Genetic variation in *PTPN1* contributes to metabolic adaptation to high-altitude hypoxia in Tibetan migratory locusts

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Animal and human highlanders have evolved distinct traits to enhance tissue oxygen delivery and utilization. Unlike vertebrates, insects use their tracheal system for efficient oxygen delivery. However, the genetic basis of insect adaptation to high-altitude hypoxia remains unexplored. Here, we report a potential mechanism of metabolic adaptation of migratory locusts in the Tibetan Plateau, through whole-genome resequencing and functional investigation. A genome-wide scan revealed that the positively selected genes in Tibetan locusts are predominantly involved in carbon and energy metabolism. We observed a notable signal of natural selection in the gene *PTPN1*, which encodes PTP1B, an inhibitor of insulin signaling pathway. We show that a *PTPN1* coding mutation regulates the metabolism of Tibetan locusts by mediating insulin signaling activity in response to hypoxia. Overall, our findings provide evidence for the high-altitude hypoxia adaptation of insects at the genomic level and explore a potential regulatory mechanism underlying the evolved metabolic homeostasis.
A
imals, including humans, have evolved both physiolo-
gical and genetic adaptation to low-oxygen availability in
high-altitude regions in their range of expansion. For
example, the Tibetan Plateau has an average altitude of
approximately 4000 m and represents one of the most extreme
environments inhabited by humans and numerous animals.
Many wild animals and indigenous human populations, as well as
their domesticated animals, have highly adapted to life with
hypobaric hypoxia. Revealing the mechanisms underlying orga-
nismal adaptation to high-altitude hypoxia is attracting con-
siderable attention and can contribute to our understanding of
hypoxia-featured human diseases, such as heart failure and var-
ious cancers.

Enhancing tissue oxygen uptake and delivery is crucial to such
adaptation to lifelong hypoxia. For example, Tibetans can main-
tain a normal hemoglobin concentration but have a higher level of
blood flow than lowlanders, whereas Andean natives have high
hemoglobin concentration1. Meanwhile, yaks in the Tibetan Pla-
ateau and deer mice in the Rocky Mountains have hemoglobin with
high-oxygen affinity2. Thus hemoglobin is undergoing natural
selection in various organisms and is associated with enhanced
oxygen delivery3–6. Genetic studies on human and other mam-
mals based on whole-genome sequencing revealed strong selection
on hypoxia-inducible factor (HIF) pathway-associated genes, such as
EPAS1 and EGLN1, which are involved in the regulation of
hemoglobin concentration and angiogenesis7–9.

Adaptation to high-altitude hypoxia involves mechanisms other
than enhanced oxygen delivery. Several studies have emphasized
the central role of metabolic changes in hypoxia adaptation. For
example, Himalayan Sherpas demonstrate metabolic adaptation at
high altitude. Compared with lowlanders, Sherpas exhibit
improved tricarboxylic acid (TCA) cycle and oxidative phos-
phorylation, as well as reduced fatty acid oxidation in the skeletal
muscles10. Genetic variant analysis has also implicated the invol-
vement of metabolic pathways in the adaptive processes, such as
those in yak11, Tibetan antelope12, Tibetan ground tit13, and some
domesticated animals14,15. However, the genetic mechanisms behind
the adaptive metabolic changes remain unclear.

Animals with wide altitudinal distribution represent a pro-
mising system for unveiling the genetic basis of hypoxia adap-
tation. However, related studies mainly involve vertebrates rather
than invertebrates. The insect tracheal system, with a completely
different gas exchange system, transports oxygen directly into
tissues. Under artificial hypoxia, insects exhibit a series of adap-
tive features, such as decreased body size, extended tracheal
terminals, enhanced hypoxia resistance, and genetic changes16–19.

The migratory locust Locusta migratoria is one of the most widely
distributed pests in the Old World and has inflicted disastrous
damage to agriculture. The Tibetan locust populations colonized
the Tibetan Plateau over 34,000 years ago and can thrive at 4200
m in altitude20. Tibetan locust populations have evolved distinct
morphological and physiological traits that render them super-
iorly able to cope with the chronic shortage of ambient oxygen.

Tibetan locusts have much stronger hypoxia tolerance and
smaller body size than conspecific lowland locusts (Fig. 1a).
Comparative analysis reveals that Tibetan locusts efficiently uti-
lize aerobic metabolism in response to extreme hypoxia21. Tibe-
tan locusts’ musculature displays a well-maintained
mitochondrial structure and enhanced cytochrome c oxidase
activity that endow them with excellent long flight and locomotor
abilities under hypoxia22. Thus Tibetan migratory locusts are an
ideal model to study the mechanisms of adaptation to high-
altitude hypoxia, although the genetic mechanism underlying
such adaptation remains unexplored.

In the current study, we performed whole-genome resequen-
cing and transcriptional analysis of Tibetan and lowland locust
populations, as well as functional investigations of adaptive
metabolic changes. Genetic analysis revealed positive selection of
genes enriched in energy metabolism pathways, and the adaptive
response of these pathways to hypoxia was corroborated by tran-
scriptional analysis. A PTPN1 coding variant shows strong
signals of differentiation between altitudes. PTPN1 encodes
tyrosine-protein phosphatase non-receptor type 1 (PTP1B), an
inhibitor of insulin receptor. The coding variant facilitates
maintaining of homeostasis of carbohydrate and energy meta-
bolism under hypoxia by regulating the activity of the insulin/
inulin-like growth peptide signaling (IIS) pathway in Tibetan
locusts. Our findings reveal a specific mechanism for metabolic
adaptation to high-altitude hypoxia by insects and improve the
understanding of the complex biological features of high-altitude
adaptation in animals.

Results
Genomic differentiation and demographic history. We per-
formed whole-genome resequencing of 22 individuals represent-
ing two geographically distinct migratory locust populations in
China; 12 individuals were from the Lhasa, Doliung, Shannan,
and Maizhikungga localities in the Tibetan Plateau, and 10 were
from the Dongfang (Dongf) and Danzhou (Danzh) localities in
Southern lowland (Fig. 1a and Supplementary Table 1). The
Tibetan locusts were approximately 20% smaller in body size than
lowland locusts, although they were taxonomically the same
species. The divergence in body size between the Tibetan and
lowland locusts persisted even after two generations’ rearing
under normoxic condition in the laboratory (Supplementary
Fig. 1).

A total of 1675 Gbp of high-quality data were uniquely mapped
to the 6.5 Gbp locust reference genom23. The average sequencing
depth of each individual was at least 10× (Supplementary Table 2
and Supplementary Fig. 2). After applying quality-control criteria,
we identified a total of 12.1 M high-quality single-nucleotide
polymorphisms (SNPs). Our analysis was based on these whole-
genome SNP variations.

The neighbor-joining (NJ) tree of the locusts featured two
distinct clusters (Fig. 1b). The Tibetan and lowland geographical
populations formed two branches and were distinctly divided
from each other. These groupings were also supported by
principal component analysis (PCA; Fig. 1c). In PCA, the first
PC explained 27.34% of the total variation that separated the
Tibetan and lowland populations. Genetic structure analysis
demonstrated a clear division between Tibetan and lowland
populations at K = 2. At K = 3, Lhasa and non-Lhasa populations
in Tibetan locusts were divided. At K = 4, Dongf and Danzh
populations in lowland locusts were separated (Supplementary
Fig. 3). Elevated genome-wide linkage disequilibrium (LD)
(Fig. 1d) and homozgyosity level (Supplementary Fig. 4) but
reduced genetic diversity ρ (Fig. 1e) were observed in the Tibetan
populations.

To reconstruct the demographic history of the Tibetan and
lowland locusts, we initially used the pairwise sequentially
Markovian coalescent (SMC) method24. Analysis results showed
that the effective population size (Ne) of the Tibetan and lowland
locusts were highly correlated 90,000 years ago. A peak of the Ne
at 1 million years ago and a constant reduction since then was
observed. The Ne of lowland locusts appeared to be less affected
by last glacial period than the Tibetan locusts. In the last glacial
maximum (LGM), the Ne of the Tibetan locusts reached the
lowest level and recovered slightly after LGM (Supplementary
Fig. 5a). We then performed multiple SMC (MSMC) approach to
evaluate divergence time25. The curve of relative cross coalescence
rates (CCRs) was 0.5 at approximately 90,000 years ago and

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transcriptomic analysis of the thoracic muscle of Tibetan and
pathways account for hypoxia adaptation, we conducted
mentary Table 3). These pathways are all related to metabolic reg-
fection in the GO terms, namely, cofactor metabolic process
Gene Ontology (GO) analysis of PSGs demonstrated high
value and variance. Maps were generated using DIVA-GIS (http://www.diva-gis.org/). Photographs of locusts were taken by D.D.
reached maximum values at about 1 million years ago. The curve
converged to zero for recent times, indicating hardly any gene
flow between the two populations (Supplementary Fig. 5b). Thus
we estimated that the two populations split at approximately
90,000 years ago.

**Evidence for metabolic adaptation in Tibetan locusts.** To
identify the genomic region that present signatures of natural
selection associated with high altitude, we performed whole-
genome genetic differentiation analysis between Tibetan and
lowland locusts. Using 5% maximum Z-transformed fixation
($Z_{ST}$) and 5% minimum pooled heterozygosity ($ZH_p$), we
identified a total of 113.8 Mb genomic regions that covered all
outliers (Fig. 2a). These regions contained 484 annotated posi-
tively selected gene (PSG) candidates (Fig. 2a). Among these
PSGs, 137 showed amino acid substitution. Kyoto Encyclopedia
of Genes and Genomes (KEGG) signaling pathway analysis
demonstrated that fatty acid biosynthesis ($P = 1.9 \times 10^{-3}$, Fisher’s
Exact Test), insulin signaling pathway ($P = 0.015$), fatty acid
metabolism ($P = 0.038$), and aldosterone-regulated sodium
reabsorption pathway (which is closely related to carbohydrate
digestion and absorption, $P = 0.040$) were the most highly enri-
ched (Fig. 2b). These pathways are all related to metabolic reg-
ulation. Gene Ontology (GO) analysis of PSGs demonstrated high
enrichment in the GO terms, namely, cofactor metabolic process
($P = 0.015$) and oxidoreduction coenzyme metabolic process
($P = 0.030$), which are involved in energy metabolism (Supple-
mentary Table 3).

To determine whether the enriched metabolic regulatory
pathways account for hypoxia adaptation, we conducted transcriptomic analysis of the thoracic muscle of Tibetan and
lowland locusts treated with 10% partial oxygen pressure ($P_{O2}$)
hypoxia. The thoracic muscle of adults was subjected to the
analysis because this tissue was actively involved in anaerobic
and aerobic metabolism (Supplementary Fig. 6). Gene expression
levels under normoxic and hypoxic conditions were compared.
Interestingly, we identified more differentially expressed genes
(DEGs) in Tibetan locusts than in lowland locusts (420 vs. 280,
respectively). More genes were upregulated than downregulated
in Tibetan locusts (231 vs. 189, respectively), a pattern in contrast
to that observed in the lowland locusts (120 vs. 160, respectively)
(Fig. 2c). KEGG analysis demonstrated that the DEGs were highly
enriched in lowland locusts’ pathways related to carbohydrate
and energy metabolism, i.e., citrate cycle ($P = 7.3 \times 10^{-5}$, Fisher’s
Exact Test), carbon metabolism ($P = 2.2 \times 10^{-3}$), oxidative
phosphorylation ($P = 0.015$), and pyruvate metabolism
($P = 0.039$). Conversely, no significant enrichment of KEGG
pathways was observed in Tibetan locusts (Fig. 2d). The DEGs
involved in energy metabolism showed overall suppressed
expression after hypoxia induction in lowland locusts but were
stably expressed in Tibetan locusts (Fig. 2e). The differential
expression of glycolysis-related genes in response to hypoxia
between the two locust populations was further validated by
quantitative PCR (qPCR) (Supplementary Fig. 7). GO analysis
also exhibited a similar pattern in metabolic processes (Supple-
mentary Fig. 8). These results indicate that energy metabolism
in lowland locusts is highly repressed by hypoxia, whereas Tibetan
locusts evolved metabolic robustness against hypoxic stress.

Therefore, both whole-genome genetic and expression analysis
pointed to the involvement of energy metabolic pathways in
hypoxia adaptation. In addition to the genes involved in
metabolism, the top 20 genes with the highest $Z_{ST}$, cross-
population composite likelihood ratio (XP-CLR), or $\Delta Z_{HP}$ value

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**Fig. 1** Phylogenetics of migratory locust based on whole-genome SNPs. a Geographical distribution of collected locust samples used for whole-genome re-
sequencing. Tibetan and lowland locusts are shown at the top-right corner. b Unrooted neighbor-joining tree of migratory locust. c Principle component
(PC) analysis. d Patterns of LD (linkage disequilibrium) decay across the genome in different geographic populations. $r^2,$ Pearson’s correlation coefficient.
e Significant difference in population nucleotide diversity ($\alpha$), as evaluated using the Wilcoxon rank-sum test ($P < 2.2E−16$). The boxplot represents mean
value and variance. Maps were generated using DIVA-GIS (http://www.diva-gis.org/). Photographs of locusts were taken by D.D.
were examined. Genes involved in tracheal growth (if and Aggf1),
wing and muscle development (osa, su(dx), CAP1, and Klhdc1),
cell differentiation and division (Efhc1, D1Pas1, Gas2l1, and
Ctdspl2), and oxidative stress response (stau2) were likely
involved in the adaptation to hypoxia or high altitude in Tibetan
locusts (Supplementary Tables 4–6).

Positive selection of the PTPNI variants. To identify the major
genetic loci responsible for metabolic adaptation in Tibetan locusts,
we analyzed the 13 PSGs that were enriched in energy metabolism-
related pathways, namely, PTPNI, PIK3CD, FAS1, FAS2, FAS3,
ACO1, FARI, SDHD1, Ndpq8, ADIPOR, ATP1A2, VLCAD, and
PPT1. Among these PSGs, only PTPNI, FAS1, FAS3, ATP1A2, and
PPT1 had nonsynonymous mutations (Supplementary Table 7).
PTPNI locus showed a high XP-CLR score, a high average LD (r²
= 0.8) and a low Tajima’s D (Supplementary Fig. 9). These results
suggest that PTPNI is under strong positive selection at high alti-
tude. To further confirm the association of PTPNI variants with
altitude but not with latitude, we performed whole-genome rese-
quencing of two additional locusts from North China lowlands
(Supplementary Tables 1 and 2). PCA demonstrated that
PTPN1 variants in Tibetan locusts were separated from the locusts in both
South and North China lowlands (Fig.3a).

PTPN1 encodes the protein PTP1B, which is conserved among
insects and humans, especially at the protein tyrosine phospha-
tase (PTP) domain (where 61% similarity was observed between
that of locusts and humans) (Supplementary Fig. 10). High-

Fig. 2 Selective sweep and expression analysis of hypoxia adaptation. a Distribution of Z-transformed fixation (ZFST) and heterozygosity (ZHPP), calculated in 100-kb sliding windows. Genomic region (red point) located at top left of the dash lines (5% of empirical maximum FST and minimum HPP) is identified as high-altitude adaptation-associated region in Tibetan locusts. Genes in this region are positively selected genes (PSGs). b KEGG enrichment of PSGs. c Number of differentially expressed genes (DEGs) in response to hypoxia induction through transcriptome sequencing. d KEGG enrichment of DEGs. Only KEGG terms with P < 0.05 are shown (Fisher’s Exact Test). e Expression pattern of DEGs involved in energy metabolism. Heat map signal indicates log2 fold-change values relative to the median expression level within the group. Yellow signal represents higher expression and blue represents lower expression relative to the median level within the group.
throughput sequencing and Sanger sequencing revealed only one nonsynonymous mutation (c.1046A>T) in PTPN1 in Tibetan locusts, which encodes the amino acid substitution p.Asn349Ile at the proline (Pro)-rich domain of PTP1B (Fig. 3b). The mutation frequency in Tibetan locusts at >3700 m altitudes (Lhasa 3700–4000 m and Shigatze 4100 m) was >98%, whereas that in Ningchi (2840 m), South China lowlands and North China lowlands dropped to 68%, 24%, and 26%, respectively (Fig. 3c). Furthermore, the PTPN1 point mutant in Tibetan locusts at the altitudes of >3700 m showed higher homozygosity than all the other populations (Fig. 3d). The results indicate that the mutation in PTPN1 is under strong directional selection for altitude adaptation in the locust.

PTPN1 mutation attenuates IIS pathway suppression in hypoxia. To determine the effect of the missense PTPN1 mutation on PTP1B enzyme activity, we examined PTP1B activity in vivo. PTP1B negatively regulates the insulin pathway through insulin receptor (InR) dephosphorylation (Fig. 4a). Under normoxic conditions, no difference in PTP1B enzyme activity between Tibetan and lowland locusts was detected. However, PTP1B enzyme activity was elevated by hypoxia induction in lowland locusts but remained stable in Tibetan locusts (Fig. 4b). PTP1B did not respond to hypoxia challenge at both the mRNA and protein levels in lowland and Tibetan locusts (Supplementary Fig. 11a). These results suggest that the alteration of PTP1B activity is due to protein structural change. To examine the causal relationship between the mutation point and enzyme activity of PTP1B, we first conducted an in vitro assay using recombinant PTP1B protein (Supplementary Fig. 11b). The purified recombinant PTP1B showed clear phosphatase activity compared with bovine serum albumin (BSA). However, no significant differences in PTP1B enzyme activity between the wild-type p.Asn349 and mutant-type p.Ile349 were observed (Supplementary Fig. 11c). We then speculated that locust PTP1B activity required hypoxia induction and the presence of cofactors. Thus we overexpressed locust PTP1B in S2 cells and subjected the cells to hypoxia for 6 h. The mutant PTP1B presented a significantly lower enzyme activity than the wild type. The in vitro result was consistent with that observed in vivo, indicating that the
high-altitude dominant mutation attenuates the hypoxic response in terms of PTP1B enzyme activity (Fig. 4c).

Afterwards, we tested the phosphorylation level of InR, the substrate that is dephosphorylated by PTP1B, in both lowland and Tibetan locusts. The phosphorylation level of InR was reduced in lowland locusts after hypoxia induction but remained unchanged in Tibetan locusts. Accordingly, the phosphorylation level of the protein kinase B AKT, an important kinase in the insulin pathway, was also suppressed under hypoxic condition in lowland locusts but remained normal in Tibetan locusts (Fig. 4d). During this process, the insulin-like peptide (Ilp) produced in the brain and thoracic muscle did not respond to hypoxia induction in both lowland and Tibetan locusts (Supplementary Fig. 12). These results demonstrate that the inhibitory effects of the PTP1B variant on IIS signaling are not caused by reduced Ilp production but by repressed InR phosphorylation.

Finally, we validated the function of locust PTP1B in InR and AKT phosphorylation regulation by PTPN1 gene expression knockdown in vivo. RNA interference (RNAi) through double-strand RNA (dsRNA) injection in lowland locusts dramatically reduced the PTPN1 expression and PTP1B enzyme activity (Student’s $t$ test, $P = 0.009$). The PTPN1 knockdown elevated the phosphorylation levels of InR and AKT under hypoxic conditions (Fig. 4e). Thus the PTPN1 mutation represses InR phosphorylation and downstream insulin signaling pathways in locusts.

**PTP1B regulates metabolic adaptation to hypoxia.** Finally, we attempted to gain insight into the adaptive changes in energy metabolism regulated by the IIS pathway under high-altitude hypoxia. Considering that insulin is an important sugar-homeostasis regulatory hormone in animals and is involved in energy metabolism control, we first examined trehalose uptake ability by measuring trehalose level in hemolymph and thoracic muscle. Trehalose content was significantly increased in the hemolymph but dropped by 40% in the muscle cells in lowland locusts after hypoxia exposure. Glucose, the hydrolysis product of trehalose, was also dramatically reduced in the muscle cells. By contrast, trehalose in hemolymph was reduced but intracellular trehalose and glucose in the thoracic muscle were maintained at normal levels in Tibetan locusts after hypoxia challenge (Fig. 5a).
Second, we measured the glycogen content after hypoxia challenge because glucose is involved in glycogen synthesis or glycogenolysis after entering a muscle cell. Glycogen level dropped by nearly 50% in lowland locusts but did not significantly change in Tibetan locusts. Third, we measured the production of lactate and acetyl coenzym A (CoA), the product of glycolysis. Lactate level was elevated in both lowland and Tibetan locusts after hypoxia induction; conversely, the acetyl-CoA content was reduced only in lowland locusts. We also measured NADH production, an important energy product in TCA cycle and glycolysis. NADH content was significantly reduced in lowland locusts but was elevated in Tibetan locusts after hypoxia induction (Fig. 5b). Meanwhile, we tested the protection role of trehalose in cell survival under hypoxia tension. In situ cell death detection assay indicated that trehalose injection rescued cells from dying induced by severe hypoxic condition in lowland locusts (Supplementary Fig. 13).

We further investigated the effects of PTP1B on energy metabolism. The expression knockdown of PTPN1 in lowland locusts significantly reduced the level of hemolymph trehalose, increased the level of intracellular glucose, and dramatically increased lactate production by nearly three times. The results indicate that the capacity of trehalose uptake in lowland locusts under hypoxia condition improved after suppressing PTP1B activity (Fig. 5c).

We finally examined the fitness effect of PTPN1 knockdown, i.e., the adult lifespan of locusts under hypoxia condition. The dsRNA interference of PTPN1 caused at least 97% reduction in the mRNA and protein levels of PTPN1 expression (Supplementary Fig. 14a). Under normoxic condition, PTPN1 repression had no effect on the lifespan of adult locusts compared with the control. However, upon hypoxic rearing, their lifespan significantly decreased by 5 days after PTPN1 downregulation (Wilcoxon rank-sum test; P = 0.004; Supplementary Fig. 14b). The results indicate that adult locusts living under hypoxia suffers a shortened lifespan upon the repression of PTPN1 gene expression.

**Discussion**

The genetic mechanism for adaptation to high-altitude hypoxia by insects, which employ a tracheal system for oxygen delivery, was investigated in this work. The majority of previous studies on high-altitude adaptation have focused on vertebrates, especially mammals and humans, which use hemoglobin for oxygen transport. Several studies have reported evidence for positive natural selection of genes associated with hemoglobin phenotypes and erythropoiesis. This finding raised an intriguing question as to whether insects have evolved strategies different from those of vertebrates to cope with the lifelong extreme hypoxia environments. We attempted to resolve the question in the migratory locust, which is featured by global distribution and high capacity for adaptation to changing environments, including variation in...
oxygen levels along altitudes. The Tibetan locusts have evolved distinct high-altitude adaptive features in their long-term habitation in the Tibetan Plateau, particularly the superior resistance to hypoxia. Accordingly, we used the Tibetan locust as a model to dissect the genetic basis of its adaptation to high-altitude hypoxia.

In addition to the robust hypoxia resistance and metabolic change of the Tibetan locust, it also exhibits a dramatic reduction in body size that is heritable. Many studies have revealed a close relation between insect body size and ambient oxygen content. In the late Paleozoic when the atmospheric $P_{O_2}$ was hyperoxic (30%), the insects were 10 times larger than those alive today. When reared under an artificial hypoxic condition, some insects such as Drosophila melanogaster and Manduca sexta showed a significantly reduced body size. These findings are consistent with the hypothesis that body size is regulated by a mechanism that senses oxygen limitation. The decrease in body size may be adaptive for insects living in hypoxia, because oxygen diffusion distances are shortened, metabolic expenditure is reduced, and flight locomotor energetics are promoted. However, artificial hypoxia did not affect the final body size of Schistocerca Americana. By contrast, the bumble bee (Bombus) and Drosophila inhabiting high altitudes exhibit larger body sizes than their lowland counterparts. The body size development of insects is regulated by a number of ecological factors such as temperature, nutrition, gut flora, and infection in addition to oxygen supply. Tradeoffs between the response to hypoxia and other factors may constrain body size evolution. Some biotic interactions, such as predation and competition, may also supersede oxygen as the most important constraint on the body size of insects.

Laboratory selection in the fruit fly has demonstrated that the evolution of hypoxia tolerance involves altered gene expression and genetic codes. However, a genomics analysis of honey bees at different altitudes (1100–2900 m) in East Africa reveals nearly no genetic differentiation between lowland and highland populations, although two extended haplotype blocks harboring nearly no genetic differentiation between lowland and highland populations, although two extended haplotype blocks harboring several octopamine receptor genes are highly diverged between the two altitudes. Our genomic variation analysis based on 12 M SNPs revealed a clear differentiation in genetic background between the Tibetan and lowland locusts, although these populations belong to the same lineage based on mitochondrial genomes. Tibetan locusts also presented lower levels of nucleotide diversity and haplotype homozygosity than lowland locusts. These variations may represent directional selection and provide a rich source of candidate selected loci that may involve in the high-altitude adaptation of the insects.

Genetic and gene expression analysis in the locusts recapitulated the evolutionary conservation of metabolic adaptation of insects, animals, and even humans living in sustained hypoxia. Among the PSGs of Tibetan locusts, many were highly enriched in energy metabolism pathways. Transcriptomic analysis demonstrated that almost all genes in carbon and energy metabolism pathways were significantly repressed by hypoxia stress in lowland locusts. By contrast, Tibetan locusts exhibited a metabolic homeostasis in gene expression. These findings are consistent with previous ones from microarray data of lowland locusts. More genes were upregulated by hypoxia in Tibetan locusts than in lowland locusts (Fig. 2c), implying a wide array of adaptive mechanisms evolved in Tibetan locusts to cope with stress. Wild locusts highly depend on energy supplement for their daily activities (e.g., mating, foraging, or predator avoiding), thereby posing a high demand for cellular metabolic homeostasis under chronic hypoxia. Himalayan Sherpas also exhibit enhanced oxygen utilization efficiency and improved muscle energetics under hypoxia. A genetics study on Tibetan ground tits has shown an expansion in genes linked to energy metabolism. Therefore, metabolic adaptation is a crucial and conserved component of adaptive features of high-altitude animals.

The genetic analysis of candidate genes involved in carbohydrate metabolism demonstrated that PTPN1 was under strong positive selection and had high divergence at a genetic level between Tibetan and lowland locusts. PTP1B, the protein encoded by PTPN1, contained a missense mutation p.Asn349Ile in the proline-rich (Pro-rich) domain in Tibetan locusts. The mutation frequency ranging between 26% and 98% is correlated with altitude elevation at 20–4100 m. PTP1B belongs to the PTP superfamily and is known as a negative regulator of the insulin pathway through the dephosphorylation of InR and its substrate insulin receptor substrate. Thus PTP1B is used as a target of drugs for insulin resistance and type-2 diabetes. PTP1B is overexpressed in one-third of human breast cancer patients and is essential for tumor survival during hypoxia by regulating non-mitochondrial oxygen consumption. PTP61F (the Drosophila ortholog of PTPN1) is responsible for the reduced InR activity under hypoxic condition in S2 cells. An evolutionary genomic study of Tibetan wild boar has also revealed that PTPN1 is in the list of high-altitude adaptation-related genes. The lifespan cost resulting from PTPN1 downregulation in a hypoxia environment recapitulates the significant role of this gene in the hypoxia adaptation of the locust. PTPN1 repression induces high IIS activity and energy metabolism, which may cause excessive accumulation of oxidative stress, as shown in Drosophila and Caenorhabditis. Thus, in addition to cellular hypoxia regulation, PTP1B can be crucial to high-altitude hypoxia adaptation.

Our functional studies confirmed that a specific missense mutation in PTP1B altered its enzyme activity of dephosphorylation and insulin pathway signaling regulation in response to hypoxia induction. Hypoxia exposure in lowland locusts caused elevated PTP1B enzyme activity, which led to suppressed insulin pathway and downstream energy metabolism. PTP1B activity in Tibetan locust remained stable upon hypoxia induction. The reduction-of-function mutation of PTP1B facilitated the maintenance of the IIS pathway and energy metabolism in Tibetan locusts under hypoxic condition. In vivo and in vitro experiments confirmed the signaling and metabolic consequences of the mutation in PTP1B. The point mutation of PTP1B is located in the Pro-rich domain, which is a regulatory domain of PTP1B. Four mechanisms are known to regulate PTP1B activity: oxidation at the N-terminal catalytic phosphatase domain, phosphorylation at both catalytic and Pro-rich domain, sumoylation at the Pro-rich domain, and calpain-mediated proteolysis at the C-terminal near the Pro-rich domain. Thus the missense mutation possibly affects the hypoxia-induced PTP1B modification level and attenuates its enzyme activity alteration. Meanwhile, PTP1B expression interference is consequential for the locust’s fitness (e.g., lifespan). Speculatively, this specific mutation has been selected for optimum hypoxia adaptation and survival under high-altitude environments. Further studies are needed to clarify the molecular mechanism of this process.

The current study revealed a role of the IIS pathway in the evolution of high-altitude hypoxia adaptation through metabolic regulation. The IIS pathway is an evolutionarily conserved nutrient-sensing pathway that modulates energy metabolism and development in metazoans. The reduced activity of IIS can lead to the systematic suppression of energy metabolism in fruit fly and this phenotype is similar to the hypoxia-induced alteration of energy metabolism in lowland locusts. Previous studies have also provided evidence of the involvement of the IIS pathway in hypoxia adaptation. Reduction-of-function mutations in the daf-2 gene (encoding an insulin/insulin-like growth factor receptor
homolog) can provide powerful protection from hypoxic injury in Caenorhabditis elegans. The IIS pathway is closely related to growth and tracheal plasticity under hypoxic conditions in Drosophila. Insulin can activate HIF-dependent transcription in both S2 cells and Drosophila embryos. AKT/phosphoinoside-3 kinase signaling in the downstream of the IIS pathway in the skeletal muscle of mice can respond to acute hypoxia. At the genetic level, altitude-related selection loci in speckled teal (Anas flavirostris) also contain genes involved in the IIS, which suggests a conserved regulatory mechanism by IIS underlying the high-altitude hypoxia adaptation. The current study further demonstrated the role of the IIS pathway in energy homeostasis regulation during hypoxia adaptation.

We report a model of hypoxia-induced insulin resistance in locusts. Insulin resistance is an abnormal physiological state that occurs when insulin cannot trigger signaling pathways in target organs, such as the liver, adipose tissues, and muscles. Such physiological models were also established in Drosophila and C. elegans but were achieved by gene knockout or diet induction. Hypothesis can inhibit insulin signaling and induce insulin resistance in adipose tissues originating from obese human or murine, although some studies have reported that high altitude is not a risk factor for insulin resistance-induced type-2 diabetes mellitus in humans. Thus, locusts are a potential model for insulin resistance-related obesity studies. Meanwhile, previous work indicates that trehalol can work as a chaperone and prevent the misfolding of substrate proteins. Our results indeed demonstrated that elevated intracellular trehalose can rescue muscle cells from death under severe hypoxic condition. Thus, in addition to energy metabolism regulation, the maintained IIS pathway in Tibetan locusts can contribute to severe hypoxia tolerance by stimulating trehalose uptake, which in turn protects cells from hypoxia-induced injury. In summary, these findings may help elucidate hypoxia-induced metabolism syndrome in humans.

Adaptation to high-altitude hypoxia requires multiple systems, pathways, and molecular mechanisms. Herein we also identified other PSIGs in multiple pathways, such as tracheal growth and wing and muscle development. Tibetan locusts have thrived in the complex habitats on the Tibetan plateau for tens of thousands of years. We speculate that Tibetan locusts have evolved a good balance between energy metabolism and other adaptive traits (e.g., oxidative-stress prevention). Further studies are needed to improve our comprehension of high-altitude adaptation by insects.

Methods

Locust sampling and maintenance in the laboratory. We sampled 24 migratory locusts for whole-genome re-sequencing, including 12 locusts from four localities in Tibetan plateau ranging between 3700 and 4000 m in altitude, 10 locusts from lowland region (<200 m) in Southern China and 2 locusts from lowland region in Northern China. For Sanger sequencing, we sampled 207 migratory locusts, including 127 from 6 localities in Tibetan plateau, ranging between 2840 and 4100 m in altitude, 39 from four lowland localities (<200 m) in Southern China, and 41 from five lowland localities in Northern China (Fig. 1a and 4c and Supplementary Table 1). For the functional study, field-collected locusts were maintained in the laboratory at the Institute of Zoology, Chinese Academy of Sciences in Beijing (<50 m). All locusts were reared in ventilated cages (50 × 50 × 50 cm) at a density of approximately 150 individuals per cage and fed with fresh wheat seedlings and wheat bran. The culturing environment was kept constant with a 14 h light (L):10 h dark (D) photo regime at 28 ± 1 °C. The cultures were maintained in the laboratory for at least two generations prior to the experiments. The 7-day-old male adults were collected for assays.

Locust body size measurement. The body size of locusts was measured with a Vernier caliper. Field-collected and laboratory-reared locusts were selected randomly for the measurement. Body length was measured from head to wing tip. Wing length was measured from wing root to tip. Femur length is the length of posterior femur. The laboratory-reared locusts were the field-collected locusts rearred in the laboratory for more than two generations. The sample numbers for each measurement: field-collected Tibetan male/female, n = 8/10; laboratory-reared Tibetan male/female, n = 10/10; field-collected lowland male/female, n = 12/12; laboratory-reared lowland male/female, n = 10/10.

Genome sequencing and quality control. A total of 1.5 μg genomic DNA per sample was extracted and used as input material for the DNA sample preparations. Sequencing libraries were generated using the TruSeq Nano DNA HT Sample Preparation Kit (Illumina, USA) following the manufacturer’s recommendations. Index codes were added to attribute sequences to each sample. DNA was then sheared into fragments of 150 bp and sequenced using the Illumina HiSeq X Ten platform. In total, whole genomes of the 24 locusts were sequenced at an average depth of exceeding 10× using a sequencing strategy similar to that applied in the 1000 Genomes Project (see http://www.1000genomes.org; The1000 Genomes Project Consortium 2010). To obtain reliable reads and avoid filter reads with artificial biases that may affect downstream mapping and other analyses, we implemented quality-control procedures to remove the following types of reads: (1) ≥10% unidentifiable nucleotides (i.e., N content); (2) >10% with >10 nucleotides aligned to the adaptor or mismatches; (3) ≥50% of the read bases with a Phred quality score (i.e., Q-score) <5, and (4) Putative PCR duplicates generated in the library construction process. Consequently, we generated 1675.19 Gb of high-quality paired-end reads for the 24 locusts with the quality of ≥94.73% of the bases for Q20 and ≥88.44% for Q30. The detailed information of high-quality data for each sample is summarised in Supplementary Table 2.

Reads mapping and SNP calling. The filtered high-quality reads were mapped to the locust reference genome using Burrows-Wheeler Aligner with the command ‘-t m -k 32 -M 395’. Unmapped and secondary alignment reads were filtered and then sorted by SAMtools (Version:1.3.1). Potential PCR duplicates were also removed using ‘rmdup’ command in the SAMtools software. After alignment, we performed SNP calling on a population scale through GATK tools v0.1.398. The command “mpileup” was used to identify SNPs and indels with the parameters set as ‘-q 1 -C 50 -S -D -m 2 -F 0.002’. To exclude SNP calling errors caused by incorrect mapping or machine error, only high-quality SNPs (coverage depth ≥3 and ≤50, root mean square mapping quality ≥20, maf ≥0.05, miss ≤0.01) were kept for subsequent analysis. Finally, SNP variations were annotated using ANNOVAR software. Genome coverage rate for each sample was calculated by SAM tools. The saturation curve was generated by calculating the coverage rate of a Tibetan sample (sample ID: Tibetan-5) at sequencing depths of 2, 4, 6, and 8 times.

Phylogenetic tree and PCA. To clarify the phylogenetic relationship from a genome-wide perspective, we constructed an individual-based NJ tree based on the p-distance using the software TreeBestv1.9.2 (http://treesofsourceforge.net/treestbest.html). PCA analysis of the 24 samples was conducted using EIGENSOFT3.0 and the significance of eigenvectors was determined using the Trace-Widom test in the EIGENSOFT 3.0.

Population structure. The population genetic structure was examined via an expectation-maximization algorithm, as implemented in the program FRAPPE1.1, which employs the maximum likelihood and expectation-maximization algorithm to estimate ancestry proportions for each individual. The number of assumed genetic clusters K ranged from 2 to 4, with 10,000 iterations for each run.

Demographic history. Changes in N of each group was inferred using a hidden Markov model approach as implemented in pairwise sequentially Markovian coalescence with parameter as follows: N0 = 300, N1 = 15, N2 = 5, N3 = 3, and N4 = 4. Time was measured in units of 2N generations, and the N0 at time t was scaled to N0. The neutral mutation rate μ was used to infer N0 and scale the TMRCa (time to the most recent common ancestor) and N0 values into chronological time. The mean generation time g was set at 0.5 year, and μ was estimated as 0.1 × 10−6. Changes in the divergence of Tibetan and lowland populations were estimated under the MSMC model for four representative samples of each group (Tibetan 6, 7, 8, and 9; Han1 1, 2, 4, and 5).

Genetic diversity and LD. Nucleotide diversity (θ) was calculated using the list of high-quality SNPs for each group. To estimate and compare the pattern of LD for different groups, we computed the squared correlation coefficient (r2) between pairwise SNPs using the software Haploview V4.2. Parameters in the program were set as ‘-n -dr-p-minMAF 0.05’. The average r2 value was calculated for pairwise markers in a 500-kb window and averaged across the whole genome. Tajima’s D values were calculated using VGTOOLS with the parameter ‘-TajimaD’.

Selective sweep. To identify genome-wide selective sweeps associated with high-altitude adaptation, we calculated the average pooled heterozygosity (H) and the genome-wide distribution of fixation index (FST) using a sliding-window approach, which involved 100 kb windows with 50 kb increments. At each detected SNP
position, we counted the number of reads corresponding to the most and least frequently observed allele (nMAI and nMIN, respectively) in each group. The HP for each window was calculated based on the equation \( HP = 2nMAI \times 2nMIN \) (\( nMAI = nMIN \)) - 1, \( n_M = 2^n - 1 \), and \( ZHP = Z-\text{transformed to obtain } ZHP \) and \( ZFbT \). We considered the windows with the top 5% \( ZFbT \) and \( ZHP \) simultaneously as candidate outliers under strong selective sweeps. All outlier windows were assigned to corresponding SNPs and genes.

Cross-population-composite likelihood approach. The XP-CLR was used to perform a genome scanning for selective signals. A 0.005-\( \text{cm}^2 \) sliding window with maximum 200 SNPs assayed was used for scanning. Genetic map of individual SNPs used here was 1 \( \text{cM} \) Mb \(^{-1} \). The command line was XPCRL -xpcrl genofile1 genofile2 outfile File -w 0.005 200 2000 chrn 0.95. Finally, the mean likelihood score was calculated in 50-\( \text{kb} \) sliding windows with a step size of 25 \( \text{kb} \) across the genome. The highest XP-CLR values, accounting for 0.5% of the genome, were considered as selected regions.

Hypoxic treatment. Hypoxic treatment was performed in a hypoxic chamber (FLYDWC-50; Fenglei Co., Ltd, China) in which the ambient temperature and air flow is in automatic control. Twenty male locusts were placed in a cage (20 x 15 x 15 cm) kept in the chamber, into which air was blown and balanced with pure nitrogen to achieve the required \( P_O_2 \). The locusts were used in functional studies was 10 kPa, which is sufficiently induced behavioral, developmental, and metabolic alterations in insects.

Hemolymph samples from at least three locusts were homogenized with 300 \( \mu l \) of \( 4 \text{M} \) potassium bicarbonate solution and cooled on ice for 5 min. We ensured that the pH of the solution was within the range of 6–8. Acetyl-CoA levels were measured using an Acetyl-Coenzyme A Assay Kit (Sigma, MAK039-1KT, USA) following the manufacturer’s protocol and then normalized to protein levels.

Acetyl-CoA measurement. Fresh thorax muscle was deproteinized by 1 M perchloric acid precipitation before thorough homogenization. The samples were centrifuged at 10,000 \( \times g \) for 10 min to remove insoluble material. The supernatant was then neutralized with 3 M potassium bicarbonate solution and cooled on ice for 5 min. We ensured that the pH of the solution was within the range of 6–8. Acetyl-CoA levels were measured using an Acetyl-Coenzyme A Assay Kit (Sigma, MAK039-1KT, USA) following the manufacturer’s protocol and then normalized to protein levels.

NADH measurement. Thorax muscle was freshly homogenized with 400 \( \mu l \) of NADH/NAD Extract Buffer and centrifuged for 10 min at 4 °C at 10,000 \( \times g \). The supernatant was then added to the 10-kD spin column (Abcam, ab93349, USA) and centrifuged at 10,000 \( \times g \) for 20 min at 4 °C. The filtrate was collected and used for NADH assay via the NADH/NAD Assay Kit (Abcam, ab65348, USA). The measured values were normalized to lysate protein levels.

Lactate measurement in hemolymph. Hemolymph samples from at least three locusts were centrifuged for 10 min at 4 °C at 1000 \( \times g \) to remove the hemocyte. The supernatant was used for lactate assay using the Lactate Colorimetric/Fluorometric Assay Kit (Bio vision, K607, USA).

RNAi. RNAi assay was performed to knock down the PTP1N gene expression. The dsRNA of GFP and PTP1N was prepared using the T7 RiboMAX Express RNAi system (Promega, USA) following the manufacturer’s protocol. The 3 \( \mu l \) of dsRNA (9 \( \mu g \)) was injected into 5-day-old male adult locusts at the second ventral segment of the abdomen. A second injection of the same dose was performed after 3 days. The injected locusts were then placed into the hypoxic chamber for hypoxia treatment. After 5 days, PTP1N expression levels were examined by qPCR and western blot. The primers for PTP1N dsRNA synthesis are listed in Supplementary Table 8.

Recombinant expression and purification. Wild- and mutant-type locust PTP1B were recombinantly expressed in Escherichia coli. The coding sequences of the above two proteins were amplified from the locust muscle tissue using the primers PTP1BexF1 and R1. The PCR products were then cloned into pET32a (Novagen, Germany) vector. The recombinant proteins were purified by affinity chromatography using His-Bind resin (GE Healthcare, USA) following the manufacturer’s instructions. The primers for PTP1N cDNA amplification are listed in Supplementary Table 8.

PTPB enzyme activity assay. PTP1B enzyme activity was measured using the PTP Assay Kit 2 (Millipore, 17-126, USA) following the manufacturer’s protocols. For the in vivo assay, the thoracic muscle of locusts was thoroughly homogenized and centrifuged at 10,000 \( \times g \) for 20 min to remove the insoluble material. The samples were then added to a 10-kD spin column (Abcam, ab93349, USA) and centrifuged at 14,000 \( \times g \) for 15 min at 4 °C to eliminate native phosphate. After centrifugation, the samples were re-suspended with 100 \( \mu l \) phosphate extraction buffer. A total of 5 \( \mu l \) supernatant was used for the enzyme activity assay. The measured values were normalized to lysate protein levels. For the in vitro assay, mutated and wild-type PTP1B were separately overexpressed in Drosophila S2 cells (ATCC CRL-1963). After 36 h, the transfected cells were treated with 1% hypoxia for 6 h. PTP1B enzyme activity was then measured by PTP Assay Kit according to the protocols. The measured values were normalized to lysate protein levels. For recombinant protein, 0.5 \( \mu g \) purified recombinant PTP1B and BSA were used for enzyme activity measurement.
TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling) staining assay. Tissue sections were obtained from the thoracic muscle of 5-day-old male locusts. The locusts were first subjected to severe hypoxia (2% P$_{O_2}$) for 6 h. In the trehalose rescue group, locusts were injected with 500 µg of trehalose solution (100 µg µl$^{-1}$ in ddH$_2$O) before hypoxic treatment. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche, 11684817910, USA) following the manufacturer’s instructions. For details, thoracic muscle tissue sections were fixed with 4% paraformaldehyde for 20 min and washed with PBS for 30 min. The fixed tissue sections were then permeabilized with 0.1% TritonX-100 for 30 min at RT and treated with 3 U ml$^{-1}$ DNase1 for 10 min to break the DNA strands. The tissue sections were then sequentially incubated with a TUNEL reaction mixture and 0.3% Hoest (Life, E3570, USA) before washing with PBS three times. The tissue sections were imaged using an LSM 710 confocal fluorescence microscope (Zeiss) at a ×10 magnification. The experiments were repeated at least five times. TUNEL intensity was measured and quantified using Image J.

Lifespan assay. Five days after molting, a total of 120 male locust adults were selected for lifespan measurement. The locusts were kept in four cages with 30 individuals each. Two of the cages were placed under normoxic condition. The other two cages were placed in a hypoxic chamber at P$_{O_2}$ = 10 kPa. The locusts were injected with diRNA three times at a 7-day interval. Survival curves were analyzed using Wilcoxon rank-sum test.

Data availability
The published reference genome of migratory locusts used for mapping is available on LocustBase [https://locustbase.org/7.243/download.html]. Fastq files of the genome sequence for each of 24 locusts are available at BioProject PRJA433458. Fastq files of the transcriptome sequence for hypoxia treatment are available at BioProject PRJA438378. Fastq files of the transcriptome sequence for gene expression in different tissues are available at BioProject PRJA436219. The GenBank accession number for the mRNA sequence of locust PTPIB is MN973608. A reporting summary for this article is available as a Supplementary Information file.

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Acknowledgements
We gratefully acknowledge Yunandan Wang for sharing the tubulin antibody and Peng-cheng Yang for data uploading. We thank Wenfeng Wang, Dawei Hong, Bo Pang, and Bo Liang for Tibetan locust collection. We also thank Mingshan Wang, Shuai Zhan, and Zhushi Huang for their helpful comments. This study was supported by the National Science Foundation of China (grant nos. 31661143048 and 31472048).

Author contributions
B.C. and L.K. conceived the project and designed the study. D.D. designed the study and performed most of the experiments. G.L. performed bioinformatics analyses. L.H. and W. G. helped with experiments. B.C., D.D., and I.K. wrote the paper. All authors read and approved the final manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-07529-8.

Competing interests: The authors declare no competing interests.

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