Demographic History and Genetic Adaptation in the Himalayan Region Inferred from Genome-Wide SNP Genotypes of 49 Populations

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Abstract

We genotyped 738 individuals belonging to 49 populations from Nepal, Bhutan, North India, or Tibet at over 500,000 SNPs, and analyzed the genotypes in the context of available worldwide population data in order to investigate the demographic history of the region and the genetic adaptations to the harsh environment. The Himalayan populations resembled other South and East Asians, but in addition displayed their own specific ancestral component and showed strong population structure and genetic drift. We also found evidence for multiple admixture events involving Himalayan populations and South/East Asians between 200 and 2,000 years ago. In comparisons with available ancient genomes, the Himalayans, like other East and South Asian populations, showed similar genetic affinity to Eurasian hunter-gatherers (a 24,000-year-old Upper Palaeolithic Siberian), and the related Bronze Age Yamnaya. The high-altitude Himalayan populations all shared a specific ancestral component, suggesting that genetic adaptation to life at high altitude originated once in this region and subsequently spread. Combining four approaches to identifying specific positively selected loci, we confirmed that the strongest signals of high-altitude adaptation were located near the Endothelial PAS domain-containing protein 1 and Egl-9 Family Hypoxia Inducible Factor 1 loci, and discovered eight additional robust signals of high-altitude adaptation, five of which have strong biological functional links to such adaptation. In conclusion, the demographic history of Himalayan populations is complex, with strong local differentiation, reflecting both genetic and cultural factors; these populations also display evidence of multiple genetic adaptations to high-altitude environments.

Key words: Himalayas, human population history, high-altitude adaptation, positive selection, Indo-European language, Tibeto-Burman language.

Introduction

The Greater Himalayan Region is a geographical area containing the world’s highest mountain peaks and a diversity of environments that have required substantial genetic adaptations by the humans who live there. This mountain barrier has also shaped the genetic, cultural, and ethnolinguistic mosaic of South and East Asia. At present, the area falls into the countries of Nepal, Bhutan, India, Pakistan, and the Tibetan Plateau in China. Opinions are divided about whether the Himalayas were used as a corridor that facilitated human migrations from the Tibetan plateau to South Asia in ancient times, or alternatively remained uninhabited due to their inhospitality until more recent times (Majumder 2008; Gayden et al. 2009, 2013; Qi et al. 2013). Archaeological data suggest that the central Tibetan Plateau was populated during the Neolithic period (Majumder 2008; Gayden et al. 2009, 2013; Qi et al. 2013). Archaeological data suggest that the central Tibetan Plateau was populated during the Neolithic period (Majumder 2008; Gayden et al. 2009, 2013; Qi et al. 2013).
earlier human occupation in the north-eastern Qinghai region (Aldenderfer 2011).

The Himalayan region is also one of the most complex linguistic areas in the world, containing six linguistic phyla with multiple languages within each phylum, and at least two language isolates (Burushaski and Kusunda) (van Driem 2001; Kraaijenbrink et al. 2014). However, the region has not been fully represented in genetic studies overall. Previous analyses have mainly focused on populations residing to the north or south of this area, or on small numbers of populations (Gayden et al. 2009; Cai et al. 2011; Kang et al. 2012; Jeong et al. 2014; Cole et al. 2017). In the first systematic survey of Himalayan populations, which used autosomal microsatellite markers (STRs) (Kraaijenbrink et al. 2014), we showed higher genetic diversification among the Himalayans compared with the populations from the surrounding regions, and observed genetic differentiation between Indo-European and Tibeto-Burman speakers, suggesting that both language and geography have influenced the genetic structure of these populations. Genomic scans in Tibetans, and Sherpa from Nepal, have previously identified genomic regions associated with high-altitude adaptation. In particular, a derived Endothelial PAS domain-containing protein 1 (EPAS1) haplotype, whose frequency is strongly correlated with altitude in the Himalayan populations, has been suggested to have been acquired from an extinct hominin species, Denisovans (Yi et al. 2010; Huerta-Sanchez et al. 2014; Lorenzo et al. 2014; Hackinger et al. 2016). In the current study, we have performed a genome-wide SNP-based analysis of 738 individuals from 49 populations in the region in order to generate a more comprehensive reference data set, further understand the population structure and demographic history of the area, as well as search more widely for positively selected genomic regions.

**Results**

**Himalayan Samples Show Distinct Patterns of Population Structure**

We first investigated the population history and demography of the region (fig. 1 and supplementary table S1, Supplementary Material online) by determining the genetic relationships among the Himalayan populations, and comparing them with published data sets of 78 worldwide populations (supplementary table S1, Supplementary Material online). Principal Components Analysis (PCA) shows that the Himalayan populations form a cline, lying between the South and East Asian samples. Populations from Nepal are close to Indians, whereas those from Bhutan and Tibet are closer to East Asians (fig. 2A and supplementary fig. S1, Supplementary Material online). This pattern of genetic affinity to South and East Asian samples is also supported by an ADMIXTURE analysis of worldwide populations (supplementary fig. S2, Supplementary Material online), where the
A genetic component from South Asia (orange) is observed particularly in the Nepalese, and the East Asian (gold) component in the Nepalese, as well as the Bhutanese and Tibetans. However, except for the Toto, all other Himalayan populations are mainly characterized by their own ancestral component (blue). We also found some detectable European and Middle Eastern ancestral components (off-white and green) in some Nepalese. On a finer scale, the first component of a PCA using only the Himalayan populations shows strong geographical clustering with the Toto population forming an outlier, while Dhimal (Nepal) and Bodo (India) also form outliers. (C) ADMIXTURE (K values of 2–6, as indicated) analysis of the Himalayan samples. Note that most increases in the value of K result in single population being distinguished. Population codes in (C) are defined in supplementary table S1, Supplementary Material online.

**Fig. 2.** Genetic structure of the Himalayan region populations from analyses using unlinked SNPs. (A) PCA of the Himalayan and HGDP-CEPH populations. Each dot represents a sample, coded by region as indicated. The Himalayan region samples lie between the HGDP-CEPH East Asian and South Asian samples on the right-hand side of the plot. (B) PCA of the Himalayan populations alone. Each dot represents a sample, coded by country or region as indicated. Most samples lie on an arc between Bhutanese and Nepalese samples; Toto (India) are seen as extreme outlier in the bottom left corner, while Dhimal (Nepal) and Bodo (India) also form outliers. (C) ADMIXTURE (K values of 2–6, as indicated) analysis of the Himalayan samples. Note that most increases in the value of K result in single population being distinguished. Population codes in (C) are defined in supplementary table S1, Supplementary Material online.
and number of ROHs similar to populations from South Asia populations, while Bhutanese populations show a total length of ROHs. Overall, the number and length of ROHs in} 

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and populations from South and East Asia ~800 and 900 years ago (supplementary table S3, Supplementary Material online). Furthermore, Chetri, Majhi, Newar, Dhimal, Bodo, and Lhasa show gene flow from Europe and the Middle East that might be attributed to the presence of these western components as part of the Ancestral North Indian component in South Asians (Reich et al. 2009; Metspalu et al. 2011; Moorjani et al. 2013). Chetri, Bodo, Majhi, and Dhimal show a signature of admixture dated to between 1,000 and 200 years ago. Newar and Lhasa display older signatures of gene flow dated between 1,000 and 2,000 years ago (fig. 4 and supplementary table S3, Supplementary Material online). TreeMix analysis shows long branches for the Toto, Mönpa, and Chepang populations in agreement with the genetic drift patterns (supplementary fig. S14, Supplementary Material online). This is supported by the lack of detectable admixture events for these populations with $f^3$-statistics and only a few significant results for Toto with ALDER, showing an admixture event ~600–800 years ago with Chinese and Indian populations (supplementary table S3, Supplementary Material online).

**FIG. 3.** Genetic structure of the Himalayan populations from haplotype analysis using fineSTRUCTURE, and comparison with language. (A) Populations are clustered according to haplotype sharing; the branching pattern represents this hierarchy, but the branch lengths have no meaning. Note the geographical clustering of populations, particularly the Bhutanese. (B) Language family annotation of the genetic clusters revealing correspondences between genetics and language. Population codes are defined in supplementary table S1, Supplementary Material online.
Asia are detectable (supplementary fig. S14, Supplementary Material online).

We explored the genetic affinity between the Himalayan populations and five ancient genomes using $f_3$-outgroup statistics. Himalayans show greater affinity to Eurasian hunter-gatherers (MA-1, a 24,000-year-old Upper Palaeolithic Siberian), and the related Bronze Age Yamnaya, than to European farmers (5,500–4,800 years ago; fig. 5A) or to European hunter-gatherers (La Braña, 7,000 years ago; fig. 5B), like other South and East Asian populations. We

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**Fig. 4.** Admixture history of five Himalayan populations. The five populations, each named on the left, could be modelled as a mixture between different source populations from two regions. One of these is shown on the vertical axis, while the second is indicated by the colour of the horizontal bar; the position of this bar represents the inferred time of admixture, and the length in time of these admixture events, according to the scale on the horizontal axis. Thus, the Chetri, for example, can be modelled as a mixture of a large number of Asian and European pairs of populations, occurring ~200–400 years ago.
further explored the affinity of Himalayan populations by comparing them with the 45,000-year-old Upper Palaeolithic hunter-gatherer (Ust’-Ishim) and each of MA-1, La Brana, or Yamnaya. Himalayan individuals cluster together with other East Asian populations and show equal distance from Ust’-Ishim and the other ancient genomes, probably because Ust’-Ishim belongs to a much earlier period of time (supplementary fig. S15, Supplementary Material online). We also explored genetic affinity between modern Himalayan populations and five ancient Himalayans (3,150–1,250 years old) from Nepal. The ancient individuals cluster together with modern Himalayan populations in a worldwide PCA (supplementary fig. S16, Supplementary Material online), and the $f_3$-outgroup statistics show modern high-altitude populations have the closest affinity with these ancient Himalayans, suggesting that these ancient individuals could represent a proxy for the first populations residing in the region (supplementary fig. S17 and table S4, Supplementary Material online).

**Signatures of Adaptation in the Himalayan Region**

We searched for variants under positive selection within Himalayan populations living at high altitudes, using four approaches: 1) genome-wide Spearman’s correlation between derived allele frequency and altitude; 2) EMMAX, a genome-wide statistical test for association between SNP frequency and altitude that accounts for population substructure (Kang et al. 2010); 3) the Population Branch Statistic (PBS) which identifies SNPs with unusually high $F_{ST}$ values between high- and low-altitude samples, compared with an outgroup population (Yi et al. 2010); and 4) BayEnv v2, a Bayesian framework for specifically testing association between allele
frequency and environmental variables, such as altitude (Coop et al. 2010; Gunther and Coop 2013).

Genome-wide Spearman’s correlations pinpointed 75 derived alleles with frequencies that correlated significantly with altitude (Spearman’s rho >0.72) (fig. 6 and supplementary table S5, Supplementary Material online) while the EMMAX analysis showed that 99.98% of the variance was explained by the kinship matrix, but identified 56 variants where the
observed allele frequency nevertheless diverged significantly from the expected frequency (fig. 6 and supplementary table S5, Supplementary Material online). The PBS analysis pinpointed 117 variants under possible selection for the derived allele, including ones in regions such as EPAS1 and Disrupted in Schizophrenia 1 (DISC1) previously identified by Tibetan exome sequencing (Yi et al. 2010) (fig. 6 and supplementary table S5, Supplementary Material online).

Twelve candidate variants lying in three different genomic regions overlap between these first three approaches (fig. 6 and supplementary fig. S20 and table S5, Supplementary Material online). Ten of them lie on chromosome 2 in a ∼330-kb genomic region that includes EPAS1, of which two are of potential functional significance. These are rs1868092, downstream of EPAS1 in a promoter-flanking region which has previously been associated with high-altitude adaptation and shown to be a single-tissue eQTL in whole blood (Petousi et al. 2014; Basang et al. 2015), and rs982414, an intronic variant ∼231 kb downstream of EPAS1, which has been associated with hemoglobin concentration in Tibetans (Beall et al. 2010). Furthermore, rs12986653, a variant in ATPase H+ Transporting V1 Subunit E2 (ATP6V1E2) which falls in a CTCF binding site, shows single-tissue eQTLs associated with the ATP6V1E2, CXXC repeat containing interactor of PDZ3 domain (CRIPT) and Transmembrane protein 247 (TMEM247) genes (The Genotype-Tissue Expression Consortium 2013) and has a high CADD score of 20.6 (supplementary figs. S21 and S22 and table S5, Supplementary Material online). The second region overlapping between the three methods is on chromosome 1 and includes the 11th candidate SNP, rs6679627, an intronic variant in the Tripartite Motif Containing 67 (TRIM67) near Egl-9 family hypoxia inducible factor 1 (EGLN1), which has previously been associated with high-altitude adaptation in Tibetans (Simonson et al. 2010; Lorenzo et al. 2014). The third overlapping region is on chromosome 5 and includes a SNP, rs4702062, which shows a strong EMMAX signal together with strong positive correlation with high altitude for the ancestral allele (between 80% and 97% in high-altitude populations, compared with a maximum frequency ≤64% in 1000 Genomes Project populations) and has a CADD score of 12.9 (fig. 6F). This variant is also in high-linkage disequilibrium LD (r² = 0.87) with another nearby variant, rs844335, that was picked up by PBS because of its high derived allele frequency, between 80% and 97% in high-altitude populations, compared with a maximum frequency ≤61% in 1000 Genomes Project populations. Rs844335 lies within a chromatin region nearby, and is also in LD (r² = 0.73) with a third variant, rs1550825, that lies in a transcription factor binding site (supplementary fig. S23, Supplementary Material online). The ANKH gene codes for a transporter that regulates the passage of inorganic phosphate through the cell and contains two hypoxia-responsive elements (HREs) in proximity to its promoter region, and thus its expression is regulated by hypoxic factors (HIFs) (Zaka et al. 2009).

Combining the P values from the first three methods provides a concise way to merge their findings, although not a measure of the type-1 error rate because the tests are not completely independent. This approach identified 398 variants with Bonferroni-adjusted P value < 0.01 (supplementary table S5, Supplementary Material online). The fourth method, BayEnv v2, could not be included in this combined P value analysis as it used an LD-pruned subset of the SNPs. The strongest signals of selection from this last analysis, with multiple significant SNPs in each, included the three regions surrounding EPAS1, EGLN1, and ANKH discussed earlier, and also a region near the major histocompatibility complex. The EPAS1, EGLN1, and HLA-DQB1 regions were also reported as associated with high-altitude adaptation in a previous genome-wide association study between Tibetans and Han Chinese using a linear mixed model approach comparable to EMMAX (Yang et al. 2017). Multiple significant SNPs lying in these regions present single-tissue eQTLs and high CADD scores (supplementary table S5, Supplementary Material online). An additional six regions with two or more significant SNPs stood out in the combined P value analysis, surrounding the RHBDD1, Zinc finger protein 532 (ZNF532), Collagen type IV alpha 4 chain (COL4A4), Solute carrier family 52 member 3 (SLC52A3), Megakaryoblastic leukemia (translocation) 1 (MKL1), and Growth factor receptor bound protein 2 (GRB2) genes (table 1 and fig. 6C). The results from BayEnv v2 were then used for further validation of the candidate genes highlighted above. It pinpointed 503 variants falling into the category “Decisive” [Bayes Factor (BF) > 100, log10(BF) > 2] (supplementary fig. S24 and table S5, Supplementary Material online). Eight of the top ten candidate regions discussed earlier overlapped with the “Decisive” ones: EGLN1, EPAS1, COL4A4, RHBDD1, ZNF532, SLC52A3, and GRB2 while the MKL1 and COL4A4 regions were overlapped strong [10 < BF < 100, 1 < log10(BF) < 2] and substantial [3.2 < BF < 10, 0.5 < log10(BF) < 1] candidates, respectively.

We highlight further features of these candidate regions. The SLC52A3 region includes a missense variant (Pro267Leu, rs3746804) with derived allele frequency > 70% in most high-altitude populations compared with a maximum frequency ≤ 35% in the 1000 Genomes Project populations, and a synonymous variant (rs3746807) with overall high derived allele frequency in Himalayan populations (42–100%) compared with a maximum frequency ≤ 24% in 1000 Genomes Project populations (fig. 6H). Rs3746804 shows single-tissue eQTLs for SLC52A3 in lung and skin, and has a CADD score of 13.3. The COL4A4 region comprises eight SNPs: the top one, rs3769641, lies in a splicing regulatory region within COL4A4, and its derived allele frequency is positively correlated with altitude (Spearman’s ρ = 0.70). This region also contains a missense variant (rs3752895) that shows single-tissue eQTLs in brain tissue for the Rhomboid domain containing 1 (RHBDD1) gene and a synonymous variant (rs2228557). These two variants show high CADD scores of 17.2 and 16.7, respectively. The GRB2 region on chromosome 17 shows four intronic SNPs and has previously been associated with hypoxia-induced oxidative stress level at the
Table 1. Genomic Regions Showing the Strongest Signals of Positive Selection in the Himalayan Populations.

| Candidate Gene | Cluster of Selected SNPs: GRCh37 Coordinates | Number of SNPs in Cluster | Top SNP: Combined P Value | Top SNP Frequency High-Altitude Populations | Top SNP Frequency East Asian Populations | Allele Under Selection | eQTLs | Comments |
|----------------|-----------------------------------------------|---------------------------|---------------------------|--------------------------------------------|-----------------------------------------|------------------------|--------|----------|
| EPAS1          | 2: 46468276–46852033                          | 26                        | 1.83E-27                  | rs4953359                                  | 68%                                     | D                      | 9      | Known high-altitude selection signal |
| EGLN1          | 1: 231204794–231897303                         | 21                        | 3.45E-14                  | rs6655954                                  | 58%                                     | A                      | 10     | Known high-altitude selection signal |
| HLA-DQB1/HLA-DPB1 | 6: 32582075–33175824                       | 15                        | 1.66E-11                  | rs10484569                                  | 77%                                     | D                      | 6      | Known high-altitude selection signal. Region associated with susceptibility to HBV infection in East Asians (Guo et al. 2011) |
| ANKH           | 5: 14908578–149285033                         | 5                         | 2.63E-11                  | rs4702062                                   | 84%                                     | A                      | –      | Novel: regulatory region. Gene has hypoxia responsive element (HREs) in promoter region and it has been reported as a candidate for high-altitude adaptation in Tibetan pigs (Ai et al. 2014) |
| RP11-384F7.2 AC068633.1 | 3: 117427214–118549344                        | 5                         | 6.68E-09                  | rs1081896                                   | 79%                                     | A                      | 2      | Novel |
| ZNF532         | 18: 56562356–56648324                          | 3                         | 8.37E-09                  | rs3826597                                   | 18%                                     | D                      | –      | Novel |
| COL4A4         | 2: 227770592–227923231                         | 8                         | 1.19E-07                  | rs3769641                                   | 46%                                     | D                      | 1      | Novel: include synonymous and splice region variants. This gene encodes one of the sub-units of collagen type IV. Collagen metabolism plays an important role in angiogenesis during hypoxia (Tajima et al. 2001; Sudhakar et al. 2005) |
| SLC52A3        | 20: 744415–745963                              | 2                         | 1.22E-06                  | rs3746804                                   | 63%                                     | D                      | 1      | Novel: missense and synonymous variants. Riboflavin transporter that could be involved in counteracting alterations of energetic metabolism under acute hypoxia (Ghosal et al. 2015) |
| MKL1           | 22: 40827319–40905072                          | 3                         | 3.97E-06                  | rs17001997                                  | 47%                                     | D                      | 3      | Novel: gene involved in the regulation of cellular response to hypoxia in the vasculature of rats (Yuan et al. 2014) |
| GRB2           | 17: 73326965–73374945                         | 4                         | 3.66E-05                  | rs4789182                                   | 94%                                     | D                      | 4      | Novel: associated with reduction of hypoxia-induced oxidative stress in Tibetan individuals (Li et al. 2016) |

NOTE.—Bold candidate genes: Decisive (log Bayes Factor >2) candidates from BayEnv v2.

*Frequency of ancestral allele.
A, Ancestral; D, Derived.
intestinal mucosal barrier in Tibetans compared with Han Chinese (Li et al. 2016) (fig. 6C, table 1, and supplementary table S5, Supplementary Material online). Two of the four variants in GRB2, rs4542691 and rs4789182, show single-tissue eQTLs. The MKL1 region on chromosome 22 carries three intronic SNPs, and has previously been associated with the regulation of the cellular response to chronic hypoxia in the vasculature of rats. All three variants in MKL1, rs2294352, rs6001931, and rs17001997, show single-tissue eQTLs in muscle-skeletal tissue (Yuan et al. 2014).

We also examined the allele frequencies of the top SNPs in our ten candidate regions (table 1) in the five ancient Himalayan genomes, and compared them with the allele frequencies in present-day Himalayans. Six variants in the EPAS1 region and 11 in the EGLN1 region show high derived allele frequencies in ancient Himalayans (>0.60). The missense variant rs3746804 in the SLC52A3 locus also shows a high derived allele frequency of 0.67 in the ancient Himalayans. Variants in COL4A4, ANKH, RP11-384F7.2/AC068633.1, and HLA-DBP1/DBP2 show derived allele frequencies in the ancient Himalayans of 0.56–1.00, while two variants, rs542691 and rs4789182, in the GRB2 locus show a derived allele frequency of 100% in the ancient samples. Finally, rs3826597 in ZNF532 region show a derived allele frequency of 0.95 in the ancient Himalayans (supplementary table S5, Supplementary Material online). None of the top selection candidate regions, apart from EPAS1 (Huerta-Sanchez et al. 2014; Hu et al. 2017), show signatures of adaptive introgression from archaic Denisovans or Neanderthals according to published introgression maps (Sankararaman et al. 2016).

We also generated a protein homology model for SLC52A3, and investigated the position of the missense variant, rs3746804. The SLC52A3 structure resembles that of a glucose transporter and rs3746804 is predicted to lie in an exposed intracellular region which could act as an interaction surface for the intracellular environment (Iancu et al. 2013) (supplementary fig. S25, Supplementary Material online). We finally generated protein–protein interaction networks for our top ten protein candidates. EPAS1, EGLN1, COL4A4, and GRB2 were predicted to be part of the same network. Prostaglandin I2 synthase (PTGIS) and Vitamin D receptor (VDR), suggested previously by Hu et al. to be under selection for high-altitude adaptation, are also predicted to be in the same protein–protein interaction network (Hu et al. 2017) (supplementary fig. S26, Supplementary Material online).

**Discussion**

We have performed the most comprehensive survey thus far of genetic variation in the Himalayan region, aiming to elucidate the genetic ancestry of these populations, including their demographic histories, and the genetic adaptations they have undergone in order to survive in the varied and challenging environments present in the region.

**Population Structure and Demography**

In the broadest sense, all the Himalayan populations share ancestry with their geographical neighbours in South and East Asia, reflecting the common pattern of the distribution of human genetic diversity dominated by geography (fig. 2A). Within this framework, we nevertheless detect an ancestral component that is abundant in most Himalayans, but rare elsewhere (fig. 2C with K values of 2–4; supplementary fig. S2, Supplementary Material online), pointing to shared ancestry for these populations, a conclusion reinforced by their similar patterns of shared genetic drift with non-Himalayan ancient samples (fig. 4). At finer resolution, we see evidence for both substructure reflecting geography within the Himalayan region, and extreme drift leading to single populations forming outliers in the PCA (fig. 2B) or specific components in ADMIXTURE analysis (fig. 2C). The most striking example is provided by the Toto from North India, an isolated tribal group with the lowest genetic diversity of the Himalayan populations examined here, indicated by the smallest long-term Ne (supplementary fig. S5, Supplementary Material online), and a reported census size of 321 in 1951 (Mitra 1951), although their numbers have subsequently increased. Despite this extreme substructure, shared common ancestry among the high-altitude populations (figs. 2C and 3) can be detected, and the Nepalese in general are distinguished from the Bhutanese and Tibetans (fig. 2C) and they also cluster separately (fig. 3). In a worldwide context, they share an ancestral component with South Asians (supplementary fig. S2, Supplementary Material online). On the other hand, the Tibetans do not show detectable population substructure, probably due to a much more recent split in comparison with the other populations (fig. 2C and supplementary fig. S6, Supplementary Material online). The genetic similarity between the high-altitude populations, including Tibetans, Sherpa, and Bhutaneses, is also supported by their clustering together on the phylogenetic tree, the PCA generated from the coancestry matrix generated by fineSTRUCTURE (supplementary figs. S10 and S11, Supplementary Material online), the lack of statistical significance for most of the D-statistics tests (Yoruba, Han; high-altitude Himalayan 1, high-altitude Himalayan 2), and the absence of correlation between the increased genetic affinity to lowland East Asians and the spatial location of the Himalayan populations (supplementary figs. S12 and S13, Supplementary Material online). Together, these results suggest the presence of a single ancestral population carrying advantageous variants for high-altitude adaptation that separated from lowland East Asians, and then spread and diverged into different populations across the Himalayan region. Genetic drift and admixture with other Himalayan, South, and East Asian populations can explain the widespread distribution of the selected EPAS1 haplotype at lower frequencies in populations at lower altitudes (Hackinger et al. 2016), and the altitude clines in the other selection candidates (fig. 6). Our findings suggest a recent split (only a proxy for population differentiation, given the limitations of the method applied) between Tibetans, Sherpa and, possibly, other high-altitude populations, rather than the Tibetans being a mixture of Sherpa and Han Chinese (Jeong et al. 2014; Bhandari et al. 2015). Whole-genome sequences from multiple high-altitude populations will provide better estimates of such divergence times and a more detailed demographic history of the region.
Himalayan populations show signatures of recent admixture events, mainly with South and East Asian populations as well as within the Himalayan region itself. Newar and Lhassa show the oldest signature of admixture, dated to between 2,000 and 1,000 years ago. Majhi and Dhimal display signatures of admixture within the last 1,000 years. Chetri and Bodo show the most recent admixture events, between 500 and 200 years ago (fig. 4 and supplementary table S3, Supplementary Material online). The comparison between the genetic tree and the linguistic association of each Himalayan population highlights the agreement between genetic and linguistic subdivisions, in particular in the Bhutanese and Tibetan populations. Nepalese populations show more variability, with genetic subclusters of populations belonging to different linguistic affiliations (fig. 3B). Modern high-altitude Himalayans show genetic affinity with ancient genomes from the same region (supplementary fig. S17, Supplementary Material online), providing additional support for the idea of an ancient high-altitude population that spread across the Himalayan region and subsequently diverged into several of the present-day populations. Furthermore, Himalayan populations show a similar pattern of allele sharing with Denisovans as other South-East Asian populations (supplementary figs. S18 and S19, Supplementary Material online). Overall, geographical isolation, genetic drift, admixture with neighboring populations and linguistic subdivision played important roles in shaping the genetic variability we see in the Himalayan region today.

High-Altitude Adaptation
The harsh environment at high altitude due to increased ultraviolet radiation, hypobaria and hypoxemia is inescapable, so it is expected to have triggered physiological and genetic adaptations including modifications in the cellular responses of the humans who settled there. Genomic scans for positive selection in Tibetans have previously implicated several genes as candidates for high-altitude adaptation, especially an extended EPAS1 haplotype (Yi et al. 2010; Peng et al. 2011, 2017; Xu et al. 2011; Lu et al. 2016) that arose by introgression from Denisovans (Huerta-Sanchez et al. 2014), and is widespread in the region (Hackinger et al. 2016). Positive selection scans can easily be confounded by population structure, and although simple correlations of SNP frequency with altitude replicated several of the candidates reported in previous studies (fig. 6 and supplementary table S5, Supplementary Material online), including those near EPAS1, DISC1, and ATP6V1E2 which are highly differentiated between lowland Han and Tibetans (Yi et al. 2010; Lu et al. 2016), additional analyses better suited to substructured populations only confirmed a subset of these. The signal on chromosome 2 is particularly strong and includes a ∼330-kb region encompassing EPAS1, ATP6V1E2, and PIGF/CRIP1 (fig. 6B and C and supplementary figs. S21 and S22, Supplementary Material online). An expected signal of selection from EGLN1 was observed via nearby variants in TRIM67 and TNSAX-DISC1 (fig. S5D) (Xiang et al. 2013; Foll et al. 2014). A novel signal of selection was found in the region upstream of ANKH on chromosome 5 (fig. 6F). This region shows extended LD, but the variant driving the selection could not be identified by our analysis (supplementary fig. S23, Supplementary Material online). Nevertheless, ANKH is itself a strong candidate because it is involved in the regulation of the transportation of inorganic phosphate and its expression is regulated by HIF2A (EPAS1) and HIF1A (Zaka et al. 2009; Skubutyte et al. 2010). ANKH is essential for maintaining cellular function and bone mineralization, and its concentration plays a central role in several metabolic pathways (Dick et al. 2011).

In order to maximize the power to identify the additional selection candidates, we calculated combined P values for three different statistics applied to our data set, and then further validated these candidate genomic regions using a fourth statistic, BayEnv2 (fig. 6G, table 1, and supplementary fig. S24 and table S5, Supplementary Material online). Some of these additional variants may play important roles in the hypoxic environment, contributing to physiological responses to hypoxia. COL4A4 encodes one of the subunits of collagen type IV, which is an essential component of basement membranes, and plays an important role in angiogenesis. Hypoxia exposure triggers vasoconstriction which requires structural remodelling of arterial vessels, especially in lung and collage metabolism is required for this process (Tajima et al. 2001; Sudhakar et al. 2005). GRB2 is involved in the regulation of reactive oxygen species (ROS) production in hypoxic environments and it has been shown that, in Tibetans, downregulation of its expression reduces ROS damage and improves glucose and fat metabolism in intestinal tissues (Li et al. 2016). MKL1 encodes a myocardin-related transcription factor and is involved in smooth muscle cell differentiation (Cen et al. 2003). Down-regulation of MKL1 reduces the pulmonary arterial pressure in response to chronic hypoxia and regulates vascular remodelling in rats (Yuan et al. 2014). SLC52A3 encodes a transporter of riboflavin, a vitamin that modulates fatty acid and amino acid metabolism and reduces cellular oxidative stress (Ghosal et al. 2015). Riboflavin supplementation of the diets of mice improves their energetic metabolism under acute hypoxia; Thus, increased riboflavin could be effective in counteracting the alteration of human metabolism in hypoxic conditions (Wang et al. 2014). SLC52A3 is a transmembrane protein and the homology-based protein model we generated resembles the structure of a glucose transporter; our top candidate variant, rs3746804 (Pro267Leu), lies in the intracellular environment in a possible interaction region of the protein surface (supplementary fig. S25, Supplementary Material online). This selection signal seems to be specific for Himalayan populations and could be related to the diet and environment, where efficient intake of riboflavin at high altitude would be advantageous (Blanck et al. 2002). Two out of three additional candidates for high-altitude adaptation (PTGIS and VDR) suggested by Hu et al. are predicted to be in the same protein–protein interaction pathway as some of our candidates, COL4A4 and GRB2, and linked with other genes (EPAS1, EGLN1, HIF1A, VHL) involved in the hypoxic response (supplementary fig. S26, Supplementary Material online) (Hu et al. 2017). ANKH has also been reported as a candidate for high-altitude adaptation in Tibetan pigs (Ai et al. 2014).
Thus, of the top ten selected candidate regions (seven novel) highlighted by our work (table 1), four are members of the most relevant protein–protein interaction network and three others have known functions relevant to high-altitude adaptation: findings that are very unlikely due to chance. Furthermore, despite the strong ascertainment bias of the SNPs included on SNP-chips, variants lying in our top ten candidate regions are associated with single-tissue eQTLs and present high CADD scores, suggesting their possible importance in gene regulation and expression. The presence of high derived allele frequencies of variants in EGLN1, EPAS1, SLC52A3, and GRB2 loci in ancient Himalayans also supports our hypothesis that these candidates may be under selection and important for high-altitude adaptation (supplementary table S5, Supplementary Material online). According to available introgression maps (Sankararaman et al. 2016), none of the top selected candidate regions, apart from the well-known EPAS1 intronic region (Huerta-Sanchez et al. 2014; Hu et al. 2017), show signatures of adaptive introgression from Denisovans or Neanderthals. In all cases, high-coverage whole-genome sequences and comparisons with other species that have adapted to similar environments should help to identify or confirm the key causal variants and suggest strategies for functional follow-up.

In conclusion, the current analyses have established the broad features of Himalayan genetic variation: a South or East Asian substrate influenced by local differentiation and mixing in ways that are now understood in outline, including extreme genetic drift in several populations. It has provided a comprehensive data set from the region for the community to use in future studies. In addition, there is evidence for early strong genetic adaptation to high-altitude living followed by spread of the adapted population. Future functional investigations will allow these phenomena to be understood in more detail.

Materials and Methods

Samples
Eight hundred and eighty-three individuals belonging to 49 Himalayan populations were genotyped and analyzed after obtaining informed consent. The data set included 26 populations from Nepal, 16 from Bhutan, two from North India sampled in Bhutan, and five from Tibet in China (fig. 1 and supplementary table S1, Supplementary Material online). The samples represent the two major linguistic families in the area: Indo-European and Tibeto-Burman (also known as Trans-Himalayan). Specifically, 44 populations comprise Tibeto-Burman speakers from Tibet, Bhutan, North India, or Nepal, and five comprise Indo-European speakers from Nepal (Chetri, Damai, Majhi, Sarki, and Sonar). The Bhutanese, North Indian, and Nepalese samples were collected as part of the “Language and Genes of the Greater Himalayan Region” project, a genetic survey of Tibeto-Burman and Indo-European speakers from these Himalayan countries, and have been described previously (Kraaijenbrink et al. 2014). Tibetan samples were selected from participating members of an epidemiological study in the Tibet Autonomous Region, China, in 2007 that was approved by the institutional ethics review board of BGI-Shenzhen. Samples were collected from healthy unrelated Tibetans from five villages based on their medical records and a comprehensive medical examination during sampling. Peripheral venous blood or saliva was collected for DNA extraction and genotyping. All participants had a self-reported family history of at least three generations living at the sampling site.

Genotyping and Quality Control

The samples were genotyped using three Illuma SNP-chips: 1) HumanOmniExpress-12 v1.0 Bead Chip (741,000 SNPs) at the Wellcome Sanger Institute; 2) HumanOmni1-Quad BeadChip (~1 M SNPs) at the Leiden University Medical Center; and 3) HumanOmniExpress-24 BeadChip (~713,000 SNPs) at BGI-Shenzhen. Genotype calling and QC on all samples were performed using the Sanger Institute’s variant calling pipelines, and SNP positions were mapped to the human reference assembly GRCh37. Genotypes from the three arrays were merged using PLINK 1.92 (Purcell et al. 2007), resulting in a data set of 600,838 SNPs. Genotyping success rate and sample missingness thresholds were set to 99% and 10%, respectively. Sex-linked and mitochondrial SNPs as well as autosomal ones with Hardy–Weinberg Equilibrium P value < 0.0000001 were removed. We also removed related samples (PI_HAT > 0.35) and outliers using EIGENSOFT 6.0 (Patterson et al. 2006; Price et al. 2006). These filters resulted in a final data set of 738 worldwide populations and 583,011 SNPs. For comparison with worldwide populations, the Himalayans were merged with published data sets (supplementary table S1, Supplementary Material online) (Li et al. 2008; Chaubey et al. 2011; Metspalu et al. 2011) resulting in 1,962 individuals and 268,861 SNPs. Two additionally pruned data sets were generated from this by filtering out SNPs in high LD ($R^2 > 0.5$). The pruned Himalayan data set consisted of 256,506 SNPs, and the pruned worldwide data set included 190,287 SNPs. For comparison with ancient samples, we generated two further data sets: 1) we merged our data with the Human Origins data, a data set comprising both modern and ancient individuals including archaic genomes from Denisovans and Neanderthals, and a chimpanzee (Patterson et al. 2012), resulting in 82,647 SNPs in common; and 2) we merged our Himalayan and worldwide data sets with published ancient Himalayan genomes from the Annapurna Conservation Area in Nepal (Jeong et al. 2016). From the published ancient BAMs, we randomly sampled (Korneliussen et al. 2014) a single sequence with a minimum quality of $g$20 to represent each SNP in our Himalayan data set, trimming 5 bp from both ends of reads to reduce the effect of ancient DNA deamination. This resulted in 582,810 SNPs in our Himalayan data set being covered by at least one of the ancient samples (supplementary table S1, Supplementary Material online).

Population Characterization and Demography

The genetic structure of the Himalayan populations was examined using several statistical approaches. Principal component analysis (PCA) using EIGENSOFT 6.0 was performed
using the LD-pruned data sets. For the worldwide data set, the eigenvectors were calculated using the global diversity and the Himalayan individuals were projected onto the plot (fig. 2). ADMIXTURE v1.2 (Alexander et al. 2009) was used on the pruned data sets for cluster analysis and the cross validation (CV) error for identifying the best K value. Estimation of long-term effective population size (Ne) for each Himalayan population and population divergence time was performed using the NeON R package (Mezzavilla and Girotto 2015), which calculates the harmonic mean of the population size at each generation and the time of divergence between populations in generations. More specifically, using LD information ($r^2$) and recombination distance ($c$) we estimated the effective population size using the nonlinear regression model: $y_i = 1/(x + \beta c_i) + e_i$, with $y_i = (r^2 - 1/n) (r^2$ adjusted for chromosome sample size) for SNP pair i at recombination distance $c_i$ (in Morgans). We estimated the change in population size over time, as LD between loci with a recombination rate of $c$ that reflects the ancestral effective population size $1/2c$ generations ago (Hayes et al. 2003). The model is based on the assumption of linear growth/decline. However, some populations might depart from the assumed model characteristics and LD patterns in these will be affected, so the relationship $t = 1/(2c)$ should be viewed as only an approximate but useful indication of timeframe (de Roos et al. 2008). Furthermore, the time of divergence estimates are based on the assumption there was a “clean” population split, and migration will create a stronger correlation of LD (larger values of $r^2$), thereby biasing the estimate of divergence time downward. Nevertheless, this method is still useful to assess isolation and difference in Ne between populations (McEvoy et al. 2011; Tassi et al. 2015). Only populations with sample size $\geq 10$ were used, as the harmonic mean is sensitive to sample size. For all analyses, we assumed a generation time of 29 years (Fenner 2005). ROHs were identified using PLINK 1.92 (Purcell et al. 2007) with specific thresholds to maximize the detection of autozygous segments in the Himalayan populations (Howrigan et al. 2011) and other worldwide populations: a pruned data set (LD, $r^2 > 0.5$) with only common variants (MAF > 0.05) was used. The minimum number of SNPs to call an ROH was set to 100, the heterozygote allowance was set up to zero, the missing SNP allowance was set to 5 (5% of SNP threshold), and the window threshold to call an ROH was set to 0.05. The coefficient of inbreeding ($F$) was calculated with PLINK (Purcell et al. 2007).

A worldwide data set using a maximum of ten individuals from every population was used in the ChromoPainter and fineSTRUCTURE-2.0.6 (Lawson et al. 2012) analyses. The haplotypes were phased using SHAPEIT (Delaneau et al. 2011) using the 1000 Genomes Project Phase 3 (The 1000 Genomes Project Consortium 2015) as a reference panel. ChromoPainter infers the ancestry of each individual by reconstructing their haplotype segments from other individuals in the data set. FineSTRUCTURE uses the coancestry matrix inferred from ChromoPainter to construct a population-relationship tree and was run with 10,000,000 burn-in steps and 10,000,000 iterations. A PCA was also performed using the coancestry matrix generated by fineSTRUCTURE.

To assess the robustness of the results from the above data set, we additionally ran fineSTRUCTURE on a data set with fewer samples (the Himalays and 1000 Genomes Project Phase 3 populations) but more markers (579,640 SNPs) using the same parameters. To test whether or not genetic similarity among high-altitude populations correlates with their geographical location, we used YRI (Yoruba in Ibadan, Nigeria) as an outgroup and calculated D-statistics (qpDstat function in ADMIXTOOLS v3 package) using the following phylogeny: $D$(Yoruba, Han; high-altitude Himalayan 1, high-altitude Himalayan 2) where high-altitude Himalayan 1 and high-altitude Himalayan 2 are pairs of Sherpa, Tibetan, or Bhutanese populations from an altitude of 2,500 m or above sea level (Patterson et al. 2012; Jeong et al. 2017). We computed D-statistics with the above phylogeny using our worldwide data set (supplementary tables S1 and S3, Supplementary Material online). Then, we tested the correlation between values of D-statistics with pairwise differences in longitude and latitude for each pair of populations using the Mantel test implemented in the “Ades” R package (mantel.test function) (Dray and Dufour 2007).

Population admixture was studied using ALDER v1.03 (Loh et al. 2013), three-population statistics ($f_3$) (Reich et al. 2009; Patterson et al. 2012), and TreeMix 1.12 (Pickrell and Pritchard 2012). Only populations with at least six individuals were included in these tests. ALDER was used with the default parameters and the threshold of LD in the reference groups was inferred by the program. A test was considered positive when both the 2-ref weighted LD curve was significant and the decay rates between the 2-ref and 1-ref curves were consistent. We considered a jack-knife block of 500 SNPs for $f_3$ statistic analyses. Shared genetic drift between modern populations and ancient samples was tested using outgroup $f_3$ statistics (ancient genome, X; Yoruba) (Patterson et al. 2012) with Yoruba as an outgroup. The ancient genomes used in this investigation were: 1) Eurasian hunter-gatherer (MA1,24,000-year-old Upper Palaeolithic Siberian) (Raghavan et al. 2014); 2) Bronze Age Yamnaya population (3,500–2,700 year old) (Allentoft et al. 2015); 3) Neolithic European farmer (LBK_EN, 5,500–4,800 year old) (Haak et al. 2015); 4) Mesolithic hunter-gatherer (La Braña, 7,000 year old) (Olalde et al. 2014); 5) Eurasian hunter-gatherer (Ust’Ishim, 45,000 year old Upper Palaeolithic Siberian) (Fu et al. 2014); 6) five ancient Himalayan genomes 3,150–1,250 years old (C1, M63, S10, S35, and S41) from the Annapurna Conservation Area, Nepal (Jeong et al. 2016). We also used the archaic Denisovan and Neanderthal genomes and the chimpanzee to study genetic affinity of Himalayan samples to these archaic individuals: we calculated principal components using Denisovan, Neanderthal, and chimpanzee, and projected modern samples onto them (Skoglund and Jakobsson 2011). We also computed D-statistics (Yoruba, X; Denisovan, Chimpanzee) where X are different modern human populations from the worldwide data set).

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Positive Selection
Signals of positive selection were evaluated in four ways. First, we considered the Spearman correlation between derived allele frequency and the residence altitude of each population (Hackinger et al. 2016), adjusting the P value for multiple tests by applying the Bonferroni correction (requiring <0.05/number of tests). Second, we calculated a genome-wide association between allele frequency and altitude using a mixed model approach implemented in the Efficient Mixed-Model Association eXpedited program (EMMAX) (Kang et al. 2010). EMMAX detects variants where the observed allele frequency is significantly divergent from the expected frequency, and accounts for population stratification and sample relatedness through a variance component approach. A kinship matrix was constructed to account for population structure and implemented in a linear mixed model. Variants with was constructed to account for population structure and sample relatedness through a variance component approach. A kinship matrix was constructed to account for population structure and implemented in a linear mixed model. Variants with P value <5 \times 10^{-8} were considered significantly associated with altitude. Although these methods detect associations between allele frequency and altitude, they are not able to distinguish between high- and low-altitude selection signals. Third, we also calculated the Fixation Index \(F_{ST}\) (Reynolds et al. 1983) for each SNP position between Himalayan, European (CEU; Utah Residents [CEPH] with Northern and Western European Ancestry) and East Asian (CHB; Han Chinese in Beijing, China) from the 1000 Genomes Project Phase 3 populations, and searched for unusual values using the Population Branch Statistic (PBS) (Yi et al. 2010). To reduce noise due to population structure within the Himalayan populations and differences in sample sizes, we ran PBS following the approach described (Yi et al. 2010), assuming that the CHB is the most closely related population to Tibetans and looked specifically for signals of high-altitude adaptation (Yi et al. 2010). For this analysis, we only used the populations from Bhutan and Tibet that clustered together in the fineSTRUCTURE analysis compared with CEU and CHB. Variants above the 99.99th percentile of the empirical distribution were considered statistically significant (Ayub et al. 2015; Fumagalli et al. 2015). The top hits from each method were assessed and the overlap collated. LD estimations for the regions containing the top candidates were calculated and plotted using Haploview (Barrett et al. 2005). We also used Fisher’s method (Fisher 1950) for combining P values of the three statistics used for detecting positive selection: 1) we calculated a rank P value of the PBS values (values were ranked in decreasing order from the most significant value and divided by the total number of SNPs used in the analysis); 2) we combined the P values of the three statistics genome-wide; and 3) we adjusted the P value for multiple tests by applying the Bonferroni correction (requiring < 0.01/number of tests). Finally, to further validate our selection signals, we calculated genome-wide associations between allele frequency and altitude using BayEnv v2 (Coop et al. 2010; Gunther and Coop 2013), a Bayesian framework specifically designed to detect correlation between allele frequencies and environmental factors taking population structure into account. The input files for BayEnv2 v2 were generated from a LD \(r^2 > 0.5\) pruned SNP file using PGDSpider (Lischer and Excoffier 2012) and altitude (the environmental variable) was standardized according to the BayEnv2 v2 manual. BayEnv v2 was run with the default parameters and the Bayes Factors interpreted according to previous recommendations (Kass and Raftery 1995); only candidate variants falling into the category “Decisive” [Bayes Factors (BF) > 100, log_{10}(BF) > 2] were considered significant. Where possible, we also calculated allele frequencies in the five Himalayan ancient genomes for our top candidates of selection.

We generated a protein homology model for SLCS2A3 using Phyre2 software (Kelley et al. 2015) and mapped the mis-sense variant found in SLCS2A3 onto the protein structure using PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC). We predicted protein–protein interaction networks using the STRING software (v. 10.5) (Szklarczyk et al. 2015) for our top selection candidates. Finally, we used the Ensembl Variant Effect Predictor (VEP) (McLaren et al. 2016) to predict the consequences of variants of interest on gene expression and protein sequence. We retrieved the Combined Annotation Dependent Depletion v.1.2 (CADD) scores (Kircher et al. 2014) of our top candidates and also overlapped our results with the Genotype-Tissue Expression (GTEx) database (The Genotype-Tissue Expression Consortium 2013).

Data Availability
All the genotype data are available from European Genome-phenome Archive under accession number EGAS0000102731.

Web Resources
Ensembl VEP: https://grch37.ensembl.org/info/docs/tools/vep/index.html, last accessed May 10, 2018
GTEx Portal: https://www.gtexportal.org, last accessed May 10, 2018
Phyre2: http://www.sbg.bio.ic.ac.uk/phyre2, last accessed May 10, 2018
PLINK: https://www.cog-genomics.org/plink2, last accessed May 10, 2018
PyMOL: https://www.pymol.org/, last accessed May 10, 2018
STRING: http://string-db.org, last accessed May 10, 2018
Combined Annotation Dependent Depletion (CADD): http://cadd.gs.washington.edu/, last accessed May 10, 2018

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

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