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Archaic hominin genomics provides a window into gene expression evolution

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Differences in gene expression are thought to account for most phenotypic differences within and between species. Consequently, gene expression is a powerful lens through which to study divergence between modern humans and our closest evolutionary relatives, the Neanderthals and Denisovans. Such insights complement biological knowledge gleaned from the fossil record, while also revealing general features of the mode and tempo of regulatory evolution. Because of the degradation of ancient RNA, gene expression profiles of archaic hominins must be studied by indirect means. As such, conclusions drawn from these studies are often laden with assumptions about the genetic architecture of gene expression, the complexity of which is increasingly apparent. Despite these challenges, rapid technical and conceptual advances in the fields of ancient genomics, functional genomics, statistical genomics, and genome engineering are revolutionizing understanding of hominin gene expression evolution.

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Introduction

Over the past decade, high-coverage genome sequences from archaic hominins have provided a catalog of genetic changes that distinguish modern humans from our extinct evolutionary relatives, the Neanderthals and Denisovans [1,2,3]. These studies identified ~150 000 single nucleotide substitutions and short insertions or deletions that are fixed (or nearly fixed) in modern humans, but absent from Neanderthal, Denisovan, and non-human great ape genomes. Despite this knowledge, drawing causal links between genetic and phenotypic differences has proven challenging. Few substitutions lie in coding regions of the genome, and of those, even fewer are predicted to consequentially alter protein structure or function. Protein-coding changes are thus unlikely to account for the bulk of phenotypic divergence between modern humans and archaic hominins. Regulatory mutations that influence gene expression provide an appealing alternative, as such mutations are thought to drive most phenotypic differences within [4–6] and between [7,8] species.

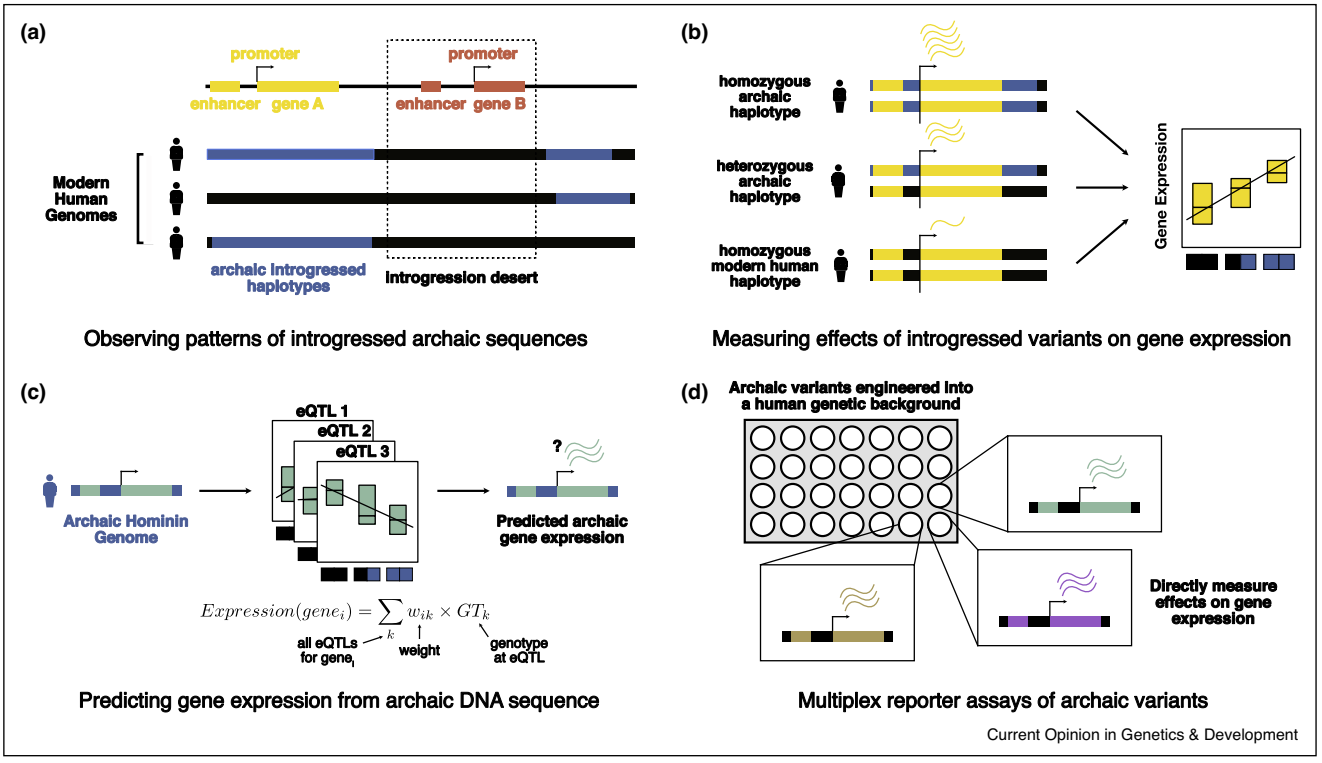
With few exceptions [9], the rapid degradation of ancient RNA prevents researchers from directly assaying differences in gene expression between modern and archaic samples. Several complementary indirect approaches have therefore been developed to leverage functional genomic data to infer these differences, as well as their underlying regulatory mechanisms. Here, we discuss recent approaches that use archaic introgression, genome engineering, and/or statistical methods to study gene expression divergence in the hominin lineage (Figure 1). We examine their unique strengths and limitations in turn.

Archaic introgression in regulatory regions

A variety of statistical methods have been developed to discover Neanderthal and Denisovan haplotypes that persist in modern human genomes [11–15]. Application of these methods to genomic data from large human population reference panels allowed researchers to map the genome-wide distribution of introgressed haplotypes across populations. These haplotypes are reduced or absent in some areas of the genome, termed 'deserts' of introgression, and enriched in others, suggesting that both negative and positive selection influenced the fate of archaic introgressed sequence during the generations following admixture [12,16,17]. Characterizing the heterogeneity of introgressed sequence with respect to annotated genes and regulatory elements provides insight into the functional basis of phenotypic divergence, as well as the evolutionary forces that have shaped the landscape of persisting introgressed haplotypes.

Such studies have revealed that archaic introgressed sequences are depleted in regions under strong selective constraint, including coding and non-coding sequences that are conserved among primates [16,18•]. Fine-grained examination of regulatory elements further demonstrated that archaic introgression is reduced in promoters, miRNAs, and certain classes of enhancers [18•,19•,20•]. Specifically, Telis *et al.* found that enhancers harbor fewer introgressed variants on average, despite having a higher

Figure 1



Approaches for studying archaic gene expression. Modern humans diverged from Neanderthals and Denisovans approximately 500 kya, with subsequent admixture among these lineages <100 kya [10]. Differences in gene expression between modern and archaic hominins likely contributed to their phenotypic differences, as well as the phenotypic impacts of introgressed sequences. Because of the degradation of ancient RNA, assaying the expression of archaic alleles requires indirect methods. **(a)** Heterogeneity in the genomic landscape of introgressed haplotypes in modern human populations serves as a signature of positive, negative, and balancing selection targeting genes and regulatory elements following introgression. **(b)** Associations between introgressed haplotypes and modern human gene expression can be tested directly in contemporary samples. **(c)** Statistical methods can be used to predict archaic gene expression by training models with features such as genotype-expression associations, DNA sequence features, and methylation status. **(d)** Massively parallel reporter assays allow thousands of archaic (and non-archaic) alleles to be introduced episomally or by genome editing into human cell lines or model organisms, then tested simultaneously for effects on gene expression.

tolerance for new mutations than other genomic regions [19•]. In apparent contradiction, Silvert *et al.* found that enhancers are enriched for common archaic introgressed alleles, particularly in adipose tissue, where some enhancers also exhibit signatures of adaptive introgression [20•]. These conflicting results highlight statistical challenges in determining whether particular genomic elements are enriched or depleted of introgression. Interpreting such enrichment tests is complicated by disparate methods of discovering introgressed alleles, defining control sets of variation, annotating regulatory elements, correcting for multiple hypothesis testing, and controlling for confounding factors such as linkage disequilibrium (LD), allele frequency, and expression level. As knowledge of genome function continues to grow, so does the urgency for studies focused on identifying and mitigating relevant biases in enrichment tests as applied to evolutionary processes such as introgression.

One interpretation of the broadly reduced archaic introgression in regulatory regions is that it reflects differences in the efficacy of purifying selection in small Neanderthal and Denisovan populations. Once introgressed into larger modern human populations, these weakly deleterious mutations would be exposed to negative selection [21,22]. Indeed, introgressed Neanderthal ancestry is lowest in regions of low recombination, consistent with historical purifying selection against weakly deleterious introgressed alleles [23]. An alternate explanation for negative selection against Neanderthal alleles invokes regulatory epistasis — the concept that Neanderthal genes and regulatory elements are adapted to function within the Neanderthal genetic background. Rapid evolution of such regulatory incompatibilities has been observed in other systems and is one proposed mechanism contributing to hybrid incompatibility and eventual speciation [24–26]. In modern humans, genes that are

highly expressed in the testes are depleted of archaic ancestry, potentially consistent with male hybrid infertility [16]. In addition, a large proportion of human accelerated regions (HARs) — sequences that are conserved in mammals but divergent in humans — are thought to function as enhancers, suggesting that the corresponding regulatory networks may have evolved rapidly within the hominin lineage [27,28]. While providing circumstantial support for regulatory epistasis, direct tests are hampered by the statistical intractability of pairwise association, as well as the lack of reciprocal data measuring the expression of modern human alleles within archaic genetic backgrounds.

Effects of introgressed variants on gene expression

Introgressed archaic haplotypes allow researchers to assay associations with gene expression, providing insight into the characteristics of gene expression divergence throughout hominin evolution. Like their non-introgressed counterparts, many introgressed variants are expression quantitative trait loci (eQTLs), significantly associated with the expression of nearby genes in *cis*. Quach *et al.* [29], Dannemann *et al.* [30••], and Natri *et al.* [31] identified introgressed eQTLs of genes involved in immune functions. These included multiple eQTLs exhibiting strong allele frequency differentiation in specific populations — a signature of historical positive selection following introgression. McCoy *et al.* developed a complementary approach to examine allele-specific expression (ASE) of Neanderthal haplotypes in heterozygous samples, finding that approximately one quarter of all loci tested exhibited significant ASE [32••]. The authors also discovered anecdotal examples of ASE mediated by changes in patterns of alternative splicing (i.e. isoform-specific gene expression). Such introgressed splicing QTLs (sQTLs) have been characterized in further detail with respect to their impacts on immune response [33], including several examples associated with signatures of adaptive introgression [34,35]. Beyond the context of immune function, the impacts of introgressed sQTLs and their implications for hominin splicing divergence merit further investigation.

Genetic effects on expression are ubiquitous, and a large proportion of modern human variants constitute eQTLs [36]. One question of interest is whether introgressed archaic variants as a group are unique in their functional impacts, due to their unique evolutionary histories. Given the aforementioned hypotheses about genetic load accumulated within small Neanderthal populations, one may predict that Neanderthal-introgressed alleles would be enriched for effects on modern human gene expression. While some studies have confirmed such an enrichment [20•,30••,33] others have not [32••]. As with assessing the enrichment or depletion of introgressed sequences in regulatory regions, these discrepancies likely arise from methodological discrepancies in defining case and control groups and accounting for relevant covariates.

Perhaps more insidiously, because of the nature of LD in human populations, identifying an archaic-introgressed eQTL is not sufficient for establishing its causality. The archaic variant may simply tag one or more causal variants (introgressed or non-introgressed) through LD. Fine-mapping approaches can be used to assess causality [4,37], but have not been widely applied to studies of archaic gene expression. These considerations are especially important in light of recent findings that Neanderthal sequences in modern humans make limited contributions to variation in phenotypes, including gene expression. Rare and singleton variants explain the majority of gene expression heritability in humans, but with a negligible contribution from Neanderthal-introgressed singletons [38]. Many phenotypic associations previously attributed to archaic alleles are better explained by linked non-archaic variation [39•]. Further nuancing this picture, introgressed haplotypes may indirectly contribute to phenotypic variation by reintroducing ancestral alleles that were previously lost in the human lineage [40]. Together, these results suggest that future research into the gene expression impacts of archaic introgression must carefully consider the potential effects of linked alleles with distinct evolutionary histories.

Predicting gene expression from archaic DNA sequence

Even when scaling to large samples, less than half of the Neanderthal and Denisovan genomes are represented by introgressed fragments that persist within modern humans today, thus limiting insights about regulatory divergence that can be deduced from introgressed haplotypes [12]. Recent methodological advances in predicting gene expression from DNA sequences have enabled the study of archaic expression beyond the landscape of introgression.

Colbran *et al.* used the method PrediXcan [41], which imputes the *cis*-regulatory component of gene expression from genotype data, to predict the expression of archaic DNA sequences. They found that Neanderthal and modern human gene expression diverged substantially in genes without introgression, suggesting that this divergence may have served as a barrier to introgression [42••]. Similarly, by inferring DNA methylation from archaic hominin genome sequencing data (obtained from bones) [43], Gokhman *et al.* [44] and Gokhman *et al.* [45] identified differentially methylated regions between modern humans and archaic hominins. Upon extrapolating to downstream effects on expression and organismal phenotypes, the authors recapitulated several known features of archaic hominin anatomy, including a wide anterior mandible in Denisovans, and a low forehead and wide pelvis in both Neanderthals and Denisovans [44,45].

While highly innovative, the indirect nature of these studies poses a challenge for validation. One key concern regards generalizability to divergent hominin populations, as statistical models are generally trained on predictor (genotype or methylation) and response (gene expression or other phenotypes) data from contemporary human populations. Underscoring this concern, the accuracy of polygenic risk scores inferred from European populations decreases by more than 50% in non-European populations [46]. Maintaining strict criteria to filter out false positives is one possible solution to this issue. For example, Colbran *et al.* showed that the polygenic prediction method PrediXcan tends to underestimate divergence in more distant populations, leading to more false negatives than false positives when identifying divergent archaic loci [42••]. The accuracy of these 'imputation' methods can also be evaluated by benchmarking in extant species, such as non-human great apes [45].

Ongoing studies are clarifying the complex genetic architecture of gene expression, including widespread allelic heterogeneity (i.e. multiple causal variants) and abundant but weak *trans* effects. Future statistical models of gene expression evolution will be tasked with the challenge of incorporating these features while maintaining model tractability.

Multiplex assays of archaic gene expression

Recent *in vitro* methods offer promising approaches for disentangling LD and testing the causal effects of individual archaic hominin substitutions on gene expression. For example, in unpublished work, scientists reportedly used CRISPR to introduce the Neanderthal allele of the splicing factor *NOVA1* in a brain organoid cell line, characterizing downstream effects on global gene expression and cell morphology [47]. Reporter assays have also been used to demonstrate that some ancestral hominin alleles reintroduced into humans through Neanderthal introgression have strong effects on gene expression, even when tested independently of other alleles in perfect LD [40].

Beyond such candidate mutations, high-throughput methods such as massively parallel reporter assays (MPRAs) provide hypothesis-free approaches to simultaneously quantify the expression impacts of hundreds of thousands of loci genome wide [48,49,50]. In these assays, individual variants and their surrounding DNA sequence are genetically engineered into reporter constructs, allowing the gene expression effects of each variant to be measured experimentally. MPRAs were recently used to identify HARs that act as enhancers in neuronal cell lines, and whose expression differs significantly between human and chimpanzee sequences [28]. Further application of these methods to assess the effects of fixed archaic hominin substitutions will provide important insight into the role of regulatory changes in hominin evolution.

By necessity, most MPRA studies are performed on human cell lines, and the tested variants are often introduced episomally, outside of their endogenous loci. As a result, these studies cannot account for epistatic interactions, differences in nuclear architecture, or epigenetic modifications. CRISPR-based genome editing, which incorporates mutations into their native genomic contexts, will allow for more comprehensive assays of variant effects [51].

Conclusions

Changes in gene expression are a key driver of phenotypic innovation. Studies of archaic hominin gene expression thus provide a window into the genetic basis of phenotypic divergence in the hominin lineage, as well as the evolution of modern human-specific phenotypes. Hominin evolution may also serve as a useful model for investigating broader questions about regulatory evolution, given the wealth of available data. Such data can be leveraged to address fundamental questions such as the role of positive and purifying selection in shaping gene expression evolution; the mode and tempo of regulatory divergence among genes expressed in different cells, tissues, and developmental contexts; and the relative contributions of protein-coding and regulatory changes to phenotypic diversity and divergence across evolutionary timescales. The growth of ancient DNA, functional genomic, and phenotypic datasets, as well as the development of novel assays and analytical approaches, will fuel further understanding of gene expression evolution over the coming decades.

Conflict of interest statement

Nothing declared.

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