Human evolved regulatory elements modulate
genes involved in cortical expansion and
neurodevelopmental disease susceptibility

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Modern genetic studies indicate that human brain evolution is driven primarily by changes in
gene regulation, which requires understanding the biological function of largely non-coding
gene regulatory elements, many of which act in tissue specific manner. We leverage chro-
matin interaction profiles in human fetal and adult cortex to assign three classes of humans-
evolved elements to putative target genes. We find that human-evolved elements involving
DNA sequence changes and those involving epigenetic changes are associated with human-
specific gene regulation via effects on different classes of genes representing distinct bio-
logical pathways. However, both types of human-evolved elements converge on specific cell
types and laminae involved in cerebral cortical expansion. Moreover, human evolved ele-
ments interact with neurodevelopmental disease risk genes, and genes with a high level of
evolutionary constraint, highlighting a relationship between brain evolution and vulnerability
to disorders affecting cognition and behavior. These results provide novel insights into gene
regulatory mechanisms driving the evolution of human cognition and mechanisms of vul-
nerability to neuropsychiatric conditions.

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Human evolution is hypothesized to be driven primarily by changes in gene regulation rather than divergence in protein-coding sequences. Recent comparative genomic and epigenomic studies have identified regions on the human lineage having either an accelerated sequence, referred to as human accelerated regions (HARs), or epigenetic changes, referred to as human gained enhancers (HGEs). One class of human evolved genomic elements, HARs, are enriched in developmental enhancers, suggesting that they may drive evolution of human-specific traits via developmental gene regulation. Recent targeted sequencing of HARs in consanguineous autism spectrum disorder (ASD) families identified significant enrichment of rare bi-allelic variants, highlighting a potential role of HARs in susceptibility to neurodevelopmental disorders. While we were able to assign 638 and 717 HARs to 972 and 1021 genes using contacts defined by three-dimensional chromatin conformation in cortical plate (CP, neocortical laminae containing post-mitotic neurons) and germinal zone (GZ, neocortical laminae consisting primarily of neural progenitors) in the fetal brain, respectively (Methods; Supplementary Fig. 2). When combined with intragenic HARs, we were able to assign 1028 HARs to 1648 putative target genes (Supplementary Tables 1–2, Supplementary Fig. 2, subsequently referred to as putative target genes for HARs). Only 26.3% of these physically interacting genes were the genes nearest to a HAR (Fig. 1b), which indicates how risky it is to perfunctorily assign regulatory elements to the nearest gene without other evidence. This is also consistent with emerging evidence that chromatin interactions often link genes to quite distal regulatory elements and are not related to linear distance or genetic recombination, as defined by linkage disequilibrium.

The putative target genes of HARs are enriched for genes that regulate pathways involved in human brain development, regionalization, dorsal-ventral patterning, cortical lamination, and proliferation of neuronal progenitors (Fig. 1d, Supplementary Fig. 3), suggesting that multiple aspects of human brain development are subject to human-specific regulation. This includes genes driving the dorsal–ventral patterning of the telenencephalon (EMX2, PAX6, GLI3, NKX6.1, and NKX6.2), genes playing major roles in cortical neurogenesis (PAX6, HES1, SOX2, GLI3, and TBR2), genes that specify lamination identity of cortical neurons (TBR1, CUX1, POU3F2, POU3F3, ROB1, MDGA1, and ETV1), and genes involved in axonal pathfinding (DSCAML1 and ROBO). While the majority of genes are involved in forebrain development, a few putative target genes regulate mid- or hindbrain development, including GLI2, EN1, and GBX2.

Doan et al. performed targeted sequencing of HARs in consanguineous families containing probands diagnosed with ASD, finding enrichment of rare sequence variants within HARs in patients with ASD. They also used chromatin interaction profiles in multiple non-neuronal cell types to identify putative target genes of HARs, a portion (20.8%) of which overlap with targets identified based on Hi-C from developing brain. Although this overlap is significant ($P = 2.09 \times 10^{-17}$, OR = 2.5, Fisher’s exact test) and implicates a high confidence core set of target genes (Supplementary Table 1), most target genes are discordant (Fig. 1c), which may be due to differences between tissue specific gene regulation, or methodologic factors.

To reduce confounding due to use of different analytic pipelines, we next examined genes that interact with HARs in non-neuronal cell types (embryonic stem cells: ES cells and fetal lung fibroblasts: IMR90 cells) using the same analytic pipeline previously applied to fetal brain. We found that HARs interacted with 823 and 467 protein-coding genes in ES cells and IMR90 cells, respectively (Supplementary Fig. 4a, b). The majority of genes (~60%) interacted with HARs in a cell type-specific manner, again highlighting the cell type specific nature of chromatin interactions, consistent with the notion of tissue specific gene regulation (Supplementary Fig. 4). Notably, many genes known to play major roles in cerebral cortex development and dorsal–ventral/anterior–posterior pattern specification, including SOX2, PAX6, POU3F2, GLI3, EN1, and TBR2, interacted with HARs in the developing cortex (Supplementary Fig. 4c), consistent with the model that chromatin contact maps in developing brain will likely provide more biologically relevant targets for human brain evolution than other tissues.
Comparing different classes of human evolved elements. We next analyzed another major type of regulatory element predicted to play a role in human brain evolution – the class of human gained enhancers (HGEs) and human lost enhancers (HLEs) – genomic regions that exhibit increased and decreased enhancer activity, respectively, as assessed through changes in active epigenomic regions that exhibit increased and decreased enhancer odds ratio calculated by Fisher’s exact test. Error bars denote for 95% confidence intervals (CI). **b** Overlap between nearest genes to HARs (closest) with genes that interact with HARs in developing brain (fetal brain). **c** Overlap between genes that interact with HARs in developing brain (fetal brain), and non-neuronal cell types (non-neurons)\(^{30}\). Protein-coding genes were used for Venn diagrams. **d** Gene ontology enrichment for HAR-associated genes

We next identified predicted target genes for HGEs and HLEs (Methods). We used previously identified target genes for HGE\(_{AB}\)\(^{11}\), while we leveraged new chromatin interaction profiles from the adult prefrontal cortex (PFC\(^{21}\)) to identify putative target genes of HGE\(_{AB}\) and HLEs, since they were defined in adult brain. We were able to assign 1518 HGE\(_{AB}\) and 1779 HLEs to 1513 and 1547 putative target genes, respectively, based on chromatin interaction profiles. We first observed that the predicted target genes of HARs, HGEs, and HLEs exhibit minimal genome-wide overlaps, consistent with the notion that different classes of human evolved elements regulate different biological processes (Fig. 2b). Whereas HAR-associated genes are involved in cerebral corticogenesis and cortical lamination as described above, putative target genes for HGE\(_{AB}\) are enriched for GTPase regulators and the GPCR signaling pathways\(^{11}\). In contrast, HGE\(_{AB}\) interact with genes involved in collagen metabolism, TOR signaling, immune function, and lipid storage, and HLEs interact with genes involved in oxygen transport, autophagy, and thymus development (Supplementary Fig. 5a). It is particularly interesting that HGE\(_{AB}\) interact with genes...
involved in lipid storage, as humans display an increased capacity to metabolize a lipid-rich diet, which is accompanied by the larger brain size that requires high energy demands. We then explored developmental expression trajectories of putative target genes for HARs, HGEs, and HLEs. We observed distinct average expression trajectories between these groups, especially during prenatal stages, consistent with the differential migration, during the period of synaptic formation and gliogenesis, peaking near mid-gestation, a period marked by pronounced postnatal enrichment for HGE- and HLE-associated genes. HAR-associated genes exhibit any laminar specificity, and a pattern of more gradual upregulation throughout prenatal development, manifesting their highest expression after the peak of neuronal genesis, peaking near mid-gestation, a period marked by neuronal migration, early neuronal phenotype definition, and dendritic arborization. HGE- and HLE-associated genes do not show prenatal enrichment. HGE- and HLE-associated genes are more highly expressed during postnatal development, with more pronounced postnatal enrichment for HGE- and HLE-associated genes (Fig. 2c). HGE- and HLE-associated genes show a pattern of more gradual upregulation throughout prenatal development, manifesting their highest expression after the peak of neuronal migration, during the period of synaptic formation and gliogenesis. We next examined whether genes associated with human evolved elements exhibit any laminar specificity. Remarkably, all classes of human evolved element-associated genes, especially HARs and HGEs, were enriched in superficial cortical layers, layers 2 and 3, which form the inter-and intrahemispheric
connections between cortical regions and are significantly expanded in primates (Fig. 2d)\textsuperscript{23–27}. In contrast, HGE\textsubscript{AB}- and HLE-associated genes are also enriched in layer 6 (Fig. 2d), which projects to subcortical regions, primarily thalamus. The expansion of the superficial, supragranular layers is hypothesized to contribute to the elaboration of gyriﬁcation, as it displays the largest increase in the number of neurons and thickness in primates compared with rodents and carnivores\textsuperscript{26–31}. These data therefore directly connect the expansion of hemispheric regions and their connectivity with speciﬁc molecular pathways and regulatory elements.

To further reﬁne their functional annotation, we next determined whether human evolved element-associated genes are expressed in speciﬁc cell types by leveraging data from single-cell sequencing in the developing human neocortex and adult PFC\textsuperscript{32,33} (Fig. 2e). In the developing cortex, all classes of human evolved element-associated genes were enriched in the outer radial glia, which comprise a major class of neural stem cells in the germinal layer that shows substantial expansion on the primate lineage\textsuperscript{34} (Fig. 2e). This suggests that even though these different classes of human evolved elements regulate divergent biological processes, they converge on human cortical expansion, a striking ﬁnding. This observation is also consistent with the laminar patterns of enrichment described above, which highlight superficial cortical layers\textsuperscript{30}. In the adult PFC, all classes of human evolved element-associated genes were enriched in astrocytes, while neuronal enrichment was detected for HAR- and HGE\textsubscript{FB}-associated genes (Fig. 2e). This cell-type speciﬁcity reﬂects gene ontology enrichment, as HAR-associated genes are involved in neuronal proliferation and differentiation, whereas HGE\textsubscript{FB}- and HLE-associated genes are involved in immune function. Astrocytic enrichment is particularly interesting, as human astrocytes are morphologically more complex and transcriptomically distinct from murine astrocytes\textsuperscript{35,36} and glial co-expression networks are less preserved between rodents and humans than neuronal networks\textsuperscript{37}. Taken together, different classes of human evolved elements potentially regulate distinct biological pathways during different developmental windows and in different cell types, although they do converge on cell types and layers responsible for cortical expansion and gyriﬁcation on the primate and human lineages.

**Human evolved elements and human-speciﬁc gene regulation.**

We had previously shown that HGE\textsubscript{FB} interact with protein-coding genes that are under purifying selection in primates and humans\textsuperscript{11}, so we next tested the hypothesis that protein-coding genes linked to human evolved elements are under similar selection pressures. Indeed, HAR-, HGE\textsubscript{FB}-, and HLE-associated genes are also under purifying selection when compared with the genome background (Methods; Supplementary Fig. 6). HAR- and HGE\textsubscript{FB}-associated genes are not only evolutionary constrained, but also enriched with genes that are intolerant to predicted loss of function (LoF) variation (pLI \(\geq 0.9\)) in human populations\textsuperscript{38} (Fig. 2f). In summary, human evolved genomic elements are associated with protein-coding genes that are evolutionary conserved and intolerant to haploinsufﬁciency, supporting the hypothesis that non-coding regulatory elements drive evolutionary divergence by species-speciﬁc transcriptional regulation of often essential, highly conserved genes\textsuperscript{1}.

As candidate genes for human evolved elements are subject to human-speciﬁc regulation, we tested whether they are differentially regulated in humans compared with non-human primates by exploiting a transcriptional atlas of human and non-human primate brain (Methods)\textsuperscript{39,40}. Candidate genes for human evolved elements were not enriched for developmental human-speciﬁc genes that show distinct developmental expression trajectories in human vs. rhesus macaque based on a recent study\textsuperscript{40} (HAR, \(P = 0.70, \text{OR} = 1.11\); HGE\textsubscript{FB}, \(P = 0.37, \text{OR} = 1.23\); HGE\textsubscript{AB}, \(P = 0.27, \text{OR} = 1.42\); HLE, \(P = 0.86, \text{OR} = 0.84\), Fisher’s exact test). In contrast, HGE\textsubscript{AB}-associated genes showed modest, but signiﬁcant, enrichment for differentially expressed genes in the adult brain tissue between humans and non-human primates (Fig. 3c, OR = 1.51, \(P = 0.022\), Fisher’s exact test)\textsuperscript{40}.

Because genes associated with human evolved elements do not display distinct human-speciﬁc regulation during brain development, we hypothesized that they may be under more precise developmental control. To assess more reﬁned developmental regulation, we ﬁrst calculated the difference in relative developmental expression levels (\(\Delta\) expression Z-score) at a matching developmental stage between human and rhesus macaque (Methods)\textsuperscript{40}. We found that during prenatal and early postnatal periods (20 post-conception week (PCW) – 5 months after birth), HGE\textsubscript{FB}-associated genes show a small, but signiﬁcant increase in \(\Delta\) expression Z-score (mean \(\Delta = 0.128, P = 8.2 \times 10^{-4}\), two-sided t-test), suggesting that HGE\textsubscript{FB} genes show selective expression during that stage in human brain relative to rhesus macaque brains (Fig. 3a). Genes associated with other human evolved elements do not show any deviation of \(\Delta\) expression Z-scores, indicating that they do not show stage-speciﬁc enrichment in human compared with rhesus macaque (Supplementary Fig. 7).

Another characteristic of human-speciﬁc transcriptional regulation is the observation of early breakpoints during brain development, which denote a group of genes that display abrupt expression changes in human compared to rhesus macaque\textsuperscript{40}. Genes with early breakpoints are thought to represent an earlier onset of developmental processes that are potentially extended in human brain compared with non-human primates\textsuperscript{40}. Notably, HAR-associated genes tend to have earlier breakpoints (Fig. 3b, Methods), implying that HARs may contribute to the expansion and elaboration of human cortex by inducing early peaks and more protracted expression of essential regulators of neuronal proliferation and differentiation. HAR-associated genes with earlier breakpoints include CPLX2, whose protein product functions in synaptic vesicle exocytosis\textsuperscript{41} and ITPR1, which harbors mutations found in spinocerebellar ataxia\textsuperscript{42}. In contrast, HGE\textsubscript{AB}-associated genes show delayed breakpoints (Fig. 3b), suggesting that human evolved elements active in adult brain regulate genes with later peak expression during development. These genes include SI100B, a marker for astrocytes\textsuperscript{43}. HAR\textsubscript{FB}- and HLE-associated genes do not exhibit signiﬁcant changes in breakpoints in the genes they regulate (Supplementary Fig. 7), indicating that the activity of different classes of human evolved regulatory elements manifest distinct developmental trajectories.

We also hypothesized that human evolved elements might mediate human-speciﬁc gene co-regulation. To address this question, we leveraged recently identiﬁed human-speciﬁc co-expression modules\textsuperscript{44} to gain further insights into human-speciﬁc gene regulation mediated by human evolved elements. Indeed, HGE\textsubscript{FB}- and HGE\textsubscript{AB}-associated genes were enriched in human-speciﬁc modules, M162 (Methods, OR = 3.12, \(P = 2.06 \times 10^{-4}\), Fisher’s exact test) and M122 (OR = 7.12, \(P = 3.45 \times 10^{-5}\), Fisher’s exact test), respectively (Fig. 3c). Genes in M162 are associated with alternative splicing and expressed in a speciﬁc subgroup of excitatory neurons, while M122 is not associated with a speciﬁc gene ontology or cell type\textsuperscript{44}. Given that alternative splicing is hypothesized to play an essential role in transcriptomic complexity and diversity and subject to dynamic regulation in humans compared with non-human primates\textsuperscript{45}, it is of note that HGE\textsubscript{FB}-associated genes are differentially regulated in human and associated with alternative splicing in a subset of excitatory neurons.
Human cortical evolution and neurodevelopmental disorders.
We next hypothesized that regulatory elements that drive human brain evolution may affect susceptibility to neurodevelopmental disorders via their target genes because (1) LoF-intolerant genes are enriched for de novo LoF variation in ASD and developmental delay (DD)\(^46\), (2) HARs are enriched with biallelic mutations in consanguineous ASD families\(^10\), and (3) HGE\(_{FB}\) interact with genes associated with intellectual disability\(^11\). Indeed, we observed that HAR- and HGE\(_{FB}\)-associated genes are enriched with LoF-intolerant genes that harbor de novo mutations in ASD (ASD constrained genes) and DD risk genes\(^47\) (Fig. 2f, Supplementary Fig. 5c). In contrast, HGE\(_{AB}\) and HLE-associated genes are enriched with genes affected by copy number variation (CNV) in schizophrenia\(^48\), a disorder that is strongly associated with intellectual disability\(^11\). Indeed, we observed that HAR- and HGE\(_{AB}\)-associated genes are enriched with LoF-intolerant genes that harbor de novo mutations in ASD (ASD constrained genes) and DD risk genes\(^47\) (Fig. 2f, Supplementary Fig. 5c). In contrast, HGE\(_{AB}\) and HLE-associated genes are enriched with genes affected by copy number variation (CNV) in schizophrenia\(^48\), a disorder that is strongly associated with intellectual disability\(^11\). Indeed, we observed that HAR- and HGE\(_{AB}\)-associated genes are enriched with LoF-intolerant genes that harbor de novo mutations in ASD (ASD constrained genes) and DD risk genes\(^47\) (Fig. 2f, Supplementary Fig. 5c). In contrast, HGE\(_{AB}\) and HLE-associated genes are enriched with genes affected by copy number variation (CNV) in schizophrenia\(^48\), an adolescent- and adult-onset disorder. It is also interesting to note that although both HGE and HAR regulated genes are implicated in ASD, the overall patterns predict slightly different relationships to neurodevelopmental disease. Relative to HGE\(_{FB}\), genes putatively regulated by HARs show more enrichment in ASD constrained genes and de novo LOF variation, whereas HGE\(_{FB}\) appear more enriched for constrained DD genes, or genes harboring LOF mutations in DD (Supplementary Fig. 5c).

Functional validation of HAR-associated genes. Although chromatin contacts provide a powerful tool to identify long-range physical chromatin interactions necessary for gene regulation, experimental validation would increase confidence that these chromatin contacts were functional. We therefore experimentally validated the functional impact of a subset of HARs active in developing human brain using primary human neural progenitor cells (phNPCs), which are a well validated in vitro model system for human neural development. We chose 3 enhancer-gene predictions based on the known role of the target gene in neurodevelopment, GLI2, GLI3, and TBR1 and present all of the results for these predicted interactions. We targeted catalytically inactive Cas9 linked to the synthetic VP64 activation domain (dCas9-VP64) to three HARs in phNPCs whose putative target genes include GLI2, the promoter of which interacts with HAR-01246 (Fig. 4a). GLI2 encodes a C2H2-type zinc-finger protein that mediates Sonic hedgehog (Shh) signaling and is critical for the induction of neural tube in mice\(^49,50\). Targeting dCas9-VP64 to the HAR using two guide RNAs (gRNAs) resulted in a ~60% increase in the expression level of GLI2 in phNPCs (Fig. 4a). CRISPR/Cas9-mediated transcriptional activation of HAR-02296 that interacts with GLI2 also led to a 30-40% increase in its expression (Fig. 4b). GLI3 is required for dorsal–ventral patterning of telencephalon including the formation of the cortical hem in mouse and humans\(^51,52\) and GLI3 null mice display a substantially smaller neocortex and absence of the hippocampus\(^53\). TBR1, a marker for deep layer projection neurons in the developing cortex that specifies laminar identity of cortical neurons\(^54,55\), interacts with HAR-01298, which is ~170 kb distal (Fig. 4c). Due to the small size of HAR-01298 (16 bp), we could not find gRNAs directly targeting this element, so instead we designed gRNAs flanking the region. One gRNA targeting the region 88 bp upstream of HAR-01298 increased TBR1 expression up to 80%, while the other gRNA targeting the region 92 bp downstream of HAR-01298 did not affect TBR1 expression (Fig. 4c).
Discussion

We leveraged chromatin architecture in fetal and adult human cerebral cortex to identify the putative regulatory targets of non-coding elements that have been previously identified as those most changing on the human lineage. By comparing the two major different classes of human evolved elements, those based on sequence changes, and those based on functional epigenetic alterations, we found that multiple modalities of regulatory relationships likely drive human brain evolution by orchestrating different molecular programs in distinct developmental windows and cell types. For example, HAR-associated genes are prenatally enriched, HGE AB- and HLE-associated genes are postnatally enriched,
while HGEFB-associated genes do not show developmental-stage specific enrichment and are expressed across development as a group. In postnatal brain, HAR target genes are predominantly expressed in excitatory neurons, while HGE- and HLE-associated genes are expressed in astrocytes and neural stem cells.

Critically, despite the observation that different human evolved elements are predicted to modulate distinct biological pathways, there are areas of convergence. In developing brain, genes regulated by both HGE and HAR converge on radial glia, a major neurogenic niche in developing human cortex9. In adult brain, they converge on the supragranular layers, which are most expanded in primates, especially humans, and mediate connections between different cortical regions, as well as between the two cerebral hemispheres12. The expansion of supragranular layers in human is attributed to the enlargement of outer subventricular zone (OSVZ), the layer in which the majority of human radial glia are located13,14, suggesting that human specific gene regulation converges on human cortical expansion and its subsequent intra- and inter-cortical connectivity, which is the major anatomical feature of human brain evolution9. Since it is gene regulation, rather than changes in protein-coding sequences that is the major distinguishing feature between humans and non-human primates1, these data provide a fundamental molecular map linking human specific gene regulation to brain evolution.

Human evolved elements do not only differ in the biological processes they regulate, but the types of human-specific regulation to which they are subject. HAR-associated genes show earlier onset of expression that continues throughout brain development, while HGEAB-associated genes show later onset of expression in human compared with rhesus macaque. On the contrary, HGEFB-associated genes show higher relative prenatal and early postnatal expression in human than rhesus macaque. This observation suggests that multiple forms of gene regulation contribute to the evolution of uniquely human traits.

The functional differentiation between elements whose evolution is based on sequence vs. those based on epigenetic changes also extends to their relationship to human brain disorders. Genes regulated by human evolved elements in developing brain (HARs and HGEFAB) are both associated with neurodevelopmental disorders. However, HAR genes appear more likely to be disrupted in ASD, while HGEFAB genes are more substantially enriched in constrained or LOF DD genes although enriched in constrained ASD genes (Supplementary Fig. 5). In contrast to elements active in fetal brain15, human evolved elements in adult brain (HGEAB and HLEs) are associated with later onset psychiatric disorders, suggesting that the susceptibility to neuropsychiatric disorders is related, at least partially, to human-specific developmental gene regulation. These data not only identify the genomic elements and their target genes that underlie these human specific features, but also explain in part why behavioral outcome measures in rodent models may not be directly translatable to human disease in many cases.

We employed CRISPR/Cas9-mediated transcriptional activation system to functionally validate the effects of HARs on putative target genes (GLI2, GLI3, and TBR1) that encode essential regulators of forebrain development and cortical lamination. Both GLI2 and GLI3 are involved in patterning and growth of the central nervous system (CNS) regulated by Sonic Hedgehog (SHH). Knockdown of Gli2 in neuroepithelial cells inhibits the expression of neural stem cell markers and induces premature differentiation of neural stem cells59, whereas Gli3 hypomorphic mutant mice display perturbed cortical lamination60. Further, single-cell transcriptomic profiles on developing human cortex demonstrated that both GLI2 and GLI3 are enriched in radial glia, where GLI2 is specifically enriched in young outer radial glia, and GLI3 is an essential component of gene expression cascades in early cortical neurogenesis32. Therefore, HAR-02296 and HAR-01246 may be involved in human brain evolution by regulating cortical expansion and lamination. Another example is TBR1, a well-known marker for deep layer neurons. Rostral markers are substantially downregulated in Tbr1 null mice, highlighting its potential role in establishing frontal cortex identity64. Moreover, recurrent de novo loss-of-function (LoF) variants in TBR1 have been identified in individuals with ASD65,66, and TBR1 itself regulates other ASD risk genes65. Thus, HAR-01238, which we show regulates TBR1 expression may coordinate patterning of the frontal cortex, the disruption of which can lead to neurodevelopmental disorders. Notably, HARs and HGEFB also interact with genes that regulate the size of the frontal cortex such as FGF17 and EMX269, a functional link between human-evolved elements and evolutionary expansion of the frontal cortex in human12.

Collectively, these findings illustrate how changes in gene regulation mediated by rapid evolution of non-coding regions contribute to phenotypic differences between human and non-human primates despite the high degree of similarities and high level of constraint in protein-coding sequences. These data are consistent with a model whereby newly evolved biological mechanisms driving human cerebral cortical evolution increase vulnerability for a range of neuropsychiatric and neurodevelopmental conditions. Further, they provide a framework that links regulatory elements to target genes that will be of substantial utility for mechanistic studies and disease modeling.

Methods

Enrichment of HARs in regulatory elements. We employed GREAT66 to analyze chromatin states/histone marks enrichment for HARs. We calculated the proportion of a chromatin state over the genome (p), the number of HARs (n), and the number of HARs that overlap with a given chromatin state. The significance of enrichment was calculated by the binomial probability of \( P = Pr_{binom}(k \geq n|n, p) \), where \( k \) is fold enrichment as the ratio between the fraction of HARs in the genome that overlap with a chromatin state (c) and (the proportion of HARs in the genome \( \times \) the proportion of chromatin state in the genome). Because HARs are evolutionary conserved elements, evolutionary conservation can affect enrichment patterns in regulatory elements. We conducted a secondary enrichment analysis controlling for evolutionary conserved elements. We defined evolutionary conserved regions as genomic regions larger than 20 bp with a phastCons score >0.40 (http://compgen.cshl.edu/phast/, R library phastCons100way.ucscFich19). Then, we performed a Fisher’s exact test with the following contingency table (Table 1).

In addition, we randomly selected 10,000 sets of genomic regions that have matched size and phastCons scores with HARs (hereby referred as default regions). We then overlapped these default regions to DNase I hypersensitivity sites (DHS) in each tissue/cell type and obtained an odds ratio (OR) for each Fisher’s exact test (same contingency table used as above). This leads to a set of 10,000 ORs for each tissue type, which was plotted in Supplementary Fig. 1a. Collectively, we used three metrics (GREAT enrichment, Fisher’s test while controlling for evolutionary conservation, 10,000 permutations) to confirm that HARs show highest enrichment for DHS in the fetal brain compared with other tissue/cell types.

DHS in multiple cell/tissue types and 15-chromatin states (Table 2) in fetal brain and adult prefrontal cortex (PFC) were obtained from Roadmap Epigenome20, and chromatin accessibility peaks in cortical plates (CP) and germinal zone (GZ) were obtained from de la Torre-Ubieto et al.13. We detected strong enrichment signals for HARs in regulatory elements of the developing brain, but not in the adult cortex. However, it is difficult to distinguish the effects of the development from the effects of the cellular heterogeneity and/or regional differences. This is because (1) tissue-level DHS lack cellular resolution, and (2) fetal brain DHS lack specific regional coordinates12. To address this issue, we leveraged ATAC-seq peaks obtained from two cortical layers with well-established cellular identities (CP is comprised of post-mitotic neurons, while GZ is comprised of neural progenitors and regional coordinate, lateral prefrontal cortex)13. We were able to detect robust enrichment for HARs in ATAC-seq peaks in fetal cortex, implicating strong developmental effects. However, given that accessible chromatin in GZ was more enriched for HARs than accessible chromatin in CP, we believe cellular heterogeneity also plays a role. The distinction will become clearer once more celluarily, regionally defined epigenomic landscape becomes available.

Identification of the putative target genes of HARs. HARs were categorized into (1) coding HARs that reside in exons, 5′ untranslated regions (UTR), 3′ UTR, promoters (1 kb upstream to transcription start sites), and downstream flanking
(1 kb downstream to transcription end sites), and (2) non-coding HARs that reside in intergenic and intronic regions. Coding HARs were directly assigned to their target genes based on their genomic coordinates, while non-coding HARs were annotated based on chromatin interactions. As the highest resolution available for Hi-C data was 10 kb, we assigned non-coding HARs to 10 kb bins, and obtained Hi-C interaction profiles of the 1 Mb flanking regions for each HAR-containing bin.

We also obtained background Hi-C interaction profiles from randomly selected genomic regions that share similar properties with HARs. For each HAR, we randomly selected a region within the same chromosome that has the same length and GC content (<5% difference) with the HAR. We repeated this 40 times to construct 2351 x 10 = 109,480 randomly matched regions. As some of these regions were overlapping, we ended up having 109,408 randomly selected genomic regions with matched GC content and length as HARs (Supplementary Table 4), which we used to construct a null distribution. Using these background Hi-C interaction profiles, we fit the distribution of Hi-C contacts at each distance for each chromosome using the Weibull distribution in flexistriplus package.

Significance for a given Hi-C contact was then calculated as the probability of observing a stronger contact under the null distribution matched by chromosome and distance. P-values were adjusted to the number of HARs (non-coding, 2634) and bins (198 bins per locus), and Hi-C contacts with FDR < 0.05 were considered as significant interactions. Putative target genes were identified by overlapping HAR interacting regions with promoter coordinates (2 kb upstream to TSS, Gencode v19). The same analysis was performed on Hi-C interaction profiles for each chromosome that has the same length and GC content (<5% difference) with the HAR.

Identification of the putative target genes of HGEs and HLEs. HGEsAB and HLEs were defined as genomic regions that underwent regulatory changes between human and chimpanzee in developing cortex. To assign HGEsAB to their target genes, we used previously identified target genes of HGEsAB with a slight modification. We first categorized HGEsAB into ones located in promoters and ones that are not. Promoter HGEsAB were directly assigned to their target genes based on their genomic coordinates, while non-promoter HGEsAB were assigned to their target genes based on chromatin interaction profiles in fetal brain. We only used non-promoter HGEsAB and HLEs for target gene assignment. To assign HGEsAB and HLEs to their target genes, we first converted genomic coordinates of HGEsAB and HLEs from hg38 to hg19 using liftOver (https://genome.ucsc.edu/cgi-bin/hgliftOver). HGEsAB and HLEs were then assigned to their target genes using newly generated chromatin interaction profiles in the adult PFC. As HGEs and HLEs were respectively defined by gain and loss of H3K27ac marks, we only used promoter-based interactions.

| Table 1 | Contingency table for calculating cell-type specific enrichment of HARs |
|---------|-------------------------------------------------|
| HAR     | DHS # of HARs that overlap with DHS in a given cell type = A | Not DHS # of HARs that do not overlap with DHS in a given cell type = A + C |
| Background (evolutionary conserved regions) | # of evolutionary conserved regions that overlap with DHS in a given cell type = B | # of evolutionary conserved regions that do not overlap with DHS in a given cell type = B + D |
| Column total | A + B | C + D |
| Row total | N = (A + B + C + D) |

CACAT content (1/2 of the splicing score) above 0.25 was considered as a significant score. In this way, we obtained 214 genes with high statistical power.

Table 2 | Annotations for chromatin states |
|---------|---------------------------------|
| TssA    | Active Transcription start sites (TSS) |
| TssAFnk | Flanking active TSS |
| TxFlnk  | Transcription at gene 5' and 3' |
| Tx      | Strong transcription |
| TxWk    | Weak transcription |
| EnhG    | Genic enhancers |
| Enh     | Enhancers |
| ZNF/Rpts| ZNF genes & repeats |
| Het     | Heterochromatin |
| TSSBiv  | Bivalent/totally TSS |
| BivFlnk | Flanking Bivalent TSS/Enhancers |
| EnhBiv  | Bivalent Enhancer |
| ReprPC  | Repressed PolyComb |
| ReprPCWk| Weak Repressed PolyComb |
| Quies   | Quiescent |

Gene enrichment analysis and cell-type enrichment analysis. Gene ontology (GO) enrichment was performed by GO-Elite Pathway Analysis (EnsatMart77, http://www.genmapp.org/go_elite/). Genes that reside within 1 Mb flanking regions from each human evolved element were used as a background gene list.

For disease enrichment analysis, we used the Allen Brain Reference Gene Set (ABRG, version 6) as a background gene list. A total of 20,715 genes were used, which included all genes with human orthologs in the rhesus macaque. For cell-type enrichment analysis and enrichment analysis with human-specific elements, we used 39,046 genes based on Allen Brain Reference Gene Set.

To perform the enrichment analysis, we used the GO-Elite Pathway Analysis (EnsatMart77, http://www.genmapp.org/go_elite/) tool. A total of 200,000 randomly selected regions in the human genome were used as a background gene list. For cell-type enrichment analysis and enrichment analysis with human-specific elements, we used 39,046 genes based on Allen Brain Reference Gene Set.

For disease enrichment analysis, we used the Allen Brain Reference Gene Set (ABRG, version 6) as a background gene list. A total of 20,715 genes were used, which included all genes with human orthologs in the rhesus macaque. For cell-type enrichment analysis and enrichment analysis with human-specific elements, we used 39,046 genes based on Allen Brain Reference Gene Set.

Table S1 contains the list of Putative target genes of HGEs and HLEs. Table S2 contains the list of genes that underwent regulatory changes between human and rhesus macaque. Table S3 contains the list of genes that are conserved between human and chimpanzee. Table S4 contains the list of genes that are conserved between human and rhesus macaque. Table S5 contains the list of genes that are conserved between human and rhesus macaque.

We performed enrichment analysis with human-specific genes and expression modules reported from Bakken et al.40, Sousa et al.41, and Brawand et al.39. For example, Bakken et al. reported 197 genes that show human-specific developmental expression patterns. As Bakken et al. used a microarray-based platform in rhesus macaque, we used 10,715 genes where human orthologs were available as a background gene list. Sousa et al.44 used RNA-seq, and we used 19,154 protein-coding genes as a background list. Moreover, we leveraged brain expression data (total 22 samples, 6 human vs. 16 primates including Gorilla, Pan Troglodytes, Pongo Pygmaeus, and Rhesus Macaque) from Brawand et al.39 to run differential expression analyses between human and primates using lm (expression–species + sex). In total, 590 genes were differentially expressed in human vs. primates at an FDR<0.05. We used 13,080 primate orthologs as a background gene list. For cell-type enrichment analysis and enrichment analysis with human-specific elements, we did not add covariates in the logistic regression. The test becomes equivalent to Fisher’s exact test. After calculating the enrichment P-values, we performed a multiple correction by counting for both curated gene sets and classes of evolutionary elements.

Since HARs are evolutionary conserved elements and HGEs are enhancers, the enrichment with neurodevelopmental disorder risk genes could be simply due to their genomic features (i.e. evolutionary conservation and being a regulatory element). To further confirm that this enrichment is not merely due to their genomic features, we performed a disease enrichment analysis with HAR-associated genes compared with other genes associated with evolutionary conserved elements (3,083,588 evolutionary conserved elements with phastCons score >0.40 (similar to most HARs) were mapped to 16,676 protein-coding genes), and HGEF-associated genes over genes associated with fetal brain enhancers (186,304 enhancers reported in Reilly et al.33 were mapped to total 15,693 protein-coding genes), where we obtained similar results (Supplementary Fig. 5c).

Developmental and cellular expression profiles. The spatiotemporal transcriptomic atlas data from human brain was obtained from Kang et al.36. As this dataset contains expression values from multiple brain regions, we selected transcriptomic profiles of cerebral cortex with developmental epochs that span prenatal (6–37 post-conception weeks, PCW) and postnatal (4 months–42 years) periods. Expression values were log-transformed and centered to the mean expression level for each sample using a scale(center = 0, scale = F) + 1 function in R. Genes associated with human evolved elements were selected for each sample and their average centered expression values were calculated and plotted.
Expression values of each gene were normalized to developmental time points using the scale function in R to calculate normalized expression Z-scores. Thus, normalized expression Z-scores denote relative developmental expression enrichment at a given developmental epoch. For example, if the Z-score of a gene is high at post-conception week (PCW) 20, it means that the gene is highly expressed in that developmental stage compared with other developmental stages. We then subtracted normalized expression Z-scores at available matching developmental time points (based on developmental event scores40). Table 3 between human and rhesus. A positive Δ Z-score indicates that the gene is more enriched at a given developmental epoch in human than in rhesus. We then compared the distribution of Δ expression Z-scores for HAR-, HGE-, and HLE-associated genes with non-HAR-, non-HGE-, and non-HLE-associated genes, respectively.

We also leveraged recently published species-specific weighted gene co-expression correlation network analysis (WGCNA) modules44 to test whether genes associated with human evolved elements are enriched in co-expression networks with human-specific expression signatures. Logistic linear regression with background list of genes included in the WGCNA analysis44 was used to calculate the significance of enrichment. We did not regress out exome or genome length, as promoter-based interactions were used.

CRISPR/Cas9-mediated transcriptional activation of HARs. To experimentally validate Hi-C predicted candidate target genes of HARs, we chose HARs that (1) overlap with H3K27ac marks in fetal brain and (2) interact with developmentally important genes expressed in human neural progenitors (Supplementary Fig. 8), and that are 3′ predicted to regulate only one gene. These three HARs (HAR-01246, HAR-02296, HAR-01298) that interact with GLI2, GLI3, and TBR1, respectively. Two sets of guide RNAs (gRNAs) targeting different regions of TBR1, respectively. We designed by benchling (https://benchling.com/). As HAR-01298 is 16 bp in size, we could not design gRNAs targeting such a small region. Therefore, we targeted two gRNAs flanking this HAR. These gRNAs were cloned into an EF1a-cas9-Vp64-2A-GFP-sgRNA vector (modified from Addgene, 61422). An empty vector without any gRNA insertion was used as control. Virus was generated by co-transfection of CRISPR vectors with PVSv9 (Addgene, 8454) and PspAX2 (Addgene, 12260) in HEK293 cells. Primary human neural progenitor cells (pNPC) were infected with viruses (empty vectors, gRNA1, gRNA2 for each HAR) on the day of split and differentiated with Neurobasal A (Invitrogen) supplemented with B27 (Gibco), GlutaMAX (Gibco), antibiotics and antimitotics (Gibco), BDNF (10 ng/mL), Pепroteч, and NT-3 (10 ng/mL; Peprotech). Half of the media was replaced three times per week during the differentiation (also see ref 11). After 2.5 weeks of differentiation, cells that were infected (GFP+) were sorted by FACS. RNA was extracted by mirvNeasy Mini Kit (Qiagen) and the expression level of putative target genes (GLI2, GLI3, and TBR1) was measured by qPCR (LightCycler 480 SYBR Green Master, Roche) and normalized to GAPDH. gRNA and primer sequences for both genomic DNA and qPCR are described in Supplementary Table 3.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Hi-C data from fetal brain are available through GEO and dbGaP under the accession number GSE16655 and phs001161.v1.p1, respectively. Hi-C data from adult brain are available through https://www.synapse.org/#!Synapse:syn4921369/wiki/390671 and the PsychENCODE knowledge portal http://resource.psychencode.org/. Promoter-based interaction maps for fetal brain and adult brain are available in Supplementary Tables 22–23 of Won et al.41 and the PsychENCODE knowledge portal, respectively.

Code availability
Codes used to analyze and plot the results are available in the Supplementary Software 1.

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