Neanderthal Introgression Shaped Human Circadian Traits

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ABSTRACT

Introduction: When the ancestors of modern Eurasians migrated out of Africa and interbred with Eurasian archaic hominins, namely Neanderthals and Denisovans, DNA of archaic ancestry integrated into the genomes of anatomically modern humans. This process potentially accelerated adaptation to Eurasian environmental factors, including reduced ultra-violet radiation and an increased variation in seasonal dynamics. However, whether these groups differed substantially in circadian biology, and whether archaic introgression adaptively contributed to human chronotypes remains unknown.

Results: Here we traced the evolution of chronotype based on genomes from archaic hominin and present-day humans. First, we inferred differences in circadian gene sequences, splicing, and regulation between archaic hominins and modern humans. We identified 28 circadian genes containing variants likely to alter splicing in archaics (e.g., *CLOCK*, *PER2*, *RORB*, *RORC*), and 16 circadian genes likely divergently regulated between present-day humans and archaic hominins, including *RORA*. These differences suggest the potential for introgression to modify circadian gene expression. Testing this hypothesis, we found that introgressed variants are enriched among eQTLs for circadian genes. Supporting the functional relevance of these regulatory effects, we found that many introgressed alleles have strong associations with chronotype. Strikingly, the strongest introgressed effects on chronotype increase morningness, which is consistent with adaptations to high latitude in other species. Finally, we identified 26 circadian loci with evidence of adaptive introgression, including *PER2* and *MYBBP1A*.

Conclusions: These findings identify differences in circadian gene regulation between modern humans and archaic hominins and support the contribution of introgression via coordinated effects on variation in human chronotype.

Keywords: circadian biology, chronotype, Neanderthals, adaptive introgression

SIGNIFICANCE STATEMENT

Interbreeding between modern humans and Neanderthals created the potential for adaptive introgression as humans moved into new environments that had been populated by Neanderthals for hundreds of thousands of years. Here we discover substantial lineage-specific genetic differences in circadian genes and their regulatory elements between humans and Neanderthals. We then show that introgressed archaic alleles are enriched for effects on circadian gene regulation and consistently increase propensity for morningness in modern Europeans. These results substantially expand our understanding of how the genomes of humans and our closest relatives responded to living in environments with different light/dark cycles, and they demonstrate a coordinated contribution of archaic admixture to modern human chronotype in a direction that is consistent with adaptation to higher latitudes.

INTRODUCTION

All anatomically modern humans (AMH) trace their origin to the African continent around 300 ka (Hublin et al. 2017; Stringer 2016), where environmental factors shaped many of their biological features. Approximately seventy-thousand years ago (Bae, Douka, and Petraglia 2017), the ancestors of modern Eurasian AMH began to migrate out of Africa, where they were exposed to diverse new environments. In Eurasia, the novel environmental factors included greater seasonal variation in temperature and photoperiod.

Changes in the pattern and level of light exposure has biological and behavioral consequences in organisms. For example, *D. melanogaster* that are native to Europe harbor a polymorphism in *timeless*, a key gene in the light response of the circadian system, that follows a latitudinal cline in allele frequency (Sandrelli et al. 2007; Tauber et al. 2007). This polymorphism is an insertion in the 5' region of the gene, resulting in two initiation codons and as a result two isoforms of the protein: L-TIM with prevalence in southern Europe and S-TIM with prevalence in northern Europe. The isoforms have different levels of affinity to cryptochrome (CRY), creating a change in photosensitivity and altering the length of the period. Another example is found in pacific salmon. The Chinook salmon (*Oncorhynchus tshawytscha*) populations show a latitudinal-cline in the frequency and length of repeat motifs in the gene *OtsClock1b* (O'Malley, Ford, and Hard 2010; O'Malley and Banks 2008). Thus, this locus is under selection associated with latitude and photoperiod. The evolution of circadian adaptation to diverse environments has been widely studied in insects, plants (Q. Zhang et al. 2008; Michael et al. 2003), and fishes, but it is understudied in humans. Adaptive processes could have helped to align human biology and chronotype to new natural conditions.

Previous studies in humans found a correlation between latitude and chronotype (morningness vs. eveningness) variation (Leocadio-Miguel et al. 2017; Lowden et al. 2018; Randler and Rahafar 2017) and a latitudinal-cline in circadian allele frequencies (Putilov et al. 2019; Putilov, Dorokhov, and Poluektov 2018; Dorokhov et al. 2018), highlighting the contribution of the environment to circadian behavior and circadian biology. Many human health effects are linked to the misalignment of chronotype (Knutson and von Schantz 2018), including cancer, obesity (Gan et al. 2018; Gyarmati et al. 2016; Papantoniou et al. 2016; 2017; Shi et al. 2020; Yousef et al. 2020), and diabetes (Gan et al. 2015; Larcher et al. 2015; 2016). There is also evidence of a correlation between evening chronotype and mood disorders, most notably seasonal affective disorder (SAD), depression, and worsening of bipolar disorder episodes (Srinivasan et al. 2006; Taylor and Hasler 2018; Kivelä, Papadopoulos, and Antypa 2018). Thus, we hypothesize that the differences in geography and environment encountered by early AMH populations moving into higher latitudes created potential for circadian misalignment and health risk.

Although AMHs arrived in Eurasia ~70 ka, other hominins (e.g. Neanderthals and Denisovans) lived there for more than 400 ka (Meyer et al. 2016; 2014; Arnold et al. 2014). These archaic hominins diverged from AMHs around 700 ka (Gómez-Robles 2019; Nielsen et al. 2017; Meyer et al. 2012; Prüfer et al. 2014; 2017; Mafessoni et al. 2020), and as a result, the ancestors of AMHs and archaic hominins evolved under different environmental conditions. While there was substantial variation in the latitudinal ranges of each group, the Eurasian hominins largely lived at consistently higher latitudes and, thus, were exposed to higher amplitude seasonal variation in photoperiods. Given the influence of environmental cues on

circadian biology, we hypothesized that these separate evolutionary histories produced differences in circadian traits adapted to the distinct environments.

When AMH migrated into Eurasia, they interbred with the archaic hominins that were native to the continent, initially with Neanderthals (Green et al. 2010; Villanea and Schraiber 2019) around 60 ka (Sankararaman et al. 2012; Skoglund and Mathieson 2018) and later with Denisovans (Jacobs et al. 2019). Due to this, a substantial fraction (>40%) of the archaic variation remains in present-day Eurasians (Skov et al. 2020; Vernot and Akey 2014), although each human individual carries only ~2% DNA of archaic ancestry (Vernot et al. 2016; Prüfer et al. 2017). Most of the archaic ancestry in AMH was subject to strong negative selection, but some of these introgressed alleles remaining in AMH populations show evidence of adaptation (Gower et al. 2021; Racimo et al. 2015). Archaic alleles have been associated with differences in hemoglobin levels at higher altitude in Tibetans, immune resistance to new pathogens, levels of skin pigmentation, and fat composition (Dannemann and Kelso 2017; Racimo et al. 2015; 2017; Racimo, Marnetto, and Huerta-Sánchez 2017; Huerta-Sánchez et al. 2014; McArthur, Rinker, and Capra 2021). Introgressed alleles are also associated with chronotype (Dannemann and Kelso 2017), suggesting a potential adaptive pressure stemming from migration to higher latitudes. Summarizing effects across introgressed alleles, chronotype was also moderately enriched for heritability in introgressed regions (McArthur, Rinker, and Capra 2021).

Motivated by the potential for a role of archaic introgression in AMH circadian variation, we explore two related questions: 1) Can comparative genomic analysis identify differences in AMH and archaic hominin circadian biology?, and 2) Do introgressed archaic alleles influence human circadian biology? Understanding the ancient history and evolution of chronotypes in humans will shed light on human adaptation to high latitudes and provide context for the genetic basis for the modern misalignment caused by the development of technology and night shiftwork.

RESULTS

Did archaic hominins and modern humans diverge in circadian biology?

Following divergence ~700,000 years ago (Gómez-Robles 2019; Nielsen et al. 2017), archaic hominins and AMH were geographically isolated, resulting in the accumulation of lineage-specific genetic variation (Figure 1). In the next several sections, we evaluate the evidence for divergence in circadian biology between archaic hominin and modern human genomes.

Identifying archaic-hominin-specific circadian gene variation

With the sequencing of several genomes of archaic hominins, we now have a growing, but incomplete, catalog of genetic differences specific to modern and archaic lineages. We evaluated archaic-specific variants (Kuhlwilm and Boeckx 2019) for their ability to influence proteins, splicing, and regulation of 246 circadian genes (Methods). The circadian genes were identified by a combination of literature search, expert knowledge, and existing annotations (Table S1; Figure S1; Methods).

We identified 1,136 archaic-specific variants in circadian genes, promoters, and candidate distal cis-regulatory elements (cCREs). The circadian genes with the most archaic-

specific variants are *CLDN4*, *NAMPT*, *LRPPRC*, *ATF4*, and *AHCY* (125, 112, 110, 104, 102 respectively) (Table S2).

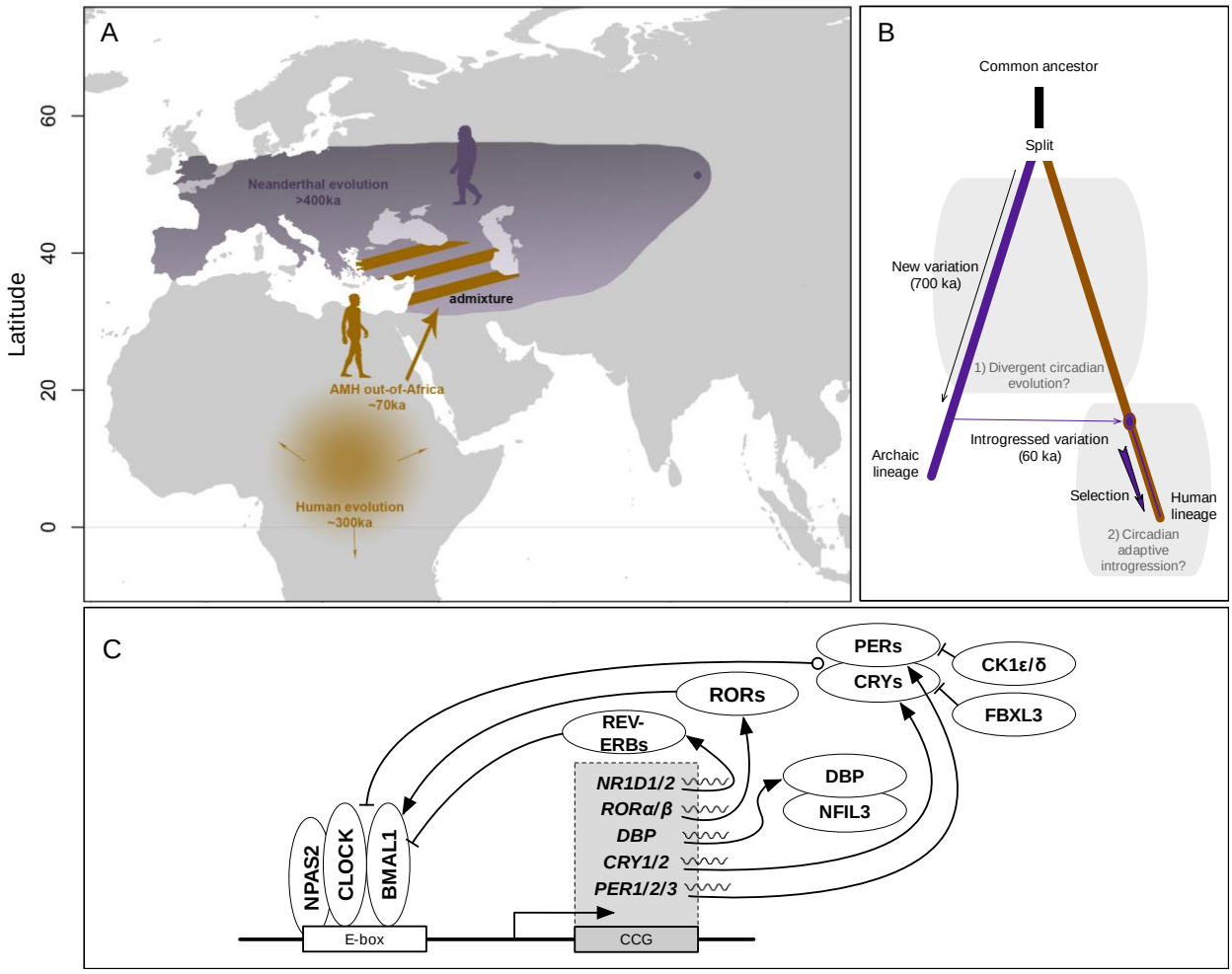

Figure 1. Did the sharing of functionally diverged alleles from archaic hominins influence human circadian biology? A) Anatomically modern humans and archaic hominins evolved separately at different latitudes for hundreds of thousands of years. The ancestors of modern Eurasian humans left Africa approximately 70 thousand years ago and admixed with archaics, likely in southwestern Asia. The shaded purple range represents the approximate Neanderthal range. The purple dot represents the location of the sequenced Denisovan individual in the Altai Mountains; the full range of Denisovans is currently unknown. Silhouettes from phylopic.org. **B)** After the split between the human and archaic lineages, each group accumulated variation and evolved in their respective environments for approximately 700 thousand years. We first test for evidence for divergent circadian evolution during this time. Humans acquired introgressed alleles from Neanderthals and from Denisovans around 60 and 45 thousand years ago, respectively. These alleles experienced strong selective pressures; however, ~40% of the genome retains archaic ancestry in some modern populations. The second question we explore is whether introgression made adaptive contributions to human circadian biology. **C)** The core circadian clock machinery is composed of a dimer between the CLOCK and ARNTL (BMAL1) transcription factors, which bind to E-box enhancer elements and activate the expression of the

Period (*PER1/2/3*) and Cryptochrome (*CRY1/2*) genes. PERs and CRYs form heterodimers that inhibit the positive drive of CLOCK-BMAL1 on E-boxes, inhibiting their own transcription in a negative feedback loop. CLOCK-BMAL1 also drive the expression of *NR1D1/2* (Nuclear Receptor Subfamily 1 Group D Member 1 and 2), *RORA/B* (RAR Related Orphan Receptor A and B), *DBP* (D-Box Binding PAR BZIP Transcription Factor), and other clock-controlled genes (CCG). ROR and REV-ERB are transcriptional regulators of BMAL1. CK1 binds to the PER/CRY heterodimer, phosphorylating PER and regulating its degradation. Similarly, FBXL3 marks CRY for degradation.

Fixed human- and archaic-specific variants are enriched in circadian genes and associated regulatory elements

After the archaic and AMH lineages diverged, each group accumulated genetic variation specific to each line. Variants fixed in each lineage are likely to be enriched in genomic regions that influence traits that experienced positive selection. We tested whether human- and archaic-specific fixed variants are enriched compared to other sites with archaic variants in circadian genes, their promoters, and in annotated regulatory elements within 1 Mb (Figure 2). We found that human- and archaic-specific fixed variants are enriched in circadian genes (Fisher's exact test; human: OR=1.84, P=7.06e-12; archaic: OR=1.13, P=0.023) and distal regulatory elements (Fisher's exact test; human: OR=1.25, P=8.39e-4; archaic: OR=1.16, P=6.15e-5). Although promoter regions have a similar enrichment pattern as that in gene and regulatory regions, the p-values are high (Fisher's exact test; human: OR=1.21, P=0.65; archaic: OR=1.09, P=0.63). This is likely due to the small number of such variants in promoters. These results suggest that both groups had a greater divergence in genomic regions related to circadian biology than expected.

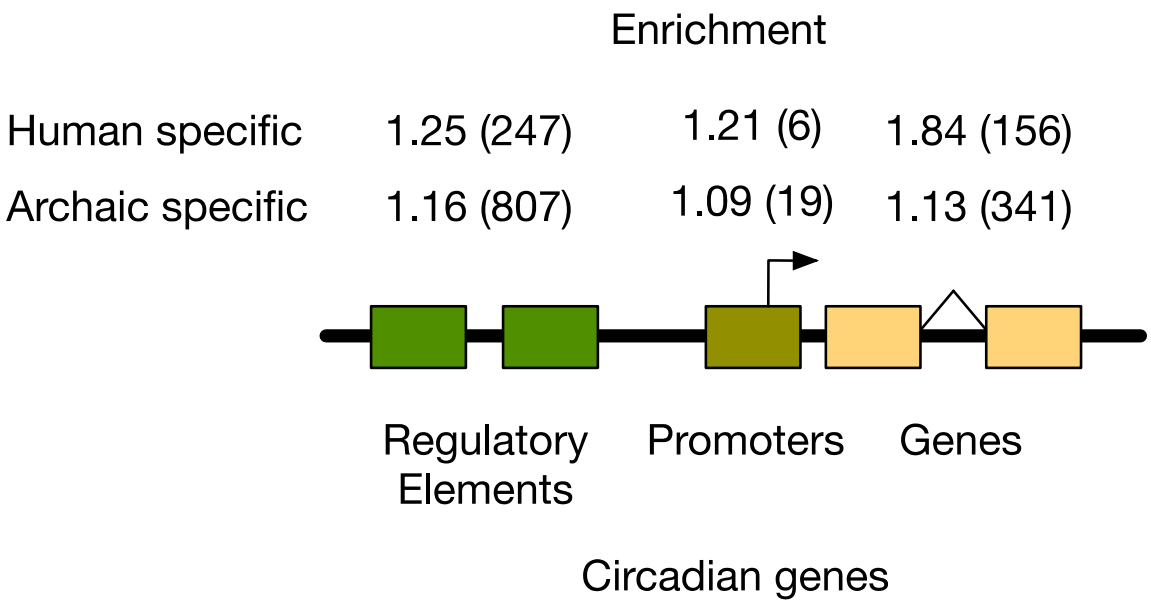

Figure 2. Human- and archaic-specific fixed variants are enriched in circadian regulatory, promoter, and gene regions. Human-specific fixed variants are significantly enriched in circadian regulatory elements (Fisher's exact: OR=1.25, P=8.39e-4) and gene regions (Fisher's exact: OR=1.84, P=7.06e-12) compared to variants that are not fixed. Promoters show a similar enrichment, but the higher p-value is the result of the small number of variants (Fisher's exact

test: OR=1.21, P=0.65). Likewise, archaic-specific variants are enriched in circadian regulatory regions (Fisher's exact: OR=1.16, P=6.15e-5) and gene regions (Fisher's exact: OR=1.13, P=0.023), with the promoters showing a similar trend (Fisher's exact test: OR=1.09, P=0.63). The number in parentheses gives the number of variants observed in each type.

Several core circadian genes have evidence of alternative splicing between humans and archaic hominins

We find only two archaic-specific coding variants in circadian genes: one missense and one synonymous. The missense variant (hg19: chr17_46923411_A_G) is in the gene *CALCOCO2*, calcium-binding and coiled-coil domain-containing protein 2. SIFT, PolyPhen, and CADD all predict that the variant does not have damaging effects. The second variant (hg19: chr7_119914770_G_T) is in the gene *KCND2*, which encodes a component of a voltage-gated potassium channels, but it is synonymous and the variant effect predictors suggest it is tolerated.

To explore potential splicing differences in circadian genes between humans and archaics, we applied SpliceAI to predict whether any sequence differences between modern humans and archaics are likely to modify splicing patterns. Four archaic individuals were included in this analysis (the Altai, the Vindija, the Chagyrskaya Neanderthals, and the Altai Denisovan). We found that 28 genes contained at least one archaic-specific variant predicted to result in alternative splicing in archaics. These included several of the core clock genes *CLOCK*, *PER2*, *RORB*, *RORC*, and *FBXL13* (Figure 3A,C; Table S3). For example, the variant chr2:239187088-239187089 in the 1st intron of *PER2* is predicted to result in a longer 5' UTR. The splice-altering variants were largely specific to the two different archaic linages (Figure 3A), with 13 specific to the Denisovan, eight shared among the three Neanderthals, and only one shared among all four archaic individuals.

Circadian gene regulatory divergence between humans and archaic hominins

Given the enrichment of variants in regulatory regions of circadian genes, we sought to explore the potential for differences in circadian gene regulation between humans and archaics with causes beyond single lineage-specific variants. We leveraged an approach we recently developed for predicting gene regulatory differences between modern and archaic individuals from combinations of genetic variants (Colbran et al. 2019). The approach uses PrediXcan, an elastic net regression method, to impute gene transcript levels in specific tissues from genetic variation. Previous work demonstrated that this approach has a modest decrease in performance when applied to Neanderthals, but that it can accurately applied between humans and Neanderthals for thousands of genes. Here, we quantify differences in predicted regulation of the 246 circadian genes between 2,504 humans in the 1000 Genomes Project (1KGP) and three archaic hominins (the Altai Neanderthal, the Vindija Neanderthal, and the Altai Denisovan). The predicted regulation values are normalized to the distribution in the training set from the Genotype Tissue Expression Atlas (GTEx).

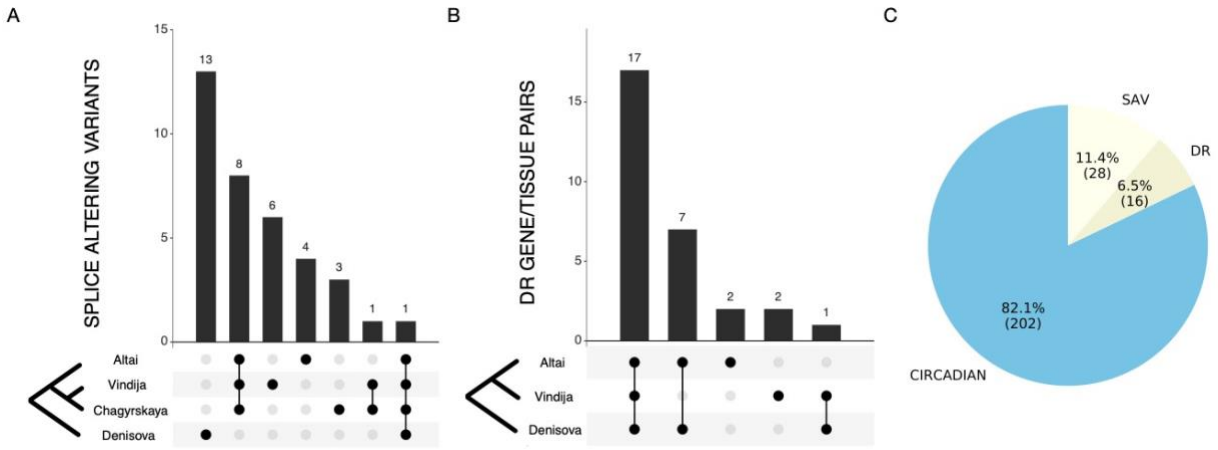

Figure 3. Many circadian genes have evidence of alternative splicing and divergent regulation between modern and archaic hominins. A) The sharing across archaic individuals of the 28 predicted archaic-specific splice-altering variants (SAV) in circadian genes. Most were specific to either the Denisovan or Neanderthal lineage (Table S3). **B)** The sharing of predicted divergently regulated (DR) gene/tissue pairs across three archaic individuals. (Predictions were not available for the Chagyrskaya Neanderthal.) Seventeen divergently regulated gene/tissue pairs were present in all three archaics (representing 16 unique genes). Additionally, 7 gene/tissue DR pairs are shared between the Altai Neanderthal and the Denisovan individual. One pair is shared between the Vindija Neanderthal and the Denisovan (Table S4). **C)** The proportion of circadian genes containing archaic splice-altering variants predicted by SpliceAI (SAV; 11.4%) or divergently regulated circadian genes predicted by PrediXcan (DR; 6.5%). Thus, 17.9% of the circadian genes are predicted to contain differences to AMH via these mechanisms.

We first analyzed gene regulation predictions in the core circadian clock genes. Archaic gene regulation was at the extremes of the human distribution for many core clock genes including *PER2*, *CRY1*, *NPAS2*, *RORA*, *NR1D1* (Figure 4; Figure S2). For example, the regulation of *PER2* in the two Neanderthals is lower than 2,491 of the 2,504 (99.48%) modern humans considered. The Denisovan has a predicted *PER2* regulation that is lower than 2,410 (96.25%). Expanding to all circadian genes and requiring archaic regulation to be more extreme than all humans (Methods), we identified 24 circadian genes across 23 tissues with strong divergent regulation between humans and at least one archaic hominin (Figure 3B; Table S4). For example, all three archaic individuals' regulation values for *RORA*, a core clock gene, are lower than for any of the 2,504 modern humans. We found that 16 of these genes (Figure S3; Table S4), including *RORA*, *MYBBP1A*, and *TIMELESS*, were divergently regulated in all archaic individuals. This represents 6.5% of all the circadian genes (Figure 3C). Surprisingly, the two Neanderthals only shared one DR gene not found in the Denisovan, while the Altai Neanderthal and Denisovan shared seven not found in Vindija (Figure 3B). The Altai and Vindija Neanderthals represent deeply diverging lineages, and this result suggests that they may have experienced different patterns of divergence in the regulation of their circadian genes.

Given these differences in circadian gene regulation between humans and archaics, we tested whether circadian genes are more likely to be divergently regulated than other gene sets. Each archaic individual shows nominal enrichment for divergent regulation of circadian genes,

but given the small sample size, the P-values are moderate (Permutation test; Altai: OR=1.21, P=0.19, Vindija: OR=1.05, P=0.43, Denisovan: OR=1.20, P=0.24). The enrichment was stronger (~1.2x) in the Altai Neanderthal and Denisovan individual.

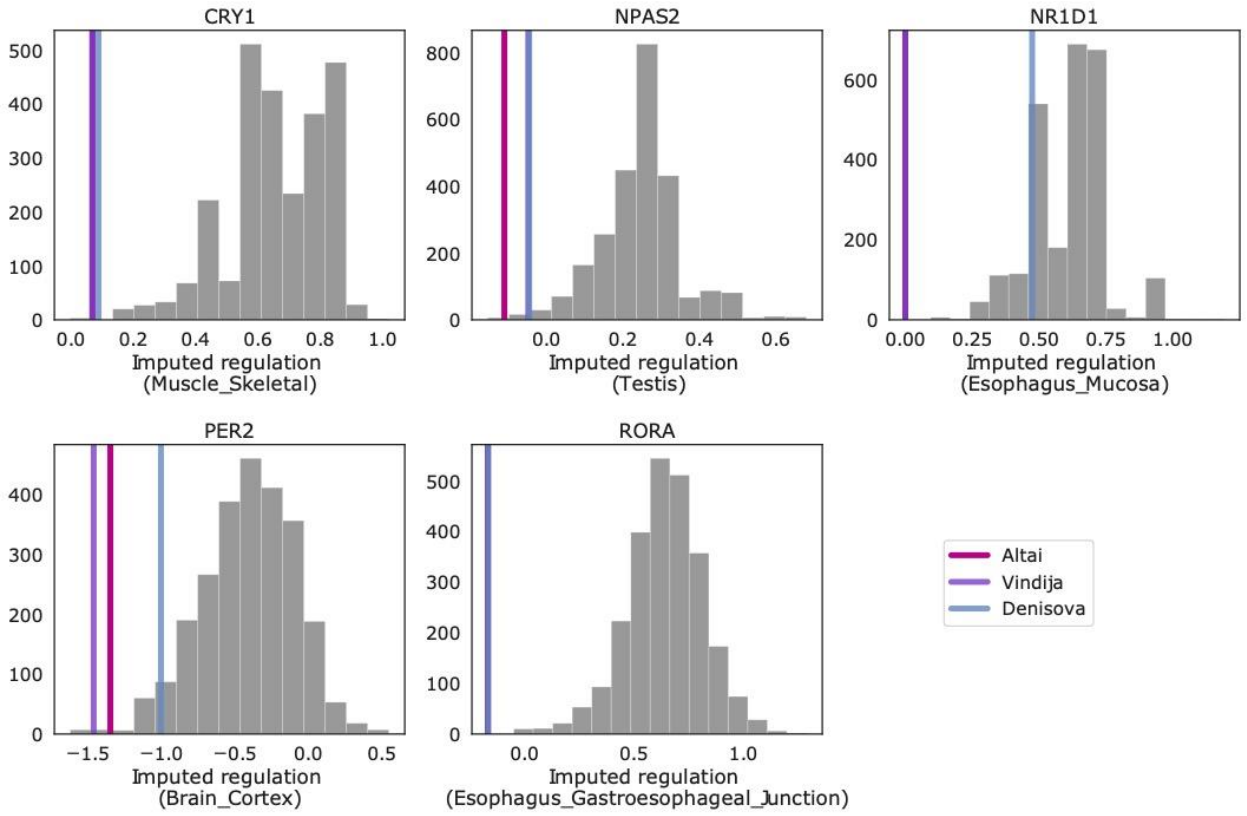

Figure 4. Many circadian genes are divergently regulated between modern humans and archaic hominins. Comparison of the imputed regulation of core circadian genes between 2504 humans in 1000 Genomes Phase 3 (gray bars) and three archaic individuals (vertical lines). For each core circadian gene, the tissue with the lowest average P-value for archaic difference from humans is plotted. Archaic gene regulation is at the extremes of the human distribution for several core genes: *CRY1*, *PER2*, *NPAS2*, *NR1D1 RORA*. See Figure S2 for all core clock genes and Figure S3 for all divergently regulated circadian genes.

Did introgressed archaic variants influence modern human circadian biology?

The previous sections demonstrate lineage-specific variation in many genes essential to the function of the core circadian clock and related pathways. Given this evidence of functional differences between archaic hominins and AMH in these systems, we next evaluated the influence of archaic introgression on AMH circadian biology.

Introgressed variants are enriched in circadian gene eQTL

Given the differences between archaic and modern sequences of circadian genes and their regulatory elements, we investigated whether Neanderthal introgression contributed functional circadian variants to modern Eurasian populations. We considered a set of 863,539 variants with

evidence being introgressed from archaic hominins to AMH (Browning et al. 2018). These variants were identified using the S* algorithm, which searches for regions containing a high density of alleles in common with Neanderthals and not present or at very low frequency in Africans.

We identified 3,857 introgressed variants associated with the regulation of circadian genes in modern non-Africans; i.e., they are eQTL for circadian genes in at least one of the tissues in GTEx (Table S5). The genes *PTPRJ*, *HTR1B*, *NR1D2*, *CLOCK*, and *ATOH7* had the most eQTL (304, 273, 262, 256, and 252 respectively). The tissues artery tibial, cells cultured fibroblasts, skin sun exposed lower leg, adipose subcutaneous, and thyroid had the most eQTL (1361, 962, 876, 871, 716, respectively); though, these patterns are driven in large part by different power between tissues. Notably, several of these circadian genes (e.g., *NR1D2* and *CLOCK*) with introgressed eQTL were also found to be divergently regulated in our previous analyses. This indicates that some of the archaic-derived variants that drove divergent regulation were retained after introgression and continue to influence circadian regulation in modern humans.

Introgressed variants are significantly more likely to be eQTL for circadian genes than expected by chance (Figure 5A; Fisher's exact test: OR=1.45, P=9.71e-101). Examining these associations in each tissue, we found that introgressed eQTL showed significant enrichment for circadian genes in most tissues (34 of 49; Figure 5B; Table S6) and trended this way in all but five. Again, the differences between tissues likely influenced by differential power. These results suggest that the circadian pressures were widespread across tissues. Given the previously observed depletion for introgressed variants in regulatory elements and eQTL (Petr et al. 2019; Rinker et al. 2020; Telis, Aguilar, and Harris 2020), this enrichment for circadian genes among introgressed eQTL is surprising and suggests that the archaic circadian alleles could have been beneficial after introgression.

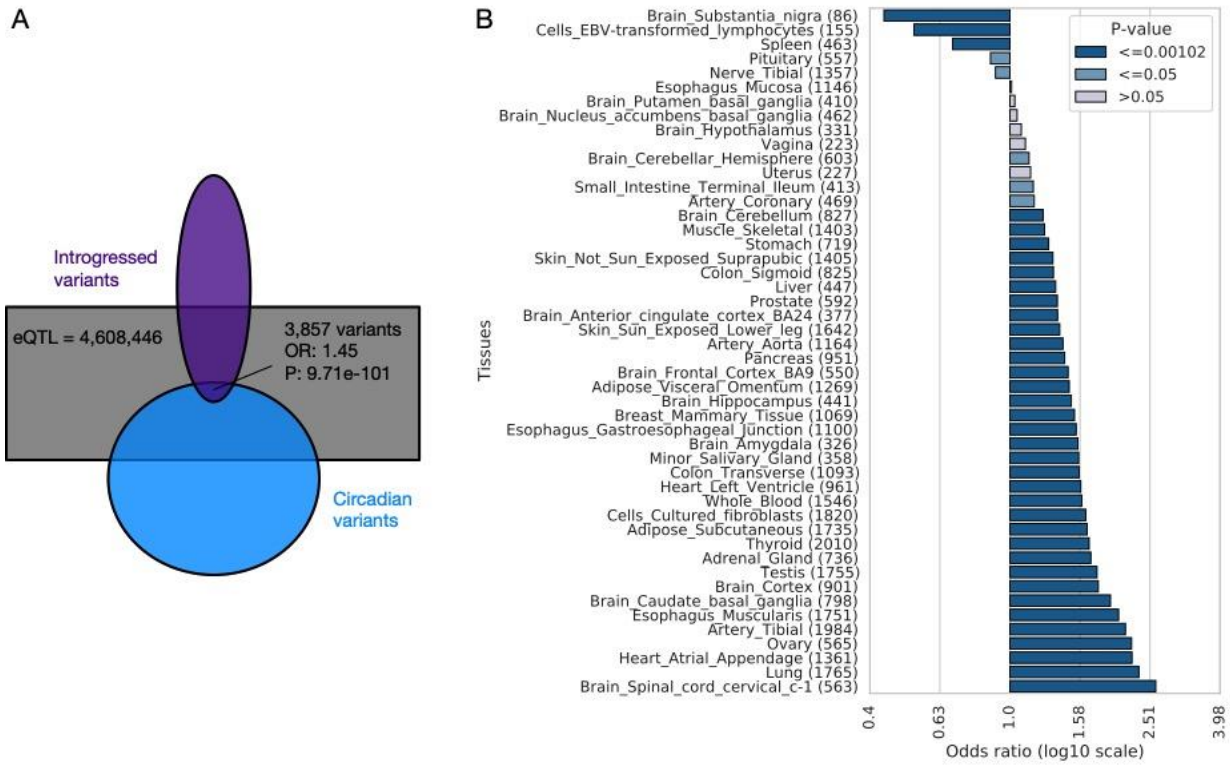

Figure 5. Circadian genes are enriched for introgressed eQTL. A) Archaic introgressed variants are more likely to be eQTL for circadian genes in GTEx than for non-circadian genes (Fisher's exact test: OR=1.45, P=9.71e-101). Purple represents the set of introgressed variants; blue represents the set of circadian variants; 3,857 are introgressed eQTL in circadian genes. The overlaps are not to scale. **B)** The enrichment for circadian genes among the targets of introgressed eQTLs in each GTEx tissue. Introgressed eQTL in most tissues show significant enrichment for circadian genes (Fisher's exact test; Table S6). Kidney cortex did not have any circadian introgressed eQTLs and thus is not shown. Numbers inside the parenthesis indicate the count of variants in each tissue. Gray bars indicate lack of statistical significance; light blue bars indicated nominal significance (p <= 0.05); and dark blue bars indicate significance at the 0.05 level after Bonferroni multiple testing correction (p <= 0.00102).

Introgressed variants predominantly increase propensity for morningness

After observing that circadian gene expression is influenced by archaic variants, we evaluated whether these effects are likely to result in a change in organism-level phenotype. To do this, we evaluated evidence that introgressed variants influence chronotype. The heritability of chronotype has been estimated in a range from 12 to 38% (Lane et al. 2016; Jones et al. 2019; 2016). Previous studies have identified individual introgressed loci associated with chronotype (Dannemann and Kelso 2017). We recently found modest enrichment for heritability of chronotype (morning/evening person phenotype in a GWAS of the UK Biobank) among introgressed variants genome-wide using stratified LD score regression (heritability enrichment: 1.58, P=0.25) and a suggestive bias toward increasing morningness (McArthur, Rinker, and Capra 2021).

To test comprehensively for a directional effect, we calculated the cumulative fraction of introgressed loci associated with chronotype in the UK Biobank that increase morningness (after clumping based on LD at R^2=0.9 in CEU). Each of the nine introgressed loci most strongly associated with chronotype increase propensity for morningness (Figure 6; Table S7; Table S8; 9 loci, P<=1.35e-13). As the strength of the association with morningness decreases, the bias begins to decrease, but the effect is maintained well past the genome-wide significance threshold (P<5e-8). When focusing the analysis on introgressed variants in proximity (<1 Mb) to circadian genes, the pattern becomes even stronger. The bias toward morningness remains above 80% at the genome-wide significance threshold. This suggests that introgressed variants act in a consistent direction on chronotype, especially when they influence circadian genes.

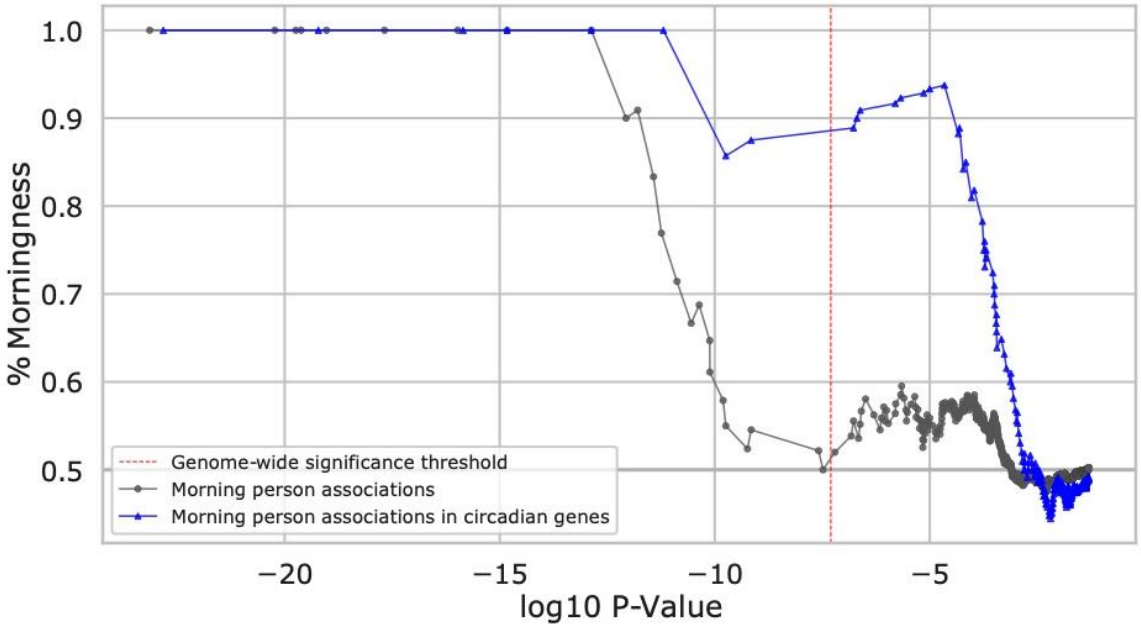

Figure 6. Introgressed variants associate with increased morningness. The cumulative fraction of introgressed loci significantly associated with the morning vs. evening person trait in the UK Biobank that increase morningness (y-axis) at a given p-value threshold (x-axis). Introgressed loci associated with chronotype are strongly biased towards increasing morningness, and this effect is strongest at the most strongly associated loci. Introgressed variants nearby (<1 Mb) circadian genes (blue) are even more strongly biased towards increasing morningness than introgressed variants overall (gray). Each dot (triangle) represents an associated locus; variants were clumped by LD for each set (R^2=0.9 CEU).

Circadian rhythms are involved in a wide variety of biological systems. To explore other phenotypes potentially influenced by the introgressed circadian variants, we evaluated evidence for pleiotropic associations. First, we retrieved all the genome-wide associations reported for introgressed variants in the Open Targets Genetics (https://genetics.opentargets.org) database, which combines GWAS data from the GWAS Catalog, UK Biobank, and several other sources. Introgressed circadian variants are associated with traits from a diverse range of categories (Table S9). Associations with blood related traits are by far the most common; however, this is

likely because they have more power in the UK Biobank. Overall, circadian introgressed variants are significantly more likely to have at least one trait association than introgressed variants not in the circadian set (Fisher's exact test: OR=1.25, P=7.03e-25) (Figure S4A). The circadian variants also associate with significantly more traits per variant than the non-circadian set (Mann-Whitney U: P=9.93e-14) (Figure S4B; Table S10). These results suggest effects for introgressed circadian variants beyond chronotype.

Evidence for adaptive introgression at circadian loci

The gene flow from Eurasian archaic hominins into AMH played a role in adaptations to some of the new environmental conditions encountered outside of Africa (Racimo et al. 2015). The above analyses demonstrate the effects of introgressed variants on circadian gene regulation and chronotype. To explore whether these circadian regions show evidence of adaptive introgression, we considered two sets of introgressed regions predicted to have contributed to AMH adaptation by two recent machine learning algorithms: *genomatnn* (Gower et al. 2021) and *MaLAdapt* (X. Zhang et al. 2023). We intersected the circadian introgressed variants with the adaptive introgression regions from each method.

We identified 26 regions in or near circadian genes with evidence of adaptive introgression—seven from genomatnn and 19 from MaLAdapt (including 53 and 116 SNPs, respectively; Table S11). One of these adapive introgression regions overlaps the upstream region of the gene *PER2*, a core clock gene involved in the generation of circadian rhythms through feedback loops. This gene is also predicted to have lower gene regulation in archaics hominins than most humans. We also found several introgressed eQTL for *PER2*, though they do not overlap the predicted adaptive introgression region. At the phenotype level, *PER2* introgressed variants have been associated with having an effect on morning/evening preference in humans in the UK Biobank. The Vindija Neanderthal also carries a lineage-specific variant in this gene that has splice-altering effects; this suggests that *PER2* may have experienced multiple functional changes in different modern and archaic lineages, with potential adaptive effects.

Another region with evidence of adaptive introgression overlaps the circadian variants in the gene *MYBBP1A*. In mouse, MYBBP1A acts as co-repressor of PER2 by binding to its promoter in combination with Cryptochrome-1 (CRY1). *MYBBP1A* is predicted to be divergently regulated between AMH and archaic hominins, with decreased expression in the archaics, and it also contains introgressed variants that are circadian eQTLs.

DISCUSSION

The Eurasian environments where Neanderthals and Denisovans lived for several hundred thousand years are located at higher latitudes with more variable photoperiods than the landscape where AMH evolved before leaving Africa. Evaluating genetic variation that arose separately in each of the archaic and AMH lineages after their split ~700 MYA, we identified lineage-specific genetic variation in circadian genes, their promoters, and flanking distal regulatory elements. We found that both archaic- and human-specific variants are observed more often than expected in each class of functional region. This result suggests that, while each group evolved separately during hundreds of thousands of years in divergent environments, both experienced pressure on circadian related variation. Leveraging sequence-based machine learning methods, we identified

many archaic-specific variants likely to influence circadian gene splicing and regulation. For example, core clock genes (*CLOCK*, *PER2*, *RORB*, *RORC*, and *FBXL13*) have archaic variants predicted to cause alternative splicing compared to AMH. Several core genes were also predicted in archaics to be at the extremes of human gene regulation, including *PER2*, *CRY1*, *NPAS2*, *RORA, NR1D1*. Surprisingly, the Altai Neanderthal shared more divergent regulation in the circadian genes with the Denisovan individual than the Vindija Neanderthals. The two Neanderthals represent populations that were quite distantly diverged with substantially different histories and geographical ranges. The Denisovan and Altai Neanderthal also come from the same region in Siberia, while the Vindija Neanderthal came from a region in Croatia with slightly lower latitude.

Introgression introduced variation that first appeared in the archaic hominin lineage into Eurasian AMH. While most of this genetic variation experienced strong negative selection in AMH, a smaller portion is thought to have provided adaptive benefits in the new environments (Racimo et al. 2015). Given the divergence in many circadian genes' regulation, we explored the landscape of introgression on circadian genes. We first looked at introgressed circadian variants that are likely to influence gene regulation in AMH. Variants in this set are observed more often than expected, suggesting the importance of maintaining circadian variation in the population.

We then evaluated the association of these introgressed variants with variation in circadian phenotypes of Eurasians. We previously reported a modest enrichment among introgressed variants for heritability of the morning/evening person phenotype. Here we further discovered a consistent directional effect of the introgressed circadian variants on chronotype. The strongest associated variants increase the probability of being a morning person in Eurasians.

While it is not immediately clear why increased morningness would be beneficial at higher latitudes, considering this directional effect in the context of clock gene regulation and the challenge of adaptation to higher latitudes suggests an answer. In present day humans, behavioral morningness is correlated with shortened period of the circadian molecular clockworks in individuals. This earlier alignment of sleep/wake with external timing cues is a consequence of a quickened pace of the circadian gene network (Brown et al. 2008). Therefore, the morningness directionality of introgressed circadian variants may indicate selection toward shortened circadian period in the archaic populations living at high latitudes. Supporting this interpretation, shortened circadian periods are required for synchronization to the extended summer photoperiods of high latitudes in *Drosophila*, and selection for shorter periods has resulted in latitudinal clines of decreasing period with increasing latitude, as well as earlier alignment of behavioral rhythms (Hut et al. 2013). In addition, *Drosophila* populations exhibit decreased amplitude of behavioral rhythms at higher latitudes which is also thought to aid in synchronization to long photoperiods (Hut et al. 2013).

Our finding that introgressed circadian variants generally decrease gene regulation of circadian genes suggests that they could lead to lower amplitude clock gene oscillations. However, when assayed in present day humans there is not a strong correlation between the overall expression level of *NR1D1* and the transcriptional amplitudes of other clock genes within individuals (Brown et al. 2008), and quantitative modeling of the mammalian circadian clockworks suggests that stable clock gene rhythms can result across a wide range of absolute levels of gene expression as long as the stoichiometric ratios of key positive and negative clock genes are reasonably conserved (Kim and Forger 2012). Interestingly, lower transcriptional

amplitude of *NR1D1* does confer greater sensitivity of the present-day human clockworks to resetting stimuli, a potentially adaptive characteristic for high latitudes (Brown et al. 2008).

Thus, given the studies of latitudinal clines and adaptation from *Drosophila* and the nascent understanding of clock gene contributions to behavioral phenotypes in present day humans, the directional effects of introgressed circadian gene variants toward early chronotype and decreased gene regulation we observed can be viewed as potentially adaptive. More complex chronotype phenotyping and mechanistic studies of the variants of interest are needed to fully understand these observations.

Finally, to explore evidence for positive selection on introgressed variants in AMH, we analyzed results from two recent methods for detecting adaptive introgression: *genomatnn* and *MaLAdapt*. Both methods identified circadian loci as candidates for adaptive introgression. However, we note that the predictions of these two methods have only modest overlap with one another, underscoring the difficulty of identifying adaptive introgression.

Several limitations must be considered when interpreting our results. First, multiple sets of predictions of introgressed variants are available. While these sets are qualitatively similar in their genomic attributes, additional introgressed circadian variants could be discovered by including other lines of evidence for introgression. Second, it is challenging to quantify the complexity of traits with a large behavioral component (like chronotype) and infer their variation from genomic information alone. Nevertheless, we believe our approach of focusing on molecular aspects (splicing, gene regulation) of genomic loci with relevance to circadian biology, in parallel to GWAS-based associations, lends additional support to the divergence in chronotype between archaic hominins and modern humans. Third, we also note that circadian rhythms contribute to many biological systems, so the variants in these genes tend to be associated with a variety of phenotypes. Thus, there is also the potential that selection acted on other phenotypes influenced by circadian variation than those related directly to chronotype. Fourth, recent adaptive evolution is challenging to identify, and this is especially challenging for introgressed loci. Nonetheless, we find several circadian loci with evidence of adaptive introgression from each of the scans we considered. Finally, given the many environmental factors that differed between African and non-African environments, it is difficult to definitively determine whether selection on a particular locus was the result of variation in light levels vs. other related factors, such as temperature. Nonetheless, given the observed modern associations with chronotype for many of these variants, we believe it is the most plausible target.

In conclusion, studying how humans evolved in the face of changing environmental pressures is necessary to understanding variation in present-day phenotypes and the potential tradeoffs that influence propensity to different diseases in modern environments (Benton et al. 2021). Here, we show that genomic regions involved in circadian biology exhibited substantial functional divergence between separate hominin populations. Furthermore, we show that introgressed variants contribute to variation in AMH circadian phenotypes today in ways that are consistent with an adaptive benefit.

METHODS

Circadian gene selection

Circadian biology is a complex system due to its high importance in the functioning of biological timing in diverse biological systems. For that reason, determining which genes are crucial for selection to environment response related to light exposure is not a straight forward process. To address this issue, we look at different sources of genome annotation databases and searched for genes and variants associated with circadian related phenotypes. We considered all human protein-coding genes in the Gene Ontology database annotated with the GO:0007623 ("circadian rhythm") term or terms annotated with relationship "is_a", "part_of", "occurs_in", or "regulates" circadian rhythm. We also considered genes containing experimental or orthologous evidence of circadian function in the Circadian Gene Database (CGDB), the GWAS Catalog genes containing "chronotype" or "circadian rhythm" associated variants, and a curated set of genes available in WikiPathways [https://www.wikipathways.org/index.php/Pathway:WP3594, https://doi.org/10.1093/nar/gkaa1024]. The final set of circadian genes was curated by Dr. Douglas McMahon.

To select the candidate circadian genes with the highest confidence, we defined a hierarchy system where genes annotated by McMahon or annotated in 3 out of 4 other sources receive a "High" level of confidence. Genes with evidence from 2 out of 4 of the sources are assigned a "Medium" level of confidence. Genes annotated as circadian only in 1 out of 4 sources are assigned to Low confidence and not considered in our circadian gene set. We then defined our set of circadian variants from the 1000 Genomes Project using the official list of circadian genes. The variants are included in analysis of coding, non-coding, regulatory, eQTL, human-specific, archaic-specific, and introgressed variants.

Definition of lineage-specific variants

To identify candidate variants that are specific to the human and the archaic lineages, we used a set of variants published by Kuhlwilm and Boeckx (Kuhlwilm and Boeckx 2019) (https://doi.org/10.1038/s41598-019-44877-x). The variants were extracted from the high-coverage genotypes of three archaics: a 122,000-year-old Neanderthal from the Altai Mountains (52x coverage), a 52,000-year-old Neanderthal from Vindija in Croatia (30x coverage), and a 72,000-year-old Denisovan from the Altai Mountains (30x coverage). The total variant sites retrieved from this set is 4,437,803. We used a filtering method similar to the one proposed in this publication. That is, a human-specific is defined as a position where all the humans carry the derived allele and all the archaics carry the ancestral allele. An archaic-specific is defined as a position where all the archaics carry the derived allele and the human allele frequency is not greater than 0.00001 (1e-5). These filters resulted in 9,424 human specific and 33,184 archaic-specific variants.

Enrichment of lineage-specific variants among functional regions of the genome

We intersected the sets of lineage-specific variants with several sets of annotated functional genomic regions. Inside circadian gene regions (Gencode v29), we found 156 human-specific variants and 341 archaic-specific variants. In circadian promoter regions, we found 6 human-specific variants and 19 archaic-specific variants. Promoters were defined as regions 5 kb up- to 1 kb downstream from a transcription start site. In distal regulatory elements, we found 247 human-specific variants and 807 archaic-specific variants. For this last set, we considered candidate cis-regulatory elements (cCREs) published by ENCODE (Moore et al. 2020) within 1 Mb of the circadian genes.

To compute whether lineage-specific variants are more abundant than expected in circadian genes, we applied a Fisher's exact test to the sets of human- and archaic-specific variants in regulatory, promoter, and gene regions. Human and archaic-specific variants are significantly enriched in both regulatory (Human: OR=1.25, P=8.39e-4; Archaic: OR=1.16, P=6.15e-5) and gene (Human: OR=1.84, P=7.06e-12; Archaic: OR=1.13, P=0.023) regions. The enrichment observed in the promoters of both lineages is not supported by a significant p-value (Human: OR=1.21, P=0.65; Archaic: OR=1.09, P=0.63).

Genes containing archaic variants with evidence of alternative splicing

We used a set of archaic variants annotated with the splice altering probabilities to identify circadian genes that may be differentially spliced between archaic hominins and AMH (Brand, Colbran, and Capra 2022). We considered variants from four archaic individuals: the Altai, Chagyrskaya, and Vindija Neanderthals and the Altai Denisovan. These archaic variants were annotated using SpliceAI (Jaganathan et al. 2019) and we considered any variant with a maximum delta, or splice altering probability, > 0.2. We identified 36 archaic-specific splice altering variants, defined as those variants absent from 1KGP, among 28 circadian genes. Next, we tested for enrichment among this gene set using an empirical null approach (Brand, Colbran, and Capra 2022; McArthur et al. 2022). We shuffled the maximum deltas among 1,607,350 variants 10,000 times and counted the number of circadian genes with a splice altering variant each iteration. Enrichment was calculated as the number of observed genes (N = 28) divided by the mean gene count among 10,000 shuffles. In addition to all genes with archaic-specific variants, we considered six other subsets among these variants: 1) genes with variants private to the Altai Neanderthal, 2) genes with variants private to the Chagyrskaya Neanderthal, 3) genes with variants private to the Altai Denisovan, 4) genes with variants private to all Neanderthals, 5) genes with variants shared among all archaic individuals, and 6) genes with variants private to the Vindija Neanderthal. Finally, we considered a subset of splice altering variants that were identified as tag SNPs by Vernot et al. (Vernot et al. 2016).

PrediXcan

To understand the difference in circadian biology between present-day humans and archaic hominins, we analyzed predictions on gene regulation. We considered the results from PrediXcan gene regulation predictions across 44 tissues from the PredictDB Data Repository (http://predictdb.org/). The models were trained on GTEx V6 using variants identified in 2,504 present-day humans in the 1000 Genomes Project (1KGP) phase 3 within 1 Mb of each circadian gene. The original analysis includes predictions for 17,748 genes for which the models explained a significant amount of variance in gene expression in each tissue (FDR < 0.05). The prediction models were also applied to the Altai and Vindija Neanderthals and the Denisovan. The resulting predictions are normalized values of the distribution observed in GTEx individuals used to train the original prediction models. Each prediction contains an empirical P-value which was calculated for each gene and tissue pair to define genes that are divergently regulated between archaic hominins and humans. The P-value is obtained by calculating the proportion of humans from the 1KGP that have predictions more extreme compared to the human median than the archaic individual. Significantly DR genes are defined as those where the archaic prediction falls outside the distribution of humans in the 1KGP predictions.

We tested whether the circadian genes in our set are more likely to be DR compared to an empirical null distribution from random gene sets of the same size. We account for the fact that

some genes are modeled in more tissues than others by matching the distribution of tissues in which each gene could be modeled in the random sets to our set. Among 1,467 DR genes in the Altai Neanderthal we find 23 DR circadian genes out of the total 236 genes in the circadian set. We iterate through the permutation analysis 1,000,000 times and find an enrichment of 1.21 (P=0.19). A similar analysis is done in the Vindija Neanderthal (1,536 total DR, 21 circadian DR, enrichment of 1.05, P=0.43) and the Denisovan individual (1,214 total DR, 19 circadian DR, enrichment of 1.20, P=0.24). In this study, we define a set of DR genes as the intersection between DR genes in all three archaics, resulting in a set of 16 genes.

Enrichment of introgressed variants in eQTL

We performed an enrichment analysis using Pearson's chi-squared test to evaluate if there is overrepresentation of introgressed alleles in our set of circadian variants using the GTEx dataset. We did a liftOver of the GTEx v8 dataset from hg38 to hg19. The original hg38 set contains 4,631,659 eQTLs across 49 tissues. After the LiftOver, 4,608,446 eQTLs remained, with the rest not mapping. We used the archaic introgressed variants dataset from Browning 2018. The set contains 863,539 variants that are introgressed in humans originating in archaic hominins. We performed an intersection between the set of genes containing evidence for eQTLs and our set of 246 circadian genes to retrieve a subset of variant sites with evidence of being eQTL in circadian genes. The resulting subset contained 97,441 circadian eQTLs in 49 tissues and 239 genes. We further intersected the introgressed variants and the set of eQTL, resulting in 128,138 introgressed eQTLs. The final set of eQTLs that are circadian and also introgressed is 3,857.

Direction of effect of chronotype associations

To explore the effect of archaic introgression in circadian dreams on human chronotype, we quantified the direction of effect of variants associated to a Morning/Evening person trait in a GWAS analysis of the UK Biobank (http://www.nealelab.is/uk-biobank/). The variants were LD clumped using PLINK v1.9 (R2 0.9). We generated cumulative proportion values on the beta values assigned to each associated variant on an ascending order of P-values.

Detection of pleiotropy in the set of introgressed circadian variants

To understand the extent of different phenotypes associated with the introgressed circadian variants, we first extracted genome-wide associations from Open Targets Genetics (https://genetics.opentargets.org/) for each of the variants with evidence of introgression (Browning et al. 2018). Only the variants with significant p-values were analyzed. The p-value threshold was set at the genome-wide significance level (P=5e-8). We split the variants in two sets: introgressed circadian and introgressed non-circadian. Many of these variants are not associated with any phenotype. We performed a Fisher's exact test to analyze which of the two sets contains a higher ratio of SNPs with at least one association versus SNPs with no association. The result showed that the circadian set had a significantly higher ratio (OR=1.36, P=5e-29). Then we calculated the total of unique traits associated with each of the variants, given that the SNP has at least one association. We used a Mann-Whitney U test to understand which set is represented by a higher level of traits per SNP. The circadian set was slightly more pleiotropic, and the result is supported by a significant p-value (P=5.4e-3).

Identifying introgressed circadian variants with evidence of adaptive introgression

We sought out to identify circadian variants that contain evidence of adaptive introgression (AI). To achieve this, we collected two sets of genomic regions that were measured for their likelihood to be under AI by two machine learning methods: *genomatnn* and *MaLAdapt*. *genomatnn* is a convolutional neural network trained to identify adaptive introgression based on simulations (Gower et al. 2021). *MaLAdapt* is a machine learning algorithm trained to find adaptive introgression based on simulations using an extra-trees classifier (ETC) (X. Zhang et al. 2023). Following the thresholds used in each paper, a region is considered to be under AI if the prediction value assigned to it meets a threshold of 0.5 or 0.9, respectively. Because the two methods are different, we performed two separate analyses instead of integrating them. To find the variants that fall into AI regions, we intersected the set of introgressed circadian SNPs with the genomatnn and the MaLAdapt regions individually. The set of introgressed circadian variants contains variants inside circadian genes, in circadian promoter regions (5 kb up- and 1 kb downstream of the TSS), and variants with regulatory function (cCREs) flanking circadian genes by 1 Mb. We found 53 introgressed circadian variants in 7 genes defined as AI by genomatnn, and 116 variants in 19 genes defined as AI by MaLAdapt.

DATA AVAILABILITY

The data underlying this article are available in the article and in its online supplementary material.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

We thank members of the Capra Lab for helpful comments on this work. This work was conducted in part using the resources of the Advanced Computing Center for Research and Education at Vanderbilt University, Nashville, TN. This work was supported by the National Institutes of Health [R35GM127087 to JAC, R01GM117650 to DM, F30HG011200 to EM, T32GM080178 to Vanderbilt University (EM), and T32HG009495 to the University of Pennsylvania (LLC)].

AUTHOR CONTRIBUTIONS

Conceptualization: KV, JAC; Methodology: KV, LC, EM, CB, JS, DM, JAC; Investigation: KV, LC, EM, CB, JAC; Writing – Original Draft: KV, JAC; Writing – Review & Editing: KV, LC, EM, CB, DR, DM, JAC; Funding Acquisition: JAC; Resources: JAC; Supervision: JAC.

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