Recent Regulatory Changes Shaped Human Facial and Vocal Anatomy

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Identifying changes in gene regulation that shaped human-specific traits is critical to understanding human adaptation. Here, we use ~70 DNA methylation maps of chimpanzees and different human groups, both present-day and ancient, to detect regulatory changes that emerged in modern humans. We show that genes affecting vocalization and facial features went through particularly extensive changes in methylation. Especially, we identify expansive changes in a network of genes regulating skeletal development (SOX9, ACAN and COL2A1), and in NFIX, which controls facial projection and voice box (larynx) development. We propose that these changes played a key role in shaping the human face, and in forming the human 1:1 vocal tract configuration that is considered optimal for speech. Our results provide insights into the molecular mechanisms that shaped the modern human face and voice, and suggest that they arose after the split from Neanderthals and Denisovans.
The advent of high-quality ancient genomes of archaic humans (the Neanderthal and Denisovan) opened up the possibility to identify the genetic basis of some unique modern human traits. A common approach is to carry out sequence comparisons and identify non-neutral sequence changes. However, out of ~25,000 fixed substitutions and indels that separate present-day from archaic humans, only 48 directly alter amino acid sequence, and as of today our ability to estimate the biological effect of the rest of these changes is limited. While most of these sequence changes are probably nearly neutral, some may affect gene regulation, especially those in regulatory regions such as promoters and enhancers. Such regulatory changes may have sizeable impact on human evolution, as alterations in gene regulation are thought to account for much of the phenotypic variation between closely related groups. Thus, direct examination of DNA regulatory layers such as DNA methylation is critical in understanding human-specific traits.

A key trait that sets humans apart from other apes is our unique ability to communicate through speech. This capacity is attributed not only to neural changes, but also to structural alterations to the vocal tract. The relative role of anatomy in our speech skills is still debated, but it is nevertheless widely accepted that even with a human brain, other apes could not reach the human level of articulation. Non-human apes are restricted not only in their linguistic capacity (e.g., they can hardly learn grammar), but also in their ability to produce the phonetic range that humans can. Indeed, chimpanzees communicate through sign language and symbols much better than they do vocally, even after being raised in an entirely human environment. Phonetic range is determined by the different conformations that the vocal tract can produce. These conformations are largely shaped by the position of the larynx, tongue, lips and mandible. Modern humans have a 1:1 proportion between the horizontal and vertical dimensions of the vocal tract, which is unique among primates (Fig. 1a). It is still debated whether this configuration is a prerequisite for speech, but it was nonetheless shown to be optimal for speech. The 1:1 proportion was reached through a relative shortening of the human face, together with the descent of the larynx. Attempts to use anthropological remains to determine whether Neanderthals and modern humans share similar vocal anatomy proved hard, as cartilaginous tissues do not survive long after death and the only remnant from the Neanderthal laryngeal region is the hyoid bone. Based on this single bone, on computer simulations and on tentative vocal tract reconstructions, it is difficult to characterize the full anatomy of the Neanderthal vocal apparatus, and opinions remain split as to whether it was similar to modern humans.
To gain insight into the genetic regulation that underlies human evolution, we have previously developed a method to reconstruct pre-mortem DNA methylation maps of ancient genomes based on analysis of patterns of damage to ancient DNA. We have used this method to reconstruct the methylomes of a Neanderthal and a Denisovan, and compared them to a present-day osteoblast methylation map. However, the ability to identify differentially methylated regions (DMRs) between the human groups was confined by the incomplete reference map, the differences in sequencing technologies, the lack of an outgroup and the restricted set of skeletal samples (see Methods). Here, we sought to identify DMRs based on a comprehensive assembly of skeletal DNA methylation maps. To the previously reconstructed Denisovan and Altai Neanderthal maps, we added the methylome of the ~40,000 years old (yo) Vindija Neanderthal, and four methylomes of anatomically modern humans: the ~45,000 yo Ust'-Ishim individual, the ~8,000 yo Loschbour individual, the ~7,000 yo Stuttgart individual, and the ~7,000 yo La Braña 1 individual whose genome we sequenced to high-coverage. To obtain full present-day maps, we produced whole-genome bisulfite sequencing (WGBS) methylomes from the bones of two present-day individuals (hereinafter, bone1 and bone2). To this we added 54 publically available partial bone methylation maps from present-day individuals, produced using reduced-representation bisulfite-sequencing (RRBS) and 450K methylation arrays. Hereinafter, ancient and present-day modern humans are collectively referred to as modern humans (MHs), while the Neanderthal and Denisovan are referred to as archaic humans. As an outgroup, we produced six chimpanzee methylomes (WGBS, RRBS, and four 850K methylation arrays; Extended Data Table 1). Together, these data establish a unique and comprehensive platform to study DNA methylation dynamics in recent human evolution.

**Identifying DMRs**

In order to minimize artifacts that might arise from comparing methylation maps produced through different technologies, we used the reconstructed Ust'-Ishim methylome as the MH reference, to which we compared the Altai Neanderthal and the Denisovan. We identified 18,080 loci that showed methylation differences between these individuals. Notably, these DMRs do not necessarily represent differences between the human groups. Rather, many of them could be attributed to factors separating the three individuals (e.g., Ust'-Ishim is a male whereas the archaic humans are females), or to variability within populations. To account for this, we used the ~50
additional human maps to filter out regions where variability in methylation is detected. Importantly, our samples come from both sexes, from individuals of various ages and ancestries, and from a variety of skeletal parts (tooth, femur, knee, phalanx, skull, rib; Extended Data Table 1), hence allowing the removal of DMRs that might arise due to any of these variables. This left a set of 6,371 DMRs that discriminate between the human groups, which we ranked according to their significance level (Extended Data Table 2).

Next, using the chimpanzee samples, we were able to determine for 3,869 of these DMRs the lineage where the methylation change occurred. Of these DMRs 1,667 were MH-derived, 1,103 were archaic-derived, 597 were Neanderthal-derived, and 502 were Denisovan-derived (Fig. 1b). The large number of MH samples used to filter out within population variability led us to focus in this work on MH-derived DMRs. We assigned them to three hierarchies based on which samples were used to filter out variable loci: (i) The list of 1,667 DMRs was derived using all full (WGBS and reconstructed) MH and chimpanzee bone methylomes (hereinafter, full bone MH-derived). (ii) For 1,100 DMRs we also had information from the partial (450K and 850K methylation arrays) MH and chimpanzee bone methylomes (hereinafter, bone MH-derived). (iii) For 881 DMRs we used information from all MH and chimpanzee skeletal methylomes, including teeth (hereinafter, skeletal MH-derived, Extended Data Table 2).

**Voice-affecting genes are derived in MHs**

We defined differentially methylated genes (DMGs) as genes that overlap at least one DMR along their body or up to a distance of 5 kb upstream (Extended Data Table 2). To gain insight into the function of these DMGs, we analyzed their gene ontology. As expected from a comparison between skeletal tissues, all three hierarchies of MH-derived DMGs are enriched with terms associated with the skeleton (e.g., chondrocyte differentiation, proteoglycan biosynthetic process, cartilage development, embryonic skeletal system development and ossification). Also notable are terms associated with the skeletal muscle, cardiovascular and nervous system (Extended Data Table 3).

To get a more precise picture of the possible functional consequences of these DMGs, we used Gene ORGANizer\textsuperscript{22} (geneorganizer.huji.ac.il). This is a tool that links genes to the organs where their phenotypes are observed, and was built based on curated gene-disease and gene-phenotype
associations. We used Gene ORGANizer to identify body parts that are significantly over-represented in our DMG lists. We found that within MH-derived DMGs, genes that affect the voice are the most enriched (Fig. 1c, Extended Data Table 4). For example, when running the list of skeletal MH-derived DMRs, we identified 14 significantly enriched body parts, with the strongest enrichment in the vocal cords (x2.18, FDR = 0.01), followed by the voice box (larynx, x1.74, FDR = 0.029) and then by body parts belonging primarily to the face, spine and pelvis. Interestingly, these parts are considered to be among the most morphologically derived regions between Neanderthals and MHCs. When limiting the analysis only to DMGs where the most significant DMRs are found (top quartile), the over-representation of voice-affecting genes becomes even more pronounced, with the vocal cords being enriched over 3-fold (FDR = 4.2x10^{-3}), and the larynx over 2-fold (FDR = 6.1x10^{-3}, Fig. 1d, Extended Data Table 4). This enrichment is also apparent when examining patterns of gene expression (Extended Data Table 5). Disease-causing mutations in these voice-affecting genes are associated with various phenotypes, ranging from slight changes of the pitch and hoarseness of the voice, to a complete loss of speech ability (Table 1). These phenotypes were shown be driven primarily by alterations to the laryngeal skeleton (the cartilaginous structures to which the vocal cords are anchored) and vocal tract (the pharyngeal, oral and nasal cavities, where sound is filtered to specific frequencies). Importantly, the laryngeal skeleton, and particularly the cricoid and arytenoid cartilages which are central in vocalization, are very close developmentally to limb bones, as both of these skeletal tissues derive from the somatic layer of the lateral plate mesoderm. Given that DMRs in one tissue often extend to other tissues, it is likely that many of the DMRs identified in limb samples in this study exist in the larynx as well.

Using randomization tests and promoter-specific analyses, we ruled out the possibility that the enrichment of the larynx and face stems from potentially unique characteristics of genes affecting these body parts (such as unique distributions of gene length or genomic position). In addition, we found no enrichment of voice-affecting genes in DMGs along the other lineages, nor when reconstructing methylation maps based on simulated data (Extended Data Fig. 1, Extended Data Table 4). Finally, DMRs that separate chimpanzees from all humans (archaic and MH) do not show enrichment for genes that affect the voice, larynx or the vocal tract, compatible with the notion that this trend emerged only along the MH lineage. We conclude that voice-affecting genes are the most over-represented DMGs along the MH lineage, regardless of intra-skeletal variability,
coverage by methylation array probes, the extent to which a DMR is variable across human or chimpanzee populations, or the significance level of the DMRs.

**Expansive changes in voice-affecting genes**

Our results show that methylation levels in many voice-affecting genes have changed since the split from archaic humans, but they do not provide information on the extent of changes within each gene. To do so, we scanned the genome in windows of 100 kb and computed the fraction of CpGs which are differentially methylated in MHs (hereinafter, MH-derived CpGs). We found that this fraction is more than twice as high within genes affecting the voice compared to other genes (0.142 vs. 0.055, \( P = 3.7 \times 10^{-5} \), t-test). Moreover, three of the five DMGs with the highest fraction of MH-derived CpGs affect the laryngeal skeleton\(^{25-28}\) (\( ACAN \), \( SOX9 \) and \( COL2A1 \); Fig. 2a,b). The fact that so many of the most derived genes affect the larynx is particularly surprising considering that only \( \approx 2\% \) of genes (502) are known to affect it.

The extra-cellular matrix genes \( ACAN \) and \( COL2A1 \), and their key regulator \( SOX9 \), form a network of genes that regulate skeletal growth, pre-ossification processes, and spatio-temporal patterning of skeletal development, including that of the facial and laryngeal skeleton\(^{25,26,29}\). Hypermethylation of the \( SOX9 \) promoter was shown to down-regulate its activity, and consequently, its targets\(^{30}\). \( SOX9 \) is also regulated by a series of upstream enhancers\(^{31}\). We show that its promoter and proximal (20kb upstream) enhancer\(^{31}\) (covered by DMR #30), as well as its targets – \( ACAN \) (DMR #224) and \( COL2A1 \) (DMR #1, the most significant MH-derived DMR) – have all become hypermethylated in MHs (Fig. 2c). Additionally, a more distant putative enhancer, located \( \approx 350kb \) upstream of \( SOX9 \), was shown to bear strong active histone modification marks in chimpanzee craniofacial progenitor cells, while in humans these marks are almost absent (\( \approx 10\times \) stronger in chimpanzee)\(^{32}\). In human and chimpanzee non-skeletal tissues, however, these genes exhibit very similar methylation patterns. Notably, the amino acid sequence coded by each of these genes is identical in the different human groups\(^{2}\), suggesting that the changes along the MH lineage are purely regulatory, whereby \( SOX9 \) became down-regulated in skeletal tissues, followed by hypermethylated and possibly down-regulation of its targets, \( ACAN \) and \( COL2A1 \) (Fig. 2c).
The phenotypic effects of SOX9, ACAN and COL2A1

In light of the role of facial flattening in determining speech capabilities, it is illuminating that flattening of the face is the most common phenotype associated with reduced activity of SOX9, ACAN and COL2A1\(^{22}\). Heterozygous loss-of-function mutations in SOX9, which result in a reduction of ~50% in its activity, were shown to cause a retracted lower face, and to affect the pitch of the voice\(^ {25,26}\). ACAN was shown to affect facial prognathism and hoarseness of the voice\(^ {27}\). COL2A1 is key for proper laryngeal skeletal development\(^ {28}\), and its decreased activity results in a retracted face\(^ {33}\).

Given that these genes are key players in skeletal, and particularly facial development, we turned to investigate MH-derived facial features. One of the main features separating archaic from modern humans is facial retraction. It was shown that the lower and midface of MHs is markedly retracted compared to apes, Australopithecines, and other Homo groups\(^ {10}\). The developmental alterations that underlie the flattened ontogeny of the human face are still under investigation. Cranial base length and flexion were shown to play a role in the retracted face\(^ {10}\), but reduced growth rate, and heterochrony of spatio-temporal switches are thought to be involved as well\(^ {34}\). Importantly, SOX9 and COL2A1 were implemented in the elongation and ossification of the basicranium\(^ {35,36}\), and SOX9 is a key regulator of skeletal growth rate, and the developmental switch to ossification\(^ {25,26}\).

Effects of down-regulation of NFIX

To further explore expression changes driven by changes in methylation, we scanned the DMRs to identify those whose methylation level is strongly correlated with expression. Particularly noteworthy is NFIX, one of the most derived genes in MH (Fig. 2b), which controls the balance between lower and upper projection of the face\(^ {37}\). NFIX harbors two skeletal MH-derived DMRs, whose level of DNA methylation explains 73.9% and 81.7% of NFIX expression variation (FDR = 6.2x10\(^{-3}\) and 7.5x10\(^{-4}\), Fig. 3a-d). This strong association between NFIX methylation and expression was also shown previously\(^ {38}\), and suggests that the hypermethylation reflects down-regulation that emerged along the MH lineage (Fig. 3b). Interestingly, NFI proteins were shown to bind the upstream enhancers of SOX9\(^ {39}\), hence suggesting a possible mechanism to the simultaneous change in the voice- and face-affecting genes.
To test whether changes in NFIX expression could explain morphological changes in MHs, we examined its skeletal phenotypes. Mutations in NFIX were shown to be behind the Marshall-Smith and Malan syndromes, whose phenotypes include various skeletal alterations such as hypoplasia of the midface, retracted lower jaw, and depressed nasal bridge\(^\text{37}\), as well as limited speech capabilities\(^\text{40}\). In many cases, the syndromes are driven by heterozygous loss-of-function mutations that could be paralleled to partial silencing, hence suggesting that the phenotypes associated with NFIX are dosage-dependent\(^\text{37}\). Given that reduced activity of NFIX drives these symptoms, a simplistic hypothesis would be that increased NFIX activity in the Neanderthal would result in changes in the opposite direction. Indeed, we found this to be the case in 18 out of 22 Marshall-Smith syndrome skeletal phenotypes, and in 8 out of 9 Malan syndrome skeletal phenotypes. In other words, from the NFIX-related syndromes, through healthy MHs, to the Neanderthal, the level of phenotype manifestation corresponds to the level of NFIX activity (Fig. 3c, Extended Data Table 7). Interestingly, many cases of laryngeal malformations in the Marshall-Smith syndrome have been reported\(^\text{41}\). Some of the patients exhibit positional changes of the larynx, changes in its width, and structural alterations to the arytenoid cartilage – the anchor point of the vocal cords, which controls their movement\(^\text{41}\). In fact, these laryngeal and facial anatomical changes are thought to underlie the limited speech capabilities observed in some patients\(^\text{40}\).

**Discussion**

A limitation in DNA methylation analyses is that some loci differ between cell types and sexes, change with age and might be affected by factors like environment, diet, etc. It is important to note that we account for this by limiting the stratification of samples. In MH-derived DMRs, for example, we take only DMRs where chimpanzees and archaic humans form a cluster that is distinct from the cluster of MHs. In both clusters there are samples from females and males, and a variety of ages and bones (most coming from femurs in both groups, Extended Data Table 1). Therefore, the observed differences are unlikely to be driven by these factors, but rather add credence to the notion that they reflect a true MH-specific evolutionary shift. This is further supported by the phenotypic observation that facial prognathism in general, and facial growth rates in particular, are derived and reduced in MH\(^\text{42}\).

SOX9, ACAN, and COL2A1 are active mainly in early stages of osteochondrogenesis, making the observation of differential methylation in mature bones puzzling at first glance. This could be
explained by two factors: (i) The DMRs might reflect earlier changes in the mesenchymal progenitors of these cells that are carried on to later stages of osteogenesis. (ii) Although these genes are downregulated with the progress towards skeletal maturation, they were shown to persist into later skeletal developmental stages in the larynx, vertebrae, limbs, and jaw, including in their osteoblasts\textsuperscript{29,43,44}. Interestingly, these are also the organs that are most affected by mutations in these genes\textsuperscript{25–28,33}.

We have shown here that genes affecting vocal and facial anatomy went through particularly extensive regulatory changes in recent MH evolution. These alterations are observed both in the number of diverged genes and in the extent of changes within each gene, and they are also evident in MH phenotypes. Our results support the notion that the evolution of the vocalization apparatus of MHs is unique among hominins and great apes, and that this evolution was driven, at least partially, by changes in gene regulation.
References


27. Tompson, S. W. *et al.* A Recessive Skeletal Dysplasia, SEMD Aggrecan Type, Results from a Missense Mutation Affecting the C-Type Lectin Domain of Aggrecan. *Am. J. Hum. Genet.* **84**, 72–
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# Tables and Figures

**Table 1.** DMGs that affect the voice and the larynx.

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Fig. 1. Genes affecting the voice are the most over-represented within MH-derived DMRs. 

a. Vocal anatomy of chimpanzee and MH. Colors mark central body parts: larynx and vocal cords (green), epiglottis (orange), hyoid bone (yellow), and tongue (pink). The vocal tract is the cavity from the lips to the larynx. In MHS, the flattening of the face together with the descent of the larynx (marked by dashed blue lines), led to approximately 1:1 proportions of the horizontal and vertical portions of the vocal tract, whereas chimpanzees have a longer horizontal and a shorter vertical vocal tract. 

b. The number of DMRs that emerged along each of the human branches. Split times are in years before present (ybp). 

c. A heat map representing the level of enrichment of each anatomical part within the skeletal MH-derived DMRs. Only body parts that are significantly enriched (FDR < 0.05) are colored. Most enriched parts are within the head and neck region, with the vocal cords and voice box (larynx, marked with arrows) being the most over-represented.

d. Enrichment levels of the most significant (first quartile) skeletal MH-derived DMRs, showing an even more pronounced over-representation of genes affecting vocal anatomy.
Fig. 2. The extent of differential methylation in MHs is highest among genes affecting the larynx. a. The number of MH-derived CpGs per 100 kb centered around the middle of each DMR. Genes were ranked according to the fraction of derived CpG positions within them. Genes affecting the voice are marked with red lines. In MH-derived DMGs, these genes went through more extensive changes compared to other genes, and tend to be ranked higher. Although these genes comprise ~2% of the genome, three of the top five MH-derived windows overlap genes affecting the voice. In archaic-derived DMRs and in simulated DMRs, voice-affecting genes do not harbor more changes compared to the rest of the genome. b. The fraction of MH-derived CpGs along chromosomes 12, 15, 17 and 19. The most extensive changes are found within the genes COL2A1, SOX9, ACAN, and NFIX. All of these genes control facial projection and the development of the larynx. c. Methylation levels in the skeletal MH-derived DMRs in SOX9, ACAN and COL2A1. MH samples are marked with red lines, archaic human samples are marked with blue lines and chimpanzee samples are marked with grey lines. The distribution of methylation across 52 MH samples (450K methylation arrays) is presented as a red distribution.
Fig. 3. NFIX became down-regulated after the split from archaic humans, and possibly underlies the flattening of the face in MHs. 

a. Methylation levels along NFIX, color-coded from green (unmethylated) to red (methylated). The top six panels show ancient and present-day MH samples, where NFIX is mostly methylated. The bottom three panels describe the Denisovan, Neanderthal and chimpanzee, where the gene is mostly unmethylated. Full bone MH-derived DMRs are shown in dashed rectangles, skeletal MH-derived DMRs are shown in plain rectangles. Chimpanzee and present-day samples were smoothed using the same sliding window as in ancient samples to allow easier comparison. 

b. The inferred schematic regulatory evolution of NFIX, showing when the shift in the NFIX DMRs from unmethylated (green) to methylated (red) had occurred. 

c,d. Methylation levels in DMRs #417 and #67 vs. expression levels of NFIX across 21 tissues in MHs. In both DMRs, higher methylation is associated with lower expression of NFIX. 

e. Craniofacial features of the Neanderthal, healthy MH, and MH with Marshall-Smith or Malan syndromes. NFIX controls the upper vs. lower prognathism of the face. Individuals where NFIX is partially or completely inactive present phenotypes that are largely the opposite of the Neanderthal facial features. For each facial part we show the phenotype of the Marshall-Smith and Malan syndromes (S), as well as the corresponding Neanderthal (N) phenotype. Phenotypes are compared to a healthy MH. Opposite phenotypes are marked with dark grey rectangles, and shared phenotypes are marked with light grey rectangles.
Extended Data Fig. 1. Genes affecting endocrine glands and the skeleton are the most enriched within archaic-derived DMRs. A heat map representing the level of enrichment of each anatomical part within archaic-derived DMGs. Only body parts that are significantly enriched (FDR < 0.05) are colored. a. Enrichment within full bone MH-derived DMRs. b. Enrichment within full bone archaic-derived DMRs. c. Enrichment within bone archaic-derived DMRs. d. Enrichment within skeletal archaic-derived DMRs.
Extended Data Fig. 2. **a.** Simulations of cytosine deamination, followed by reconstruction reproduce DNA methylation maps. Deamination was simulated for each position based on its methylation level, read coverage and the observed rate of deamination in each hominin. Then, DNA methylation maps were reconstructed and matched against the original map. The number of DMRs found were used as an estimate of false discovery rate. Three exemplary regions are presented. **b.** The HOXD cluster is hypermethylated in archaic humans, and in the Ust'-Ishim individual. Methylation levels are color-coded from green (unmethylated) to red (methylated). The top seven panels show ancient and present-day MH samples, the lower three show the Denisovan, Neanderthal and chimpanzee. The promoter region of \textit{HOXD9} is hypermethylated in the Neanderthal and the Denisovan, but not in MHs. The 3’ ends of the three genes are hypermethylated in the Neanderthal, Denisovan, Ust'-Ishim and chimpanzee, but not in other MH samples. The
promoter of HOXD10 is methylated only in the Denisovan. c. The procedure of merging overlapping DMRs. Plain red lines represent overlapping regions. Dashed red lines represent regions that were identified as a DMR in one of the lists, but not the other. These regions were added to the overlapping region if they clustered significantly closer to the reference human. d. COL2A1, ACAN, SOX9, and NFIX are hypermethylated in MH femurs compared to chimpanzee femurs. Even when comparing methylation in the same bone, measured by the same technology, and in the same positions, MH show consistent hypermethylation in these genes.