Ancient selection for derived alleles at a GDF5 enhancer influencing human growth and osteoarthritis risk

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Variants in GDF5 are associated with human arthritides and decreased height, but the causal mutations are still unknown. We surveyed the Gdf5 locus for regulatory regions in transgenic mice and fine-mapped separate enhancers controlling expression in joints versus growing ends of long bones. A large downstream regulatory region contains a novel growth enhancer (GROW1), which is required for normal Gdf5 expression at ends of developing bones and for normal bone lengths in vivo. Human GROW1 contains a common base-pair change that decreases enhancer activity and colocalizes with peaks of positive selection in humans. The derived allele is rare in Africa but common in Eurasia and is found in Neandertals and Denisovans. Our results suggest that an ancient regulatory variant in GROW1 has been repeatedly selected in northern environments and that past selection on growth phenotypes explains the high frequency of a GDF5 haplotype that also increases arthritis susceptibility in many human populations.

Recent genome-wide association studies (GWAS) have mapped many loci associated with complex traits and diseases. Although the relative risks conferred by GWAS loci are often small, the high frequency of some alleles can nonetheless account for a substantial health burden in human populations1. For example, SNPs linked to GDF5, which encodes growth differentiation factor 5, are among the best-replicated risk factors for adult-onset osteoarthritis2. These alleles increase osteoarthritis risk for specific joints by ~1.2- to 1.8-fold3,4 and are also linked to lumbar-disk degeneration5, hip dysplasia4, Achilles tendonopathies7, and meniscus tears8. Interestingly, risk variants are present at high frequencies in many populations3,9. Although the relative risk due to GDF5 variants may be moderate, their high prevalence contributes a substantial fraction of osteoarthritis risk in many human populations10.

High-frequency GDF5 risk alleles show multiple molecular signatures of positive selection during recent human evolution11–14. Although the causal basis of selection is still unknown, advantageous changes have presumably occurred at one or more body sites influenced by GDF5 activity.

Previous studies have identified multiple structures controlled by GDF5. In mice, Gdf5-null mutations cause shorter feet and limbs; missing joints in digits, wrists, and ankles; altered tendons; missing knee ligaments; and an increased risk of osteoarthritis15–19. Likewise, human GDF5 mutations cause short stature; short digits; joint dislocations or fusions; and hip- and knee-joint dysplasia often concurrent with osteoarthritis20–23. Interestingly, two common SNPs in the 5′ untranslated region (5′ UTR) of the human gene (rs143383 and rs143384) are associated with a 1.3- to 1.8-fold increased risk of osteoarthritis in Japan and China3. Functional studies have shown that T alleles at these SNPs decrease reporter-gene expression in cultured articular cells3 and are associated with decreased GDF5 transcript levels in the articular cartilage of people with osteoarthritis24. Both 5′-UTR variants are also associated with height variation in humans3,25–29. Homozygosity for T alleles decreases height by approximately 1 cm (ref. 9), one of the largest effects identified for GWAS height loci30. The decreased height, and increased osteoarthritis risk, may be due to the 5′-UTR mutations or to other linked variants3.

Despite high interest in GDF5 5′-UTR variants, the candidate genomic regions found in most association studies extend from several kilobases upstream (5′) to 100 kb downstream (3′) of GDF5 coding exons (Supplementary Fig. 1). To better characterize the overall regulatory structure of GDF5, we surveyed upstream and downstream regions of the human and orthologous mouse loci by using transgenic reporter assays and functional rescue experiments in vivo. We found that a large region 3′ of GDF5 is required for both normal joint development and bone length. Interestingly, the selection signatures found in humans were centered on a growth enhancer that we identified in the 3′ region, not the 5′ UTR. The selection peak included an ancient genetic variant present in Neandertals, Denisovans, and modern human populations that we found to decrease the activity of the growth enhancer.

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Likewise, deletion of the growth enhancer from mice decreased limb length and Gdf5 expression levels in vivo. We propose that selection on the derived variant in this growth enhancer underlies adaptive evolution of the GDF5 gene in both ancient and modern humans, and has led to the high prevalence of a linked risk haplotype that contributes to common skeletal diseases in many populations.

RESULTS  

Regulatory and functional scans of Gdf5  
To identify regulatory sequences surrounding the mouse Gdf5 locus, we used a BAC scanning approach31. Two BAC clones, each containing functional Gdf5 coding sequences, were modified by insertion of a lacZ reporter into the Gdf5 transcription unit and were used to generate transgenic mouse lines (Fig. 1). As we have recently reported32, regulatory elements within the upstream BAC, spanning 110 kb upstream to 30 kb downstream of Gdf5 exons, drove lacZ expression predominantly in embryonic proximal limb joints and axial structures, whereas elements in the downstream BAC, including an additional 110 kb downstream of Gdf5, drove expression in proximal and distal limb joints, including strong digit expression. More detailed analysis showed that the downstream BAC also drove additional stripes of lacZ expression in the growing ends of long bones rather than joints (Fig. 1). This newly identified expression specifically resided along a lateral margin of the developing chondrocyte growth plate within and subjacent to the perichondrium, a region where the endogenous Gdf5 gene is also expressed (Fig. 1 and Supplementary Figs. 2 and 3). Mice with Gdf5-null mutations have decreased limb lengths, and this expression is consistent with the known requirement for GDF5 in control of growth-plate dynamics17.

To test whether different regulatory regions are required for different Gdf5 functions, we introduced the BAC transgenes, each carrying functional Gdf5 coding regions, separately into a Gdf5-null background provided by mouse brachypodism (bp) mutations16. Mice with bp mutations, compared with their wild-type and heterozygous littermates, have approximately 10% shorter long bones and a 50% decrease in metaphodal lengths15. The upstream BAC allele did not rescue long-bone growth phenotypes, although it did improve metaphodal lengths (Fig. 2 and Supplementary Fig. 4). In contrast, the downstream BAC allele completely restored all major long bones and metaphodal lengths to control lengths (Fig. 2 and Supplementary Fig. 4). Therefore, key functional cis-regulatory sequences controlling limb growth reside at least 30 kb downstream of Gdf5.

Identification of a growth-collar enhancer  
To further delineate downstream regulatory sequences, we tested whether smaller human or mouse clones drove consistent lacZ expression in transgenic mice. Different human sequences independently controlled expression in limb joints (41 kb) or growth collars (37 kb) (Fig. 3). The 41-kb human clone drove expression in multiple joints and contains multiple joint-control elements, as recently described32. In contrast, the 37-kb human clone drove expression in growth-collar domains similarly to the downstream BAC (Fig. 1). Growth-zone patterns were also found with a smaller ~12-kb mouse sequence identified by a separate scan across the interval (data not shown). Truncation mapping of the orthologous human region identified a 2.54-kb enhancer (Fig. 3 and Supplementary Fig. 5), which we denoted GROW1 (for GROWTH 1). GROW1 has a chromatin signature typical of enhancers in developing mouse limbs and human chondrocytes (Supplementary Note). Two regions of evolutionary conservation were seen within GROW1: one conserved through amniotes (GROWIA) and one conserved through placental mammals (GROW1B) (Figs. 3 and 4 and Supplementary Fig. 5). Enhancer

Figure 1 A regulatory scan of the Gdf5 region. (a) Top, genomic positions of BAC constructs covering upstream (yellow) and downstream (green) sequences surrounding the mouse (mm9) Gdf5 locus. Vertical blue lines denote positions of the lacZ cassette engineered in the Gdf5 transcript. Chr, chromosome. Bottom, the downstream BAC, compared with the upstream BAC, drives a broader lacZ expression pattern in long bones and joints, and uniquely controls lacZ expression in growth collars (GC) and broad expression domains in articular surfaces (asterisks) of the femur and tibia. Whole-embryo panels shown at embryonic day E15.5 (n = 3 embryos from 3 distinct founder lines). Dissected long bones at E16.5 (ventral views). The following joints are indicated: s, shoulder; e, elbow; w, wrist; h, hip; k, knee; a, ankle; d, digit. Scale bars, 2 mm (embryo) and 1 mm (bone). (b) Localization of the downstream BAC Gdf5 lacZ signal in the proximal femoral head (top) and proximal tibia (bottom) growth collars at E16.5. The Gdf5 lacZ signal is present in the chondrocyte cell collar, within and subjacent to perichondrial and adjacent tendon cells. The Gdf5 lacZ signal is also present in the femoral head and proximal tibia articular cartilage. Scale bars, 200 μm (sections).
tests of each sequence showed that both drove long-bone expression, although the GROW1B pattern was stronger and extended across the growth collar (Supplementary Fig. 6).

Human sequence variants in GROW1
Given the association of GDF5 with height variation and osteoarthritis risk, we queried 1000 Genomes Project data\textsuperscript{34,35} to identify common human SNPs in a 400-kb region surrounding GDF5 (Supplementary Table 1). In agreement with the results of other studies\textsuperscript{3,9,12,14,34}, we found no common protein-coding substitutions that could explain the associations (Supplementary Table 1). Instead, we identified many noncoding variants, 19 of which existed within the newly mapped GROW1 enhancer. Of these, 14 were rare (minor allele frequency (MAF) > 0.05) and 1 was a common variant (MAF > 0.05) (Supplementary Table 1). The common variant was a G>A substitution (rs4911178) mapping within a highly conserved sequence within GROW1B (Supplementary Fig. 5). Strikingly, this G is perfectly conserved in placental mammals, whereas A occurs at high frequencies in some human populations (Figs. 4 and 5a).

To further examine the functional consequences of this substitution, we recreated the rs4911178 base-pair change in GROW1B-expression constructs and compared lacZ expression patterns in transgenic embryos (Fig. 4b). Strikingly, the derived A variant significantly decreased the expression of the growth enhancer in long bones but had no observable effect on facial expression, which served as an internal control.

We also cloned each GROW1B variant upstream of a luciferase-encoding gene and introduced constructs into a human fetal femoral-growth-plate chondrocyte cell line (Fig. 4c and Supplementary Fig. 7). The ancestral GROW1B sequence, compared with control vectors, drove strong luciferase expression, thus confirming its regulatory activity in human cells. The derived GROW1B-enhancer variant drove significantly lower expression than the ancestral variant (0.43- to 0.82-fold change; 500 ng average 0.72-fold change; n = 7 biological replicates; \( P = 7.7 \times 10^{-10} \)), thus indicating that the human G>A substitution decreases the activity of the GDF5 enhancer in human growth-collar chondrocytes (Supplementary Note).

Distribution of GDF5 variants in modern and ancient humans
We next explored the relationship of the rs4911178 variant to geographic patterns, trait associations, and signatures of selection in humans (Figs. 5 and 6 and Supplementary Note). The lower-activity A variant of GROW1 occurs at high frequencies in Eurasians but at
low frequencies in Africans (Figs. 5a and Supplementary Table 1). In Eurasians, the A allele is found near the center of an ~130-kb haplotype that extends from the GDF5 3′ UTR through the downstream region that controls limb length in mice (Supplementary Figs. 8 and 9). SNPs within this region showed high linkage disequilibrium with one another (\(D^\prime >0.98\); Supplementary Table 2), including tight linkage between rs4911178 and other SNPs previously associated with increased osteoarthritis risk (rs1433838, \(r^2 = 0.89\); rs6088813, \(r^2 = 1\); rs729908, \(r^2 = 1\); rs6060373, \(r^2 = 0.96\); rs6088792, \(r^2 = 0.64\); rs6060369, \(r^2 = 0.96\)) (Supplementary Fig. 10). Although rs4911178 has not been genotyped in all GWAS, several reports have confirmed that the derived A allele is significantly associated with shorter height (Supplementary Note).

To further examine the evolutionary history of GDF5, we used phased 1000 Genomes data to analyze 1,486 haplotypes spanning the 130-kb linkage block (Fig. 6a and Supplementary Table 3). Removal of low-frequency, putative recombinant haplotypes resulted in 1,091 haplotypes, which we used to construct a maximum-likelihood phylogenetic tree. This tree showed two well-supported clades differing at rs4911178 (Fig. 6a, Supplementary Fig. 11 and Supplementary Tables 4 and 5). Phylogenetic analyses using all haplotypes yielded similar results (Supplementary Figs. 12 and 13). Clade A, consisting exclusively of haplotypes containing the ancestral allele at rs4911178 (398/398), showed marked diversity within and between continents and was the most frequent haplogroup in Africans (145/202; 71.8%). In contrast, clade B consisted of many closely related haplotypes that all possessed the lower-activity derived growth variant at rs4911178 (693/693). This clade showed less sequence diversity and was the predominant haplogroup in Eurasians (i.e., 636/889; 71.5%) (Supplementary Table 4).

A subgroup of haplotypes within the B clade, which we denote B*, was particularly common in Eurasians but was also found at low levels in Kenya (1/202; 0.5% African haplotypes). Analysis of this African individual with Ancestry Informative Markers showed exceptionally low to no detectable evidence of admixture with Europeans (data not shown). Additional Africans (Luhyan and Yoruban) with B* haplotypes were present in the full data set, including low-frequency haplotypes (n = 5 haplotypes) (Supplementary Figs. 12 and 13). Interestingly, other Luhyans and Yorubans had haplotypes showing many but not all of the derived SNPs characteristic of B* (Fig. 6a). These haplotypes were candidate protohaplotypes that may have given rise to the B* haploptype.
The high differentiation of GDF5-allele frequencies among populations, as well as the prevalence, size, and low sequence diversity of the B* haplotype in non-Africans, all suggested that B* has been subject to positive selection in Eurasians\textsuperscript{11–14}. The Composite of Multiple Signals (CMS) method combines multiple selection signatures to map base-pair changes that have probably served as the causal

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**Figure 6** Evolutionary history of the GDF5 locus in humans. (a) Maximum-likelihood tree (left) and visual genotype (right) of phased 1000 Genomes, Neandertal, Denisovan, and chimpanzee (chimp) haplotypes. Left, two clades (A and B) with strong bootstrap support exist, both containing haplotypes from Europe (black), Asia (orange), and Africa (green). Within clade B, the high-frequency haplotype B* occurs in Eurasian individuals. Right, visual genotype across the 130-kb GDF5 regulatory locus. Clades A and B are partitioned on the basis of the variant at rs4911178. Ancestral (red) or derived (blue) states are shown for SNPs across the interval. Thin line demarcates clade A and clade B haplotypes; dashed line demarcates B and B*. Within clade B, Neandertal and Denisovan haplotypes are most related to haplotypes only in Africa, and are ancestral to the ‘short height’ B* haplotype found in Eurasians. (b) Three models of haplotype evolution at GDF5 locus. Blue X, shared mutations; black X, independent mutations. (c) Multiple GDF5 haplotypes were present in Africa in the middle to late Pleistocene, including haplotype B, containing the derived lower-activity growth variant. Neandertal and Denisovan ancestors carried this or a related shorter-height haplotype into northern latitudes (dashed lines). During the late Pleistocene, haplotypes (B*) related to (B) arose and were positively selected during more recent out-of-Africa migrations. The B* haplotype leads to decreased height via the derived rs4911178 variant in GROW1B (yellow). This variant travels with linked 5′-UTR functional variants (for example, rs143384)\textsuperscript{3} as well as variants within adjacent R2 (light purple) and downstream R3, R4, and R5 joint enhancers (light green)\textsuperscript{32}. 

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basis of positive selection. When applied to GDF5, CMS showed a selection peak far downstream of GDF5 coding exons, in the region where we mapped GROW1B (Fig. 5 and Supplementary Fig. 14). Notably, rs4911178 was one of the top three highest-scoring SNPs in the entire 130-kb region. The two other top-scoring SNPs resided in sequences that did not show reproducible enhancer activity in transgenic mice (Supplementary Fig. 5 and data not shown). Our findings thus suggested that the noncoding change at rs4911178 is the most likely molecular basis for the recent strong selection of GDF5 in non-Africans.

Whereas modern Eurasian populations arose from ‘out-of-Africa’ migrations beginning 130–50 thousand years ago (ka), Neandertals and Denisovans descended from an older migration out of Africa that occurred more than 600 ka. We analyzed Neandertal and Denisovan sequences to reconstruct GDF5 variants present in extinct hominins (Supplementary Table 3). Like many Eurasians, Neandertals and Denisovans show the lower-activity A allele of GROW1B rather than the ancestral G allele, which is highly conserved in other mammals (Supplementary Table 3). Phylogenetic analysis indicted that Neandertal and Denisovan haplotype clusters within clade B with haplotypes present in Yorubans and Luhyans of Africa, in agreement with sequence-divergence comparisons (Fig. 6a and Supplementary Table 6). Notably, this clade B subgroup is outside the B* cluster that is prevalent in Eurasians, and it does not share the same derived-SNP changes in the GDF5 5′ UTR (for example, rs143384) that have been identified in osteoarthritis association studies. These data suggested that an ancient low-growth variant was present in the human lineage before the last common ancestor of Neandertals, Denisovans and modern humans, and has become the predominant GDF5 variant in both archaic and modern Eurasians.

Functional consequences of GROW1 inactivation

To further examine the phenotypic consequences of changes in the Gdf5 growth enhancer, we used CRISPR–Cas9 editing to delete the GROW1 enhancer from the mouse locus (Supplementary Note). The GROW1-deletion mice lacked a 1.8-kb region, including the mouse ortholog of the rs4911178 site and highly conserved surrounding sequence. Like other mammals, all sequenced wild-type laboratory strains of mice carry a G at the rs4911178 position, similarly to the human ancestral sequence common in Africans. GROW1-deletion mice were viable, fertile, and born in expected Mendelian ratios (Supplementary Note). To identify possible effects of enhancer loss on gene regulation, we generated F1 hybrid mice heterozygous for both GROW1-deletion and control alleles, and compared the levels of expression of surrounding genes in RNA samples prepared from the growing ends of long bones. Loss of GROW1 significantly decreased the expression of the linked Gdf5 allele but not the linked upstream Cep250 allele or downstream Uqcc1 allele (deletion-allele Gdf5 expression 85 ± 1.48% of control-allele expression, P = 0.02857; deletion-allele Cep250 expression 108 ± 1.78% of control-allele expression, P = 0.02; deletion-allele Uqcc1 expression 88 ± 1.29% of control-allele expression, P = 0.6286; permutation tests; n = 4 biological replicates; Online Methods). These results confirmed that GROW1 is a cis-acting regulatory enhancer required for normal levels of Gdf5 expression at ends of bones.

To test for additional phenotypic consequences of the GROW1 deletion, we analyzed bones of GROW1+/− and control mice with microcomputerized tomography (μCT) (Fig. 7). The overall lengths of the femoral neck, femur, and tibia were shorter by ~7.5% (P = 0.007), femoral lengths (P = 0.02), femoral-head heights (P = 0.02), and tibial lengths (P = 0.01). The P values reflect results obtained from unpaired two-tailed t-tests; n = 12 mice per genotype. Box plots are as in Figure 2.

DISCUSSION

Previously, the most studied functional SNPs in the human GDF5 gene have been rs143383 and rs143884 in the 5′ UTR. Changes at these positions decrease gene expression in cultured cells and have been proposed to be the likely basis of the association with osteoarthritis and height in humans. Because they are located within the mature GDF5 transcript, changes in the 5′ UTR SNPs would probably affect most GDF5 functions. In contrast, our results showed that GDF5 is controlled by a distributed set of regulatory elements that allow for separate regulation of joints and the growing ends of bones (Figs. 1 and 3).
Several different enhancers are required to drive GDF5 expression in different joints in the skeleton. In addition, the 3′ region also contains the GROW1 enhancer characterized here, which is expressed in the growing ends of long bones, rather than joints between bones (Fig. 3). BAC rescue experiments showed that the large 3′ region, containing GROW1, is required for restoration of normal bone lengths in Gdf5 mutants (Fig. 2 and Supplementary Fig. 4). Targeted deletion of the GROW1 enhancer also decreases Gdf5 expression and the lengths of long bones in mice, thus confirming that this enhancer functions as a growth-controlling sequence in vivo (Fig. 7).

Interestingly, the orthologous GROW1 sequence in humans contains a common derived SNP (rs4911178) at an otherwise highly conserved position. This change alters a predicted binding site for PITX1, a quintessential regulator of hindlimb growth and patterning in many animals (Supplementary Fig. 15 and Supplementary Table 7). Experimental assays showed that the derived sequence change decreased PITX1 binding interactions in vitro (Supplementary Fig. 15 and Supplementary Table 7) and decreased GROW1 enhancer activity in both transgenic mice and human growth-plate chondrocytes (Fig. 4). We therefore propose that rs4911178 is a causal variant contributing to height variation in humans.

Interestingly, haplotypes containing the derived GROW1 variant show clear evidence of positive selection in Eurasians, and in many animals (Supplementary Fig. 15 and Supplementary Table 7). Experimental assays showed that the derived sequence change decreased PITX1 binding interactions in vitro (Supplementary Fig. 15 and Supplementary Table 7) and decreased GROW1 enhancer activity in both transgenic mice and human growth-plate chondrocytes (Fig. 4). We therefore propose that rs4911178 is a causal variant contributing to height variation in humans.

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The presence of this haplotype has notable human health implications, because the selected B* haplotype is associated with an increase in osteoarthritis by approximately 1.2- to 1.8-fold, thus representing one of the largest and most replicated genetic associations found for this prevalent form of human joint disease2–4. Although past positive selection for a disease-causing haplotype may seem paradoxical, osteoarthritis typically develops at postreproductive ages. Because evolutionary fitness requires successful reproduction, alleles that confer benefits at young or reproductive ages may be positively selected in populations, even if they have some deleterious consequences in postreproductive ages56.

The particular variants within B* haplotypes that cause increased osteoarthritis susceptibility remain unclear. The 5′-UTR rs143383 variant decreases reporter-gene expression in cultured cells3 and is also associated with lower expression of linked genes in patient articular cartilage24. However, the B* haplotype extends over an ~130-kb region that also includes a large and distributed system of regulatory enhancers required for normal expression and function of GDF5 in both joints32 and growing bones (Fig. 6a). Genetic variants throughout this haplotype travel in linkage disequilibrium with the selected GROW1 variant rs4911178 and may contribute to changes in osteoarthritis susceptibility. Interestingly, previous studies have suggested that the risk of GDF5-associated arthritis differs in hip, knee, wrist, and finger joints4. Some of this anatomical specificity may be related to genetic changes in different joint-specific enhancers, a possibility made more likely by the multiple GDF5 control regions regulating expression in different joints in the proximal and distal regions of the developing limb32. Future studies can focus on testing additional human sequence variants within these proximal and distal joint enhancers that may contribute to the very widespread health and morphological effects of the GDF5 locus in modern populations.

URLs. Jackson Laboratory, http://www.jax.org/; MIT CRISPR Tools, http://crispr.mit.edu/; HaploRegV4.1, http://archive.broadinstitute.org/mammals/haploreg/haplophp.php; UniPROBE Database, http://thebrain.bwh.harvard.edu/uniprobe/about.php; VisiGene, https://genome.ucsc.edu/cgi-bin/hgVisiGene?command=start; Eurexpress, http://www.eurexpress.org/e/e; Genepaint, http://www.genepaint.org/Framest.html; Mouse Genome Informatics, http://www.informatics.jax.org/; 1000 Genomes Project raw data, ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20110521/ALL.chr20.phase1_release_v3.20110123.snpss.indels_svs.genotypes.vcf.gz; high-coverage Neandertal sequence data, http://cdn.eva.mpg.de/neandertal/altai/AltaiNeandertal/VCF/; high-coverage Denisovan sequence data, http://cdn.eva.mpg.de/denisova/VCF/; Neandertal Sequencing Project, http://genome.ucsc.edu/Neandertal/; SAMtools, http://samtools.sourceforge.net/.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.D.C. and D.M.K. conceived and oversaw the project. H.C. and M.S. designed BAC transgenic mice. M.S., H.C., and T.D.C. performed mouse rescue experiments and phenotyping. J.C. and T.D.C. performed GROW1 CRISPR–Cas9 gene editing, mouse breeding, genotyping, and phenotyping. A.M.K. and T.D.C. performed morphometric analyses on GROW1 mict specimens. T.D.C. performed in situ hybridization expression experiments, identified and fine-mapped the growth-enhancer region, conducted coding SNP analyses, UniPROBE analyses, and HaploRegV4.1 analyses, and performed all in vitro and in vivo tests of the effects of the rs4911178 polymorphism. T.D.C. and A.C.D. performed allele frequency, initial haplotype detection, and CMS analyses. A.C.D. processed 1000 Genomes and archaic hominin data sets; performed haplotype and visual genotype analyses, and tree-building experiments; and provided input into all computational assays. T.D.C. and M.K.C. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

BAC, fosmid, and Hsp68 LacZ plasmid constructs. The 140-kb upstream BAC (306G24) and 200-kb downstream BAC (RP23-316K12), spanning the Gdf5 region, were isolated from mouse libraries and modified by insertion of an internal ribosome-entry site–β-galactosidase reporter cassette (pIREs-B-Geo in the 3′ UTR of the last exon of Gdf5 (ref. 32). The size and orientation of the BAC inserts, as well as the sequence of the intact Gdf5 coding exons and targeted 3′-UTR regions, were verified with restriction mapping, pulsed-field gel electrophoresis, Southern blotting, and Sanger sequencing.

Two fosmids, ABC8-2134240-H3 and ABC12-46947700-I24, generated by the Human Genome Structure Variation Project (ref. 33), were chosen on the basis of their overlap with conserved noncoding regions of downstream of the upstream BAC (Supplementary Table 8). Fosmid ABC8-2134240-H3, ~37 kb in size, has an ancestral human haplotype, as determined by sequencing, and comes from a Yoruban individual. ABC12-46947700-I24, ~41 kb in size, also has an ancestral haplotype but comes from a CEPH individual heterozygous at this locus. Each fosmid bacterial stab was streaked out on chloramphenicol plates, and individual colonies were picked and grown in LB plus chloramphenicol. After purification with Nucleobond Xtra Maxi Plus kits (Macherey-Nagel, 740416.10), each fosmid was linearized with the NotI restriction enzyme (New England Biolabs, R0189L), purified via three rounds of microinjection buffer exchange with Centriprep-30 concentrators (Amicon, A4306), and run on a CHEF gel overnight. Successfully digested clones were then SNP- and end-sequence-verified, and passed through Centrex MF-15 filters (Schleicher and Schuell, 10467004). Finally, each prepared fosmid was co-injected and co-integrated with a NotI-linearized Hsp68 basal promoter–lacZ reporter vector.

Evolutionarily conserved noncoding regions tested for enhancer activity were either PCR-amplified with primers containing appropriate linker sites from C57BL/6 mouse genomic or human fosmid DNA, or synthesized by GenScript. Each individual region was cloned into a modified (described below) or unmodified p5'-NotI-Hsp68-lacZ expression vector containing a minimal heat-shock promoter and a lacZ cassette. Supplementary Table 8 lists all the constructs used in this study, along with their primers and source DNA. For concatenated constructs, primers containing SfiI linker sequences were used. Their PCR products were briefly ligated to form tandem copies before cloning into a modified p5'-NotI-Hsp68-lacZ vector containing a SfiI restriction site that was inserted between two NotI sites upstream of the lacZ cassette. Ligated DNA was next transformed into DH10 cells and streaked on LB plates containing ampicillin; single colonies were then picked, PCR screened, sequenced, and grown in the presence of ampicillin with standard Maxi-Prep kits (Qiagen). Finally, successful clones were processed for microinjection as described below.

Transgenic mice. Transgenic mice were generated through pronuclear injections carried out by the Stanford Transgenic Facility or by Taconic/Xenogen Biosciences. All constructs were linearized with NotI and then purified for microinjection as described above. Constructs were next microinjected into derived FVB or C57BL/6/CBA1F1 oocytes. Founder transgenic embryos were collected at E14.5 or E15.5 and X-gal stained. For each transgene, multiple embryos derived from independent injections were compared for their expression patterns, and only reproducible patterns were counted as significant. Stable transgenic mouse lines were also generated for both BAC clones (n = 3 for upstream BAC; n = 5 for downstream BAC). For each stable line, timed matings were established, and the resulting progeny were X-gal stained. The results were directly compared with results from multiple independent founder transgenic embryos. Multiple stable lines were made for each construct, as previously described35. For time-course studies, embryos from stable transgenic lines were collected from E14.5 through E16.5 and at specific postnatal time points up to 6 months. All mouse procedures in this and other sections were done in accordance with protocols approved by the Stanford University and Harvard University Institutional Animal Care and Use Committees.

X-gal staining. Whole-mount staining for β-galactosidase activity was performed as previously described35. Embryos were hemisedected and fixed in 4% paraformaldehyde (PFA) (Sigma, 158127) at 4 °C, according to gestational-day guidelines. Fixed embryos were washed three times in wash buffer and stained for 16–24 h in the dark with 1 mg/ml X-gal (Sigma, B4252) in staining buffer at room temperature. For comparisons between ancestral and derived variants, embryos were processed with identical protocols. After staining, embryos were briefly rinsed in wash buffer and postfixed in 4% PFA for 5 h.

In situ hybridization experiments. Antisense and sense digoxigenin-labeled probes for in situ hybridization were generated for Gdf5, Col1a1, and Col2a1 as previously described52,59. To compare Gdf5 lacZ expression, whether driven by the upstream BAC, downstream BAC, or GROW1 regulatory sequence, to endogenous gene expression, E15 lacZ+ embryos were harvested, snap-frozen, and embedded in Tissue Tek OCT compound. Embedded embryos were then serially sectioned with a cryostat. Adjacent sections (n = 3 per experiment) were either stained for lacZ expression with the X-gal staining methods described above or used in standard in situ hybridization protocols as previously described52.

BAC rescue experiments and genotyping of progeny. Independent mouse lines carrying either the upstream BAC or the downstream BAC were crossed to animals homozygous for the brachypodism allele (URLs; described below). Multiple different downstream-BAC (n = 5) and upstream-BAC (n = 3) founders were used in the above crosses to control for the integration site of each BAC in the genome. Brachypodism mutations result in fr ameshifts and premature translational termination in the Gdf5 open reading frame16. Animals that were bpk+; Gdf5−/− were then crossed to nontransgenic bpk/bpk mice. The progeny were genotyped for the lacZ transgene and the brachypodism mutation in separate PCRs, with the primers listed in Supplementary Table 8. Notably, PCRs for the Gdf5 locus amplified the endogenous Gdf5 rather than the BAC sequences specifically, because the reverse primer was positioned downstream of the IRES-B-Geo insertion at the 3′ UTR of the Gdf5 transgene, thus making amplification inefficient for the BAC. The brachypodism mutation was identified by sequencing around the bp site in the resulting PCR product. Mice that were bpk+/bpk; BAC−, bpk/bpk, BAC+ or bpk+/BAC− were compared as described below.

CRISPR–Cas9 gene targeting of the GROW1 enhancer. Dual sgRNAs surrounding the mouse GROW1 regulatory element were designed with MIT CRISPR Tools (URLs), synthesized by Integrated DNA Technologies, and cloned into the PX458 vector according to a previously described protocol41 (step 5, part B). The sequences of both sgRNAs are listed in Supplementary Table 8. Guide RNAs were initially tested for their ability to induce efficient deletions of the mouse GROW1 enhancer in cultured mouse NIH3T3 cells. NIH3T3 cells were maintained in DMEM (Gibco) supplemented with 10% FCS (Gibco) and 1% penicillin–streptomycin (0.025%), and 0.3 × 106 cells were seeded per well of a six-well plate 1 d before transfection. On the day of transfection, cells were incubated for 30 min in 0.5 ml of fresh culture medium, to which we added 0.5 ml of a solution containing 1 μg sgRNA1–PX458 and 1 μg sgRNA2–PX458 in 250 μl Opti-MEM (Invitrogen), and 6 μl Lipofectamine 2000 (Thermo Fisher Scientific) with 250 μl Opti-MEM (Invitrogen). After 2 d of culture at 37 °C, we scanned cells under a fluorescence microscope to verify successful transfection and GFP expression. DNA was then extracted with an E.Z.N.A Tissue DNA Kit, and the GROW1 region was amplified with touchdown PCR and primers surrounding the guide-RNA-design sites (external primers F1 and R1, Supplementary Table 8). Amplification products were isolated from 1% agarose gels (E.Z.N.A Gel Extraction Kit) and sequenced (Eton Bioscience) to verify GROW1 deletion.

After confirmation that the sgRNAs worked effectively in vitro, we performed in vitro transcription of sgRNAs by following a previously described method40 to generate guides useful for DNA microinjection. These sgRNAs along with transfection-ready Cas9 mRNA (CASS500A-1, Systems Biosciences) were provided to the Harvard Genome Modification Facility for microinjection in wild-type C57BL/6J pronuclei. After F0 founder mice (n = 3) were obtained, we extracted DNA from tail clippings and performed genotyping with the F1 and R1 PCR primers as above. The wild-type amplification product was 2,373 bp, and a subset of founder mice showed a deletion band of ~550 bp. Positive F0 mice were bred to C57BL/6J mice to transmit the mutations. F1 progeny from a single stable line were then intercrossed to generate F2 progeny with wild-type, heterozygous, or homozygous GROW1 genotypes.
which were verified with PCR amplification with F1/R1, F1/R2, and F2/R1 primers (Supplementary Table 8). All genotypes were present at the expected Mendelian ratios. Sequencing confirmed that the tested GROW1 deletion removed 1,830 noncoding base pairs, corresponding to chromosome 2: 155726835–155725006 (mm9).

Skeletal preparation and measurements. Male adult mice (P30 and P56) were stained with alcin blue and alizarin red and cleared as previously described41. The skin and visceral organs were removed, and the animals were fixed in 95% ethanol. The specimens were then stained with a mixture of alcin blue, acetic acid, and ethanol. After being washed several times in 95% ethanol, the samples were cleared in KOH and then stained with alizarin red in KOH solution. Skeletons were stored in glycerol and, when necessary, photographed.

For the brachypodism BAC rescue experiment, skeletal-element length and cranial length were measured with a microcaliper under a dissecting microscope. Ten specimens (for example, femora) for each genotype (i.e., homozygous bp animals without the BAC, homozygous bp/bp animals with the BAC, and bp/+ animals without the BAC) were measured. Numbers of animals were chosen to provide multiple independent samples of all key genotypes. No randomization of animals to different treatment groups was necessary, given the nature of the experiments. No animals were excluded from analysis, and blinding was not part of the study design. Analytical details are described below.

To detect phenotypic differences in GROW1-deletion mice, 30-day old male specimens (n = 12) for each genotype were collected, and skeletons were prepared as above and sent to the Center for Skeletal Research Imaging and Biomechanical Testing Core at the Massachusetts General Hospital for scanning by μCT. The right femur and tibia of each animal were scanned with a high-resolution desktop μCT imaging system (μCT40, SCANCO Medical AG, Brüttisellen, Switzerland) to assess bone morphology. The bones were imaged with a 12 μm³ isotropic voxel size, 70-kVp peak X-ray-tube intensity, 114-mA X-ray-tube current, and 200-ms integration time. Scans were reconstructed with SCANCO GPU Reconstruction software, and DICOM images were then exported for bone morphology measurements with OsiriX MD V.7.5 (Pixmeo SARL, Bernex). Next, in a blind experimental design in which the measurer (AMK) did not have access to the genotypes, μCT scans of each femur and tibia were measured for femoral-neck length, femoral length, femoral-head height, and tibial length. In a separate round of measurements, investigator T.D.C. measured long bone lengths on the same specimens with a microlapler under a dissecting microscope. Measurements were normalized in two different ways, on the basis of either (i) the long axis of the third caudal vertebrae, a bone of the axial skeleton that was easily dissected and was not under the control of the GROW1 element, and whose measurements were not statistically different between genotypes, or (ii) the length of the clavicle, a skeletal element that forms predominantly via intramembranous ossification and is not under the control of the GROW1 element, and whose length estimates were both highly reproducible and not statistically different between genotypes. Analytical details are described below.

Allele-specific expression analyses. Timed matings were established between C57BL/6j GROW1+/− heterozygous mice and 129SVJ GROW1+/− wild-type mice. Pregnant females were sacrificed according to IACUC-approved protocols to acquire E15.5 embryos. Distal femoral growth plates from both sides of an individual animal were pooled and homogenized with a tissue homogenizer (Qiagen), and RNA was isolated with TRIzol reagent (15596-026, Ambion by Life Technologies) and an RNA Clean & Concentrator-5 Kit (supplied with DNase I, Zymo) (n = 4 distal femoral growth plate biological replicates). Samples were then run on a Bioanalyzer to ensure RNA integrity numbers greater than 8. These RNA samples were then reverse transcribed with a SuperScript IV First Strand cDNA Synthesis Reaction kit (18090010, Life Technologies) according to the manufacturer’s recommendations. Independently, tails from each embryo were used for GROW1 genotyping as described above.

cDNA samples were then sent to EpigenDx for allele-specific expression-assay design and execution. SNPs in the coding regions of Cd65 (rs27340038), Cep250 (rs27339949), and Uqcc1 (rs3684985) were identified by EpigenDx. Pyrosequencing for SNP genotyping (PSQ H96A, Qiagen Pyrosequencing) is a real-time sequencing-based DNA analysis that quantitatively determines the genotypes of single or multiple mutations in a single reaction. Briefly, 1 ng of sample cDNA was used for PCR amplification. PCR was performed with 10x PCR buffer (Qiagen) with 3.0 mM MgCl₂, 200 M of each dNTP, 0.2 μM each of the forward and reverse primers (available through EpigenDx), and 0.75 U of HotStar DNA polymerase (Qiagen) per 30-μl reaction. The PCR cycling conditions were 94 °C for 15 min; 45 cycles of 94 °C for 30 s; 60 °C for 30 s; 72 °C for 30 s; and 72 °C for 5 min. One of the PCR-primer pairs was biotinylated to convert the PCR product to single-stranded DNA-sequence-templates with streptavidin beads and the PyroMark Q96 Vacuum Workstation. 10 μl of the PCR products was bound to streptavidin beads, and the single strand containing the biotinylated primer was isolated and combined with a specific sequencing primer (available through EpigenDx). The primed single-stranded DNA was sequenced with a Pyrosequencing PSQ96 HS System (Qiagen Pyrosequencing) according to the manufacturer’s instructions (Qiagen Pyrosequencing). The genotypes of each sample were analyzed with Q96 software AQ module (Qiagen Pyrosequencing).

Pyrosequencing results for each SNP were used to calculate the allelic ratios of 129SVJ (wild-type allele) to C57BL/6j (GROW1-null allele) in the heterozygous state. Each ratio of cDNA products found in heterozygous animals was then normalized by the ratio of WT 129SVJ to C57BL/6j genomic products, amplified from known 1:1 mixtures of each sequence. Analytical details are described below.

Cloning and construct preparation for in vitro assays. For in vitro transfection into Chon-002 cells (described below), primers containing NotI linker sequences were used to amplify 3x-copy-inserted ancestral human GROW1B and derived human GROW1B sequences (from each modified p5’-NotI-Hps68lacZ plasmid studied in vivo; described above and in Supplementary Table 8) and ligated into a NotI-containing pGL4.23 firefly luciferase vector. Ligated DNA for constructs containing inserted or noninserted (‘empty’) sequence were next transformed into DH10B cells. The cells were streaked on ampicillin plates, and single colonies were then picked, PCR-screened, sequenced, and finally purified with Endo-Free Maxi-Prep kits (Qiagen).

In vitro assay with Chon-002 cells. Chon-002 cells derived from human fetal female 18-week-old femoral-growth-plate chondrocytes were obtained from ATCC (CRL-2847) and cultured at 5% CO₂ at 37 °C in ATCC complete growth medium containing DMEM, 10% FBS, 50 mg/ml penicillin–streptomycin, and 0.1 mg/ml G-418 (Genetici). Medium was replaced every 2 d, and the cells were subcultured every 5 d. We received certified passage-25 cells from ATCC and used cells at passage 26–29 for transfection experiments without additional STR authentication or mycoplasma testing. Before transfection, cells were first cultured in DMEM with 10% FBS for 24 h in 24-well dishes at a seeding density of 2 × 10⁴ cells/well. Next, in DMEM only, cells were transiently transfected with varying amounts of ancestral or derived firefly luciferase reporter vector (i.e., 100 ng, 300 ng, or 500 ng) plus empty pGL4.23 luciferase vector (to a total concentration of 500 ng of pGL4.23 luciferase vector per well) along with pRL-CMV Renilla luciferase vector for transfection efficiency (Promega, E226A) with Fugene6 transfection reagent at a Fugene6/DNA ratio of 3:1 (Promega, E269A), according to the manufacturer’s recommended protocols. Enhancer activity was measured 24 h after transfection with the Dual-Luciferase Reporter Assay System (Promega, TM040), according to the manufacturer’s protocol, on a Dual Injector GloMax Multi Luminometer System (Promega, BASE 9311-011 and DUAL INJECTOR 9301-062). Analytical details are described below.

Computational methods. The following SNP HGVS accession codes are relevant to this study: rs143383, NC_000020.10:34025983A>G; rs143384, NC_000020.10:34025556A>G; rs4911178, NC_000020.10:3952620G>A; rs6088813, NC_000020.10:33975181C>A; rs725908, NC_000020.10:33968067T>C; rs6060373, NC_000020.10:33914208A>G; rs6088792, NC_000020.10:33909784C>T; and rs6060369, NC_000020.10:33907161T>C.

Predicted transcription-factor-binding sites. To identify upstream transcription factors (TF) predicted to bind at the rs4911178 site, we used HaploRegV4.1 (ref. 62) with default parameters (URLs). We also queried the UniPROBE

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individual population and continent, we computed the LD between all pairwise Linkage disequilibrium (LD) and haploblock detection. Separately for each required for normal long-bone development 66. 1000 Genomes Project data retrieval and SNP-frequency calculations. We focused on a sequence window spanning 200 kb upstream and 200 kb downstream of the GDF5 locus (chromosome 20: 33752620–34152620; hg19). Phased haplotype data were retrieved for SNPs across this region via the 1000 Genomes resource33. The raw data (VCF format) are available online (URLs).

Twelve data files were retrieved: one data set (primary) included geno- type data for all eight nonadmixed populations (CEU, GBR, TSI, CHS, CHB, JPT, YRI, and LWK); eight data sets were divided by population; and three were divided by continent (EUR, European; ASI, Asian; and AFR, African). Admixed populations (ASW, CLM, FIN, IBS, MXL, and PUR) were removed. Related individuals were also removed from all 1000 Genomes phased haplo- type data and any subsequent analyses.

The Primary data set (all eight populations) consisted of 1,486 genotypes (743 individuals x 2 genotypes per individual) and 4,066 SNPs, identified across this 400-kb window. This data set was used to compute continent- and population-specific SNP frequencies. 1,370 and 622 SNPs were present at overall frequencies 0.01 and 0.05, respectively.

We next ran Haplovie (described below) and detected a smaller haplo- block at chromosome 20: 33887955–34025983 (138,029 bases). Within this haploblock, there were 1,354 SNPs. On the basis of MAF, we acquired 174 SNPs (MAF ≥0.05) and 1,489 genotypes, and 395 SNPs (MAF >0.01) and 1,489 genotypes. After removing all rare recombinant haplotypes with MAF >0.05, we generated a reduced data set of 1,091 human haplotypes plus sequences of chimpanzee, Neandertal and Denisovan (total 1,094 haplotypes, 174 SNPs).

Within this data set, we defined a haploblock region detected above (chromosome 20: 33887955–34025983) as the haploblock region detected above (chromosome 20: 33887955–34025983) for each individual in the data set. Corresponding chimpanzee (panTro3), Neandertal and Denisovan alleles were also included.

Ancient sequences. High-coverance Neandertal39 and Denisovan40 sequence data were acquired (URLs). Additional low-coverance Neandertal sequence data were acquired from the Neandertal Sequencing Project (URLs) and used ancient DNA acquired from six distinct individuals38. Three individuals were acquired from the Vindija cave in Croatia (V133.16, ~54.1% genome coverage; V133.25, ~46.6% genome coverage; V133.26, ~45.2% genome coverage); one was from the Neandertal-type specimen from the Neander Valley in Germany (FeldI, ~0.1% genome coverage); one was from the El Sidron cave in Asturias, Spain (Sid1255, ~0.1% genome coverage); and one was from Mezmaiskaya in the Altai Mountains, Russia (Mez1, ~2% genome coverage)38.

All sequences for the GDF5 interval from these six individuals as well as the Altai Neandertal and Denisovan were extracted specifically with SAMtools with a region specifier (URLs). Extracted sequences were re-aligned to hg19, and the consensus sequences for the region for each hominin were built. Missing sequence or gaps (due to human-specific insertions) were designated with an ‘N’ in each archaic hominin consensus.

Haplotyp phylogeny and visualization. A multiple alignment of 1,094 indi- viduals (reduced data set) was constructed for SNPs with MAF ≥0.05 across the haploblock region detected above (chromosome 20: 33887955–34025983) for each individual in the data set. Corresponding chimpanzee (panTro3), Neandertal and Denisovan alleles were also included.

Positions containing a gap (–) or missing information (N) in archaic homin- in sequences (and a small number of cases in chimpanzee) were removed. This removal resulted in an alignment of 174 SNPs with complete allele infor- mation for 1,091 human genotypes, 1 Neandertal, 1 Denisovan, and 1 chim- panzee sequence (reduced data set). A maximum-likelihood (ML) tree was then constructed with RAxML with the GTR+WAG model of evolution. The chimpanzee sequence was defined as an outgroup. RAxML rapid bootstrapping was performed to provide a measure of clad support.

Visual genotypes were generated by classifying SNP values as derived or ancestral on the basis of precalculated ancestral alleles from Ensembl Compara (originally calculated with a six-way primate alignment). Visual genotypes were then mapped onto the phylogeographic trees to trace patterns of SNP evolution.

Statistical methods. All computational statistics and visualizations were performed with SPSS Statistics 17.0, Microsoft Excel, or R, after verifica- tion of normality and approximately equivalent levels of variance between sample groups.

For RAC rescue and GROW1-deletion experiments, the indicated P values were based on unpaired two-tailed t-tests. Box-and-whisker plots for each experiment (Figs. 2 and 7 and Supplementary Fig. 4) show the upper and lower quartiles spanning the interquartile range (edges of the box), the median is marked by a horizontal line within each box, and the whiskers show the maximum and minimum values from each experiment.

For cell culture experiments, to compare expression between the ancestral GROW1 construct and either the derived GROW1 construct (Fig. 4) or the empty vector construct (Supplementary Fig. 7), we performed at least five independent transfection experiments containing eight technical replicates (i.e., individual wells of a 24-well dish) per construct per concentration, and did so at four distinct reporter concentrations (i.e., 100 ng, 200 ng, 300 ng, 400 ng).

Linkage disequilibrium (LD) and haploblock detection. Separately for each individual population and continent, we computed the LD between all pairwise SNPs with Haplovie 4.2 (ref. 69) with default parameters (HW P-value cutoff, 0.001; minimum genotype percentage, 75; maximum number of Mendel errors, 1) and MAF ≥0.05 (because this parameter was also used in subsequent haploblock-detection calculations). Haploblocks were then calculated with the default algorithm72, which detects blocks with 95% of informative comparisons in ‘strong LD’. A large haploblock containing SNP rs4911178 was detected consistently across European and Asian populations. This haploblock was also the largest observed across the 400-kb interval, and it was then extended to the maximal borders detected in any population, which occurred in the JPT population and encompassed the region chromosome 20: 33887955–34025983. These results were nearly identical to those identified in much larger genomic regions with both UCSC hg18 Genome Browser LD annotations and HGDP online tools. Additional LD scores reported in this manuscript were also computed with Haplovie with the above parameters unless otherwise noted.

Analysis of protein-coding substitutions in the locus. Analysis of protein-coding substitutions involved with data collected from several existing databases (UCSC Genome Browser coding SNPs and dbSNP) as well de novo detection of polymorphisms from 1000 Genomes Project databases (pilot 1000 Genomes variant calls, URLs). Given the presence of a high number of exception- tally rare (MAF <0.0001) to rare (MAF <0.01) variants from both de novo and existing databases, functional predictions were made only for those poly- morphisms that had an MAF ≥0.05 for each continent and a global MAF >0.1, and that were not disqualified because of ascertainment and variant-calling issues33. Each coding substitution was then functionally analyzed with Poly-Phen2 (ref. 67) and SIFT48 databases (Supplementary Table 1).

Statistical methods. All computational statistics and visualizations were performed with SPSS Statistics 17.0, Microsoft Excel, or R, after verifica- tion of normality and approximately equivalent levels of variance between sample groups.

For RAC rescue and GROW1-deletion experiments, the indicated P values were based on unpaired two-tailed t-tests. Box-and-whisker plots for each experiment (Figs. 2 and 7 and Supplementary Fig. 4) show the upper and lower quartiles spanning the interquartile range (edges of the box), the median is marked by a horizontal line within each box, and the whiskers show the maximum and minimum values from each experiment.

For cell culture experiments, to compare expression between the ancestral GROW1 construct and either the derived GROW1 construct (Fig. 4) or the empty vector construct (Supplementary Fig. 7), we performed at least five independent transfection experiments containing eight technical replicates (i.e., individual wells of a 24-well dish) per construct per concentration, and did so at four distinct reporter concentrations (i.e., 100 ng, 200 ng, 300 ng, 400 ng).

Analysis of protein-coding substitutions in the locus. Analysis of protein-coding substitutions involved with data collected from several existing databases (UCSC Genome Browser coding SNPs and dbSNP) as well de novo detection of polymorphisms from 1000 Genomes Project databases (pilot 1000 Genomes variant calls, URLs). Given the presence of a high number of exception- tally rare (MAF <0.0001) to rare (MAF <0.01) variants from both de novo and existing databases, functional predictions were made only for those poly- morphisms that had an MAF ≥0.05 for each continent and a global MAF >0.1, and that were not disqualified because of ascertainment and variant-calling issues33. Each coding substitution was then functionally analyzed with Poly-Phen2 (ref. 67) and SIFT48 databases (Supplementary Table 1).

Linkage disequilibrium (LD) and haploblock detection. Separately for each individual population and continent, we computed the LD between all pairwise
and 500 ng). For each concentration, we compared the mean expression of the ancestral construct to that of the mean expression of the empty-vector construct or derived construct with a two-sample directional Student’s t-test. The P values from independent experiments were combined across either five (for the empty-vector-construct comparison) or seven (for the derived-construct comparison) experiments (at each concentration) with Fisher’s combined probability test (two-tailed). Box-and-whisker plots (Fig. 4 and Supplementary Fig. 7) show the upper and lower quartiles spanning the interquartile range (edges of the box), the median is marked by a horizontal line within each box, and the whiskers show the maximum and minimum values.

For the allele-specific expression analysis, the permutation test, a nonparametric measure, was used to determine significance between the wild-type and heterozygous allelic expression ratio with the [perm] module in R.

Data availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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