Evolving Brains with New Genes

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The human brain is distinguished by an enlarged volume of cerebral cortex that underlies much of our cognitive abilities (Hill and Walsh, 2005; Lui et al., 2011; Rakic, 2009; Wilsch-Brauninger et al., 2016). The genetic and molecular mechanisms underlying the developmental programs leading to a larger and more complex cortex in the human remain poorly understood but significant progress has been made recently. Because of a relatively high conservation of orthologous gene repertoires among most animal species, the regulatory changes in gene expression have been proposed to constitute the main driving force of evolution (Carroll, 2003, 2008; Shubin et al., 2009). Indeed many human-specific patterns of gene expression have been described during brain development, and most strikingly in the cerebral cortex (de la Torre-Ubieta et al., 2018; Fietz et al., 2012; Johnson et al., 2009; Khaitovich et al., 2005; Kronenberg et al., 2018; Lambert et al., 2011; McLean et al., 2011; Mora-Bermúdez et al., 2016; Nord et al., 2015). On the other hand, recent improvement of technology in genomics has allowed to identify dozens of genomic regions that appeared in very recent hominid evolution, mostly through chromosomal rearrangements known as segmental duplications (Dennis and Eichler, 2016; Dennis et al., 2017; Dougherty et al., 2018; Kronenberg et al., 2018). Segmental duplications tend to occur in specific locations of the genome and create hot-spots for copy number variation (CNV). Interestingly, these CNV hot-spots are frequently associated with congenital diseases, in particular neurodevelopmental and psychiatric conditions, suggesting that human lineage-specific gene duplications may have impacts on brain development, and constitute another significant driver for brain evolution (Coe et al., 2012; Dennis and Eichler, 2016; Mefford and Eichler, 2009; Sudmant et al., 2010). This fits well with a traditional hypothesis that a phenotypic evolution is driven by gene duplication (Ohno, 1970; Ohno, 1999).

Indeed, recent studies identified that several gene duplications present solely in human or in hominid species including human and great apes have pivotal functional roles during cortical development (Charrier et al., 2012; Dennis et al., 2012; Fiddes et al., 2018; Florio et al., 2015; Florio et al., 2018; Florio et al., 2016; Fossati et al., 2016; Ju et al., 2016; Liu et al., 2017; Sporny et al., 2017; Suzuki et al., 2018). Here we summarize these functional studies on human/hominid-specific genes (HS genes) regulating human cortical development, including the latest one NOTCH2NL, as well as SRGAP2, ARHGAP11, TBC1D3 and TMEM14B.

NOTCH2NL

Multiple copies of NOTCH2NL genes in human genome

NOTCH2NL was originally identified as a putative substrate of a serine protease neutrophil elastase in the blood cells and named as "NOTCH2 N terminal like" because of a very close sequence conservation to the N terminal (extracellular) part of NOTCH2 (Duan et al., 2004). At that time and even until 2015, the sequencing status of the 1q21 region of human genome containing NOTCH2NL gene was incomplete because of the highly repetitive nature of this region. The last updates of human reference genome eventually provided whole sequences of this and other repetitive regions (Steinberg et al., 2014). This allowed us to identify that three loci in 1q21 and another locus near NOTCH2 in 1p12 harbor sequences with significant homology to the originally identified NOTCH2NL transcript (Duan et al., 2004). We named NOTCH2NL-A the genomic locus of original NOTCH2NL, and -B, -C and -R the remaining loci (Figure 1A), and validated the transcriptional activity of all four genomic loci (Fiddes et al., 2018; Suzuki et al., 2018). Four NOTCH2NL genes demonstrate almost identical exon-intron structures and very high sequence homology even in introns (>98%), but they nevertheless encode structurally different proteins because of a small number of non-synonymous nucleotide substitutions.

NOTCH2NL-B encodes a longest protein, which contains N terminal signal peptide, six EGF repeats and a unique C terminal region of 24 residues (Figure 1B). The signal peptide and EGF repeats are highly conserved with NOTCH2 but the C terminal residues are unique to NOTCH2NL members. NOTCH2NL-A and C proteins lack a signal peptide and the beginning of the first EGF repeat compared to NOTCH2NL-B and NOTCH2. NOTCH2NL-R has a unique C terminal tail structure that makes the protein highly unstable, and is thus unlikely to be functional (Fiddes et al., 2018). The degree of sequence conservation among NOTCH2NL and NOTCH2 genes is similar to those of recently duplicated

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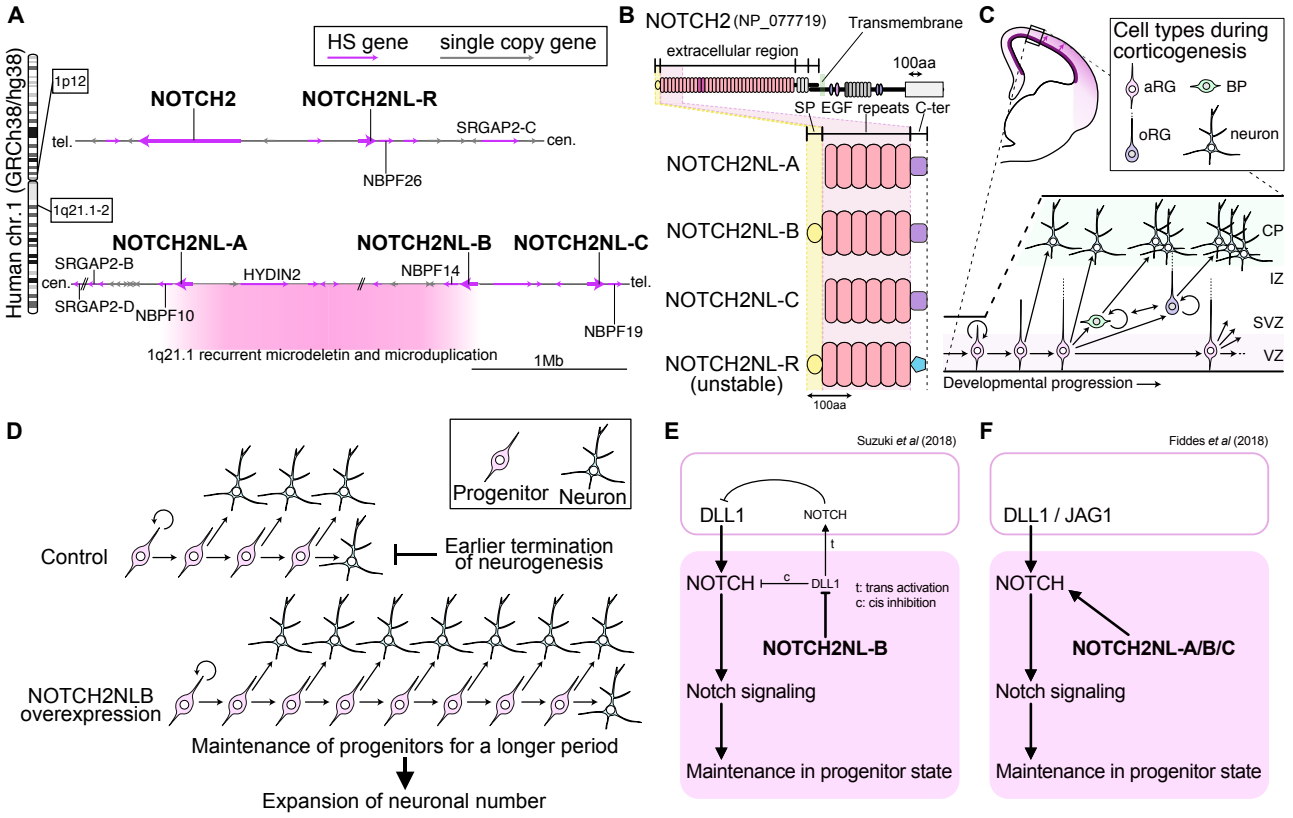

Figure 1. *(A) Genomic positions of NOTCH2NL-family genes and other human/hominid lineage-specific duplicated genes in 1p12 and 1q21. (B) Protein structures of NOTCH2NL-family genes. (C) Major cell types during corticogenesis. NOTCH2NL is heavily expressed in the VZ harboring the aRG and sparsely detected in the outer SVZ containing the oRG. (D) Proposed mechanisms of action of NOTCH2NL-B in cortical neurogenesis; NOTCH2NL-B keeps cortical progenitors for a longer period and it thus elongate the duration of neurogenesis. (E, F) Proposed molecular function of NOTCH2NL in human cortical progenitors. Suzuki et al (2018) demonstrated that NOTCH2NL-B inhibits a Notch ligand DLL1 and its suppressive action on DLL1 increases Notch signal input cell-autonomously (E). Fiddes et al (2018) showed that NOTCH2NL proteins bind to and activate Notch receptors (F). HS genes, human/hominid-specific genes; SP, signal peptide; aRG, apical radial glia; BP, basal progenitor; oRG, outer radial glia; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate.*

SRGAP2 and Hydin family genes (Dennis et al., 2012; Dougherty et al., 2017), which are also duplicated and located in 1q21 (Figure 1A), indicating that these gene families diversified almost at the same evolutionary timing of 3-4 Mya (Fiddes et al., 2018), when the cranial volume of ancestral hominins started to expand at a maximal rate (Du et al., 2018).

Evolution of NOTCH2NL genes

The comparative genomics and phylogenetic analysis point to an evolutionary scenario whereby one copy of NOTCH2NL gene had originated by a partial duplication of the ancestral NOTCH2 gene in the common ancestor of hominids, and subsequently NOTCH2NL genes further duplicated into four copies in the human lineage. It was found that NOTCH2NL-related transcripts are uniquely detected in the human, gorilla and chimpanzee, but not in orangutan and other species. Interestingly, all of NOTCH2NL-related transcripts of gorilla and chimpanzee are distinct from those of human as they are the fusion products of NOTCH2-like sequence and other genes such as PDE4DIP, and contain no reliable open reading frames (Fiddes et al., 2018). The conservation between those of chimpanzee and gorilla indicates that the status in these two non-human hominids must be the state in the common ancestor of chimpanzee, gorilla and human. These suggest that one copy of non-protein coding NOTCH2NL-like fusion gene in the common ancestor was "revived" in the human lineage by gene conversion with NOTCH2, to produce a protein-coding NOTCH2NL gene. Moreover, all the diverse human populations and the archaic humans (the Neanderthal and Denisovan) possess four NOTCH2NL genes, suggesting that the "revived" protein-coding NOTCH2NL genes were amplified into four copies in the common ancestor of modern and archaic humans and maintained subsequently. Therefore the protein-coding NOTCH2NL genes (A/B/C) are indeed human-specific and highly likely to be indispensable for some biological processes given their fixation in the human population.

Expression of NOTCH2NL genes

NOTCH2NL genes were identified as most significantly expressed candidates from a set of recently duplicated genes in the human genome during human fetal

corticogenesis (Suzuki et al., 2018). Also NOTCH2NL (-A) was identified as one of human specific gene most highly enriched in neural progenitors over differentiated neurons (Florio et al., 2018). RNA sequencing and in situ hybridization (ISH) on human fetal cortex clearly showed that NOTCH2NL genes are consistently expressed in neural progenitor cells throughout cortical development. More specifically, RNA sequencing revealed that the longest NOTCH2NL-B exhibits highest level of expression among four NOTCH2NL genes in the earlier phase of corticogenesis and ISH showed that a pan-NOTCH2NL probe recognizing all four NOTCH2NL members marked the expression in the apical radial glia (aRG) in the ventricular zone through all examined developmental stages, and in the outer subventricular zone (oSVZ) harboring the outer/basal RG (oRG/bRG) at the late stage (GW21), which is the neural progenitor subtype prominently observed during the development of highly expanded and folded cortex such as in the human species (Figure 1C) (Suzuki et al., 2018).

Functional roles of NOTCH2NL during the cortical development

NOTCH2NL genes were found to be functionally involved in the expansion of neuron production through direct influence on cortical progenitors. The first line of evidence came from gain-of-function analyses of NOTCH2NL-B (Suzuki et al., 2018). Through an overexpression screen of HS gene in the mouse embryonic cortex, NOTCH2NL-B in mouse embryonic cortex was found to strikingly increase the number of aRG in the VZ. Additionally, NOTCH2NL-B was overexpressed by a very low dosage of lentivirus in a two-dimensional culture of pluripotent stem cell-derived human cortical progenitors, the majority of which are aRG at the stage used in this assay (Espuny-camacho et al., 2013; Suzuki and Vanderhaeghen, 2015). This experiment allowed the quantitative examination of clonal growth derived from a single cortical progenitor with or without NOTCH2NL-B overexpression. Remarkably, the cortical progenitors expressing NOTCH2NL-B produced larger number of progenitors and neurons following three weeks of overexpression (Figure 1D). This effect was then found to be associated with increased self-renewal capacity of progenitors without direct effect on their cell cycle properties. Interestingly the function of NOTCH2NL-B in cortical progenitors requires its EGF repeats but not its C terminal end. Therefore, NOTCH2NL-B keeps progenitors in an undifferentiated state for a longer period through its EGF repeats, and as a consequence, elongates the period of neurogenesis, leading to the expansion of neuronal output. Conversely, and in line with this conclusion, combined loss of function of NOTCH2NL-A /B was found to result in accelerated neuronal differentiation in cortical organoids derived from human pluripotent stem cells (Fiddes et al., 2018).

In addition to NOTCH2NL-B, the shorter paralogs NOTCH2NL-A/C were also studied by overexpression. A NOTCH2NL-A/C expressing mouse ESC line was induced to form cortical organoids and revealed slower progression of differentiation (Fiddes et al., 2018). The overexpression of NOTCH2NL-A/C in the developing mouse cortex was also shown to result in increase in the number of basal progenitors (BP) (Florio et al., 2018). This suggests that different NOTCH2NL genes may have distinct cellular effects on cortical neurogenesis, though this remains to be tested more exhaustively, as it is sometimes difficult to distinguish effects on distinct types of cortical progenitors using in utero electroporation targeting a single stage.

What could be the mechanisms of the effects of NOTCH2NL genes on neurogenesis? Given the well-known roles of Notch signaling during cortical neurogenesis, interactions between NOTCH2NL and the Notch pathway were studied in several ways. NOTCH2NL-B was found to increase the levels of Notch signaling in mouse and human cortical progenitors in a cell-autonomous manner, an effect that required the presence of intact EGF repeats in the NOTCH2NL protein (Suzuki et al., 2018). The molecular substrate of the positive regulation of Notch signaling by NOTCH2NL-B was further explored using heterologous systems, leading to two distinct, yet not mutually exclusive mechanisms. (Figure 1E and F).

On the one hand, NOTCH2NL-B was found to act as a direct suppressor of Notch ligands such as DLL1, which is a major Notch ligand during mammalian cortical neurogenesis (Grandbarbe et al., 2003; Hatakeyama et al., 2014; Kawaguchi et al., 2008; Lindsell et al., 1996; Nelson et al., 2013; Yun et al., 2002) (Figure 1E)(Suzuki et al., 2018). NOTCH2NL-B was found in a protein complex with DLL1, and shown to inhibit its trafficking to the plasma membrane. Moreover NOTCH2NL could suppress the function of DLL1 cell-autonomously during cortical neurogenesis, thereby leading to enhanced self-renewal of aRG cells. The activation of Notch signaling through cell-autonomous DLL1 inhibition could be linked to the mechanism of "cis inhibition" (Miller et al., 2009; Sprinzak et al., 2010), whereby Notch ligands can inhibit Notch receptors when they are found in "cis', i.e. in the same cell. However it could be linked also to the more classical lateral inhibition mechanism, although in this case it is less clear how it would result in increased amplification of progenitors (Kageyama et al., 2008; Kawaguchi et al., 2008; Miller et al., 2009; Yoon and Gaiano, 2005). On the other hand, NOTCH2NL proteins were found to bind to Notch receptors and lead to direct stimulation of Notch signaling activity, including in a paracrine manner as they appear to be secreted in some conditions (Figure 1F) (Fiddes et al., 2018). Future work should determine the relative contribution of these mechanisms in vivo, in relation with the specific NOTCH2NL paralog involved.

Association of NOTCH2NL genes to the diseases showing human brain size abnormality

The functional importance of NOTCH2NL genes was

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also revealed by the analysis of these genes in relation with pathological CNVs. Indeed NOTCH2NL-A/B/C are found within the 1q21.1 region, which has been known to associate with abnormal brain size: 1q21.1 microduplications or microdeletions have been associated with macro- and microcephaly, respectively, together with psychiatric symptoms (Figure 1A) (Brunetti-Pierri et al., 2008; Mefford et al., 2008). More detailed sequence analyses of patients with 1q21.1 CNV now revealed that pathological changes in brain size are strongly associated with the copy number of NOTCH2NL-A and/or NOTCH2NL-B (Fiddes et al., 2018). The study also pointed out the high frequency of gene conversion between two NOTCH2NL genes, suggesting that the presence of highly conserved sequences of NOTCH2NL-A and -B in close positions could be a driving force of further genomic rearrangement. In summary, the positive correlation of copy number of NOTCH2NL genes with brain size further strengthens the functional involvement of NOTCH2NL genes in expanding neuronal number in the human cerebral cortex.

SRGAP2

SRGAP2 was the first example of a human-specific gene duplication functionally relevant to brain development and evolution (Charrier et al., 2012; Dennis et al., 2012). SRGAP2 (hereafter SRGAP2-A) is an evolutionarily conserved gene encoding a protein containing F-BAR, Rho GAP (Rho GTPase activating protein) and SH3 (SRC Homology 3 Domain) domains (Figure 2A), and regulates neuronal morphology, migration and connectivity (Charrier et al., 2012; Fossati et al., 2016; Guerrier et al., 2009). The 5′ part of the ancestral SRGAP2-A gene is uniquely duplicated into three more copies, SRGAP2-B, -C and -D, which are located in 1p11.2 and 1q21.1 loci (Figure 1A). Because of the incomplete nature of duplication, human-specific paralogs encode shorter proteins containing most of the F-BAR but not the Rho GAP domain. Detailed evolutionary analyses suggest that SRGAP2-A was duplicated once around 3.4Mya ago in the human lineage, generating a SRGAP2-B/C-like paralog, and then this paralog has been further duplicated into three paralogs more recently (Dennis et al., 2012). Subsequently, SRGAP2-C has been rapidly accumulating amino acid changing mutations and its protein has become less soluble, which could participate to its ability to inhibit SRGAP2A function (Sporny et al., 2017): because SRGAP2 family proteins can make dimers through their F-BAR domain, SRGAP2-C could decrease the solubility of SRGAP2-A by making a heterodimer. Among the human-specific SRGAP2-paralogs, SRGAP2-C is expressed in the highest level and fixed in the human population, suggesting its functional importance (Charrier et al., 2012; Dennis et al., 2012).

Experimental studies confirmed that human-specific SRGAP2-C has a dominant negative effect on ancestral SRGAP2-A in cortical neurons (Figure 2B) (Charrier et al., 2012; Fossati et al., 2016). Both SRGAP2-A and SRGAP2-C are mainly expressed in postmitotic neurons. SRGAP2-A accelerates the speed of radial migration and enhances maturation of both excitatory and inhibitory synapses in mice. Conversely the overexpression of SRGAP2-C in the mouse cortex reveals similar phenotypes as those following loss-of-function of SRGAP2-A. These data suggest that SRGAP2-C can decrease/delay neuronal maturation and maintain the immature state for a longer period through a dominant negative effect suppressing SRGAP2-A. This effect is striking to put in relation with the known neoteny, i.e. prolonged maturation, that characterizes cortical neurons in certain areas of the human cortex (Defelipe, 2011; Petanjek et al., 2011), and that is thought to be mostly intrinsic to the neurons (Espuny-camacho et al., 2013).

ARHGAP11

ARHAGAP11, whose protein also contains Rho GAP domain, has been duplicated into two genes in the human lineage, and human-specific ARHGAP11-B encodes a truncated protein conserved with N terminal part of evolutionary conserved ARHGAP11-A (Florio et al., 2015). The genomic region encoding the Rho GAP domain in ARHGAP11-A is duplicated in ARHGAP11-B, however the ARHGAP11-B protein does not appear to have Rho GAP enzymatic activity due to a point mutation causing a truncation of C terminal end of the domain by a frame shift afterwards (Figure 2C). Interestingly, the "ancestral" form of ARHGAP11B, which does not carry a mutation and maintains Rho GAP activity, has no impact on corticogenesis, suggesting that functional ARHGAP11-B gene emerged through two steps of evolution: a first duplication from ARHGAP11-A and then a loss Rho GAP activity in the -B paralogue (Florio et al., 2016).

ARHGAP11-B was identified as a gene enriched in human cortical progenitors vs. differentiated neurons (Florio et al., 2015). Overexpression of ARHGAP11-B in the mouse embryonic cortex induces an expansion of BP as well as gross morphological changes of the cortical tissue leading to the folding of the pial surface (Florio et al., 2015). These data suggest that human-specific acquisition of ARHGAP11-B may have a significant impact on the expansion of cortical progenitor expansion, as well as on the patterning of cortical folds.

TBC1D3

TBC1D3 is present uniquely in the hominid species including human and great apes, and its copy number is amplified in human (11 copies in GRCh38/hg38) (Hodzic et al., 2006). TBC1D3 family genes are tandemly aligned in two blocks in 17q12, which is a hot-spot of human segmental duplication associated with prostate tumors, renal cysts, and diabetes (Cardone et al., 2008; Sharp et al., 2006; Stahl and Wainszelbaum, 2009). TBC1D3 is named by the presence of TBC (Tre2-Bub2-Cdc16) domain, which has a Rab GAP activity regulating the intracellular vesicular transport (Figure 2D). TBC1D3

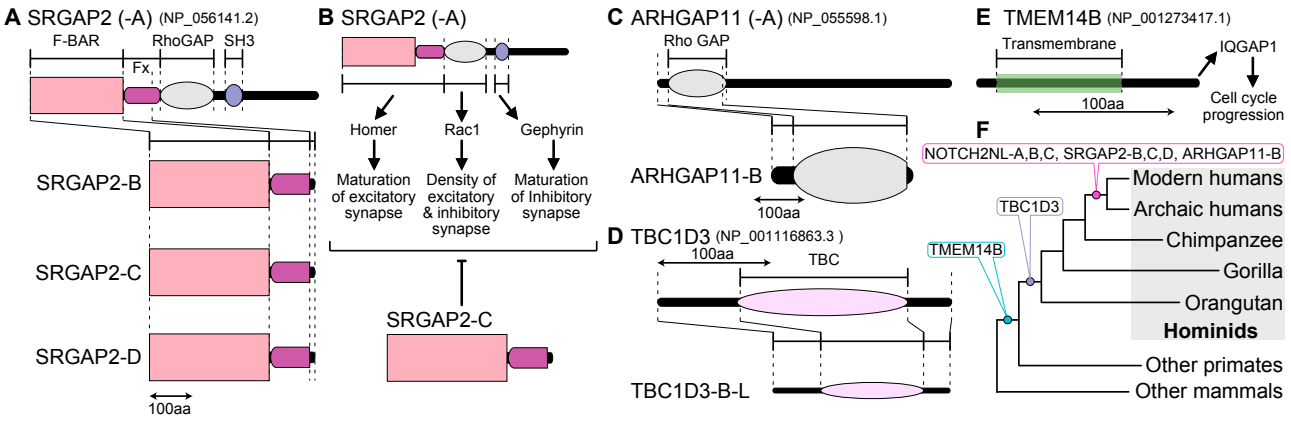

***Figure 2**. (A) Protein structures of SRGAP2-family genes. (B) Proposed molecular mechanism of SRGAP2 (-A) and SRGAP2-C. (C) Protein structures of ARHGAP11-family genes. (D) Protein structures of TBC1D3-family genes. (E) Protein structures of TMEM14B-family genes and its proposed molecular function. (F) The evolutionary timing of the emergence of human/hominid lineage-specific genes.*

was shown to control the activity of Rab5 and regulate the intracellular traffic of the EGF receptor (Frittoli et al., 2008; Pei et al., 2002; Wainszelbaum et al., 2008).

Overexpression of TBC1D3 in the mouse embryonic cortex by focal electroporation and stable knockin transgenic strategy leads to larger number of basally positioned progenitor cells resembling oRG cells, as defined by molecular expression and typical morphology, and also leads to increased folding of the cortical surface (Ju et al., 2016). Conversely, the knockdown of TBC1D3 in human fetal cortical slices leads to decreased delamination of cells from the VZ to SVZ. These data suggest that TBC1D3 may enhance the conversion of aRG to oRG cells by releasing cells from adhesion to the junction structures in the VZ. This hypothesis was supported by the molecular changes observed in gain-of-function study, such as downregulation of N-cadherin in the VZ and the upregulation of oRG gene HOPX. In summary, the evolutionary appearance of TBC1D3 may have contributed to cortical expansion in primates specifically through amplification of oRG progenitors.

TMEM14B

TMEM14B is a primate-specific and rapidly evolving gene (Liu et al., 2017). This gene is exclusively detected in primates (both in the new world monkeys and the old world monkeys including hominids) and the protein structure of TMEM14B is highly divergent between hominids and non-hominids. Furthermore, TMEM14B has a sister gene TMEM14C in the neighboring genomic position, which is conserved among the diverse vertebrates. Therefore, it is likely that the primate-specific TMEM14B emerged by a duplication of TMEM14C early on during primate evolution, although in depth evolutionary analyses of these genes are required to support more firmly this model. TMEM14B encodes a functionally unknown protein containing a single pass transmembrane region (Figure 2E).

Comprehensive screening of marker genes specifically expressed in the oRG during human corticogenesis identified this gene (Liu et al., 2017). Overexpression of TMEM14B in the mouse embryonic cortex induced more proliferating progenitors (both BP and oRG) in the SVZ and increased folding of the cortical surface, both of which are similarly observed following overexpression of ARHGAP11B and TBC1D3. One of the interactors of TMEM14B IQGAP1 enhances cell cycle progression of neural progenitors in the mouse and is activated in the downstream of TMEM14B. Therefore the emergence of TMEM14B in the early evolution of hominids could enhance the induction and proliferation of the SVZ progenitors by activating cell cycle progression through regulators including IQGAP1.

Summary

The genetic association between genomic regions containing recent segmental duplication and human neurodevelopmental diseases led to the hypothesis that the new genes originated by recent duplications may have significant impacts on brain evolution and development (Coe et al., 2012; Grayton et al., 2012; Kaminsky et al., 2011; Malhotra and Sebat, 2012; Mefford and Eichler, 2009; Stankiewicz and Lupski, 2010; Takumi and Tamada, 2018; Weischenfeldt et al., 2013). Five human/hominid-specific genes have now been shown to be involved in brain development so far, and more genes are likely to be proposed (Dennis and Eichler, 2016; Dumas et al., 2012; Florio et al., 2017; O'Bleness et al., 2012; Stahl and Wainszelbaum, 2009; Suzuki et al., 2018). There is a commonality in NOTCH2NL-B/C/D/R, SRGAP2-B/C/D and ARHGAP11-B, in which they are indeed human-specific and may have emerged at the time of cortical expansion during hominin evolution (Figure 2F). In contrast, TBC1D3 and TMEM14B originated much earlier during primate evolution (Figure 2F), and their evolutionary history remains less certain so far. From a mechanistic viewpoint, only SRGAP2 is involved in the neural circuit formation through regulation of neuronal

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maturation and connectivity, while the other four genes may regulate neuronal production and thereby brain size. Due to the limited number of cases, it is currently not possible yet to get the whole picture of brain evolution driven by human/hominid-specific genes. But given the large number of human/hominid-specific duplicated genes that have been identified by comparative analyses of recently updated hominid genomes (Kronenberg et al., 2018; Sudmant et al., 2010), and that show significant levels and dynamic patterns of expression during human corticogenesis (Suzuki et al., 2018), we will definitely identify more examples and thereby determine how much human brain evolution has relied on recent gene duplication.

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