.

Inferences about the adaptive value of environmentally induced changes in gene expression require an understanding of how the developmental and physiological integration of individual components of plasticity affect the net plasticity of higher-level performance traits that have a more direct bearing on fitness. When integration among subordinate traits involves mutual inhibition, the plastic response of individual traits may present the appearance of maladaptation even when the net plastic response of the higher-level performance trait is in the adaptive direction (152). As Lande (152, pp. 11368–69) stated, “Apparent maladaptation of a component of net plasticity in a complex character cannot be taken literally, without understanding the developmental integration of the components.”

## CONCLUSIONS AND FUTURE OUTLOOK

Population genomic data can provide indirect evidence for a past history of positive selection on a particular gene or set of genes, and studies of HIF pathway genes in human populations demonstrate the value of such data for generating hypotheses. However, in the absence of functional experiments, lists of selection-nominated candidate genes and gene ontology enrichment analyses provide no direct insight into mechanism and often lend themselves to adaptive storytelling. Future advances in the field require integration of genomic analyses with mechanistic physiological approaches, as exemplified by several recent studies. For example, identification of locus-specific signatures of positive selection on HIF genes and preliminary genotype–phenotype associations have inspired experimental efforts to investigate effects on systemic physiology, performance, and reproduction (21, 30, 35, 39, 71-73, 79, 153). As a complement to such efforts, reverse genetics experiments have been used to test hypotheses about molecular mechanisms underlying changes in gene expression or protein function in hypoxia-relevant pathways (25, 47, 48, 51, 52, 54, 57-60, 119, 154-163).

Studies of model high-altitude species such as deer mice are advancing our understanding of hypoxia adaptation by integrating population genomic and functional genomic approaches with mechanistic approaches in systemic physiology (30, 32, 34, 36, 37, 40-44, 46, 50, 66, 74-77, 103, 143, 151, 164-168). Whereas transcriptomic analyses of humans have been largely limited to cell-type surrogates, such as peripheral blood lymphocytes, studies of mice can investigate genome-wide patterns of gene expression in a diverse array of organs, tissues, and cell types from a full range of developmental stages and can therefore provide deeper insights into regulatory mechanisms of hypoxia adaptation and acclimatization. Moreover, the ability to perform genetic crosses as well as common-garden and reciprocal-transplant experiments permits rigorous analysis of environmental and genetic components of variation in phenotypes of interest (62). Many other high-altitude animal species hold similar promise for mechanistic studies of high-altitude adaptation.

In studies of high-altitude natives, the integration of genomic approaches with mechanistic studies of systemic physiology provides a means of establishing causal connections between genotype and phenotype in the context of a well-defined environmental selection pressure. Insights into mechanisms of hypoxia adaptation are critical for identifying phenotypic targets of selection and may also help translate such discoveries into medical and veterinary applications (64, 169, 170).

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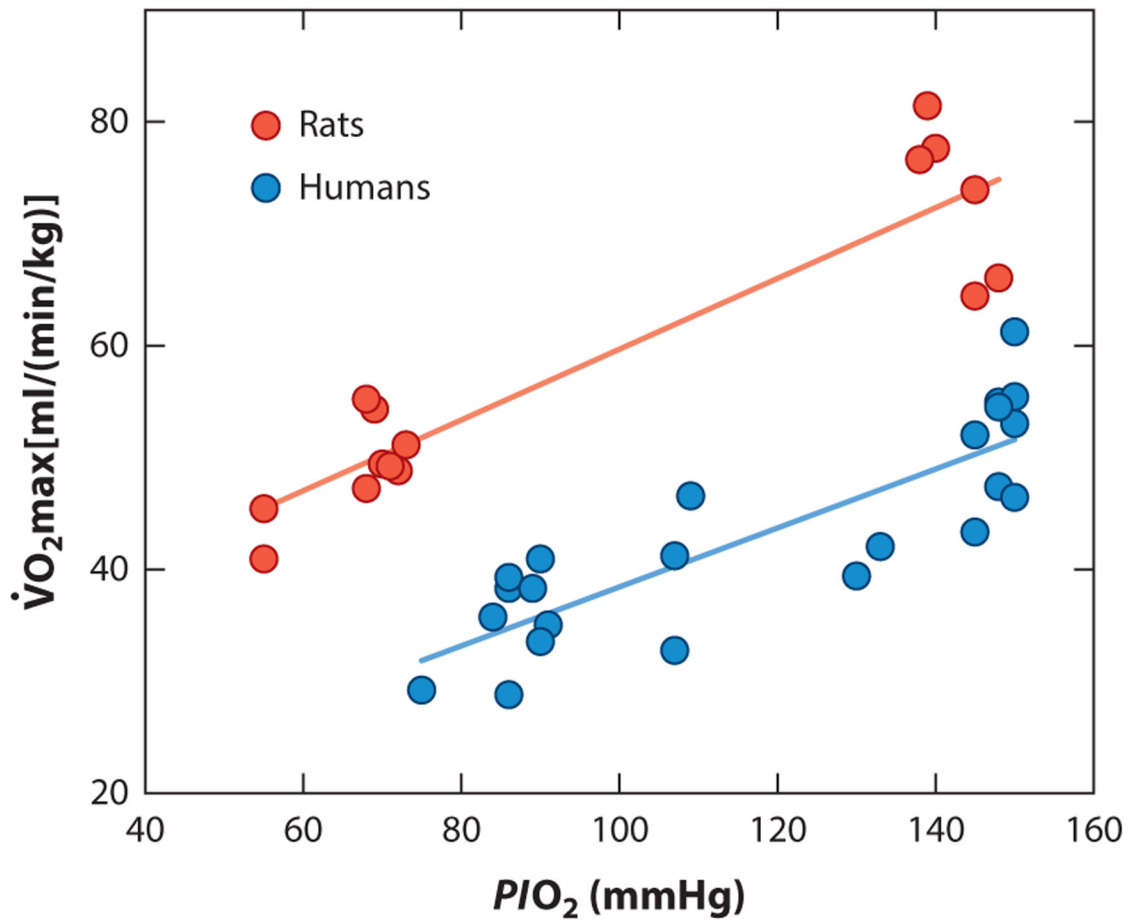
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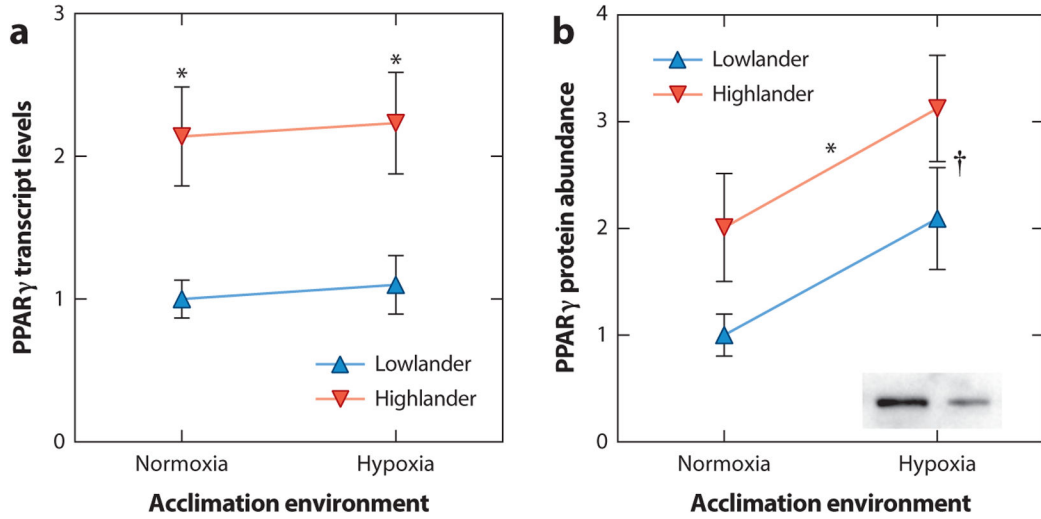
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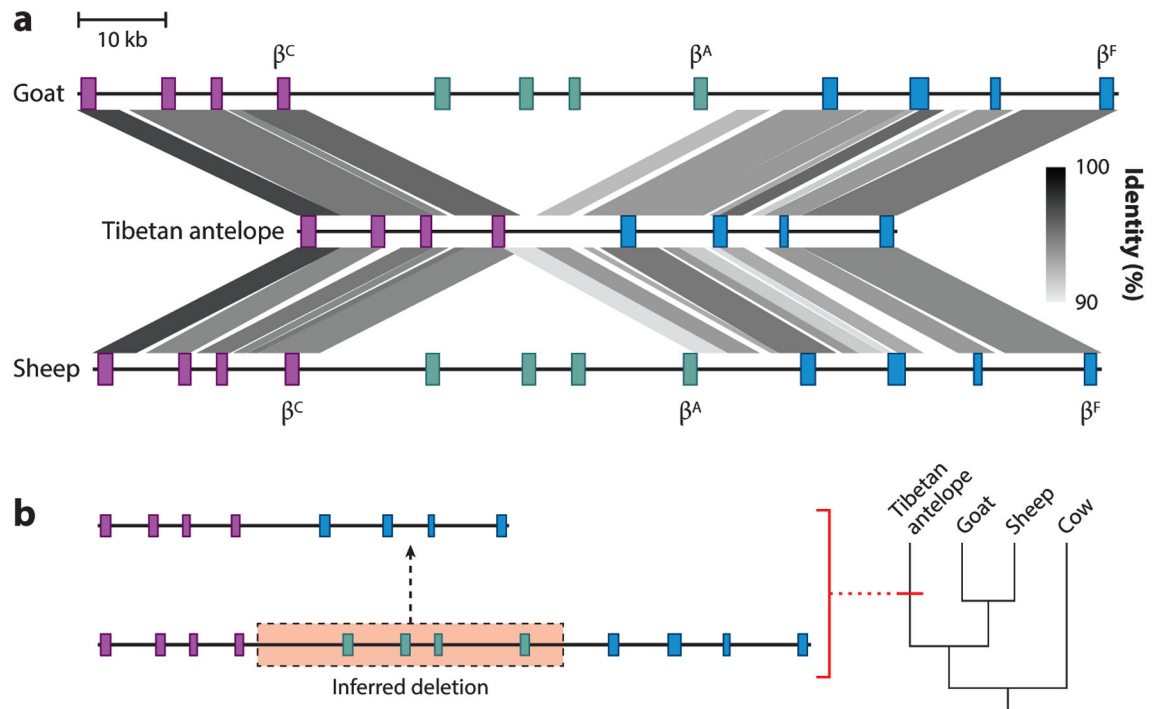
**Figure 1.** Effects of acute hypoxia on aerobic exercise capacity, as measured by maximal rates of  $O_2$  consumption ( $\dot{V}O_2\text{max}$ ), in humans and rats. The lower the partial pressure of  $O_2$  of inspired air ( $PIO_2$ ), the greater the degree of hypoxia. Data points represent average values from studies compiled by Gonzalez & Kuwahira (69). Figure adapted with permission from Gonzalez & Kuwahira (69); copyright 2018 John Wiley and Sons.





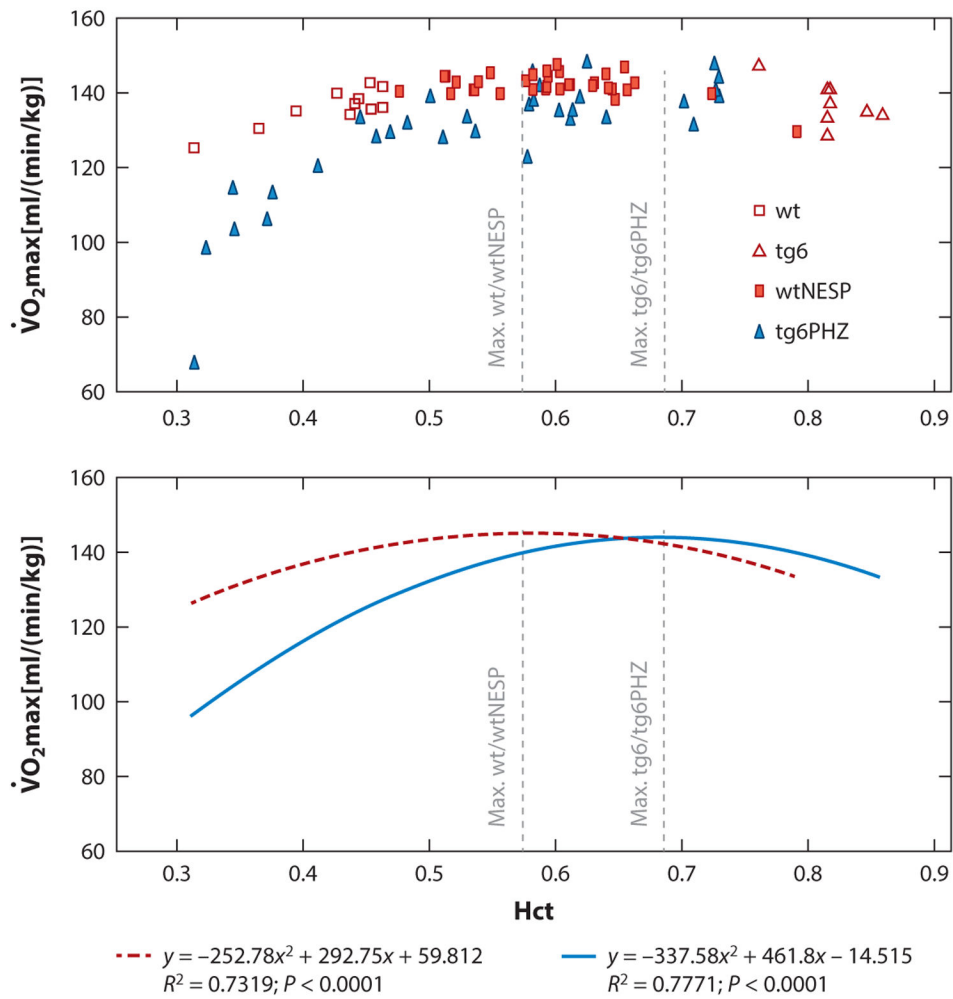
**Figure 2.**

Deer mice with highland ancestry have higher constitutive peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) expression in the gastrocnemius muscle, as revealed by common-garden experiments involving descendants of high- and low-altitude natives. (a) In the case of PPAR $\gamma$  (*Pparg*) transcript levels, native altitude had a significant main effect ( $*F_{1,20} = 14.48$ ;  $P < 0.001$ ), but acclimation treatment did not ( $F_{1,20} = 0.11$ ;  $P = 0.748$ ). (b) In the case of PPAR $\gamma$  protein abundance, there were significant main effects of both native altitude ( $*F_{1,24} = 5.36$ ;  $P = 0.030$ ) and acclimation treatment ( $\dagger F_{1,24} = 6.25$ ;  $P = 0.020$ ). There were no significant interaction effects in either case. Inset shows representative immunoreactive bands for a highlander (*left*) and a lowlander (*right*), each acclimated to hypoxia. Figure adapted with permission from Lui et al. (36); copyright 2015 American Physiological Society.



**Figure 3.**

Large-scale deletion in the  $\beta$ -globin gene cluster of Tibetan antelope is revealed by analysis of pairwise sequence matches with homologous chromosomal regions in other bovids. Purple, green, and blue colored boxes represent members of triplicated gene blocks containing the genes that encode the  $\beta$ -type subunits of juvenile ( $\beta^C$ ), adult ( $\beta^A$ ), and fetal ( $\beta^F$ ) Hb isoforms, respectively. (a) Gray shading denotes percent sequence identity between homologous  $\beta$ -globin gene clusters. (b) An ~45-kb chromosomal deletion in the  $\beta$ -globin gene cluster of Tibetan antelope resulted in secondary loss of the  $\beta^A$ -containing gene block. Figure adapted with permission from Signore & Storz (60); copyright 2020 American Association for the Advancement of Science.



**Figure 4.**

Optimal hematocrit (Hct) is the level that maximizes aerobic exercise performance (measured by  $\dot{V}O_2\text{max}$ ). To estimate optimal Hct for  $\dot{V}O_2\text{max}$  and other measures of endurance exercise performance, Schuler et al. (130) experimentally generated a broad range of Hcts by using four sets of mice: wild-type mice (wt), wild-type mice that had their Hcts increased by means of an erythropoiesis-stimulating treatment (wtNESP), transgenic mice that exhibit excessive erythrocytosis owing to overexpression of human Epo (tg6), and the same transgenic mice that had their Hcts reduced by means of a hemolysis-inducing treatment (tg6PHZ). The estimated relationship between Hct and  $\dot{V}O_2\text{max}$  revealed that exercise performance is maximized at higher Hcts in polycythemic mice that had their Hcts experimentally reduced (tg6PHZ) than in wild-type mice that had their Hcts experimentally increased (wtNESP). This likely reflects compensatory cardiovascular adjustments in the mice that experience chronic erythrocytosis. In both groups, optimal Hcts for exercise performance were substantially higher than the normal resting levels (represented by values for wild-type controls). Regression plots for the wtNESP and tg6PHZ mice are shown in the bottom panel. For both groups of mice, the relationship between  $\dot{V}O_2\text{max}$  and Hct could be described as a second-degree polynomial function. In both upper and lower panels, vertical

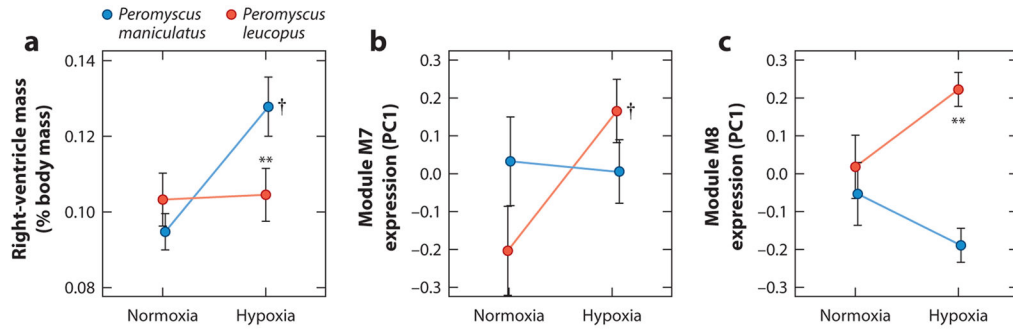
dotted lines denote the Hct values at which  $\dot{V}O_2\text{max}$  is maximized (the optimal Hcts) in both wtNESP and tg6PHZ mice. Figure adapted with permission from Schuler et al. (130); copyright 2010 National Academy of Science.

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**Figure 5.**

Maladaptive plasticity in the pulmonary vasoconstrictive response to hypoxia is blunted in highland deer mice (*Peromyscus maniculatus*). (a) Relative right-ventricle mass, expressed as a percentage of total body mass, increases with hypobaric hypoxia (60-kPa) acclimation in lowland white-footed mice (*Peromyscus leucopus*) but not in highland *P. maniculatus*. Plotted values are mean relative mass ± standard error of the mean (SEM). (b,c) Relative right-ventricle mass in both species is associated with the expression of two transcriptional modules (M7 and M8) that are significantly enriched for genes that participate in inflammatory responses and the interferon regulatory factor pathway. Overall module expressions are summarized using principal component analysis and expressed as PC1 scores. Plotted values are mean PC1 values ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$  effect of species within treatment (ANOVA). † $P < 0.05$  effect of treatment within species (ANOVA). Figure modified with permission from Velotta et al. (151); copyright 2018 John Wiley and Sons.