A Catalogue of 59,732 Human-Specific Regulatory Sequences Reveals Unique-to-Human Regulatory Patterns Associated with Virus-Interacting Proteins, Pluripotency, and Brain Development

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Extensive searches for genomic regions harboring various types of candidate human-specific regulatory sequences (HSRS) identified thousands' HSRS using high-resolution next-generation sequencing technologies and methodologically diverse comparative analyses of human and nonhuman primates' (NHPs) reference genomes. In this study, a comprehensive catalogue of 59,732 genomic loci harboring candidate HSRS has been assembled to facilitate the systematic analyses of genomic sequences that were either inherited from extinct common ancestors (ECAs) or created *de novo* in human genomes. These analyses identified thousands of candidate HSRS and HSRS-harboring loci that appear inherited from ECAs, yet absent in genomes of our closest evolutionary relatives, chimpanzee and bonobo, presumably due to the incomplete lineage sorting and/or species-specific loss or regulatory DNA. This pattern is particularly prominent for HSRS-harboring loci that have been putatively associated with human-specific gene expression changes in cerebral organoid models. A prominent majority of regions harboring human-specific mutations associated with human-specific expression changes during brain development is highly conserved in chimpanzee, bonobo, and gorilla genomes. Among NHPs, dominant fractions of HSRS-harboring loci associated with human-specific gene expression in both excitatory neurons (347 loci; 67%) and radial glia (683 loci; 72%) are highly conserved in the gorilla genome. Analysis of 4433 genes encoding virus-interacting proteins (VIPs) revealed that 95.9% of human VIPs are components of human-specific regulatory networks that appear to operate in distinct types of human cells from preimplantation embryos to adult dorsolateral prefrontal cortex. These analyses demonstrate that modern humans captured unique genome-wide combinations of regulatory sequences, divergent subsets of which are highly conserved in distinct species of six NHP separated by 30 million years of evolution. Concurrently, this unique-to-human mosaic of genomic regulatory patterns inherited from ECAs was supplemented with 12,486 created *de novo* HSRS. Genes encoding VIPs appear to represent a principal genomic target during evolution of human-specific regulatory networks, which contribute to fitness of *Homo sapiens* and affect a functionally diverse spectrum of biological and cellular processes controlled by VIP-containing liquid-liquid phase-separated condensates.

Keywords: human-specific regulatory sequences, human-specific mutations, PtERV1 retrovirus, virus-interacting proteins, human-specific transcription factor binding sites, exaptation of ancestral regulatory DNA

Introduction

RECENT ADVANCES ENABLED by the analyses of individual genomes of great apes using high-resolution sequencing technologies and methodologically diverse comparative analyses of human and nonhuman primates' (NHPs) reference genomes significantly enhanced our understanding of human-specific structural genomic variations of potential regulatory and functional significance (Chimpanzee Sequencing and Analysis Consortium, 2005; Locke

et al., 2011; McLean *et al.*, 2011; Konopka *et al.*, 2012; Prüfer *et al.*, 2012; Scally *et al.*, 2012; Shulha *et al.*, 2012; Capra *et al.*, 2013; Marchetto *et al.*, 2013; Marnetto *et al.*, 2014; Gittelman *et al.*, 2015; Prescott *et al.*, 2015; Dong *et al.*, 2016; Dennis *et al.*, 2017; Sousa *et al.*, 2017; Guffanti *et al.*, 2018; Kronenberg *et al.*, 2018; Glinsky *et al.*, 2015, 2016a–c, 2017, 2018). Previous reports were focused on analyses of subsets of loci selected from this expanding genomic universe of human-specific structural variations to highlight mechanistically distinct pathways of their

evolutionary origins (Glinsky, 2016b) (18,364 loci analyzed) or potential impacts on evolution of human-specific traits (reviewed in Levchenko *et al.*, 2018; 4428 loci analyzed). Collectively, these studies markedly expanded the compendium of candidate human-specific regulatory sequences (HSRS), which currently comprises nearly 60,000 genomic loci aligned to the most recent release of the human reference genome (Tables 1–3 and Supplementary Tables S1–S19). This remarkable progress creates a multitude of significant contemporary challenges, the centerpiece of which is a need to compile a comprehensive catalog of HSRS to facilitate identification of the high-priority panel of genetic targets for stringent functional validation experiments. One of the important considerations during the selection of high-priority genetic targets for targeted functional experiments should be focused on illuminating the most compelling evidence linking HSRS and biological pathways that contributed to phenotypic divergence of humans and nonhuman great apes. This approach might help to identify the elite set of HSRS, selection of which will be based on the expectation of high likelihood of biologically significant species-specific effects on phenotypes contributing to segregation of species. Then, in-depth structural-functional explorations of the impact of HSRS on development of human-specific traits should become feasible. One of essential steps toward addressing these problems is to gain insights into evolutionary origins of genomic regions harboring HSRS and to identify HSRS that are absent in genomes of NHPs.

It has been suggested that regulatory networks associated with the pluripotency phenotype underwent human-specific changes during evolution (Marchetto *et al.*, 2013; Blake *et al.*, 2018). Emergence of human-specific changes of pluripotency regulatory networks operating during human preimplantation embryogenesis was associated with activities of HSRS derived from various families of transposable elements (TE), including stem cell-associated retroviral sequences (Glinsky, 2016a,c, 2017, 2018; Glinsky and Barakat, 2019). These TE-derived regulatory loci include many thousands of transcription factor binding sites (TFBS) and functional enhancer elements (Glinsky, 2016a–c, 2017, 2019; Glinsky and Barakat, 2019), a large fraction of which manifests distinct activities in the adult human brain during physiological and pathological conditions (Guffanti *et al.*, 2018). Expression of several 100,000s of TE-encoded RNA molecules in postmortem samples of the adult human dorsolateral prefrontal cortex (DLPFC) has been documented (Guffanti *et al.*, 2018), more than 4000s of which represent candidate HSRS. Most recently, human-specific gene expression changes in various cell types of developing human and chimpanzee brains have been linked to regulatory loci harboring human-specific mutations (Kronenberg *et al.*, 2018). Collectively, these reports are consistent with the concept that human-specific features of pluripotency regulatory networks highlight a common regulatory nexus of pluripotency with fetal and adult brain development and functions (Glinsky, 2017, 2018), according to which functional priming of HSRS and design of the timeline of their developmental activity maps occur during human preimplantation embryogenesis. Thus, it was of interest to analyze the validity of these observations within the context of a genome-wide global network of nearly 60,000 HSRS.

Consistent with the hypothesis that viruses may represent a major driver of the human lineage adaptation, it has been estimated that interactions with viruses accounted for ~30% of protein adaptation in the human lineage (Enard and Petrov, 2018; Enard *et al.*, 2016). The diversity, abundance, and biological properties of virus-interacting proteins (VIPs) that are available for engagements during the host-virus encounter would ultimately determine the susceptibility to infection, and its progression and resolution. However, the biological significance of VIPs is not limited to the sensing and management of host-virus interactions. VIPs represent one of the ubiquitous constituents of membraneless organelles designated liquid-liquid phase-separated condensates, formation of which is mediated by cooperative interactions of multivalent proteins and nucleic acid molecules (Brangwynne *et al.*, 2009; Banjade *et al.*, 2015; Bergeron-Sandoval *et al.*, 2016; Banani *et al.*, 2017). These structures were originally defined as nuclear or cytoplasmic nonmembrane-bound structures that can be observed as independent domains using transmission electron microscopy without the requirement for antibody labeling for their visualization (Spector, 2006), and now have been functionally implicated in a wide range of biological and cellular processes (see Discussion section). Hence, VIP-encoding genes should be considered one of the major targets during the evolution of species-specific genomic regulatory networks. Consequently, it was of interest to determine whether genes encoding VIPs represent the putative downstream regulatory targets of HSRS and to assess what the potential biological effects and significance of these interactions are.

In this contribution, an up-to-date catalog of 59,732 candidate human-specific regulatory loci has been assembled and their conservation patterns in genomes of 5 non-human great apes (chimpanzee, bonobo, gorilla, orangutan, and gibbon; Tables 1–3 and Supplementary Table S1–S19) have been analyzed. Systematic comparisons of genomic regions harboring HSRS and unique to African great apes insertions of the PtERV1 retrovirus-derived sequences were carried out resulting in identification of 248 insertions sites of PtERV1 in great apes (45.9%; $p = 1.03E-44$) intersecting genomic regions harboring 442 HSRS in humans. Diverse patterns of sequence conservation of different classes of HSRS and HSRS-harboring loci were observed, reflecting quantitatively distinct profiles of inheritance from extinct common ancestors (ECAs) of the human lineage and each of the five species of nonhuman great apes. One of the prevalent modes of evolutionary inheritance is exemplified by thousands of HSRS that appear inherited from ECAs, yet absent in genomes of our closest evolutionary relatives, chimpanzee and bonobo. Significant fractions of retrotransposon-derived loci transcriptionally active in human DLPFC are highly conserved in genomes of gorilla, orangutan, gibbon, and rhesus (1688; 1371; 1148; and 1045 loci, respectively), yet they are absent in genomes of both chimpanzee and bonobo. This mode of evolutionary inheritance was demonstrated for HSRS and HSRS-harboring loci putatively associated with genes implicated in development and functions of human brain. Significantly, these analyses revealed that 95.9% of human genes encoding VIPs represent apparent targets of human-specific regulatory networks in distinct types of human cells from preimplantation

TABLE 1. MOSAICISM OF EVOLUTIONARY ORIGINS OF GENOMIC LOCI HARBORING VARIOUS CLASSES OF HUMAN-SPECIFIC MUTATIONS: HUMAN-SPECIFIC MUTATIONS TARGET HIGHLY CONSERVED SEQUENCES MAPPED TO REFERENCE GENOMES OF MULTIPLE SPECIES OF NONHUMAN PRIMATES (95% SEQUENCE IDENTITY THRESHOLDS DURING BOTH DIRECT AND RECIPROCAL CONVERSIONS)

Classification category	Human genome	Chimpanzee	Percent Bonobo	Percent Gorilla	Percent Orangutan	Percent Gibbon	Percent Rhesus	Percent Mouse	Percent						
Fixed human-specific insertions ^a	11886	7138	60.05	6261	52.68	7104	59.77	3209	27.00	3268	27.49	2036	17.13	0	0.00
Fixed human-specific deletions	5891	4748	80.60	4487	76.17	4542	77.10	3569	60.58	3288	55.81	2923	49.62	96	1.63
Human-specific STR expansions	4910	272	5.54	260	5.30	236	4.81	237	4.83	298	6.07	212	4.32	131	2.67
Human-specific STR contractions	1464	1165	79.58	1115	76.16	1165	79.58	1031	70.42	927	63.32	867	59.22	85	5.81
Human-specific mutations associated with human-specific gene expression changes in excitatory neurons ^b	517	263	50.87	206	39.85	347	67.12	21	4.06	25	4.84	22	4.26	0	0.00
Human-specific mutations associated with human-specific gene expression changes in radial glia ^b	947	476	50.26	450	47.52	683	72.12	49	5.17	41	4.33	48	5.07	0	0.00
Duplicated regions in GRCh38 space defined by WSSD	7897	5579	70.65	4969	62.92	5692	72.08	3895	49.32	3548	44.93	3236	40.98	597	7.56

^aResults of the analyses of 10 kb regions centered at the exact sites of fixed human-specific insertions are reported.

^bAssociated with changes in gene expression in human versus chimpanzee cerebral organoids. STR, short tandem repeats; WSSD, whole-genome shotgun sequence detection.

TABLE 2. MOSAICISM OF EVOLUTIONARY ORIGINS OF GENOMIC LOCI HARBORING CANDIDATE HUMAN-SPECIFIC REGULATORY SEQUENCES ASSOCIATED WITH HUMAN-SPECIFIC GENE EXPRESSION CHANGES IN HUMAN VERSUS CHIMPANZEE BRAIN ORGANOID

Classification category	Human genome	NHP genomes ^a	Percent Chimpanzee	Percent Bonobo	Percent Gorilla	Percent Orangutan	Percent Gibbon	Percent Rhesus	Percent Mouse	Percent							
Human-specific mutations associated with human-specific gene expression changes in excitatory neurons ^b	517	283	54.74	183	64.66	143	50.53	249	87.99	9	3.18	16	5.65	14	4.95	0	0.00
Human-specific mutations associated with human-specific gene expression changes in radial glia ^b	947	526	55.54	326	61.98	282	53.61	472	89.73	21	3.99	24	4.56	27	5.13	0	0.00

Only records remapped to the identical hg38 loci are reported.

^aMapped to highly conserved regions in genomes of NHP identified based on 95% sequence identity thresholds during both direct and reciprocal conversions.
^bAssociated with changes in gene expression in human versus chimpanzee cerebral organoids; percentage values for individual genomes of nonhuman species reflect fractions of all records scored in NHPs.

NHP, nonhuman primate.

TABLE 3. ASSOCIATIONS OF VIRUS-INTERACTING PROTEIN-ENCODING GENES COMPRISING HUMAN-SPECIFIC EXPRESSION SIGNATURE OF RADIAL GLIA WITH HUMAN-SPECIFIC MUTATIONS

Classification category	VIPs	Non-VIPs	All genes	VIPs, %	p ^a
Up human radial glia	124	259	383	32.38	4.74E-11
Associated with human-specific mutations	50	16	66	75.76	
Percent	40.32	6.18			
p ^a		1.36E-15			
Down human radial glia	103	182	285	36.14	0.0125
Associated with human-specific mutations	32	27	59	54.24	
Percent	31.07	14.84			
p ^a		0.0021			
All radial glia human-specific genes	227	441	668	33.98	6.50E-11
Associated with human-specific mutations	82	43	125	65.60	
Percent	36.12	9.75			
p ^a		9.44E-16			

^ap-Values were estimated using two-tailed Fisher's exact test.
VIPs, virus-interacting proteins.

embryos to adult DLPFC, suggesting that VIP-encoding genes may represent one of the principal regulatory targets during the evolution and phenotypic divergence of *Homo sapiens*. Collectively, these observations suggest that manifestations of human-specific phenotypes are controlled by unique-to-human mosaic of human-specific mutations and highly conserved genomic regulatory sequences inherited from ECAs, which is supplemented with 12,486 created *de novo* HSRS.

Materials and Methods

Data source

Candidate HSRS and African ape-specific retroviral insertions. A total of 59,732 candidate HSRS and all currently known 504 insertion sites of the African ape-specific PtERV1 retrovirus (Kronenberg *et al.*, 2018) were analyzed in this study, detailed descriptions of which and corresponding references of primary original contributions are reported in the Tables 1–3 and Supplementary Tables S1–S19.

Additional data sources and analytical protocols. Solely publicly available datasets and resources were used in this contribution as well as methodological approaches and a computational pipeline validated for discovery of primate-specific genes and human-specific regulatory loci (Kent *et al.*, 2002; Schwartz *et al.*, 2003; Tay *et al.*, 2009; Capra *et al.*, 2013; Marnetto *et al.*, 2014; Guffanti *et al.*, 2018; Glinsky, 2015, 2016a–c, 2017, 2018). The analysis is based on the University of California Santa Cruz (UCSC) LiftOver conversion of the coordinates of human blocks to corresponding nonhuman genomes using chain files of pre-computed whole-genome BLASTZ alignments with a minMatch of 0.95 and other search parameters in default setting (<http://genome.ucsc.edu/cgi-bin/hgLiftOver>). Extraction of BLASTZ alignments by the LiftOver algorithm for a human query generates a LiftOver output “Deleted in new,” which indicates that a human sequence does not intersect with any chains in a given nonhuman genome. This indicates the absence of the query sequence in the subject genome and was used to infer the presence or absence of the human sequence in the nonhuman reference genome. HSRS were manually curated to validate their identities and genomic features using a BLAST algorithm and the latest

releases of the corresponding reference genome databases for time periods between April, 2013, and September, 2018.

The significance of the differences in the expected and observed numbers of events was calculated using two-tailed Fisher's exact test. Additional placement enrichment tests were performed for individual classes of HSRS, taking into account the size in base pairs of corresponding genomic regions. Datasets of NANOG-, POU5F1-, and CTCF-binding sites and human-specific TFBS in hESCs, as well as all other classes of HSRS were reported previously (Kunarso *et al.*, 2010; McLean *et al.*, 2011; Konopka *et al.*, 2012; Prüfer *et al.*, 2012; Scally *et al.*, 2012; Shulha *et al.*, 2012; Capra *et al.*, 2013; Marchetto *et al.*, 2013; Marnetto *et al.*, 2014; Gittelmann *et al.*, 2015; Prescott *et al.*, 2015; Dong *et al.*, 2016; Dennis *et al.*, 2017; Sousa *et al.*, 2017; Guffanti *et al.*, 2018; Kronenberg *et al.*, 2018; Glinsky *et al.*, 2015, 2016a–c, 2017, 2018) and are publicly available.

Data analysis

Categories of DNA sequence conservation. Identification of highly conserved in primates (pan-primate), primate-specific, and human-specific sequences was performed as previously described (Glinsky, 2015, 2016a–c, 2017, 2018). In brief, all categories were defined by direct and reciprocal mapping using liftOver (see previous section, Additional data sources and analytical protocols). Specifically,

- Highly conserved in primates' sequences: DNA sequences that have at least 95% of bases remapped during conversion from/to human (*H. sapiens*, hg38), chimp (Pan troglodytes, v5), and bonobo (Pan paniscus, v2, in specifically designated instances, and Pan paniscus, v1 was utilized for comparisons). Similarly, highly conserved sequences were defined for hg38 and genomes of gorilla, orangutan, gibbon, and rhesus.
- Primate specific: DNA sequences that failed to map to the mouse genome (mm10).
- Human specific: DNA sequences that failed to map at least 10% of bases from human to both chimpanzee and bonobo. All candidate HSRS identified based on the sequence alignments failures to ge-

nomes of both chimpanzee and bonobo were subjected to more stringent additional analyses requiring the mapping failures to genomes of gorilla, orangutan, gibbon, and rhesus. These loci were considered created *de novo* HSRS.

To infer the putative evolutionary origins, each evolutionary classification was defined independently by running the corresponding analyses on all candidate HSRS representing the specific category. For example, human-rodent conversion identifies sequences that are absent in the mouse genome based on the sequence identity threshold of 10%. Additional comparisons were performed using the same methodology and exactly as stated in the article text and described in details below.

Genome-wide proximity placement analysis. Genome-wide proximity placement analysis of distinct genomic features co-localizing with HSRS was carried out as described previously (Glinsky, 2015, 2016a–c, 2017, 2018). Briefly, as a typical example of the analytical protocol, we examined the significance of overlaps between hESC active enhancers and human-specific Transcription Factor Binding Sites (hsTFBS) by first identifying all hsTFBS that overlap with any of the genomic regions tested in the ChIP-STARR-seq dataset (Barakat *et al.*, 2018; Glinsky *et al.*, 2018). We then calculated the relative frequency of active enhancers overlapping with hsTFBS. To assess the significance of the observed overlap of genomic coordinates, we compared the values recorded for hsTFBS with the expected frequency of active and nonactive enhancers that overlap with all TFBS for NANOG (15%) and OCT4 (25%) as previously determined (Barakat *et al.*, 2018). Our analyses demonstrate that more than 95% of hsTFBS co-localized with sequences in the tested regions of the hESC genome.

Inference of the evolutionary origin and functional enrichment analyses. Evolutionary origins of HSRS were inferred from the results of the conservation patterns of 59,732 candidate human-specific regulatory DNA sequences based on the hg38 release of the human reference genome and latest available releases of genomes of six NHPs, namely chimpanzee, bonobo, gorilla, orangutan, gibbon, and rhesus. The conservation analyses was carried out using the LiftOver algorithm and Multiz Alignments of 20 mammals (17 primates) of the UCSC Genome Browser (Kent *et al.*, 2002) on Human Dec. 2013 Assembly (GRCh38/hg38) (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&position=chr1%3A90820922-90821071&hgid=441235989_eelAivpkubSY2AxxLhSXKL5ut7TN).

All DNA sequences were converted to most recent releases of the corresponding reference genome databases and were utilized consistently throughout the study to ensure the use of the most precise, accurate, and reproducible genomic DNA sequences available to date. A candidate HSRS was considered conserved if it could be aligned from/to hg38 reference genome and either one or both chimpanzee or bonobo genomes using defined sequence conservation thresholds of the LiftOver algorithm MinMatch function and direct and reciprocal conversions protocols. Similarly, the conservation patterns were evaluated for genomes of other NHP. LiftOver conversion of the coordinates of human

blocks to nonhuman genomes using chain files of pre-computed whole-genome BLASTZ alignments with a specified MinMatch levels and other search parameters in default setting. Several thresholds of the LiftOver algorithm MinMatch function (minimum ratio of bases that must re-map) were utilized to assess the sequence conservation and identify candidate human specific (MinMatch of 0.1; 0.95; 0.99; and 1.00) and conserved in NHP (MinMatch of 0.95 and 1.00) regulatory sequences as previously described (Guffanti *et al.*, 2018; Glinsky, 2015, 2016a–c, 2017, 2018). The net alignments provided by the UCSC Genome Browser were utilized to compare the sequences in the human genome (hg38) with the mouse (mm10), chimpanzee (PanTro5), and latest available releases of bonobo, gorilla, orangutan, gibbon, and rhesus genomes. A given regulatory DNA segment was defined as the highly conserved regulatory sequence when both direct and reciprocal conversions between humans' and NHPs' genomes were observed using the MinMatch sequence alignment threshold of 0.95, requiring that 95% of bases must re-map during the alignments of the corresponding sequences. A given regulatory DNA segment was defined as the created *de novo* candidate HSRS when sequence alignments failed to both chimpanzee and bonobo genomes using the specified MinMatch sequence alignment thresholds. More stringently, these requirements were extended to include genomes of gorilla, orangutan, gibbon, and rhesus. Analyses of conservation patterns of 11,866 human-specific insertions have been performed using 11 different window sizes (Supplementary Table S12) centered at the insertion sites previously reported by Kronenberg *et al.* (2018). Numbers of records that successfully completed direct and reciprocal conversions from/to hg38 and genomes of nonhuman species (six NHPs and mouse) using sequence identity threshold 95% are reported in the Supplementary Table S12.

The Enrichr API (January 2018 version) (Chen *et al.*, 2013) was used to test genes linked to HSRS of interest for significant enrichment in numerous functional categories. To comply with the web interface, we considered the 1000 genes closest to the tested peaks for enrichments. In all plots (unless stated otherwise), we report the “combined score” calculated by Enrichr, which is a product of the significance estimate and the magnitude of enrichment (combined score $c = \log(p) * z$, where p is the Fisher's exact test p -value and z is the z -score deviation from the expected rank). When technically feasible, larger sets of genes comprising several 1000 entries were analyzed. Regulatory connectivity maps between HSGRS and coding genes and additional functional enrichment analyses were performed with GREAT (McLean *et al.*, 2010) at default settings.

Statistical analyses of the publicly available datasets. All statistical analyses of the publicly available genomic datasets, including error rate estimates, background and technical noise measurements, and filtering, feature peak calling, feature selection, assignments of genomic coordinates to the corresponding builds of the reference human genome, and data visualization, were performed exactly as reported in the original publications and associated references linked to the corresponding data visualization tracks (<http://genome.ucsc.edu>). Any modifications or new elements of statistical

analyses are described in the corresponding sections of the Results. Statistical significance of the Pearson correlation coefficients was determined using GraphPad Prism version 6.00 software. Both nominal and Bonferroni adjusted *p*-values were estimated. The significance of the differences in the numbers of events between the groups was calculated using two-sided Fisher's exact and chi-square test, and the significance of the overlap between the events was determined using the hypergeometric distribution test (Tavazoie *et al.*, 1999).

Results

Mosaicism of evolutionary origins of genomic loci harboring various classes of human-specific mutations

Recent experiments identified 24,151 genomic regions harboring various classes of human-specific mutations identified based on the comparative analyses of genomes of modern humans and nonhuman great apes (Kronenberg *et al.*, 2018). It was of interest to analyze the sequence conservation patterns of these 24,151 regions in genomes of 6 NHPs, including 5 nonhuman great apes (chimpanzee, bonobo, gorilla, orangutan, and gibbon) and rhesus macaque (Table 1). In these analyses, genomic sequences that manifested at least 95% of sequence conservations during the direct and reciprocal conversions from/to reference genomes of modern humans (hg38) and corresponding NHP species were considered highly conserved. Within the context of definition of evolutionary origins of genomic regions harboring human-specific mutations, one of the main motivations was the inference that this analytical effort would identify highly conserved DNA sequences that were inherited by the modern humans' lineage from ECAs.

Consistent with a model of the significant contribution of the ECA inheritance, a majority (66–88%) of genomic loci harboring various classes of human-specific mutations appeared in genomic regions that are highly conserved in genomes of great apes and rhesus (Table 1). Notably, only 13% of 4910 human-specific short tandem repeat (STR) expansions' regions are conserved. Consistent with the predominantly primates' origins of regulatory regions harboring human-specific mutations, less than 5% of analyzed sequences appear highly conserved in the mouse genome (Table 1). Two classes of HSRS were mapped within varying fractions of DNA sequences highly conserved in genomes of different species of NHP: DNA loci harboring 49.6–80.6% of fixed human-specific deletions and 59.2–79.6% of human-specific STR contractions were placed within highly conserved genomic regions in genomes of different species of nonhuman great apes and rhesus (Table 1).

Genomes of the ancestral evolutionary branch leading to human and African great apes show the significant increase in duplication activity (Marques-Bonet *et al.*, 2009; Sudmant *et al.*, 2013) and human-specific segmental duplications have been identified among the most promising candidate genetic loci contributing to the evolution of human-specific phenotypes (Fortna *et al.*, 2004; Charrier *et al.*, 2012; Dennis *et al.*, 2012, 2017; Florio *et al.*, 2015; Kronenberg *et al.*, 2018). It was of interest to analyze evolutionary origins of 7987 duplicated genomic regions that were mapped to the most recent hg38 release of the human reference genome using whole-genome shotgun

sequence detection algorithm (Kronenberg *et al.*, 2018). Sequence conservation analyses of genomic regions harboring loci duplicated in the human genome revealed that a vast majority of these regions (regions harboring 6826 duplicated loci; 86.4%) are highly conserved in genomes of six NHPs (Table 1 and Supplementary Table S4 and Fig. 1). Interestingly, the largest fractions of both all regions successfully remapped from/to hg38 human reference genome (Table 1 and Fig. 1) and species-specific highly conserved regions (Supplementary Table S4 and Fig. 1) were observed in gorilla and chimpanzee genomes, indicating that these two species of nonhuman great apes appear similarly close to modern humans based on conservation patterns of genomic regions harboring duplicated loci in the human genome. These observations clearly demonstrate the species-specific mosaicism of evolutionary origins of genomic regions harboring duplicated loci in the genome of modern humans (Table 1 and Fig. 1 and Supplementary Table S4).

Mosaicism of evolutionary origins of genomic loci harboring human-specific mutations associated with human-specific gene expression changes in excitatory neurons and radial glia

Overall, the fractions of highly conserved sequences harboring human-specific mutations and assigned to different NHP species seem to reflect the consensus evolutionary order of the NHP genomes' similarity to the genome of modern humans. Notable exceptions from this pattern were identified during the analyses of human-specific mutations associated with human-specific gene expression changes in excitatory neurons and radial glia (Tables 1 and 2). Among NHP species, a majority of genomic regions harboring human-specific mutations associated with human-specific gene expression changes during brain development in both excitatory neurons and radial glia are highly conserved only in genomes of our three closest evolutionary relatives: 50.9% and 50.3% in chimpanzee; 39.9% and 47.5% in bonobo; and 67.1% and 72.1% in gorilla (for excitatory neurons and radial glia, respectively). In contrast, only ~5% of genomic regions harboring human-specific mutations associated with human-specific gene expression changes detected in cerebral organoids are highly conserved in genomes of orangutan, gibbon, and rhesus. Among nonhuman great apes, most significant fractions of candidate HSRS associated with human-specific gene expression changes in both excitatory neurons (347 loci; 67%) and radial glia (683 loci; 72%) are located in genomic regions highly conserved in the gorilla genome. For highly conserved regions harboring HSRS associated with human-specific gene expression changes in excitatory neurons, differences in conservation profiles between genomes of gorilla, chimpanzee, and bonobo were highly significant as defined by the two-tailed Fisher's exact test ($p=1.434\text{E-}07$; $p=1.574\text{E-}18$; $p=0.000463$). For highly conserved regions harboring HSRS associated with human-specific gene expression changes in radial glia, differences in conservation profiles between genomes of gorilla and chimpanzee as well as gorilla and bonobo were highly significant as well ($p=1.623\text{E-}22$ and $p=7.369\text{E-}28$, respectively). In contrast, conservation profiles of HSRS associated with human-specific gene

FIG. 1. Mosaicism of evolutionary origins of 7897 duplicated regions in the hg38 release of human reference genome defined by WSSD. (A) A consensus model of the lineage speciation during the evolution of great apes. The arrows depict the hypothetical flow of genomic information inherited from ECAs and acquired through species-specific gain and loss. Evolutionary origins through ECA inheritance pathways of highly conserved sequences in genomes of modern humans and nonhuman species of great apes are postulated. (B) Mosaicism of evolutionary origins of 7897 duplicated regions in the hg38 release of human reference genome defined by WSSD (Kronenberg *et al.*, 2018). Numbers of highly conserved regions that successfully completed direct and reciprocal conversion tests are reported for each NHS. (C) Species-specific mosaicism of evolutionary origins of duplicated regions in the hg38 release of human reference genome defined by WSSD. Only highly conserved sequences identified in the human reference genome and genomes of designated NHS are reported. ECAs, extinct common ancestors; NHS, nonhuman species; WSSD, whole-genome shotgun sequence detection. Color images are available online.

expression changes in radial glia were similar in genomes of chimpanzee and bonobo ($p=0.250$; Table 1). These observations suggest that the majority of highly conserved genomic regions harboring candidate HSRS associated with human-specific differences of gene expression in both excitatory neurons and radial glia was inherited from ECAs of modern humans and gorilla. This conclusion remains valid when the analyses were performed considering either only loci remapped to/from NHP genomes to identical hg38 genomic coordinates (Table 2 and Figs. 2 and 3 and Supplementary Table S4) or only genomic loci uniquely mapped to genomes of only single species of nonhuman great apes (Fig. 3). Notably, differences in conservation profiles of genomic loci harboring HSRS associated with human-specific gene expression changes in radial glia appear par-

ticularly prominent (Table 2 and Figs. 2 and 3 and Supplementary Table S5). Consistent with the ECA inheritance model, from 85.3% to 95.1% of HSRS-harboring regions that are highly conserved in the genomes of chimpanzee and bonobo are highly conserved in the gorilla genome as well. In contrast, only from 59.0% to 59.3% and from 62.7% to 65.1% of HSRS-harboring regions that are highly conserved in the gorilla genome remain highly conserved in the genomes of bonobo and chimpanzee, respectively. These findings were corroborated by observations demonstrating common evolutionary patterns of 248 insertion sites of African ape-specific retrovirus PtERV1 (45.9%; $p=1.03E-44$) intersecting genomic regions harboring 442 HSRS, which are enriched for HSRS that have been associated with human-specific gene expression changes in cerebral

FIG. 2. Mosaicism of evolutionary origins of candidate human-specific and primate-specific regulatory loci defined by sequence conservation analyses. (A) Evolutionary patterns of inheritance, gains, and losses of 540 insertions of African great ape-specific retrovirus PIERV1. (B) Evolutionary patterns of inheritance, gains, and losses of 947 human-specific regulatory regions associated with human-specific gene expression in radial glia. Only records of identically remapped loci in NHP during direct and reciprocal conversions from/to hg38 are reported. (C) Evolutionary patterns of inheritance, gains, and losses of 517 human-specific regulatory regions associated with human-specific changes of gene expression in excitatory neurons. Only records of identically remapped loci in NHP during direct and reciprocal conversions from/to hg38 are reported. (D) Evolutionary patterns of inheritance, gains, and losses of 4645 human-specific TE loci transcriptionally active in human DLPFC. Common ancestor heritage number of 2361 loci takes into account 1045 loci highly conserved in thesis; 4645 loci failed conversion to PanTro5 and PanPan1 (sequence identity threshold of 10%); 18 loci completed direct and reciprocal conversions (sequence identity threshold of 95%) from/to hg38 and PanPan2; 4612 failed conversions to PanTro5; PanPan1; PanPan2 (sequence identity threshold of 10%). DLPFC, dorsolateral prefrontal cortex; NHP, nonhuman primate; TE, transposable elements. Color images are available online.

FIG. 3. Species-specific mosaicism of evolutionary origins of human-specific regulatory sequences associated with human-specific changes of gene expression in excitatory neurons (**A, B**) and radial glia (**C, D**). (**A**) Highly conserved sequences reciprocally mapped as identical loci to the 282 regions harboring human-specific mutations associated with human-specific gene expression changes in excitatory neurons. (**B**) Highly conserved species-specific sequences reciprocally mapped as identical loci to the genomic regions harboring human-specific mutations associated with human-specific gene expression changes in excitatory neurons. (**C**) Highly conserved sequences reciprocally mapped as identical loci to the 525 regions harboring human-specific mutations associated with human-specific gene expression changes in radial glia. (**D**) Highly conserved species-specific sequences reciprocally mapped as identical loci to the genomic regions harboring human-specific mutations associated with human-specific gene expression changes in radial glia. Color images are available online.

organoid models (Supplementary Data and Supplementary Table S6a–c). Taking into account that placements in defined genomic regions of HSRS and PtERV1 in genomes of human and African apes, respectively, reflect independent stochastic events, these observations are consistent with the hypothesis of potentially significant impact of identified genomic regions on phenotypic divergence of humans and other great apes.

Mosaicism of evolutionary origins of candidate human-specific regulatory loci defined based on the mapping failure to both chimpanzee and bonobo reference genomes

One of the approaches to the identification of candidate HSRS is based on their absence in the genomes of our closest evolutionary relatives, chimpanzee and bonobo. These analyses demonstrate that large fractions of genomic regions harboring candidate HSRS are highly conserved in genomes of our more distant evolutionary relatives, gorilla, orangutan, gibbon, and rhesus (Tables 1 and 2). These observations suggest that candidate human-specific regulatory loci, which were defined based on the mapping failures to both chimpanzee and bonobo genomes, may originate on DNA sequences highly conserved in genomes of other NHP species. To test the validity of this hypothesis, 16,730 genomic loci harboring distinct classes of HSRS were identified that failed to convert to genomes of both chimpanzee and bonobo using 10% sequence identity threshold (Supplementary Table S7). Then, highly conserved sequences in genomes of rhesus, gibbon, orangutan, and gorilla were identified and tabulated for each category of HSRS. Consistent with the bypassing patterns of evolutionary inheritance, thousands of distinct classes of candidate HSRS that failed to map to genomes of both chimpanzee and bonobo are highly conserved in genomes of gorilla, orangutan, gibbon, and rhesus (Supplementary Table S7). Most prom-

inently, significant fractions of retrotransposon-derived loci that are transcriptionally active in human DLPFC and absent in genomes of both chimpanzee and bonobo are highly conserved in genomes of gorilla, orangutan, gibbon, and rhesus (1688; 1371; 1148; and 1045 loci, respectively; Fig. 4). It has been observed that in all instances, the gorilla genome had the largest numbers of shared with modern humans highly conserved sequences that failed to map to genomes of both chimpanzee and bonobo (Supplementary Table S7).

These observations indicate that a more stringent approach for definition of candidate HSRS, which are likely to have been created *de novo* in the genome of modern humans, would be to require the conversion failures of a regulatory DNA sequence to all six NHP genomes, namely genomes of chimpanzee, bonobo, gorilla, orangutan, gibbon, and rhesus. Using this strategy, 12,486 candidate HSRS have been identified (Supplementary Table S8), indicating that 24.8% of all analyzed in this contribution HSRS-harboring genomic loci could be classified as created *de novo* candidate HSRS.

Enrichment within human-specific regulatory pathways of genes comprising expression signatures of human-specific neurodevelopmental and pluripotency transcriptional networks

It is possible that distinct types of candidate HSRS are not just unrelated elements of a random population of DNA sequences, but they might represent a coherent collection of regulatory DNA sequences assembled during evolution to facilitate execution of human-specific functions. If this hypothesis is correct, then HSRS may represent the key components of human-specific genomic regulatory pathways governing human-specific gene expression patterns observed in various phenotypic contexts associated with human-specific traits. Therefore, the validity of this hypothesis

FIG. 4. Species-specific mosaicism of evolutionary origins of human-specific regulatory sequences encoded by transcriptionally active TE in human DPLFC. (A) Numbers of sequences highly conserved in NHP genomes reciprocally mapped to human-specific DLPFC-expressed TE. (B) Highly conserved species-specific sequences reciprocally mapped from NHP genomes to human-specific DLPFC-expressed TE. Color images are available online.

could be tested using strictly defined by comparisons with nonhuman great apes human-specific gene expression signatures (GESs) associated with development of human brain in the cerebral organoid model (Kronenberg *et al.*, 2018) and induced pluripotency phenotypes of human versus NHP cells (Marchetto *et al.*, 2013). Kronenberg *et al.* (2018) identified several 100 genes that manifest human-specific expression changes in modern humans versus chimpanzee cerebral organoids' model of brain development. Significant fractions of these human-specific neurodevelopmental networks operating in both excitatory neurons and radial glia appear associated with human-specific structural variants, specifically with human-specific insertions and deletions (Supplementary Table S9). Even larger fractions of genes comprising human-specific neurodevelopmental networks have been identified as components of the GES of the Multilineage Markers Expressing Cells in human preimplantation embryo: common gene sets represent 262 genes (68.2%; $p=1.59\text{E-}93$) and 481 genes (72.0%; $p=3.95\text{E-}187$) for human-specific GES of excitatory neurons and radial glia, respectively (Supplementary Table S9).

It has been reported that the creation of the MLME cells in human preimplantation embryos is associated with increased expression of primate-specific retrotransposon-derived regulatory long noncoding RNAs termed human pluripotency-associated transcripts (HPATs) (Glinsky *et al.*, 2018). Most recently, the expansive networks of primate-specific and human-specific retrotransposons transcriptionally active in human DLPFC and associated coding genes have been identified (Guffanti *et al.*, 2018). Thus, it was of interest to determine what fractions of genes comprising human-specific neurodevelopmental gene expression networks in excitatory neurons and radial glia may overlap with coding genes coupled with active transcription of TE in human DLPFC. Remarkably, it has been observed that common gene sets represent a vast majority of genes comprising human-specific neurodevelopmental networks: they comprise 322 genes (83.9%; $p=5.57\text{E-}84$) and 561 genes (84%; $p=3.08\text{E-}146$) for human-specific GES of excitatory neurons and radial glia, respectively (Supplementary Table S9). Interestingly, both *SRGAP2C* and *ARHGAP11B* genes driving divergent cortical development between humans and chimpanzee (Charrier *et al.*, 2012; Dennis *et al.*, 2012; Florio *et al.*, 2015) harbor TE transcriptionally active in human DLPFC (Guffanti *et al.*, 2018).

Marchetto *et al.* (2013) identified human-specific GES distinguishing induced pluripotent stem cells (iPSC) engineered from cells of modern humans and NHP species. To infer the putative regulatory association patterns of genes distinguishing human iPSC versus NHP iPSC and human-specific genomic regulatory pathways, overlap enrichment analyses of corresponding gene sets have been performed. It has been observed that the association patterns of human-specific GESs of brain development and pluripotency phenotypes with compendiums of genes likely governed by human-specific regulatory pathways appear strikingly similar (Supplementary Tables S9 and S10). Overall, 88% of genes comprising human-specific expression signature of the induced pluripotency phenotype represent genes implicated in putative regulatory associations with human-specific genomic pathways. These observations support the

hypothesis that human-specific GESs of brain development and pluripotency phenotypes are associated with a collection of HSRS assembled during evolution into human-specific genomic regulatory pathways to govern transcriptional networks in human cells.

Digital validation experiments of the enrichment within human-specific regulatory pathways of genes comprising expression signatures of human-specific neurodevelopmental and pluripotency transcriptional networks

It is important to note that human-specific genomic regulatory networks utilized in these analyses were defined employing vastly different experimental, analytical, and computational approaches that were applied within the broad range of experimental settings: (1) great apes' whole-genome sequencing-guided identification of human-specific insertions and deletions (Kronenberg *et al.*, 2018); (2) genome-wide analysis of retrotransposon's transcriptome in postmortem samples of human DLPFC (Guffanti *et al.*, 2018); (3) shRNA-mediated silencing of LTR7/HERVH retrovirus-derived long noncoding RNAs in human Embryonic Stem Cells (hESC) (Wang *et al.*, 2014); and (4) single-cell expression profiling analyses of human preimplantation embryos (Glinsky *et al.*, 2018). Thus, these human-specific genomic regulatory networks appear to operate in a developmentally diverse spectrum of human cells from preimplantation embryos to adult DLPFC, which collectively represent cell types that are biologically and anatomically highly relevant to manifestations of human-specific phenotypes. To test the validity of the above findings, the independent sets of GESs of human-specific neurodevelopmental (Supplementary Tables S13–S16) (Pollen *et al.*, 2019) and pluripotency (Supplementary Table S17) (Blake *et al.*, 2018) transcriptional networks were analyzed. It has been observed that a total of 817 of 877 (93.2%) genes comprising genetic markers distinguishing human versus chimpanzee radial glia cells in the cerebral organoids appear associated with human-specific genomic regulatory networks (Supplementary Table S13). Similar association patterns were documented for all other cell types of developing human and chimpanzee brains: common gene sets comprise 708 of 758 (93.4%) human-specific marker genes of intermediate progenitors (Supplementary Table S14); 857 of 912 (94%) human-specific marker genes of excitatory neurons (Supplementary Table S15); and 340 of 383 (88.8%) human-specific marker genes of inhibitory neurons (Supplementary Table S16). Consistently, 3218 of 3267 (98.5%) genes distinguishing human versus chimpanzee iPSC overlap with human-specific genomic regulatory networks operating in human cells (Supplementary Table S17). The enrichment patterns were highly significant for all four analyzed in this study of human-specific genomic regulatory networks (Supplementary Table S13–S17). Based on these observations, it has been concluded that human-specific GESs of brain development and pluripotency phenotypes manifest significant patterns of associations with a collection of HSRS assembled during evolution into human-specific genomic regulatory pathways to govern transcriptional networks in human cells.

Enrichment within human-specific regulatory pathways of genes encoding VIPs

These analyses identified thousands of genes that are likely regulated by HSRS, among which *SERINC5*, *APO-BEC3B*, and *PIWIL2* genes are of particular interest because high expression of proteins encoded by these genes is likely to confer increased resistance to the retroviral infection and to restrict the propagation of retrotransposons in human cells (Marchetto *et al.*, 2013; Rosa *et al.*, 2015; Usami *et al.*, 2015). These observations suggest that genes involved in interactions between viruses and human cells may constitute an important category of genetic targets that are placed under control of HSRS during speciation of the human lineage. Therefore, it was of interest to determine whether human genes encoding VIPs are components of human-specific regulatory networks by analyzing a comprehensive set of more than 4000 genes encoding human VIPs recently reported by Enard and Petrov (2018). The set of VIP-encoding genes analyzed in this study comprised 4433 individual records, which were obtained after removal of 3 loci that are no longer in the current ENSEMBL database and adjustment for records that have multiple ENSEMBL identifying numbers associated with the same genomic loci defined by unique gene symbols in hg38 release of the human reference genome database. It has been observed that a significant fraction of genes encoding human VIPs (27.2%; $p = 1.03E-66$; the hypergeometric test) appears associated with human-specific structural variants, specifically with human-specific insertions and deletions (Supplementary Table S11). A prominent majority of genes encoding human VIPs have been identified as components of the expression signature of the MLME cells in human preimplantation embryo (3551 VIPs; 80.1%; Supplementary Table S11) and coding genes coupled with active transcription of

TE in human DLPFC (3574 VIPs; 80.6%; Supplementary Table S11). Intriguingly, the *LBP9/HERVH* gene expression pathway regulated in hESC by primate-specific retrovirus LTR7/HERVH-derived long noncoding RNAs appears to control the expression of 2661 VIPs (60%; Supplementary Table S11). Overall, a vast majority of genes encoding human VIPs (4253 of 4433 genes; 95.9%) have been identified as putative downstream targets of human-specific genomic regulatory networks.

VIP-encoding genes represent large fractions of genes comprising human-specific expression signatures of radial glia, excitatory neurons, and iPSC

Above considerations suggest that VIP-encoding genes may be represented among genes comprising human-specific expression signatures of neurodevelopmental and pluripotency networks (see previous section, Enrichment within human-specific regulatory pathways of genes encoding VIPs). Follow-up analyses revealed that VIPs indeed constitute large fractions of genes comprising human-specific expression signatures of radial glia (34%; $p = 1.51E-94$; the hypergeometric test; Fig. 5), excitatory neurons (30%; $p = 1.92E-41$; Fig. 5); and human iPSC (25%; $p = 1.22E-08$). Intriguingly, disproportionately large fractions of VIP-encoding genes comprising human-specific expression signatures of both upregulated and downregulated genes in radial glia appear associated with human-specific structural variants (Fig. 5 and Table 3). It has been observed that the association with human-specific mutations of VIPs is 6.5-fold greater compared to non-VIPs ($p = 1.36E-15$; two-tailed Fisher's exact test) for genes upregulated in human radial glia cells (Fig. 5 and Table 3). For genes downregulated in human radial glia cells (Fig. 5 and Table 3), the

FIG. 5. Genes encoding VIPs represent large fractions of genes comprising human-specific expression signatures of excitatory neurons and radial glia. **(A)** VIP-encoding genes comprise 34% and 30% of genes differentially regulated in human versus chimpanzee radial glia cells and excitatory neurons, respectively, isolated from species-specific cerebral organoids. Significance of the overlaps was estimated using the hypergeometric distribution test. **(B)** Disproportionally large fractions of both upregulated and downregulated VIP-encoding genes comprising human-specific expression signatures of radial glia cells are associated with human-specific mutations. Corresponding cohorts of genes encoding VIP and non-VIP transcripts associated with human-specific mutations were identified from the set of 668 genes manifesting human-specific expression changes in human versus chimpanzee radial glial cells (Kronenberg *et al.*, 2018). Significance of the differences in associations with human-specific mutations between VIP and non-VIP genes was estimated using the two-tailed Fisher's exact test. VIPs, virus-interacting proteins. Color images are available online.

association with human-specific mutations of VIPs is 2.1-fold greater compared to non-VIPs ($p=0.002$; two-tailed Fisher's exact test). In striking contrast, no increased associations with human-specific mutations have been detected for VIP-encoding genes comprising human-specific expression signatures of excitatory neurons (data not shown), suggesting that observed putatively regulatory associations might be specific to human radial glia cells. Collectively, observations reported in this contribution are highly consistent with the hypothesis that VIP-encoding genes represent important components of human-specific regulatory networks operating in phenotypically distinct human cells that are essential for biological functions of cognition and pluripotency.

Genes encoding VIPs manifest significantly higher enrichment patterns within human-specific genomic regulatory networks compared to non-VIP-encoding genes

To determine whether larger fractions of genes encoding VIPs are associated with human-specific regulatory networks operating in human cells, we first identified genes associated with each category of HSRS using GREAT algorithm (McLean *et al.*, 2010) and then compiled a set of 13,824 human genes comprising putative regulatory targets associated with HSRS. This set of genes includes 3364 (76%) VIP-encoding genes. Next, the enrichment patterns within human-specific regulatory networks were independently analyzed for the entire set of 13,824 genes (Supplementary Table S18) and 10,460 non-VIP-encoding genes (Supplementary Table S19). The results were then compared to the enrichment patterns observed for all human genes encoding VIPs (Supplementary Table S11). In all instance, the highest enrichment values were documented for VIP-encoding genes compared to either the entire set of 13,824 genes or the set of 10,460 non-VIP-encoding genes ($p<0.0001$; chi-square test with Yates' correction). These observations are consistent with the hypothesis that VIP-encoding genes represent important regulatory targets for HSRS during the evolution of human-specific genomic regulatory networks.

Discussion

Implications for future structural-functional validation studies of regulatory effects of HSRS on human-specific regulatory networks and phenotypic traits

An impressive contemporary collection of nearly 60,000 candidate HSRS assembled by the collective decade-long effort of many laboratories (see Introduction section) lends further credence to the idea that unique-to-human phenotypes might result from human-specific changes to genomic regulatory sequences (King and Wilson, 1975). This study identifies multiple high-priority candidate HSRS for in-depth structural-functional validation analyses, among which most prominent candidate HSRS appears associated with human-specific gene expression changes in excitatory neurons and radial glia as well as in human iPSC. This high-priority set of elite genetic targets include candidate HSRS putatively regulating expression of *SERINC5*, *APOBEC3B*, and *PIWIL2* genes, high expression of which in human cells

is likely to confer increased resistance to the retroviral infection and propagation of retrotransposons (Marchetto *et al.*, 2013; Rosa *et al.*, 2015; Usami *et al.*, 2015). It is tempting to speculate that these changes may have been significant genetic contributors conferring the selective fitness advantage to human lineage during primate evolution.

Observations reported in this contribution have important implications for future functional validation studies, in particular, our approaches to selection and design of experimental systems for follow-up structural-functional analyses of the effects of specific HSRS on defined human-specific phenotypes. These considerations must take into account the history of evolutionary origins of HSRS to design the specifically tailored experimental models from a panel of cells obtained from modern humans and the appropriate NHP species, genomes of which either have (thus, are appropriate for targeted knock-out and silencing experiments) or not have (thus, are appropriate for knock-in and overexpression experiments) genetic loci of interest. Results of these analyses strongly argue that these experimental systems for targeted interrogation of human-specific genomic regulatory networks should include iPSC as well as brain development models of *in vitro* induced iPSC differentiation and cerebral organoids. Ideally, these experimental systems should be based on cells obtained from modern humans, our evolutionary closest relatives, chimpanzee and bonobo, as well as gorilla, orangutan, gibbon, and rhesus.

Analyses of evolutionary origins of genomic loci harboring HSRS revealed evidence of a striking ancestral polymorphism and regions of genomic divergence of modern humans and great apes

High-confidence human-specific mutations leading to emergence of candidate HSRS should be considered rare genomic events that unlikely occur more than once during evolution at the same genomic locations. Therefore, observations that large fractions of genomic regions harboring distinct classes of HSRS represent DNA sequences highly conserved in genomes of distinct NHP species should be interpreted as strong circumstantial evidence consistent with their putative regulatory functions. Collectively, the evidence presented in this contribution revealed a complex unique-to-human mosaic of regulatory DNA sequences inherited from ECAs following separation events from multiple distinct NHP species and reflecting the striking ancestral polymorphism of modern humans. One of the novel mechanisms that may have contributed to divergence of genomic regulatory networks of modern humans and nonhuman great apes is illustrated by observations that the insertions of the African great ape-specific retrovirus PTERV1 and distinct classes of HSRS have common genomic coordinates within orthologous genomic regions of gorilla, chimpanzee, bonobo, and modern humans. Overall, these common patterns of species-specific mutations within overlapping genomic regions were observed for nearly half (248 of 540 loci) of all PTERV1 insertions and 442 HSRS, including 21 HSRS associated with genes differentially expressed in human versus chimpanzee cerebral organoid models of brain development.

Genomic divergence and speciation of modern humans reflect a complex continuing evolutionary process rather than a singular instantaneous event

Observations reported herein support the hypothesis that the speciation process during evolution of great apes is not likely to occur as an instantaneous event (Patterson *et al.*, 2006): for example, human and chimpanzee lineages could have exchanged genes following the iterative sequences of the initial lineage divergence, separation, and gathering together before the permanent segregation of two species. Since human, chimpanzee, and gorilla lineages may have diverged during the relatively short evolutionary time, this model might reflect the extended complex speciation process of these three closely related great apes, possibly involving co-evolution of their ECAs (Supplementary Fig. S1). Incomplete lineage-sorting events were intrinsic components of the genomic divergence and likely played an important role in the lineage segregation. In agreement with this hypothesis, comparative analyses of multiple alignments of sequences of human, chimpanzee, gorilla, and orangutan genomes have demonstrated that a considerable fraction of genes in the human genome is more similar to the gorilla genome than to the chimpanzee genome (Chen and Li, 2001; O'hUigin *et al.*, 2002; Yang, 2002; Wall, 2003; Patterson *et al.*, 2006; Hobolth *et al.*, 2007). Genomic regions harboring HSRS appear to follow the similar evolutionary trajectory. Observed examples of the bypassing pattern of the evolutionary inheritance highlight HSRS supporting the inference of alternative genealogies (human being most closely related to NHP other than chimpanzee) most likely reflect the incomplete lineage-sorting events. Incomplete lineage sorting has been consistently observed in the multiple alignments of the genomes for human, chimpanzee, gorilla, and orangutan, where differences in models of gene genealogies and species phylogeny were documented for up to 36% of the human autosomal genome (Chen and Li, 2001; Yang, 2002; Wall, 2003; Patterson *et al.*, 2006; Hobolth *et al.*, 2007, 2011; Kronenberg *et al.*, 2018). Similar changes could result from species-specific losses of conserved ancestral loci of regulatory DNA, a mechanism that contributed to evolution of human-specific traits (McLean *et al.*, 2011).

VIPs as intrinsic protein constituents of liquid-liquid phase-separated condensates contributing to fitness and adaptation of human cells

These analyses argue that genes encoding VIPs may represent one of the principal genomic elements of human-specific regulatory networks. However, multiple lines of experimental evidence and theoretical considerations suggest that VIPs may constitute an important regulatory component of the human proteome as well by making a vital contribution to the formation and function of multimolecular complex based on multivalent protein interactions. Intracellular biochemical reactions are spatially compartmentalized within membraneless organelles called cellular bodies, which were originally defined as nuclear or cytoplasmic nonmembrane-bound structures that can be observed as independent domains using transmission electron microscopy without the requirement for antibody labeling for their visualization (Spector, 2006). Typically, these membraneless

organelles represent liquid-liquid phase-separated condensates, formation of which is mediated by cooperative interactions of multivalent proteins and nucleic acid molecules (Brangwynne *et al.*, 2009; Banjade *et al.*, 2015; Bergeron-Sandoval *et al.*, 2016; Banani *et al.*, 2017). Cooperative interactions between multivalent proteins and nucleic acid molecules located in close spatial proximity within a cell initiate the formation of liquid-liquid phase-separated condensates appearing as membraneless intracellular organelles, which are being assembled to compartmentalize and accelerate specific biochemical reactions. Most recently, a phase separation model of transcriptional control in mammalian cells has been formulated (Hnisz *et al.*, 2017) and direct experimental evidence supporting the model has been reported (Cho *et al.*, 2018; Sabari *et al.*, 2018).

One of the essential structural features of protein constituents of phase-separated condensates is the propensity for multivalent intermolecular interactions. Notably, it has been reported that human VIPs have more interacting protein partners in the network of human protein-protein interactions than non-VIPs (Stark *et al.*, 2011; Luisi *et al.*, 2015; Enard and Petrov, 2018). These observations indicate that human VIPs may represent intrinsic constituents of many phase-separated membraneless intracellular organelles and these VIP-containing liquid-liquid phase-separated condensates might be pathogenic targets of viruses. Several independent lines of experimental evidence and theoretical considerations stemming from the concept of intrinsically disordered proteins (IDPs) support this hypothesis.

It has been underscored that IDPs are abundant in the mammalian and viral proteomes (Tarakhovsky and Prinjha, 2018). One of the characteristic features of IDPs is the flexibility of three-dimensional conformations, and this lack of structural constraints of both host and viral IDPs is likely to promote a multiplicity of viral protein interactions with host proteins, thus targeting multiple elements of the host cell defense system (Xue *et al.*, 2014). IDPs are unable to fold spontaneously into stable three-dimensional globular structures and continually fluctuate through a range of conformations (Dyson and Wright, 2005) because they contain high proportions of charged hydrophilic amino acids combined with a relatively low abundance of hydrophobic amino acids. This flexibility of conformational features allows IDPs to form dynamically heterogeneous complexes with multiple binding partners (Ferreon *et al.*, 2009; Ishiyama *et al.*, 2010). The dynamic conformational nature of IDPs is essential for increased exposure and availability of potential binding sites to multiple binding partners, thus facilitating the multivalent intermolecular interactions of IDPs within diverse sets of multiprotein complexes (Tarakhovsky and Prinjha, 2018). Collectively, these features contribute to the apparent governing role of IDPs in signaling networks (Dunker *et al.*, 2005; Dyson and Wright, 2005; Ishiyama *et al.*, 2010). It has been suggested that viral proteins interfere with host phase-separated protein condensates to maximize the impact of viruses on host protein networks and that both viral and host IDPs play a central role in these processes (Tarakhovsky and Prinjha, 2018). It is tempting to speculate that continuing interactions between myriads of viral and host IDPs might represent the major

force of the evolutionary adaptation on molecular, cellular, and organismal levels.

Taken together, the above considerations strongly argue that human VIPs are components of a large family of IDPs that function as intrinsic protein constituents of many liquid-liquid phase-separated multimolecular complexes, facilitating compartmentalization and acceleration of biochemical reactions in human cells. Consistent with this hypothesis, *BRD4*, *MED1*, and *CDK9* genes that were recently identified as the essential structural-functional elements of phase-separated condensates exerting transcriptional controls at superenhancers (Cho *et al.*, 2018; Sabari *et al.*, 2018) also have been described as VIPs (see database of VIP-encoding genes reported in Enard and Petrov, 2018). Therefore, VIP-containing phase-separated multimolecular complexes are likely to represent high-value targets for viruses and important fitness determinants during adaptation.

It has been estimated that interactions with viruses accounted for ~30% of protein adaptation in the human lineage (Enard and Petrov, 2018; Enard *et al.*, 2016), consistent with the hypothesis that viruses appear to be a major driver of adaptation in the human lineage. VIPs evolve under both stronger purifying selection and tend to adapt at much higher rates compared to similar proteins that do not interact with viruses (Enard and Petrov, 2018; Enard *et al.*, 2016). Most recent assessments of the enrichment within VIPs of introgressed segments of genomic DNA after interbreeding of Neanderthals and modern humans provide strong evidence that viruses drive adaptive introgression between species and that adaptive introgression had a substantial impact on VIPs after interbreeding (Enard and Petrov, 2018). This contribution extends the concept of critical importance of VIPs for evolution of modern humans by demonstrating that 95.9% of all human genes encoding VIPs are components of human-specific genomic regulatory networks that appear to operate in distinct types of human cells ranging from preimplantation embryos to adult DLPFC.

Conclusion

Observations reported in this contribution support the conclusion that modern humans captured unique combinations of human-specific regulatory loci, divergent subsets of which were created within genomic regions highly conserved in distinct species of six NHP separated by 30 million years of evolution. Concurrently, this unique-to-human mosaic of genomic regulatory pathways built on DNA sequences inherited from ECAs was supplemented with 12,486 created *de novo* HSRS. Collectively, these findings suggest that incremental genomic divergence of the human lineage has been continued throughout the primate's evolution concurrently with the emergence and segregation of other nonhuman great ape species. This complex continuous process of genomic divergence was gradually driving speciation of *H. sapiens*, in part, by capturing and retaining the unique mosaic of genomic signatures of ECAs. Genes encoding human VIPs are emerging as principal genomic elements of this continuing evolutionary process, contributing to the molecular, cellular, and organismal adaptations by interactions with myriads of viruses.

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Author Contributions

This is a single author contribution. All elements of this work, including the conception of ideas, formulation, and development of concepts, execution of experiments, analysis of data, and writing of the article, were performed by the author.

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Supplementary Material

Supplementary Data
 Supplementary Figure S1
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