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Evolutionary Changes in Transcriptional Regulation: Insights into Human Behavior and Neurological Conditions

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Abstract

Understanding the biological basis for human-specific cognitive traits presents both immense challenges and unique opportunities. Although the question of what makes us human has been investigated with several different methods, the rise of comparative genomics, epigenomics, and medical genetics has provided tools to help narrow down and functionally assess the regions of the genome that seem evolutionarily relevant along the human lineage. In this review, we focus on how medical genetic cases have provided compelling functional evidence for genes and loci that appear to have interesting evolutionary signatures in humans. Furthermore, we examine a special class of noncoding regions, human accelerated regions (HARs), that have been suggested to show human-lineage-specific divergence, and how the use of clinical and population data has started to provide functional information to examine these regions. Finally, we outline methods that provide new insights into functional noncoding sequences in evolution.

Contents

INTRODUCTION

Our understanding of the workings of the brain, as well as its genetic underpinnings, has rapidly advanced in recent years. This development in both neurobiological and genetic understanding has put us in the unique position to answer the fascinating but immensely challenging question of what makes us human.

From high-level comparisons of humans with other mammals, key behavioral and intellectual traits are apparent. Apart from physical differences such as bipedalism and relative hairlessness compared with other primates (Schwartz & Rosenblum 1981, Sockol et al. 2007), cognitive abilities such as communication with syntactical grammar (Hauser et al. 2002), symbolic and abstract representation (Penn et al. 2008), the production of art (Tomasello & Rakoczy 2003), and the development of the scientific method are but a few that appear unique to humans.

Although we can observe these striking behavioral differences, understanding the underlying mechanisms has been far more difficult. Neuroanatomical studies have revealed insights into alterations in brain development among species, notably gross size. Humans possess an unusually large brain compared with our nearest phylogenic relatives; the human brain is approximately three times the size of that of chimpanzees. Many studies have investigated the biological consequences of having an increased brain size, leading to mixed data in which some researchers suggest an important role in social and cognitive functioning (Deaner et al. 2007, Heldstab et al. 2016, Reader & Laland 2002, Street et al. 2017). However, larger mammals (e.g., elephants and killer whales) possess brains that are much larger than that of humans, but are not known to exhibit enhanced cognitive abilities, supporting roles of other possible factors such as total neuron number (Wright et al. 2017). Furthermore, the extended duration of brain development seems to support a special type of neurodevelopment occurring in humans because it occurs over the course of decades—longer than

the entire life span of most primates and other mammals (Silbereis et al. 2016). However, although more complex analyses accounting for body size and differing neuroanatomical connections have been informative, there have not yet been any clear human-specific structural differences in the brain that explain the observed cognitive differences among humans and other primates.

Despite the challenges in characterizing biological mechanisms behind human-specific traits, the rapid rise in comparative genomics and epigenomics has provided novel ways to search for processes relevant to interspecies differences. By comparing genomic sequences across species to examine conservation and divergence, we can identify candidate sequences that may have contributed to developmental differences, all without prior knowledge of the precise processes underlying complex human cognitive function. More recently, the use of epigenomics has provided an additional layer of information, relaxing the focus on sequence changes and instead examining epigenomic marks that can suggest changes in gene regulatory function. Together, these methods have opened alternative approaches to studying human evolution.

Moreover, there has been a growing appreciation of the potential of medical genetics in understanding the functional component of this question. Although the use of model organisms has been powerful in understanding fundamental and well-conserved biology, their use in understanding human-specific traits has been much more controversial and limited (Muotri 2016). By contrast, medical genetics provides direct evidence that a given genomic sequence possesses essential functions in human biology. This has been particularly evident in cases such as microcephaly, which affects early brain development and neuroproliferation, and where a portion of the responsible genes appear to have been targets of selective pressures during the evolution of the human lineage. Analogous cases are beginning to be made for more complex neuropsychiatric conditions such as autism spectrum disorder and schizophrenia, which have profound impacts on social behavior, suggesting that some underlying genes might have been evolutionary targets contributing to the development of human social structures.

The hope of evolutionary approaches to human biology is twofold. First, a better understanding promises to help elucidate aspects of the long-standing and fundamental fascination with what biological differences exist between humans and other mammals. Second, and perhaps more importantly, evolutionary approaches provide a unique window into the mechanistic differences that underlie these complex cognitive traits and processes.

In this review, we discuss cases from medical genetics that have identified genes that are important for brain development as well as exhibit evolutionary signatures that suggest a role in phenotypic divergence across species. Furthermore, we examine the gradual shift of human evolutionary biology to noncoding sequences of the genome, specifically focusing on human accelerated regions (HARs), genomic sequences that are conserved across mammals but that appear to diverge in humans. Finally, throughout this review we discuss some of the more recent techniques that enable high-throughput functional study of noncoding elements, and examine their role in human disease.

EVOLUTION OF GENOME STRUCTURE

Genomic Alterations Shaped Human-Specific Neural Development

Some of the most fascinating genetic advances in our understanding of why humans are distinct from other primates resulted from the combined strengths of comparative genomics, DNA sequencing, and human diseases. The Chimpanzee Genome Project aimed to identify and correlate genetic differences occurring between humans and our most recent primate ancestor, with some of the most striking human-specific characteristics including neurodevelopmental and behavioral evolution (Chimpanzee Seq. Anal. Consort. 2005, Olson & Varki 2003, Varki & Altheide 2005, Watanabe & Hattori 2006). Simultaneously, numerous vertebrate genomes, including those of

other primates, have been assembled, allowing ever-better, large-scale comparative genomic studies of human-specific phenotypes.

Comparative analyses between primates have provided many groundbreaking discoveries about human phenotypic divergence by implicating multiple mutational mechanisms. Studies have included examinations of the role of structural variation [e.g., deletions, segmental duplications (Cheng et al. 2005, Chimpanzee Seq. Anal. Consort. 2005, Dennis & Eichler 2016, Lander et al. 2001), translocations, inversions (Cheng et al. 2005, Chimpanzee Seq. Anal. Consort. 2005, Newman et al. 2005), and transposable elements (Beck et al. 2011, Friedli & Trono 2015)] in human divergence and of amino acid conservation across different species (Bakewell et al. 2007, Sabeti et al. 2006, Vallender & Lahn 2004). Furthermore, genomic analyses suggest that 1.5% (~35 Mb) of the human genome is lineage specific (Chimpanzee Seq. Anal. Consort. 2005, McLean et al. 2011). Therefore, the association of human-specific genomic alterations with cognitive development is challenging, especially given that multiple mechanisms, including selective pressures and genetic drift in nonfunctional elements (Cheng et al. 2005, Siepel & Arbiza 2014), resulted in their formation. Moreover, both cognitive and social development are likely the result of many mutations, each with a small effect size, acting in unison (Olson & Varki 2003, Varki & Altheide 2005, Wittkopp & Kalay 2011). Although many strategies have been invoked to elucidate functional from nonfunctional evolutionary changes, this review leverages the strong statistical and biological power of relevant human disease mutations to prioritize the elements identified by comparative genomic studies of single-nucleotide changes.

Segmental Duplication and Divergent Gene Conversion in Neurodevelopment

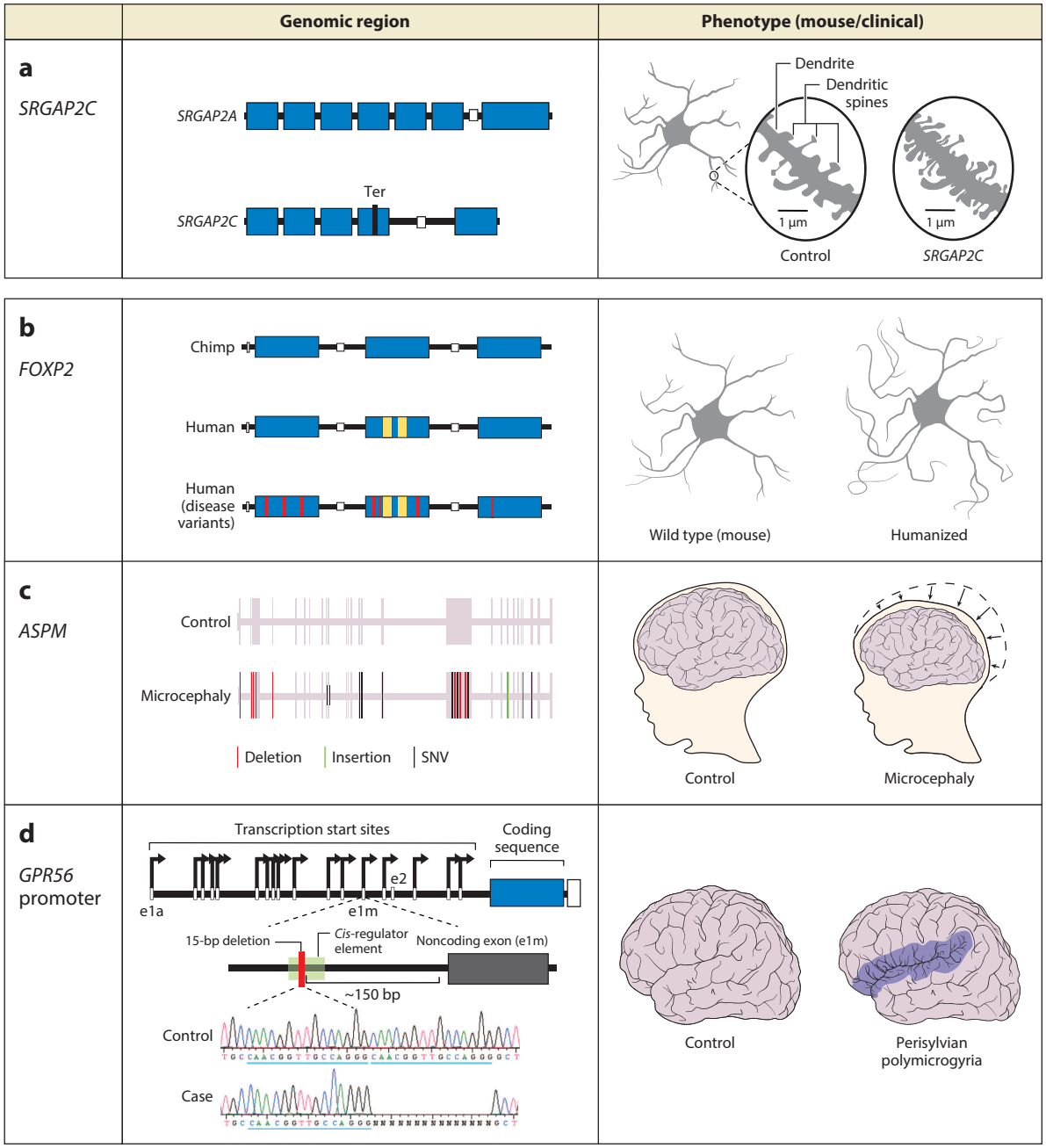
Segmental duplications, though not the focus of this review, have revealed candidate genes for several human-specific traits, including social and cognitive functioning (Ciccarelli et al. 2005; Dennis et al. 2012, 2017; Paulding et al. 2003; Zhang 2003). Therefore, we suggest other articles for further reading on the topic (Sudmant et al. 2013), but we briefly mention three genetic changes with particular evidence relevant to brain development: *DUF1220*, *BOLA2* (Sudmant et al. 2013), and *SRGAP2C*. *DUF1220* represents one of the most extensive coding sequence expansions in the human genome (Popesco et al. 2006) and has been correlated with brain size and with potentially promoting neurogenesis (Dumas et al. 2012; Keeney et al. 2014, 2015; Popesco et al. 2006). *BOLA2* is one of the most recent, human-specific segmental duplications and shows rapid fixation in the human lineage (Nuttle et al. 2016, Sudmant et al. 2013). *SRGAP2C* is a partially duplicated gene that disrupts the function of its ancestral gene, *SRGAP2A* (**Figure 1*a***). In mouse models, *SRGAP2A* expression is implicated in neuronal migration, morphology (Guerrier et al. 2009), and dendritic spine maturation (Charrier et al. 2012), whereas disruption of *SRGAP2C* prolongs spine maturation and increases spine density (Charrier et al. 2012, Fossati et al. 2016). Thus, although the complexity of gene duplication makes it difficult to adduce statistical evidence and thresholds to quantitatively evaluate its role in cognitive evolution, further knowledge of the repetitive structure of primate genomes will likely further highlight it as an important mechanism.

EVOLUTION OF CODING REGIONS

Evolution of Neurodevelopmental Genes

When characterizing genomic differences between individuals, regardless of the species, the most commonly investigated sites are amino acids in protein-coding genes, which are generally conserved among similar species (Chimpanzee Seq. Anal. Consort. 2005, Rhesus Macaque Genome Seq. Anal. Consort. et al. 2007, Scally et al. 2012). This conservation enables researchers to use

the ratio of amino acid–altering mutations (i.e., nonsynonymous) to synonymous variants in a gene (Goldman & Yang 1994) to identify using quantitative metrics potential genes undergoing positive selection. Genome scans have revealed that as many as 15% of genes exhibit evidence of positive selection by this criterion, resulting in hundreds of potential candidate genes (Lindblad-Toh et al. 2011, Nielsen et al. 2005). Some of the best-characterized examples include neurodevelopmental



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Clinically identified and candidate regions with evolutionarily intriguing sequence signatures. (*a*) Although not yet clinically linked to disease, *SRGAP2C* is a novel human-specific gene that in mouse models disrupts the function of its ancestral copy, *SRGAP2A*. The ancestral copy is associated with neuronal migration and neurite spine maturation in mice, whereas in humans it has been found within large deletions in children with developmental delays. (*b–d*) Sequences of genes that have clinical evidence for a significant effect on brain development. Panels *c* and *d* depict clinical phenotypes with deleterious variants in these regions; panel *b* illustrates a morphological difference observed in humanized mouse models of *FOXP2*. (*b*) Deleterious variants (*red*) in *FOXP2* are associated with language deficits, and human-specific sequences (*yellow*) in the gene are associated with increases in neurite and dendrite length in humanized mice. (*c*) Over 150 mutations in *ASPM* cause primary microcephaly. *ASPM* shows signs of accelerated evolution along the primate lineage. (*d*) Mutation in the e1m promoter of *GPR56* is associated with perisylvian polymicrogyria. Abbreviation: SNV, single nucleotide variant.

genes whose essential roles were first revealed in patients and were subsequently shown to exhibit signs of positive selection. Although many candidate positively selected genes lack known human disorders, those with clear associations to disease provide key insights into evolutionary changes affecting human biology.

Evolution of Human Speech and Possible Role of *FOXP2*

The best-known example of a positively selected gene linked to a behavioral disorder is perhaps forkhead box P2 transcription factor (*FOXP2*) (Ayub et al. 2013). *FOXP2* was first associated with development by researchers tracking a severe language and speech disorder in a large family with deficits in producing fine orofacial movements, word inflections, and syntax (Hurst et al. 1990, Lai et al. 2001). Despite verbal deficits, the average IQ of the affected individuals was similar to the population average. Affected individuals possessed a single missense variant (R553H) in *FOXP2* and the gene was also disrupted in an unrelated individual with similar language deficits (Lai et al. 2001).

Once identified, comparative genetics revealed three amino acid differences between the human and mouse *FOXP2* orthologs, two of which diverge between humans and chimpanzees (Enard et al. 2002) (**Figure 1*b***). The rate of change is ~60 times the expected amino substitution rate (J.Z. Zhang et al. 2002); moreover, this change appears fixed in the human population (Lai et al. 2001, J.Z. Zhang et al. 2002). In other species requiring complex vocalization (e.g., echolocating bats), there appears to be divergent selection on *FOXP2* based on sonar systems (Li et al. 2007). Interestingly, Denisovans and Neanderthals possess the same *FOXP2* gene as humans do (Meyer et al. 2012, Noonan et al. 2006), suggesting either that Denisovans and Neanderthals possess the same potential for verbal communication or, more likely, that other coding and noncoding regions also contribute to language development.

Cell lines and animal models have confirmed the functional importance of *FOXP2* and the human-specific variants in language-associated phenotypes, including different transcriptional targets (Konopka et al. 2009) in humans and chimpanzees, humanized *FOXP2* mice with accelerated learning, changes in ultrasound vocalizations (Enard et al. 2009, Schreiweis et al. 2014), incomplete vocalizations in songbirds with *FOXP2* knockdown (Haesler et al. 2007), and severe vocalization and motor issues in mice with disrupted *FOXP2* (Fujita et al. 2008, Shu et al. 2005). Although *FOXP2* is widely expressed in the developing brain (Ferland et al. 2003), humanized and disease models suggest particularly strong effects in the corticobasal ganglia circuits and cerebellum. However, a portion of mutant knock-in mice experience premature death (Fujita et al. 2008) and adult mutant mice no longer exhibit different ultrasound vocalizations, potentially highlighting some limitations of the mouse model (Hammerschmidt et al. 2015). Despite this finding, the wealth of clinical, evolutionary, and functional data exemplifies how positive selection in genes, particularly transcription factors regulating expression, can drastically alter phenotypes.

Association of *ASPM* and the Centriole Complex to Human Brain Expansion

Similar to *FOXP2*, several genes implicated in microcephaly and neurodevelopment show signs of positive selection. One of the more prominent genes with signs of positive selection is *ASPM*. *ASPM* has been studied extensively in the context of both evolution and disease, specifically microcephaly (**Figure 1*c***).

The human ortholog of *ASPM* was first described for its association to microcephaly (Bond et al. 2002). Positional cloning of the *MCPH5* locus, which was strongly associated with microcephaly (Roberts et al. 2002), revealed four different homozygous premature stop codon mutations in *ASPM* across multiple, large, affected families (Bond et al. 2002). Subsequent clinical investigation has reported more than 150 disease-causing variants in *ASPM*, with most decreasing *ASPM* levels by introducing either premature stop codons or frameshift mutations that cause protein truncation or nonsense-mediated decay (Faheem et al. 2015, Stenson et al. 2017). *ASPM* encodes a centrosomal protein (Fish et al. 2006) that together with *WDR62*, the second-most common microcephaly gene, localizes to the mother centriole and is required for normal apical complex formation (Jayaraman et al. 2016). Knockdown and knockout mouse models have demonstrated that *ASPM* is necessary for maintaining the neuroprogenitor pool in the ventricular zone during brain development, affecting brain size (Fish et al. 2006).

In terms of positive evolutionary selection, the most consistent evidence has been across primates (Montgomery et al. 2011) and placental mammals (Montgomery & Mundy 2014). However, this sign of positive selection seems to apply to numerous microcephaly-associated loci, including *CDK5RAP2*, *MCPH1*, *CENPJ*, *WDR62*, and *CEP152*. Remarkably, CDK5RAP2, ASPM, WDR62, CEP152, and CEP63, as well as other proteins encoded by microcephaly-associated genes, physically interact and assemble sequentially at the maternal centriole, suggesting that they may regulate neurogenesis through a common mechanism (Kodani et al. 2015). Sequence changes in *ASPM* and *CDK5RAP2* appear to be associated with changes in neonatal brain size across primates (Montgomery et al. 2011). Interestingly, even in outlier primate species, such as callitrichids, that have especially small brains among primates, changes in *ASPM* appear to correlate with decreases in brain size, suggesting selection on *ASPM* can act to increase or decrease brain size (Montgomery & Mundy 2012). Evolution on *ASPM*, as well as other microcephaly-centrosomal genes, may serve as a general mechanism to influence brain size.

GENE REGULATION UNDERLYING BRAIN EVOLUTION

Role of Gene Regulation in the Evolution of Social and Cognitive Behavior

Although comparative and disease-related studies have focused on the important role of amino acid divergence during evolution, this divergence is widely regarded as insufficient to account for all human-specific social and cognitive functioning, brain size, and synaptic complexity. A hypothesis gaining more attention suggests that answers to phenotypic evolution might lie in the noncoding portion of the genome. This was postulated in early comparative genetics studies, originating from the observation that most genes between humans and chimpanzees were strikingly similar, and that the amount of protein sequence divergence did not seem to explain the phenotypic differences (King & Wilson 1975). These studies revealed extremely high amino acid conservation between humans and chimpanzees, in which 29% of proteins are identical and 71% have a median of two nonsynonymous (protein altering) and three synonymous (amino acid preserving) substitutions (Chimpanzee Seq. Anal. Consort. 2005). The similarity in protein sequence, along with functional consistency across organisms, pleiotropy, and the extensive role of regulatory noncoding sequence in gene regulation have been used to support the hypothesis that other regions (e.g., noncoding

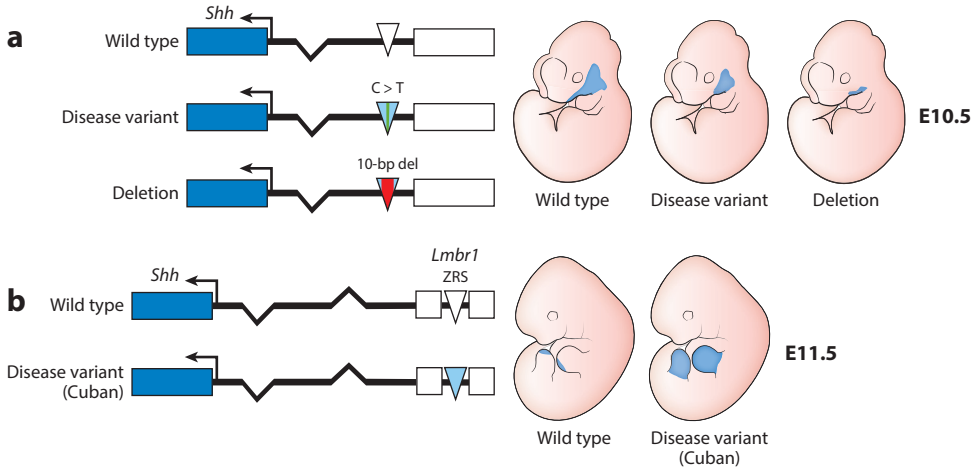


Figure 2

Example of *Shh* regulatory sequence mutations that have different clinical phenotypes depending on the enhancer affected. (*a*) Variants in the enhancer *Sbe2* are associated with a holoprosencephaly phenotype, and (*b*) mutations in the ZRS lead to a preaxial polydactyly phenotype in patients (in this case, the Cuban variant). Although these *Shh* enhancers do not show signs of human-specific evolutionary selection, they illustrate more generally how changes to regulatory elements can lead to complex phenotypic outcomes, particularly if the regulatory element is acting on important developmental genes. Depicted on the right are corresponding changes in expression seen in mouse models of the *Shh* enhancer variants. Development time points of mouse embryos are noted on the right (E10.5–11.5). Abbreviations: C > T, substitution of a cytosine with a thymine; E, embryonic day; *Sbe2*, *SHH* brain enhancer 2; *Shh*, Sonic hedgehog; ZRS, zone of polarizing activity regulatory sequence.

functional elements) had an important role in the evolution of social and cognitive development in humans (Carroll 2008, Wray 2007).

Studies of human disease have linked alterations to regulatory regions to many complex biological functions. As a particularly instructive example, different types of mutations in regulatory sequences of the morphogen Sonic hedgehog (*SHH*) have been associated with several disorders. Deleterious mutations and truncations in the *SHH* gene cause holoprosencephaly (Roessler et al. 1996), and mutations affecting a neural *SHH* enhancer, *SHH* brain enhancer 2 (*SBE2*), located more than 400 kb away, cause a similar phenotype (Jeong et al. 2008) (**Figure 2**). When examined in transgenic mice, the enhancer point mutation significantly decreased activity (**Figure 2*a***), resulting in reduced affinity for *SIX3*, a transcriptional regulator of *SHH*. Moreover, a mutant form of *SIX3* associated with holoprosencephaly was unable to bind the wild-type enhancer *SBE2* sequence (Jeong et al. 2008). Further increasing the complexity of enhancers is that mutations can have different effects depending on the tissue specificity of the enhancer. For instance, mutations in another enhancer of the same *SHH* gene, active in the limb bud, manifest as a polydactyly phenotype in patients (Lettice et al. 2003) (**Figure 2*b***). Importantly, these loss-of-function mutations reflect but a small subset of possible types of changes that may affect regulatory sequences (**Figure 3**). So, this example, along with a range of other cases of regulatory sequences associated with disease (for a review, see Epstein 2009, Maston et al. 2006), has demonstrated the important roles of mutational alterations of regulatory sequences and the evolutionary complexity for which these elements allow.

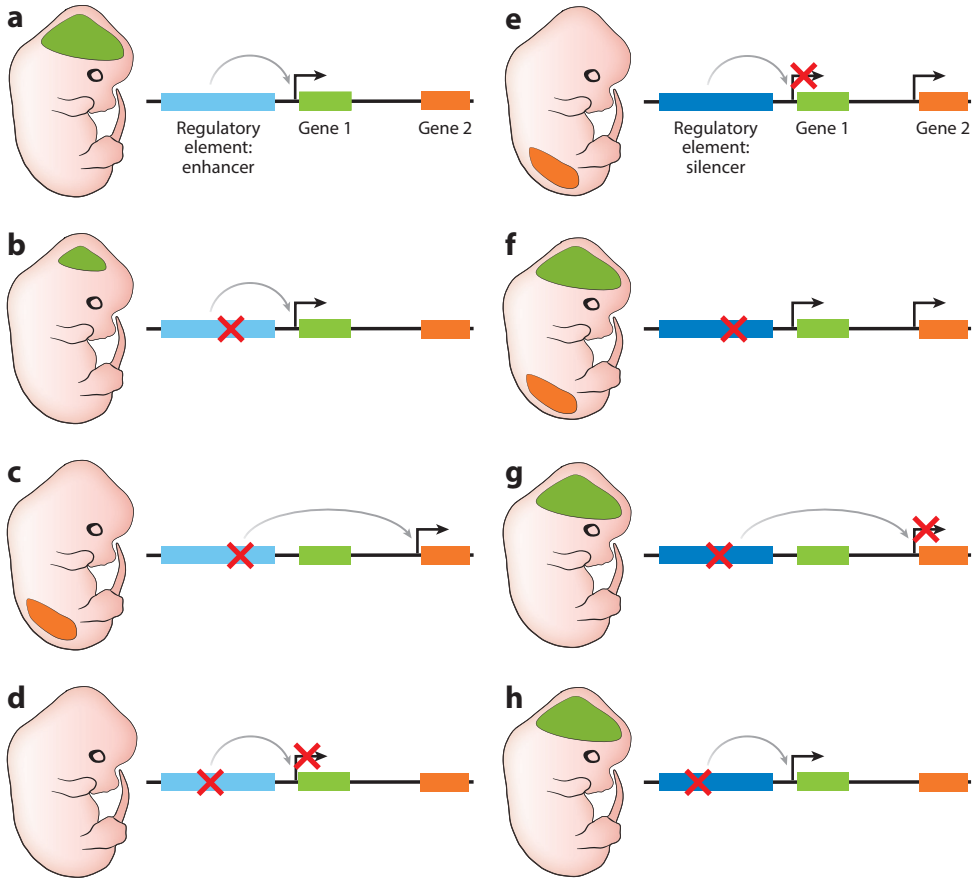


Figure 3

Models of regulatory sequence changes and their effect on gene expression. (*a–d*) Changes in enhancer sequences. Enhancers promoting expression of a target gene (*a*) can have their activity altered by mutations in their sequence, with potential gain or loss of transcription factor–binding sites. This can lead to changes in activity such as (*b*) loss-of-function, (*c*) changes in target specificity, and (*d*) gain of silencing. (*e–h*) Changes in silencer sequences. Similar to changes in enhancer sequences, for silencers (*e*) mutations leading to protein binding sites can lead to (*f*) loss of silencing, (*g*) change of repressor target, or (*h*) gain of enhancer activity.

Primate-Specific *GPR56* Gene Promoter Involved in Regulating Gyrification in Humans

However, given that millions of putative regulatory elements in humans have been identified (ENCODE Proj. Consort. 2012, Roadmap Epigenomics Consort. et al. 2015, Zhu et al. 2013), selecting noncoding sequences involved in human-specific developmental processes has been immensely challenging. In this regard, assessing mutations in clinical cases of relevant neurodevelopmental disorders and then overlaying them with evolutionary data is a powerful tool to narrow down functionally important elements. One recent example is an alternative promoter of the *GPR56* gene. *GPR56* is a G protein–coupled receptor required for normal cortical development (Piao et al. 2004). Its expression levels have been associated with the regulation of progenitor proliferation. Prior clinical cases have demonstrated that loss-of-function mutations in *GPR56* cause polymicrogyria by affecting the entire neocortex (bilateral frontoparietal polymicrogyria)

(Bahi-Buisson et al. 2010, Piao et al. 2004). Loss of *GPR56* disrupts radial glia and causes breaches in pial basement membrane; as a result, neurons either over- or undermigrate in different regions, leading to irregular cortical layers.

Bae et al. (2014) examined more than 1,000 individuals with gyral abnormalities, from which they identified 5 individuals from 3 families who shared strikingly similar disruption restricted to a cortical area surrounding the Sylvian fissure and including the primary language area (Broca's area). Upon linkage analysis and sequencing, the researchers discovered that the 5 patients shared a homozygous 15-base pair deletion mutation in the promoter of e1m, a noncoding exon of *GPR56* (**Figure 1*d***). Transgenic mouse models demonstrated that this mutation led to the loss of gene expression in the lateral cortex and the lateral ganglionic eminence, which mimics the *GPR56* loss phenotype in these regions. Moreover, the 300-base pair region that contained the site of the mutation possessed species-specific regulatory activity, so that when inserted into transgenic mice, the sequence from a range of mammalian species drove different expression patterns (Bae et al. 2014), suggesting an example of an evolutionarily divergent noncoding element with essential developmental functions in controlling regional cortical development in humans.

CIS-ACTING REGULATORY ELEMENTS IN NEURAL DEVELOPMENT

Using Evolutionary Signatures to Enrich for Functional Enhancer Elements

One of the most widely used analyses for identifying functional regulatory regions in the genome involves examining levels of sequence conservation across vertebrates, including humans (Pennacchio & Rubin 2001). Much like with coding sequences, the conservation of noncoding sequences across species suggests that these elements may have some function that would be disrupted by mutations. Using this approach, early studies identified many conserved functional regulatory elements, including neurodevelopmental enhancers (de la Calle-Mustienes et al. 2005, Nobrega et al. 2003, Prabhakar et al. 2006, Visel et al. 2008, Woolfe et al. 2005).

Although sequence conservation has helped identify many regulatory elements, elements contributing to human-specific traits require investigation of regions where conservation is not maintained among humans and other species. The most dramatic example would be human-specific deletions, which include approximately 13.5 million base pairs (Sudmant et al. 2013). Within primates and other species, olfactory- and immune-related genes are most frequently lost and gained, owing in part to instability caused by large clusters of gene families. In terms of human-specific deletions, many candidates exist but few have been functionally linked to a specific trait.

One challenge to studying human-specific deletions affecting genes and regulatory elements is the reliance on less complete genome assemblies of nonhuman species (Alkan et al. 2011, Rogers & Gibbs 2014, Zhang et al. 2012). Despite this challenge, several regulatory regions, including two deletions affecting an enhancer of the human androgen receptor (*AR*) gene and an enhancer of the tumor suppressor gene growth arrest and DNA-damage-inducible gamma (*GADD45G*), have been identified. The loss of the *AR* enhancer raises fascinating hypotheses regarding sexual evolution, as it is associated with the loss of sensory vibrissae and penile spine (McLean et al. 2011, Reno et al. 2013). However, with respect to brain development, the deletion of the *GADD45G* enhancer is particularly interesting. *GADD45G* encodes a DNA methylase that represses the cell cycle repressor and induces apoptosis (Zerbini et al. 2004). Somatic loss of *GADD45G* is strongly associated with tissue growth in human pituitary adenomas, with many pituitary tumors having decreased *GADD45G* expression (Binse et al. 2014, X. Zhang et al. 2002). Although further characterization is necessary, it raises the possibility that a change of *GADD45G* expression may be relevant to cell proliferation in human brain development.

Identification of Elements with Accelerated Primate–Human Divergence

An alternative approach to identify elements contributing to human-specific traits focuses on sequences that show conservation across species but accelerated sequence divergence along the human lineage. This enriches for regulatory elements that have undergone recent evolutionary changes at the nucleotide level in humans, possibly resulting in altered activity, conversion to a different mechanism (e.g., enhancer to silencer), or changes in tissue specificity. Such human-specific changes would theoretically affect unique human traits arising concurrently with the mutation. One class of such elements is known as human accelerated regions (HARs). HARs are genomic segments that are highly conserved in many mammals but show unusually high (accelerated) divergence between humans and other mammals; hence, they are thought to represent potential targets of recent evolutionary selection of *Homo sapiens* (Lindblad-Toh et al. 2011; Pollard et al. 2006a,b).

In 2006 Pollard et al. (2006a) leveraged human and primate genome assemblies to calculate selective pressures and conservation of genomic loci to prioritize regions with a divergence greater than that predicted by a neutral mutation rate in humans, thus suggesting the potential for positive selection and novel functions. Since the release of the first map of HARs by Pollard et al. (2006a,b), several studies have identified accelerated coding and noncoding regions (Bird et al. 2007, Bush & Lahn 2008, Gittelman et al. 2015, Lindblad-Toh et al. 2011, Prabhakar et al. 2008). The most recent approach utilized additional markers for functional regulatory sequences, such as DNase hypersensitivity sites to select functional human accelerated sites, revealing that up to 70% of their substitutions are the result of positive selection (Gittelman et al. 2015). However, HARs identified in each study often do not overlap each other, with few loci shared across the studies (Bird et al. 2007, Capra et al. 2013, Doan et al. 2016, Gittelman et al. 2015). The lack of congruency among HARs identified in different studies is likely due partly to incomplete high-quality vertebrate genome assemblies, including that of nonhuman primates, available at the time of each study.

Properties of HARs

Despite the differing methods of identification and a lack of congruency, HARs as a group exhibit many features that suggest involvement in neurodevelopmental alterations that occurred in the human lineage. The depletion of HARs in promoter regions and the enrichment within 50–500 kb from transcriptional start sites suggest a long-distance *cis*-acting regulatory mechanism (Capra et al. 2013, Doan et al. 2016). Furthermore, HARs are preferentially located proximal to genes enriched for neuronal processes, brain development, transcription, neuronal cell adhesion, and axon processes, which are sensitive to gene dosage, as indicated by loss-of-function intolerance data (Lek et al. 2016) and haploinsufficiency scores (Capra et al. 2013, Doan et al. 2016, N. Huang et al. 2010, Prabhakar et al. 2008). Similar proximal enrichment to neural genes has been observed in several studies linking noncoding variants, though not HARs specifically, to neuropsychiatric disorders including schizophrenia, attention deficit hyperactivity disorder, and intellectual disability (Devlin & Scherer 2012, ENCODE Proj. Consort. 2012, Haraksingh & Snyder 2013, Lee & Young 2013, Makrythanasis et al. 2012, Maurano et al. 2012, Nair & Howard 2013, Ward & Kellis 2012). Interestingly, it is estimated that 58% of HAR-gene interactions do not include flanking genes but instead distant genes as far as 1 Mb away (Doan et al. 2016, Goh et al. 2012, Heidari et al. 2014, Li et al. 2012, Lieberman-Aiden et al. 2009), suggesting that although proximity is often a good predictor of target genes, additional global mapping of chromatin interactions with Hi-C sequencing (Lieberman-Aiden et al. 2009) and chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) (Fullwood et al. 2010; Li et al. 2010, 2012) will further expand our functional understanding of HARs.

Regulatory potential is often correlated with the presence and density of transcription factor (TF)-binding motifs (Crowley et al. 1997, Harbison et al. 2004, Heintzman et al. 2007, Wasserman & Sandelin 2004). In order for HARs to exhibit species-specific regulatory functions, the human-specific nucleotides must affect essential motifs in such a way that their activity would be altered in neural cell types during neural development. Several studies have shown an enrichment of TF-binding motifs in HARs (Capra et al. 2013, Doan et al. 2016, Pollard et al. 2006a) and a significant increase in their density, resulting in clusters of motifs (Doan et al. 2016). In support of a role in neurogenesis, binding motifs for neural development–associated TFs such as myocyte enhancer factor 2A (MEF2A) and SRY-related HMG-box gene 2 (SOX2) are highly enriched in HARs (Doan et al. 2016). *SOX2* is essential for the renewal of neural progenitors (Ferri et al. 2004, Kelberman et al. 2008), and when expression is reduced, neural progenitors differentiate into neurons. Interestingly, several enriched TFs identified by Doan et al. (2016) have roles as transcriptional repressors, based on annotations present in the TRANScription FACtor (TRANSFAC) database, suggesting that some HARs may be *cis*-acting transcriptional silencers, not enhancers. Although less is known about silencers, they act through a mechanism similar to that of enhancers except that bound TFs block or inhibit transcription of the target gene (Maston et al. 2006, Ogbourne & Antalis 1998). Moreover, human-specific nucleotides alter TF-binding motifs by creating or removing sites for TFs essential for neural development, splicing regulation, and neural differentiation, such as REST, CTCF, and NFIA, illuminating possible mechanisms behind their proposed human-specific functions (Doan et al. 2016).

The role of HARs as regulatory sequences is also supported by the distance between HARs and their closest genes and by their enrichment for CTCF binding, suggesting the predominant mechanism relies on physical looping between gene promoters and distant HARs (Capra et al. 2013, Doan et al. 2016). Although HAR1, the first studied HAR, encodes a human-specific noncoding RNA expressed in Cajal–Retzius neurons (Pollard et al. 2006a,b), subsequent examinations of HARs have focused primarily on their role as enhancers. The first epigenomic evidence for a biological role of HARs in human behavior and brain development was based on their marks of regulatory activity, with up to 50% of HARs believed to exhibit active regulatory functions in neural tissues (Doan et al. 2016), including many associated with developmental roles (Capra et al. 2013). In further support of functional roles of variation within HARs, there appears to be a significant correlation between HapMap2 single nucleotide polymorphisms (SNPs) and differences in gene expression for 16% of variants in HARs (Bird et al. 2007), suggesting that mutations in HARs through either evolution or disease could alter gene expression.

Functional Validation of Regulatory Activity of HARs

Although the combination of human disease and epigenetic and comparative genomic screening has led to the identification of over 3,000 HARs, further functional studies are necessary to understand their biological role. Amino acid–altering mutations often result in loss- or gain-of-function, but regulatory regions and their mutations have a much greater complexity in their functional role. Noncoding elements have the potential to regulate multiple genes in different tissues while acting as either enhancers or silencers of gene transcription (**Figure 3*a*,*e***). Moreover, when the elements are mutated, the cell specificity can be altered, causing aberrant expression in the same or new tissues (**Figure 3*b*–*d*,*f*–*h***). Because mutations in these elements cannot always be simply classified as pure loss-of-function, breakthrough technologies such as massively parallel reporter assays (Melnikov et al. 2012) and self-transcribing regulatory region sequencing (Arnold et al. 2013) have allowed for the functional interrogation of thousands of DNA regulatory fragments. The difficulties and low-throughput nature of in vitro and in vivo functional analyses have limited

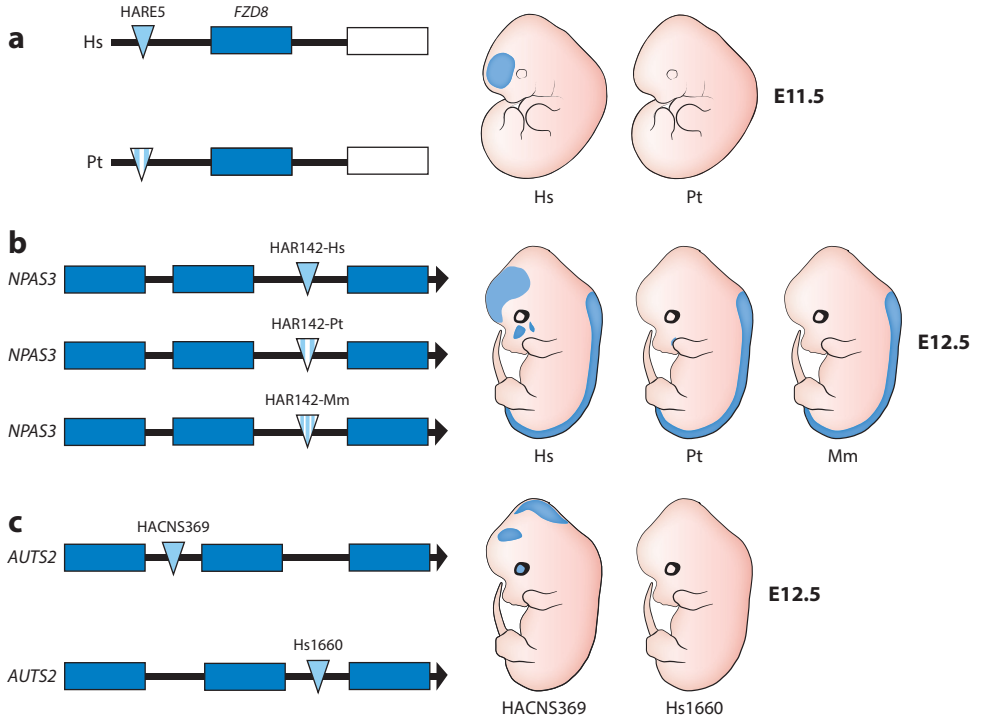


Figure 4

Examples of regulatory sequence changes and species-specific expression profile changes. (*a–c*) Examples of species-specific sequence differences in human accelerated regions (HARs) and their impact on expression patterns in reporter mouse models. Development time points of mouse embryos are noted on the right (E11.5–12.5). Abbreviations: E, embryonic day; Hs, *Homo sapiens* (human); Mm, *Mus musculus* (mouse); Pt, *Pan troglodytes* (chimpanzee).

the number of HARs with additional functional characterization (Boyd et al. 2015, Capra et al. 2013, Doan et al. 2016, Gittelman et al. 2015, Oksenberg & Ahituv 2013, Oksenberg et al. 2013). However, HARs that have been validated have demonstrated several drastic impacts on activity from just a few human-specific nucleotides (Boyd et al. 2015, Capra et al. 2013, Gittelman et al. 2015, Shibata et al. 2012). The human-specific changes not only altered expression levels but also, in some cases, changed the anatomical regions of gene expression (Capra et al. 2013).

One of these candidate HARs, 2xHAR142, is near the *NPAS3* gene (Kamm et al. 2013a) (**Figure 4*b***). *NPAS3* encodes a member of the basic helix-loop-helix transcription factor family (Brunskill et al. 1999), and it is linked to roles in brain development and synaptic function in knockout mice (Brunskill et al. 2005, Erbel-Sieler et al. 2004). In humans, *NPAS3* is expressed in the fetal brain during the three trimesters, with changing spatial patterns of nuclear activity in the ventricular zone, hippocampus, cerebellum, and maturing neocortex (Gould & Kamnasaran 2011). It has also been associated with a risk for schizophrenia and bipolar disorders in family-based studies (Kamnasaran et al. 2003), cohort studies (Pickard et al. 2008), and genome-wide association studies (GWAS) (J. Huang et al. 2010). In terms of evolutionary selection, what makes *NPAS3* particularly interesting is that it has more than 14 HARs in its proximity, making it the densest HAR-surrounded locus in the genome (Kamm et al. 2013b).

In transgenic mouse models, HAR 2xHAR142 was examined and the human sequence exhibited broad activity early in development, at embryonic day (E) 10.5 and E12.5 in the hindbrain, midbrain, and forebrain (Kamm et al. 2013a). From E12.5 to E14.5, forebrain expression is predominantly in regions of the developing cortex, hippocampus, ventral thalamus, and hypothalamus, whereas midbrain and hindbrain expression is more restricted. In comparison, the mouse and chimp sequences differed in activity pattern, particularly a lack of activity in the developing forebrain (Kamm et al. 2013a). In fact, the expression patterns of the mouse and chimp sequences were more similar to each other than they were to the human version of 2xHAR142. This result suggests that mutations in HARs may not act solely by changing expression levels, but may in fact alter the regions and timing of gene expression in accordance with the changes in the TF-binding sites. Although further functional characterization is necessary, the *NPAS3* locus and its density of HARs, demonstration of human-specific activity in the reporter mouse model, and association to neuropsychiatric disorders make it a promising candidate for understanding human-specific differences in brain development (Kamm et al. 2013b).

Despite the progress made in identifying expression changes, translating reporter assays and potential regulatory changes into functional impact has been particularly difficult, especially as genes are often regulated by multiple enhancers. This form of regulation often leads to significant redundancy. However, recent studies have identified particularly illuminating cases of noncoding evolutionary changes that have functional impact on brain development. One of these studies involves HARE5, a HAR that acts as an enhancer for *FZD8*, a Wnt signaling receptor. The HARE5 locus is 12 kb in length and has 16 base pair differences between the human and the chimp sequences (**Figure 4*a***). Ten of these mutations are fixed in the human branch and 6 mutations are fixed in the chimpanzee branch since the divergence from our last common ancestor (Boyd et al. 2015). On the basis of the sequence divergence, TF binding is predicted to be affected, particularly at the human-derived sites, with the sequence predicted to have gained binding sites for *myc* and lost binding sites for *myc* repressors. HARE5 interacts directly with a core promoter of *FZD8* that is ~305 kb away, and its regulatory activity is confirmed by reporter assays, recapitulating *FZD8* activity patterns. Interestingly, when chimpanzee and human HARE5 sequences were tested in *LacZ* transgenic mice, the human-specific element showed significantly stronger and tighter expression in the lateral telencephalon beginning at E10 (Boyd et al. 2015). The chimpanzee sequence, although overall spatially similar in terms of expression, induced a more diffuse and weaker expression profile.

Functionally, HARE5 seems to affect neural progenitor cells. Both chimpanzee and human enhancers were active in most Pax6-positive neuroepithelial cells, with a smaller portion active in TuJ1-positive neurons at E10.5, and these enhancers appear active in the ventricular zone at E12.5. Furthermore, in transgenic mice in which human HARE5 drove *FZD8* expression, there was increased cell cycling in neural progenitors and more FoxP1-positive neurons. An analysis of E18.5 transgenic mice in which the *FZD8* gene is controlled by either the human or the chimpanzee HARE5 sequence showed an increase in cortical size of ~12% due to the human-specific alleles (Boyd et al. 2015). This change in brain size consisted of an increase in tangential length rather than cortical layer thickening, similar to cases of β-catenin (Chenn & Walsh 2002), further supporting a progenitor proliferation mechanism. Although further work is necessary to demonstrate function in the human brain, the study by Boyd et al. (2015) demonstrates the potential functional role that some HARs may have in neurodevelopment.

As an orthogonal approach, recent comparative epigenomic analyses have examined the enhancers in the developing (Reilly et al. 2015) and adult (Vermunt et al. 2016) brain, as well as induced pluripotent stem cell (iPSC)-derived neural crest cells (Prescott et al. 2015). These studies have generated sets of promising candidates that appear to display species-specific activity,

particularly given the tissue-specific activity used to identify the elements. In bulk sequencing approaches, observed human-specific differences in enhancer activity can sometimes be due to a mix of enhancer activity differences and cell population changes across species (Vermunt et al. 2016); however, in all three studies (Prescott et al. 2015, Reilly et al. 2015, Vermunt et al. 2016) HARs appear as candidates. As comparative epigenomics rapidly improves, particularly in its ability to capture cell specificity and diversity, the use of histone mark–based chromatin immunoprecipitation sequencing to find conservation will provide researchers a powerful method for uncovering species-specific enhancers, proving valuable for both assessing enhancers derived from conservation-based methods and identifying enhancers that are not well conserved and hence would be missed by traditional comparative genomic methods. Together with the expansion of iPSC-derived in vitro models with respect to cell type diversity as well as the number of nonhuman mammals (particularly primates), these methods may provide insightful tools to study comparative epigenomics in organisms that have been traditionally difficult to study.

Role of HARs in Social and Cognitive Development from Studies of Autism Spectrum Disorder and Schizophrenia

As demonstrated throughout this review, one of the best tools for mapping elements to their function is by studying relevant human phenotypes and their underlying mutations. However, studies are just beginning to utilize large-scale sequencing and SNP array data to assess HARs in neurological disorders. Early studies provided some clues to their roles in autism spectrum disorder (ASD) and schizophrenia. Oksenberg and colleagues (Oksenberg & Ahituv 2013, Oksenberg et al. 2013) confirmed the existence of enhancers within noncoding HARs of *AUTS2*, a gene linked to ASD, and postulated that mutations in these elements might contribute to risk of ASD (**Figure 4*c***). Taking a GWAS approach, Xu et al. (2015) revealed that genes associated with HARs are enriched for loci with significant associations to schizophrenia. Furthermore, GABAergic and glutamatergic genes were enriched among the HAR-associated schizophrenia genes. Interestingly, HAR-associated genes were enriched for processes involved in synaptic formation, and exhibited a higher connectivity to regulatory networks in the prefrontal cortex (Xu et al. 2015). By directly identifying highly penetrant risk alleles in ASD, Doan et al. (2016) sequenced a cohort of consanguineous families with ASD whose diagnoses could not be accounted for by underlying coding region mutations or copy number variants. Using an approach similar to those widely used in large-scale de novo exome and genome studies of ASD, this study provided the first evidence that mutational excess of recessively acting point mutations in HARs has a role in ASD. These HAR mutations were suggested to alter essential pathways that regulate brain development such as gene splicing, synaptogenesis, and others, including *MEF2C*, *CDKL5*, *PTBP2*, and *GPC4*, with known association to ASD and intellectual disability (ID) (Doan et al. 2016). With a combination of transient transgenic mice and luciferase assays, Doan et al. (2016) showed an interaction of HAR426 with the promoter of *CUX1*, a gene that regulates dendritic spine density in a dosage-sensitive fashion in mice (Cubelos & Nieto 2010; Cubelos et al. 2010, 2014). Furthermore, HAR426 has in vitro and in vivo enhancer activity, and a mutation in HAR426 found in several individuals with ASD or ID results in increased enhancer activity (Doan et al. 2016). Therefore, mutations in HAR426 may affect synaptic complexity through overexpression of *CUX1*, which alters dendritic spines in mice. Although these studies provide an intriguing insight into several interesting HARs, the data supporting their roles need much additional work. Moreover, these studies do not distinguish the strength of the effects of the mutations in HARs, whether they are low-penetrance risk alleles, or whether they are highly penetrant alleles with the potential for Mendelian conditions. Furthermore, as the study leveraged the elevated rates of recessive disease in consanguineous

families, it has yet to be shown whether such a contribution exists in other populations or whether other mutational classes such as de novo point mutations have a role.

CONCLUSIONS

Our understanding of the biological basis of evolutionary changes in human social and cognitive functioning has dramatically increased owing to recent breakthroughs in whole-genome sequencing of hundreds of species and tens of thousands of human genomes, including those in both healthy individuals and individuals with neurological disorders. Although the question of what makes us human remains unsolved, the combination of human disease genetics and comparative genomics has revealed important contributions by amino acid–altering coding mutations in genes such as those involved in neural development and brain size. Moreover, the discovery of disease-associated mutations in both promoters and HARs provides intriguing new elements with strong functional ties to brain development. Through limited studies HARs have already been linked to synaptic complexity, brain size, and social and cognitive disorders such as ASD and schizophrenia. Together, each evolutionarily distinct genomic coding and noncoding region in humans provides key pieces to the puzzle of why humans possess such unique social and cognitive abilities and behaviors.

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