EPIGENETIC MECHANISMS OF NEUROGENESIS IN THE DEVELOPING NEOCORTEX

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EPIGENETIC MECHANISMS OF NEUROGENESIS IN THE DEVELOPING NEOCORTEX

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DNA-Methylation: Master or Slave of Neural Fate Decisions?

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The pristine formation of complex organs depends on sharp temporal and spatial control of gene expression. Therefore, epigenetic mechanisms have been frequently attributed a central role in controlling cell fate determination. A prime example for this is the first discovered and still most studied epigenetic mark, DNA methylation, and the development of the most complex mammalian organ, the brain. Recently, the field of epigenetics has advanced significantly: new DNA modifications were discovered, epigenomic profiling became widely accessible, and methods for targeted epigenomic manipulation have been developed. Thus, it is time to challenge established models of epigenetic gene regulation. Here, we review the current state of knowledge about DNA modifications, their epigenomic distribution, and their regulatory role. We will summarize the evidence suggesting they possess crucial roles in neurogenesis and discuss whether this likely includes lineage choice regulation or rather effects on differentiation. Finally, we will attempt an outlook on how questions, which remain unresolved, could be answered soon.

Keywords: DNA methylation, neurogenesis, DNA modification, epigenomics, epigenetics

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DNA METHYLATION AND OTHER FORMS OF DNA MODIFICATIONS

In 1948, Rollin Hotchkiss used paper chromatography to separate and quantify the components of DNA. To his surprise he detected not only the four nucleo-bases thymine, adenine, cytosine, and guanine, but also a "minor constituent designated epicytosine [with] a migration rate somewhat greater than that of cytosine" (Hotchkiss, 1948). As Hotchkiss had already suspected, epicytosine turned out to be a methylated form of cytosine. Thus, the first description of an epigenomic mark occurred only few years after DNA has been identified as the carrier of genetic information (Avery et al., 1944) and years before its structure has been resolved (Watson and Crick, 1953). Coincidently to these biochemical insights, first conceptual ideas arose attempting, to explain, how a single set of genetic information could give rise to the pleiotropy of cellular phenotypes (Waddington, 1957). From these early days on, epigenomic marks and epigenetic phenotypes have been closely intertwined, which lead to great discoveries but also to misconceptions, such as the perception, these two terms, *epigenetic* ("heritable traits that have their origin not in the DNA sequence") and *epigenomic* ("reversible marks, modifications and features of DNA-implicated in epigenetic traits") would be equivalent.

Today we know that many more DNA modifications exist. Additionally to the mark usually meant by the phrase "DNA methylation" [the methylation of cytosine at position C5

(C5-methylcytosine, 5mC)], the same base can also occur methylated on other positions [e.g., N3-methylcytosine (3mC)]. 3mC is, however, thought to represent rather a product of DNA damage than a bona fide information carrier (Sadakierska-Chudy et al., 2015). But not only cytosine can be targeted by methylation, also adenine [N6-methyladenine, (6mA); (Wu et al., 2016)]. On top, new DNA modifications on the position C5 have been discovered recently, which are generated by DNA demethylation pathways (Booth et al., 2015, **Figure 1**). The first of these 5mC oxidation products to be reported was 5hmC (C5-hydroxymethylcytosine) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009); 5fC (C5-formylcytosine), and 5caC (C5-carboxylcytosine) followed later. Although 5hmC has been described to occur in animal tissues (e.g., mouse brains) already in the 70s (Penn et al., 1972), its relevance was not recognized as it was widely interpreted as a product of DNA damage (Privat and Sowers, 1996). Today we know that 5hmC and 5caC are not necessarily transient marks occurring solely in a sequence of chemical reactions; instead they can appear quite stable at least under some circumstances (Bachman et al., 2014, 2015).

In the following, we will give a short overview about the distribution of DNA modifications and discuss how they are established. We will then present the suggested roles for DNA modifications in gene expression control and review how those have been implicated into regulating lineage decisions during brain development. We finish with re-evaluating the scientific evidence for DNA methylation marks controlling neurogenesis and discuss recent technical advances to study their function at precise sites in the genome. Although we mention several biological processes and all known DNA modifications in this review, we will focus on the role C5-methylcytosine plays in neurogenesis and neuronal maturation.

EPIGENOMIC DISTRIBUTIONS OF DNA MODIFICATIONS

Although DNA modifications are common in bacteria (e.g., m6A, N6-methyladenine; m5C, C5-methylcytosine; m4C, C4-methylcytosine) (Chen et al., 2014), many eukaryotic model systems have no or only traces of 5C methylation. Neither *Saccharomyces cerevisiae* nor *Caenorhabditis elegans* possess this epigenomic mark (Shin et al., 2014). In *Drosophila melanogaster* it is very rare and has only lately been confirmed (Lyko et al., 2000). This remarkable absence of canonical DNA methylation in the three most frequently used genetic model systems might be one reason its universal significance for gene expression and cellular phenotypes is still not known. Consequently, today, 70 years after its discovery, the discussion about how frequent *functional* DNA methylation marks are, is still ongoing (Stricker et al., 2017). In this context it should be mentioned, however, that the genomes of *Caenorhabditis elegans* and *Drosophila melanogaster* have recently shown to possess significant levels of m6A (Greer et al., 2015; Zhang et al., 2015).

In mammalian cells m5C is rather frequent it occurs mainly in pairs of CpGs, in which between 80 and 90% of cytosines are methylated (Hon et al., 2013). Interestingly, those 10–20% CpGs found to be unmethylated are not distributed randomly throughout the genome, but concentrate on so called CpG islands, which mostly coincide with gene promoters. Indeed, around half of mammalian transcripts begin in a CpG island (Bird, 2002). Until recently, it was believed that 5mC occurs in mammalian cells exclusively in the CpG context. That this is not necessarily the case has been shown with the help of new methods for epigenomic analysis of DNA modifications (**Figure 2**): first, in human pluripotent stem cells, in which 25% of m5C occurs at CpH sites (with H = A, C, or T) (Lister et al., 2009), later this has been found also in (mouse and human) brain samples. Other tested somatic cells are, as far as we know, mostly devoid of such non-CpG methylation as far as we know (Xie et al., 2012; Lister et al., 2013). Similarly to non-CpG methylation, C5-hydroxymethylcytosine has also been first described in DNA derived from pluripotent and brain cells (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Especially hypothalamus, cerebral cortex, and hippocampus have been reported to be rich sources of hm5C (Munzel et al., 2010), occurring almost at the rate of one sixth of m5C (Shin et al., 2014), often on enhancers (Yu et al., 2012). N6-methyladenine was found in mouse ES cells, in which

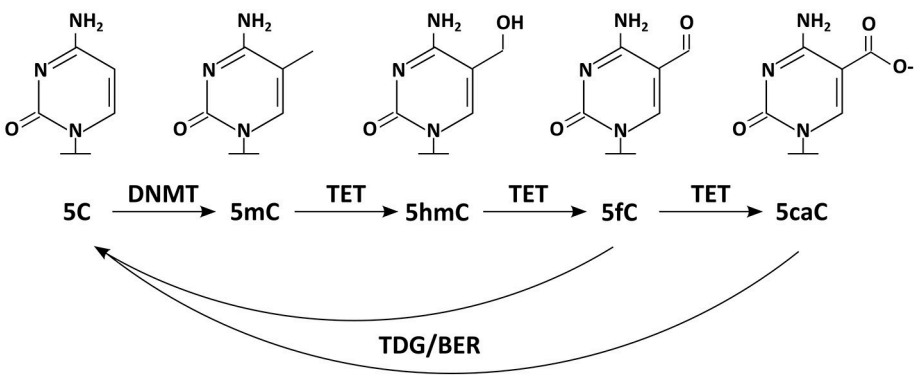

FIGURE 1 | Chemical structures of DNA modifications: five DNA modifications and relevant enzymes are depicted. DNMTs methylate 5C resulting in 5mC, which can be further modified by TET enzymes to 5hmC, 5fC, and 5caC. Enzymes of the TDG/BER pathway have been implicated in removal of the DNA modifications.

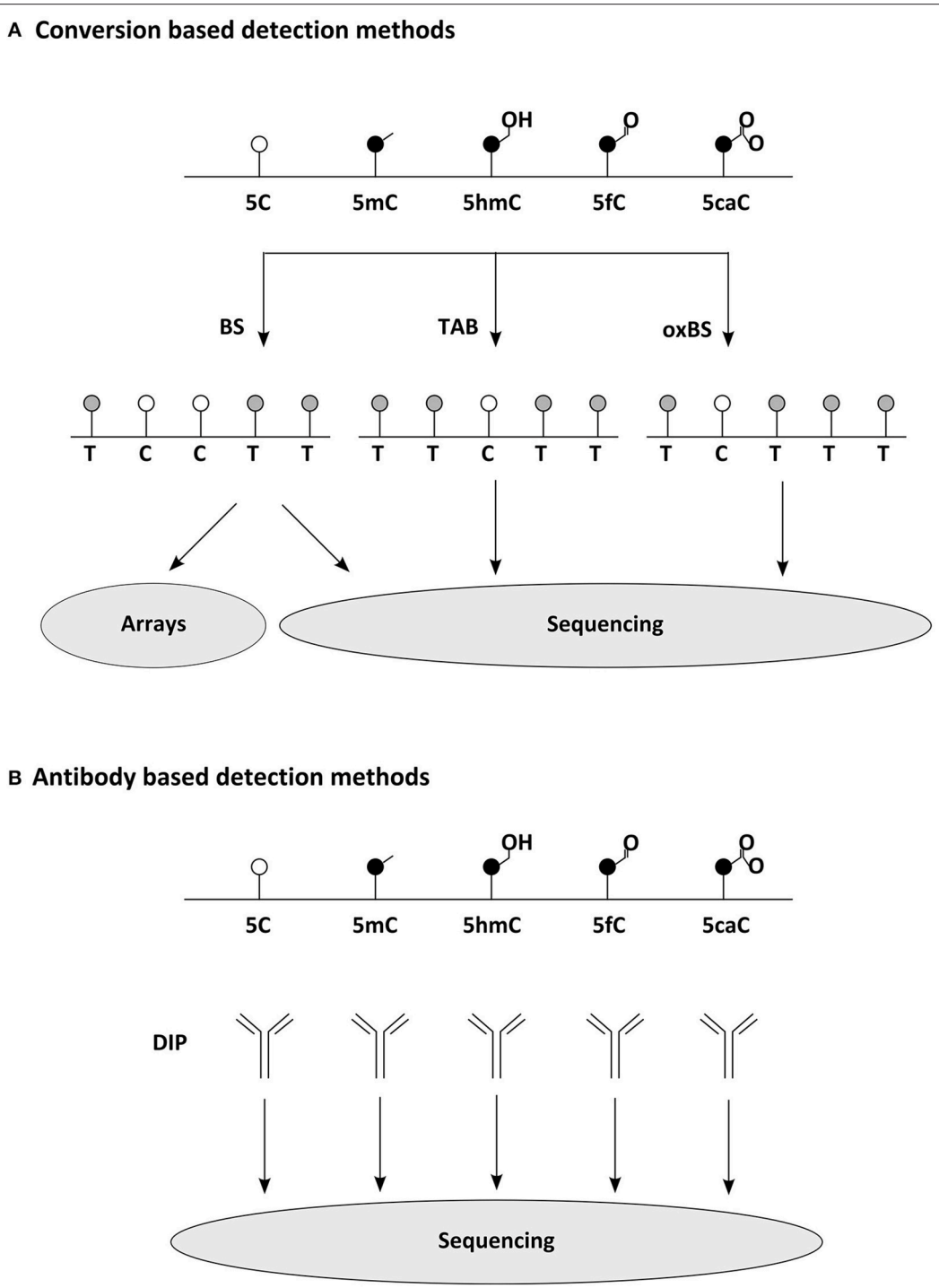

FIGURE 2 | Common methods for widespread detection of DNA modifications. **(A)** Conversion based detection methods. Bisulfite (BS) sequencing, oxidative bisulfite (oxBS) sequencing, and Tet assisted BS (TAB) sequencing enable the epigenomic distinction of 5C, 5mC, and 5hC, while similar techniques separating 5fC and 5caC have been developed as well (Plongthongkum et al., 2014). Sequence below indicates readout expected in NGS. For comprehensive analysis of DNA modifications several detection methods must be combined. **(B)** Antibody based detection methods. DNA Immunoprecipitations (DIP) using modification specific antibodies allow the quantitative analysis of epigenomic distribution (making use of NGS or arrays). meDIP (methylated DNA immunoprecipitation) has been the archetype of this methodology (Weber et al., 2005), but several variants for other DNA modifications have been reported as well recently (comprehensively reviewed in Plongthongkum et al., 2014).

it occurs particularly on young LINE elements; they themselves are enriched on the X-chromosome (Wu et al., 2016).

ESTABLISHMENT AND REMOVAL OF DNA MODIFICATIONS

DNA methylation is catalyzed by a group of enzymes, the DNA methyltransferases, which catalyze the transfer of a methylation residue from S-adenosyl-L-methionine to C5 of cytosine. In mammals these consist of Dnmt3a and Dnmt3b, the *de novo* methyltransferases and Dnmt1, that maintains methylation through the cell cycle by copying CpG methylation patterns from the mother to the newly synthesized strand. Rodents have recently been shown to possess an additional *de novo* DNA methyltransferase, Dnmt3c, evolved through a gene duplication of Dnmt3b (Barau et al., 2016). The mammalian enzyme responsible for adenine methylation is currently unknown. Dnmt3a has been reported to occur in two different forms, due to alternative promoter usage. Although this is not uncommon for protein coding genes, it might be relevant for the methylome, since in cell lines Dnmt3a1 (the full length protein) and Dnmt3a2 (the short isoform) have been reported to occupy very different locations in chromatin. While Dnmt3a1 is found mainly in heterochromatin, Dnmt3a2 is associated with euchromatic regions (Chen et al., 2002). The two remaining members of the Dnmt family, 3L and 2, are paralogs, which either lost enzymatic activity or methylate RNA (Goll et al., 2006; Ooi et al., 2007). While the *de novo* enzymes Dnmt3a and Dnmt3b are necessary to set DNA methylation marks (on CpG and likely also non-CpG positions) (Guo et al., 2014), Dnmt1 ensures its long term inheritance. It is acting on hemi-methylated DNA, occurring after DNA replication (or DNA repair) and transfers a methyl group to the cytosine on the unmethylated strand. Obviously, this depends on the palindromic base composition of CpG di-nucleotides. mCpH sites lack a cytosine residue on the second DNA strand and thus, are certainly asymmetrically inherited to the progeny of pluripotent and neural stem cells. Whether this has however, any functional consequence has remains to be shown.

That Dnmt1 constantly antagonizes passive DNA demethylation is widely accepted. Whether there are any active processes selectively removing DNA methylation marks from certain epigenomic locations has been a controversial issue for a long time. Over the last decades there have been a series of reported findings of DNA demethylases (wittily summarized by Ooi and Bestor, 2008). In contrast to those, recent candidates have been received more favorably (Wu and Zhang, 2010). Today it is widely accepted, that a number of enzymes contribute on the de-methylation of 5mC. First of all the members of the ten-eleven translocation family of enzymes (Tet1, Tet2 and Tet3) oxidize 5mC to 5hmC. But Tet activity does not necessarily stop at this point, as these enzymes can further oxidize 5hmC to 5fC and subsequently to 5caC (**Figure 1**) (He et al., 2011; Ito et al., 2011). These marks are then thought to be lost passively or removed by the thymine DNA glycosylase (TDG), a forerunner of the base excision repair (BER) (Yu et al., 2012; Zhang et al., 2012). Also other proteins and enzymes involved in DNA repair (e.g., GADD45/AID/APOBEC) have frequently been implicated in active DNA de-methylation (Rai et al., 2008; Bhutani et al., 2010, 2011), although their contributions to global methylomic changes are still being discussed (Nabel et al., 2012).

GENERAL CONCEPTS FOR POTENTIAL FUNCTIONS OF DNA MODIFICATIONS

DNA methylation has been implied in regulation of gene transcription already in the late 60s (Harrisson, 1971; Scarano, 1971; Holliday and Pugh, 1975; Riggs, 1975) and often still is; although it has become clear that it likely plays a much less general role than believed originally. But why has DNA methylation become the one epigenomic mark most frequently connected to epigenetic gene silencing in the first place? There are plenty of answers to this question, which we are neither able to discuss fairly, nor to list comprehensively; we think however, that most of the concepts and experimental evidence gained during the decades can be grouped into four types, which we will address below. First, the biochemical features of DNA methylation, its life cycle and inheritance make it a prime candidate for a developmental epigenetic mark; second, global correlations between the presence of DNA methylation and the activity state of DNA in the nucleus do occur; third, DNA methylation is necessary for normal animal development and finally, on some individual model loci a functional effect of DNA methylation on restricting transcription is clearly evident. Hereafter, we will discuss the evidence for the above criteria in establishing the previous model, namely a role of DNA-methylation in repressing alternative fates. Subsequently we will proceed to discuss experimental evidence testing this model. Data from pluripotent stem cell differentiation and mouse models *in vivo* (section Mouse Models) demonstrate that no fate switch to an alternative fate occurs even when most or all of methylation marks have gone (see section Mouse Models). Conversely, phenotypes appear late in brain development, often at postnatal stages, indicating rather that maturation processes are affected (**Tables 1**, **2**).

The Life Cycle of DNA Methylation Levels and Its Inheritance

Since decades it is relatively undisputed that mammalian development has to provide a molecular memory restricting the options of each individual cell to express or adopt cell identities. Until recently, cellular potency was believed to be a one way street, with continuously less choices as development progresses. This has been put in a nutshell by the iconic depiction of the epigenetic landscape conceived by Waddington (1957). Although we know today, that we can revert development (Takahashi and Yamanaka, 2016) or provide direct shortcuts (Masserdotti et al., 2016), the basic question remains: What informs and restricts cellular identity during development? Very early on DNA methylation has been considered to be the prime candidate fulfilling this role. The reason for this has much to do with the dynamics of the mark itself as m5C is a quite stable

TABLE 1 | Published knockout mouse models and their reported phenotype during brain development and in the adult brain.

Gene	Type of Mutant	Cells/Time	Phenotype	References
Dnmt1	Nestin-Cre	NPCs/E12	Premature glial marker induction, neuron loss.	Fan et al., 2005
	CamK-Cre	Neurons	No obvious effect.	Fan et al., 2001
	Nestin-CreER^{T2}	NPC/adult	Decreased survival of hippocampal neurons.	Noguchi et al., 2015
	CamK2a-Cre93	Excitatory neurons in the mouse forebrain	Deficits in learning and memory (+Dnmt3a).	Feng et al., 2010
	Emx1-Cre	Early cerebral cortex	Cortical degeneration, neuronal loss.	Hutnick et al., 2009
	Olig1-Cre	Early OPC progenitors	Oligodendrocyte Maturation defect, ER Stress.	Moyon et al., 2016
	Chx10-Cre	Retinal NSCs	Defective photoreceptor differentiation.	Rhee et al., 2012
	Rx-Cre	Early retina anlage	Photoreceptor degeneration (+Dnmt3a,b).	Singh et al., 2017
Dnmt3a	Nestin-Cre	NPCs/E9-E10	Motor neuron loss.	Nguyen et al., 2007
	Full K.O.		Impaired postnatal differentiation, repression of neurogenic genes.	Okano et al., 1999; Wu et al., 2010
	CamK2a-Cre93	Excitatory neurons in the mouse forebrain	Deficits in learning and memory (+Dnmt1).	Feng et al., 2010
	Plp-CreER(t)	Adult OPCs	Remyelination impaired.	Moyon et al., 2017
Dnmt3b	Full K.O.	E11.5	Rostral neural tube defects.	Okano et al., 1999
Uhrf1	Emx1-Cre	E10–E12	Postnatal neurodegeneration, IAP activation.	Ramesh et al., 2016
Tet1	Full K.O.		Impaired adult hippocampal neurogenesis, Activity induced gene activation affected, Memory formation and extinction affected, When outbred, embryonic lethal (forebrain defects).	Rudenko et al., 2013; Zhang et al., 2013; Khoueiry et al., 2017
MBD1	Full K.O.		Reduced adult hippocampal neurogenesis, Expression of endogenous viruses, Aneuploidy, Impaired LTP in DG.	Zhao et al., 2003
MBD2	Full K.O.		Maternal behavior affected in adult mothers.	Hendrich et al., 2001
MeCP2	Full K.O.		Impaired neuronal maturation in Hippocampus.	Smrt et al., 2007
GADD45b	Full K.O.		Reduced activity induced proliferation of progenitor cells in the hippocampus.	Ma et al., 2009

modification. Many m5C marks are set early in development (some even in the germ line, e.g., the imprints), but can often still be found in somatic cells. This stability is mainly provided by Dnmt1, which faithfully copies the methylation signature from the mother strand after each round of DNA replication. Despite its heritability over cell divisions, DNA methylomes also undergo significant changes during development, both globally and locally. A good example for global methylation changes is occurring during early embryogenesis. Sperm and oocyte each show high overall methylation levels. During the first cell divisions of the zygote, DNA methylation gets remodeled. Both, the maternal and the paternal epigenome get de-methylated, interestingly, however, with very different dynamics. While the paternal genome is immediately actively demethylated, the maternal genome undergoes passive DNA demethylation during continuous DNA replications (Messerschmidt et al., 2014). Thereafter, rapid re-methylation occurs on both genomes with the blastocyst stage, coincidently at the time cells loose totipotency and specify (Reik et al., 2001). Even though such dramatic changes are not recurring later in development; there are plenty local DNA methylation changes occurring in each cellular lineage, resulting in rather specific methylomes (Bernstein et al., 2007), which can not only be used to predict cell type, but even age (Horvath, 2013).

Correlations between DNA Methylation and Transcription

Early on it has been noticed that some DNA methylation changes occurring during development can correlate to transcriptional changes. The most impressive example, maybe because of its scale, is the hypermethylation on CpG island promoters found on inactivated X-chromosomes in female mammalian cells (Lock et al., 1986; Singer-Sam et al., 1990), while the genetically identical copies on the active X-chromosome remain unmethylated. But also promoters of lineage specific genes,

TABLE 2 | Predictions and experimental support of two models for main function of DNA-methylation in neurogenesis.

Predictions model 1	Met (+)/unmet (-)	Predictions model 2	Met/unmet
Early phenotype	–	Late (postnatal) phenotype	+
Appearance of alternative fate	–	Maintenance of immature hallmarks	+
mRNA up-regulation of alternative cell fate genes	–(except GFAP)	Failure to down-regulate progenitor-specific mRNas	+

Model 1: DNA-methylation represses alternative fates vs. Model 2: DNA-methylation represses immature hallmarks to allow full maturation.

like MyoD or various globins, being studied since decades in primary and immortalized cells, have been found to attract DNA methylation when the respective genes get downregulated (Jones et al., 1990). More recently, these concepts have been refined, as it has been reported that those DNA methylation changes occurring during development and correlating to transcriptional differences among tissue types, do rarely involve entire CpG islands, but more often their mere borders (Irizarry et al., 2009). It should, however be mentioned, that most methylated sites in the genome lack predictive value and quite some methylated loci correlate rather to active transcription than gene silencing (Niesen et al., 2005; Irizarry et al., 2009; Bahar Halpern et al., 2014; Zhu et al., 2016).

Genetic Manipulation of DNA Modifications

Further hints into the functional relevance of DNA modifications were given by the generation of genetically modified mouse lines lacking parts of the machinery necessary for their deposition or removal. Thus, it has been shown that the ability to set and propagate DNA methylation marks is absolutely essential to undergo normal embryonic development, since animals lacking the *de novo* DNA methyltransferases Dnmt3a and b or the maintenance methyltransferase Dnmt1 are not viable (Li et al., 1992; Okano et al., 1999). In contrast to this, the consequences of losing members of the Tet family of enzymes seem less severe. ESCs and mice lacking Tet1 [showing a considerable loss of 5hmC (∼20–40%)] are overall viable and only few genes are significantly mis-regulated (Dawlaty et al., 2011), although it has been reported that in non-inbred mice Tet1 is essential for embryogenesis (Khoueiry et al., 2017). A combined loss of Tet1 and 2 lead to a larger number of intermittent phenotypes, but mice lacking both proteins can be born viable and fertile (Dawlaty et al., 2013). Only when all three Tet proteins are depleted differentiation of pluripotent cells is largely impaired possibly due to dysregulation of important developmental genes (Dawlaty et al., 2014). Also depletion of the TDG affects animal development and accumulation of erroneous DNA methylation marks which is compatible with its suggested role in the DNA de-methylation pathway. However, reported changes are comparatively moderate and involve mostly genes known to swiftly attract DNA methylation, like the Hox genes (Cortazar et al., 2011).

Model Systems of DNA Methylation Function

Several model systems have over the years suggested a direct role for DNA methylation in transcriptional regulation. An early example is the *in vitro* methylation of DNA which has been shown to prevent transcription of exogenous copies of globin genes when transfected into mammalian cells (Busslinger et al., 1983). But also the discovery of genomic imprinting, a phenomenon of parental specific gene expression in the embryonic or adult offspring (Barlow et al., 1991; Bartolomei et al., 1991; Ferguson-Smith et al., 1991), delivered much needed evidence. It has been found that loci containing genes with imprinted expression contain a differentially methylated region, established through differences in gametic methylation patterns, which serve as imprinting control regions (ICEs). Genetic approaches resulting in loss of ICEs, imprinted DMRs or global DNA methylation eliminate parental specific gene expression, strongly suggesting a direct functional role for these DNA methylation marks in imprinted gene regulation (summarized in Barlow and Bartolomei, 2014).

THEORETICAL MODELS OF TRANSCRIPTIONAL REGULATION BY DNA MODIFICATIONS

How DNA methylation influences transcription was long elusive. The mechanisms by which DNA methylation of ICEs regulate imprinted gene expression vary and span from controlling expression of long non-coding RNAs (Lyle et al., 2000; Seidl et al., 2006) to interfering with the binding of the common chromatin protein CTCF on insulator elements (Bell and Felsenfeld, 2000). The most popular model for the effect of DNA methylation entails (consistent with active genes containing many 5mC residues in their bodies) that DNA methylation is not directly interfering with transcription. One common assumption is that it is rather the DNA binding affinity of transcription factors which is influenced by DNA methylation (Tate and Bird, 1993; Zhu et al., 2016). While many transcription factors are thought to be impaired by DNA methylation, some special transcription factors bind methylated DNA specifically (**Figure 3**). In this model, the group of proteins involved in gene regulation by DNA methylation can be divided in writer (e.g., the aforementioned DNMTs), eraser (e.g., TET proteins), and reader proteins. The latter can sense the presence of DNA methylation marks and respond with altered DNA binding affinity. Characterization of methyl binding proteins was a tedious task that is still ongoing today. Firstly discovered was a family of transcription factors defined by the possession of a protein domain, shown to prevalently bind to 5mC containing DNA *in vitro*. This so called MBD (methyl CpG binding domain) family of transcription factors has five known members (MBD1, MBD2, MBD3, MBD4, and MeCP2) (Hendrich and Bird, 1998; Zhu et al., 2016). Recent technological development has enabled the genome-wide characterization of their DNA binding features and elucidated methylation dependent and (particularly in the

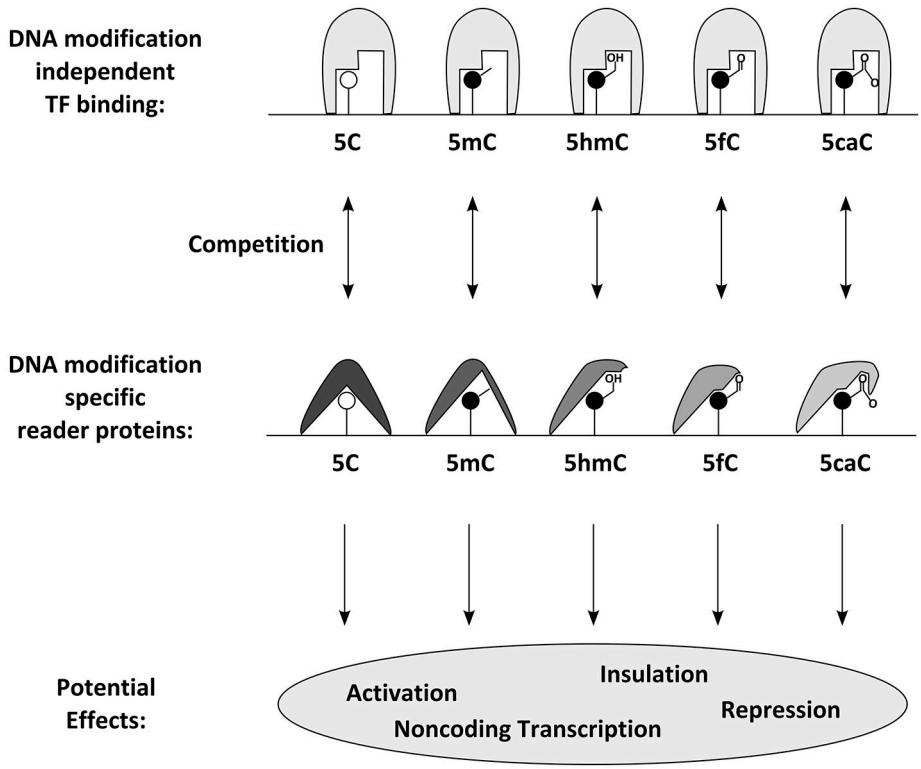

FIGURE 3 | Proposed molecular effects and consequences of DNA modifications: DNA modifications can be specifically bound by reader proteins. Those can either have a direct effect or compete with DNA modification independent transcription factors and thus influence transcription through gene activation, repression, non-coding transcription or insulation.

case of MBD3) independent DNA binding (Baubec et al., 2013). Complementary approaches helped discovering a large series of new candidate proteins that *in vitro* bind at least some of their possible binding motives specifically in the methylated form (comprehensively reviewed in Zhu et al., 2016), including many classical transcription factors like the pioneering factors Klf4 (Hu et al., 2013) and Kaiso (Prokhortchouk et al., 2001). A specialty among known methylation binding transcription factors is Uhrf1, a critical partner of Dnmt1, as it has been shown to recognize *hemi-methylated* DNA in its binding motive (Fang et al., 2016). Recent approaches aiming to discover reader proteins also for other DNA modifications. These efforts resulted in candidate lists for 5hmC- (e.g., Uhrf1 and Uhrf2), 5fC- (e.g., members of the NuRD complex), and 5caC-binding proteins (Frauer et al., 2011; Iurlaro et al., 2013; Spruijt et al., 2013) and indicated that MeCP2 is binding 5mC in both, CpG and CpH sites as well as other cytosine modifications (Mellen et al., 2012; Guo et al., 2014; Gabel et al., 2015). While much needs to be learnt about the *in vivo* roles of these reader proteins and few have been investigated in the context of neurogenesis and DNA methylation so far, recent analysis of Uhrf1 supports its key role in DNA-methylation homeostasis during development and reveals key requirements for later neuronal differentiation processes (Ramesh et al., 2016).

Arguments against Global Roles

Classical epigenetic research on model loci has provided functional examples and mechanistic models; through epigenomic approaches we can acknowledge how complex and dynamic epigenomes present themselves. Thus, to date the most pressing question in epigenetics is not so much, whether chromatin models of epigenetic gene regulation are correct, but rather how ubiquitous their functional relevance is; it is, for example, completely unclear, how many genes (and phenotypes) are significantly regulated by DNA modifications during development and disease. This is especially relevant for DNA methylation, which is rather frequent throughout the genome and has been extensively mapped. Interestingly, however, quite some data argues against the idea that the aforementioned models could be easily translated to any locus or transcriptional unit.

One of the earliest arguments against a ubiquitous role for DNA methylation in gene regulation was the finding that most epigenetically silenced promoters do not appear heavily methylated during development and, related to this, that those that do, often gain DNA methylation after gene expression is lost (Bird, 2002). But there is not only evidence that developmental gene silencing does not depend on DNA methylation changes, recent approaches using cancer tissue derived induced pluripotent stem cells suggest also that removal

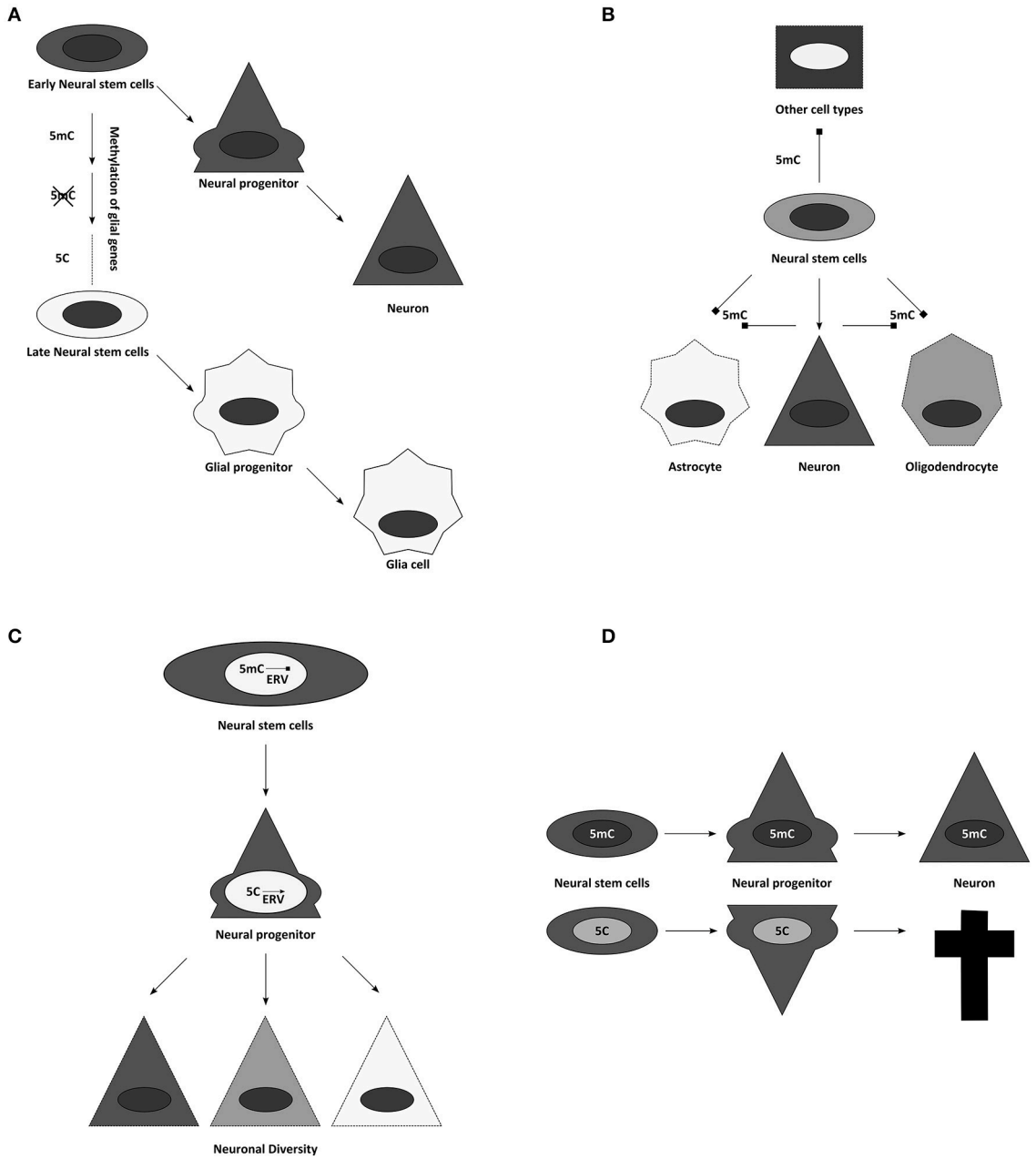

FIGURE 4 | Suggested influences of DNA methylation on neurogenesis. **(A)** Temporal progression of DNA methylomes might influence the potential of neural stem and progenitor cells. **(B)** Cell specific methylomes, here 5mC for simplification, might be responsible for neural cell identities. They could not only influence lineage choices, but might also simultaneously block alternative fates. **(C)** Through controlling activity of transposon derived sequences, DNA methylation has been implicated in contributing to neuronal diversity. **(D)** Global alterations of DNA modifications often result in cell death during differentiation.

of disease associated DNA methylation marks does not influence tumorigenicity of cancer cells significantly (Stricker et al., 2013; Chao et al., 2017). The most convincing argument might, however, come from genetically engineered embryonic stem cells lacking all six active copies of DNA methyltransferases [Dnmt1, Dnmt3a and Dnmt3b, Dnmt3c is not expressed in embryonic stem cells (Barau et al., 2016)]. These triple knockout ESCs have undetectable levels of DNA methylation in their genome. Surprisingly these cells are not only viable and macroscopically normal; they also possess very few mis-regulated genes. Moreover, as subsequently revealed by DNAse hypersensitive site analysis, very few transcription factors change their binding spectrum once DNA methylation is lost in these cells (Domcke et al., 2015). Similarly unexpected is the finding, that the complete loss of TET proteins in differentiating ESCs only results in a moderate increase of 5mC (Dawlaty et al.,

2014). These and other findings suggest that our current models of epigenetic gene regulation might be incomplete and have to be revisited in order to elucidate the function of DNA modifications.

DNA METHYLATION IN NEUROGENESIS

The above data prompt the question of how important DNA methylation would be in development. Development can be seen as a series of cellular fate restrictions and hence DNA methylation has been suspected to be involved in these processes. For example, neural stem cells (NSCs) become progressively restricted in the generation of neurons and later retain only the potential to generate glial cells in most brain regions (**Figure 4A**). Interestingly, the earliest restriction in fate is spatial and special to the nervous system as it is mediated by patterning and occurs in regard to the region the NSCs reside in (Kiecker and Lumsden, 2005). Even prior to the generation of neurons or glial cells, NSCs are already committed to generate region-specific subtypes, e.g., excitatory projection neurons in the cerebral cortex. The second fate restriction is temporal, with neurons of deep cortical layers generated earlier than neurons of the upper layers of the mammalian neocortex (Molyneaux et al., 2007). Indeed, transplantation experiments revealed that early NSCs have the potential to generate neurons of all cortex layers while late NSCs loose the potential to generate deep layer neurons (Frantz and McConnell, 1996), suggesting progressive fate restriction in regard to neuronal subtype specification. As recently confirmed with new tools for clonal analysis (Gao et al., 2014), this occurs via an asymmetric mode of division, by which a NSC generates sequentially different neuronal subtypes sometimes directly and sometimes via intermediate, transit-amplifying progenitor cells. Only after generating all these neurons, the NSC eventually switches to generate glial cells. Thus, the developmental time point predicts whether the stem cell progeny commits to a neuronal or a glial fate (Götz et al., 2016). Not well-understood is, however, how the sequential fate specification is achieved, how the previous fates are repressed and how new lineages are installed.

DNA modifications have been attributed diverse roles in this process. For example, it has been suggested that gliogenesis occurs late, because glial genes are repressed during most of neurogenesis by DNA methylation (Takizawa et al., 2001). This finding could be expanded to the concept that cellular methylomes define cell identities directly (**Figure 4B**). A certain combination of DNA methylation marks might safeguard the faithful expression of adequate cellular programs, while simultaneously repressing inappropriate transcriptional networks (**Figure 4B**) (Lee et al., 2014). Accordingly, temporal changes in DNA methylation may then also allow the sequence of neuronal fates generated during development (**Figure 4A**) (Takizawa et al., 2001; Sanosaka et al., 2009). In agreement with this is the recent finding that human GABAergic interneurons and glutamatergic projection neurons indeed differ vastly in their distribution of DNA modifications (Kozlenkov et al., 2016). This concept of DNA-methylation fixing fates and repressing alternatives predicts ectopic fates to be generated upon interference with DNMTs or TETs, and we will see below that evidence from mouse mutants does not support this prediction.

But first we will consider another important role for DNA methylation, where its repressive role is clearly evident, namely repressing endogenous retroviral elements (ERVs, Groh and Schotta, 2017). In all mammalian cells, the highest proportion of DNA methylation is found on repetitive regions, representing transposons, retrotransposons, or sequences derived from these (Crichton et al., 2014). Therefore, it has been frequently suggested that the main function of DNA methylation might be to silence these intragenomic parasites (Yoder et al., 1997). However, these elements might also have important roles during neurogenesis (**Figure 4C**). On one hand active transposition could contribute (Erwin et al., 2014), on the other many regulatory elements in the genome are evolved from or influenced by endogenous retroviruses (ERVs) domesticated for gene expression (Rebollo et al., 2012; Fasching et al., 2015). Thus, it is not unlikely that genome protective and gene-regulatory roles of DNA methylation follow similar principles. It has been suggested that ERVs contribute to the enormous neuronal diversity and plasticity of the neuronal lineage (Rebollo et al., 2012; Erwin et al., 2014). Epigenetic mechanisms control ERV activity and thus regulate local chromatin remodeling, transcription and potentially their translocation (**Figure 4C**). This would imply an important evolutional role to the pronounced increase of viral elements in the genome during mammalian phylogeny. However, there are only few experimental options to unequivocally assess the function of DNA modifications during cortical development and thus to strengthen these hypotheses, including: the characterization of the availability of the enzymatic machinery during development; the epigenomic analysis of DNA modifications during cortical neurogenesis; and finally, the use of genetically modified mouse models, possessing altered amounts or distribution of DNA modifications.

Expression of the DNA Modifying Machinery during Cerebral Cortex Neurogenesis

The developing as well as the adult brain expresses most proteins implicated in the regulation of DNA modifications. Dnmt1 is ubiquitously present in fetal and full grown mouse brains (Goto et al., 1994); i.e., even in postmitotic neurons and glia. But also the *de novo* methyltransferases are detectable in the nervous system. Dnmt3a is prominently expressed e.g., in neural stem and progenitor cells of the ventricular and subventricular zone of the developing cerebral cortex (E10.5–E17.5), as well as in postnatal neurons and the oligodendrocyte lineage (Moyon et al., 2016), while it is mostly absent in astrocytes (Feng et al., 2005). Dnmt3b can only be detected in the SVZ early (E10.5–13.5), not later during development (E15.5) (Feng et al., 2005; Moyon et al., 2016). The newly discovered rodent Dnmt3c lacks expression in brain as far as we know (Barau et al., 2016). Neural expression of the three Tet proteins has been reported as well (Khoueiry et al., 2017), with Tet3 most dominant and Tet1 most feeble, and

with little modulation between newborn and adults (Szulwach et al., 2011) or brain regions (Szwagierczak et al., 2010), but dynamic changes during oligodendrocyte differentiation (Zhao et al., 2014). Interestingly, Tet3 expression has been found to be amplified by synaptic activity in cultured hippocampal neurons (Yu et al., 2015). Additionally, many methyl binding proteins are present in the nervous system, sometimes in remarkably selective patterns. A typical example is Mbd1, expressed commonly in neurons, but not detectable in astrocytes (Zhao et al., 2003). Thus, the availability and the (at least partially) dynamic expression of the DNA methylation and de-methylation machinery during cell fate commitment and differentiation is indeed in line with potential roles for this epigenomic mark in these processes.

Epigenomic Distribution of DNA Modifications during Neurogenesis

First indications about the cell type specific distribution and dynamics of DNA methylation during neurogenesis (and its relation to other epigenomic marks and transcription factor binding) have been gained from differentiation of embryonic stem cells or neural progenitor cells (Meissner et al., 2008; Stadler et al., 2011). Profiling of pluripotent and neural stem cells revealed for example, that regions with low methylation show the most dynamic DNA methylation changes during development. Moreover, these are frequently overlapping with regulatory sequences of important cell fate factors (like Pax6) and are dependent on transcription factor activity in some tested cases, as DNA binding (of the neural repressor REST for example) is necessary and sufficient to evade high DNA methylation levels on its binding sites (Stadler et al., 2011).

The recent development of affordable technology for DNA methylome analysis made the investigation of human brain samples practicable as well (**Figure 2**). Large cohorts of human prefrontal cortex samples revealed the dynamic changes occurring during development and aging of the brain (Hernandez et al., 2011; Numata et al., 2012; Jaffe et al., 2016). These studies indicate that, although methylation differences are occurring in different scales, either at individual CpGs, at differentially methylated regions (DMRs) or at even larger domains, most changes are established during development and childhood, while methylomes are less plastic later in life. These findings likely point to differences in cellular composition rather than developmental dynamics and thus demonstrate the predicaments when heterogeneous cell populations are examined. Analysis of more homogeneous cell populations allow deeper insights, e.g., revealing how in the developing and adult frontal cortex 5mC patterns distinguish cell types (Lister et al., 2013) or that methylated CpH sites are almost absent from (NeuN negative) non-neuronal cells (Lister et al., 2013). Instead, CpH methylation is generated *de novo* during neuronal maturation both in mouse and human cells (Lister et al., 2013; Guo et al., 2014) and parallels synaptogenesis and neuronal diversity (Lister et al., 2013; Mo et al., 2015). Remarkably, studies also indicate that methylation marks occurring in regulatory regions are more indicative of transcriptional repression when falling on CpH rather than on CpG sites (Mo et al., 2015). The first characterization of 5hmC dynamics was linked to the development of reliable methods mapping this mark epigenome-wide (**Figure 2**). Using hMeDIP for example has shown that in contrast to 5mC, the cellular amount of 5hmC is significantly increasing when neural stem and progenitor cells are differentiating to neurons (Hahn et al., 2013). A similar developmental dynamic has also been detected during *ex vivo* analysis of mouse cortices and human brain samples (Szulwach et al., 2011; Lister et al., 2013; Wen et al., 2014; Vogel Ciernia and LaSalle, 2016). Interestingly, newly acquired 5hmC often associates with regulatory elements of neuronal genes (Szulwach et al., 2011; Wang et al., 2012) and are solely detectable at CpG sites (Lister et al., 2013). Bisulfite sequencing of DNA derived from adult mouse dentate granule neurons before and after synchronous neuronal activation *in vivo*, revealed that some DNA methylation marks do not behave as stable as commonly expected and rather suggested that around 1% of analyzed 5mC sites fulfill the criteria of activity induced de-methylation (Guo et al., 2011) with yet elusive function. Taken together profiling of DNA methylation in mammalian brain cells from both *in vitro* and *ex vivo* models indicate that diverse cell populations differ significantly in their methylome and that these changes can swiftly emerge at meaningful sites, indicating that they could contribute to shape cellular functions.

Mouse Models

Genetically modified mouse models of all known writers of the DNA methylation machinery have been generated to functionally test the global relevance of this epigenomic modification. The full knockout for the *de novo* methyltransferase Dnmt3a for example appears overall normal at birth (Li et al., 1992; Okano et al., 1999), but mice die 4 weeks after birth due to multiple developmental defects (Okano et al., 1999). It has been suggested that this is in part due to a disturbed neurogenesis in the SEZ of the forebrain and the hippocampal dentate gyrus, as NSCs loose DNA methylation on the gene bodies of neuronal genes and fail to activate those during differentiation (Wu et al., 2010). While defects in adult neurogenesis are unlikely to cause death of the entire organism, these data did reveal a key role of DNA-methylation in NSC differentiation with a clear decrease in postnatal neurogenesis. The authors also suggest that this was due to an increase in gliogenesis and hence a fate switch, but this is less clear as postnatal and adult NSCs also express astroglial markers, such as GFAP and some level of S100b (Beckervordersandforth et al., 2010), making it impossible to decide whether the increased cell population are NSCs or astrocytes. Conditional deletions of Dnmt3a in the developing nervous system (Nes1-Cre) have been reported to have a shortened lifespan as well, which has been attributed to postnatal motor neuron loss (Nguyen et al., 2007). Mouse embryos lacking Dnmt3b exhibit multiple developmental abnormalities, including rostral neural tube defects, and are not delivered to term (Okano et al., 1999). Thus, normal neural development is (at least partially) dependent on the presence of both *de novo* methyltransferases. Although full knockouts of

Tet1 have been reported to be born overall normal (Dawlaty et al., 2011), recently new mutant alleles have been generated that are lethal during embryogenesis when outbred, at least partially due to "deformities in forebrain development associated with incomplete closure of the anterior neuropore" (Khoueiry et al., 2017).

Dnmt1 full knockout embryos have strong phenotypes and are early embryonic lethal (Li et al., 1992), while conditional deletions show a remarkably specific effect. Depletion of this methyltransferase in postmitotic neurons, using the CamK-Cre line, neither affected DNA methylation levels significantly, nor influenced postnatal survival of the animals, raising questions, which role Dnmt1 expression might play in postmitotic cells (Fan et al., 2001). Deletion of Dnmt1 in neural progenitors during development results in animal death (hours after birth in animals with high recombination rates; and significant neuronal loss in animals with reduced Cre activity) (Fan et al., 2001). Although after deletion of DNMT1 in the developing CNS up-regulation of some glial genes, like GFAP, have been observed, this occurred only at the end of neurogenesis and hence onset of gliogenesis *in vivo*, despite much earlier loss of DNMT1 using the Nestin-Cre line (Fan et al., 2005). Importantly, genomewide expression analysis should reveal best whether true fate changes occur—nowadays ideally done at single cell level. However, RNA-seq data do not reveal any indication for a fate switch when done early (E15 cortex $Emx1^{Cre}$/Uhrf1, Ramesh et al., 2016) and highlight rather neuronal death as the main phenotypic consequence of hypomethylation in the brain and the GFAP increase as an indication of gliosis due to postnatal neuronal cell death when done later (Hutnick et al., 2009; Ramesh et al., 2016). Cortical degeneration appears not to be a consequence of altered fates, but rather due to another key role of DNA methylation: to stably silence repetitive elements [for Dnmt1 in particular ERVs like the intracisternal A-particle retroviruses (IAPs); (Walsh et al., 1998; Hutnick et al., 2009)]. Conditional deletions of the Dnmt1 partner Uhrf1 during cortical neurogenesis confirmed these findings and showed that despite profound demethylation primarily IAPs were de-repressed and that this is accompanied by postnatal neuronal degeneration (Ramesh et al., 2016). Interestingly, IAPs were up-regulated already at E12, yet neuronal death occurred only after the first postnatal week when neurons become functionally active. Indeed, many genes encoding for proteins involved in neuronal activity were dysregulated supporting again a role of DNA methylation in regulating neronal differentiation processes. Notably, despite loss of at least 25% of global DNA methylation no ectopic fates such as premature gliogenesis were observed in these mutants. Moreover the data indicated, that it is not the loss of DNA methylation, but rather the gain of 5hmC, which results in IAP activation during brain development, since the process can be rescued by simultaneous reduction of Tet2 and Tet3 (Ramesh et al., 2016). Thus, depleting key enzymes for DNA methylation maintenance or removal throws the epigenome out of balance, resulting in rather specific consequences for neuronal maturation and survival (**Figure 4D**). Similar to the phenotypes observed in brain development, deletion of DNMT1 in the retina and in oligodendrocyte progenitor cells show profound defects in the final maturation of photoreceptors and oligodendrocytes, respectively, but no generation of alternative fates (**Table 1**).

Development continues to some extent also in the adult brain, both in adult neurogenesis but also in the ongoing synaptic plasticity that constantly re-forms new synaptic connections. DNA modifications have also accredited functional roles in these processes including information storage and providing (in adult NSC niches) new mature neurons (Ninkovic and Götz, 2013). In the late 60s, an open debate was started, how neurons would be able store memory information for life, while the stability of the molecular building blocks of these cells is many orders of magnitudes shorter. Interestingly, DNA modifications, due to their mode of inheritance, have been frequently suggested as prime candidates for memory storage (Griffith and Mahler, 1969; Crick, 1984). Already in 1969 J.S. Griffith suggested "that the physical basis of memory could lie in the enzymatic modification of the DNA of nerve cells. It might be worth looking to see if there are unusual bases specific to nerve cell DNA, but in the absence of evidence to that effect, a plausible suggestion would be that the modification consists of methylation (or demethylation)" (Griffith and Mahler, 1969). During the last decades this concept has been regularly revived (Meagher, 2014). Indeed we know now, that the brain is, compared to other organs, especially active in remodeling DNA methylation patterns and a prominent source of scarce DNA modifications. For example, non-CpG methylation is common in neurons in contrast to other differentiated cell types (Guo et al., 2014), its occurrence is highly linked to the neuronal expression of Dnmt3a, as knockdown of this *de novo* methyltransferase abolishes CpH methylation (but not CpG methylation, which is mainly dependent on Dnmt1) (Guo et al., 2014). However, maybe the most surprising results stem from genetically modified, overexpression or knockdown mouse models of writer, reader, and eraser proteins of DNA modifications, resulting either in phenotypes affecting memory formation or consolidation [Tet1 (Kaas et al., 2013; Rudenko et al., 2013; Zhang et al., 2013), Dnmt1, Dnmt3a, and Dnmt3a2 (Miller and Sweatt, 2007; Feng et al., 2010; Oliveira et al., 2012, 2016)], emotional or maternal behavior [Dnmt3a (LaPlant et al., 2010), Mbd2 (Hendrich et al., 2001)], LTP [Mbd1 (Zhao et al., 2003)], or adult neurogenesis in the hippocampus [GADD45b (Ma et al., 2009), Tet1 (Zhang et al., 2013), Mbd1 (Zhao et al., 2003), Mecp2 (Smrt et al., 2007)] indicating that neuronal maturation or specific neuronal functions in particular neuronal plasticity might indeed be dependent on normal availability of the DNA modification machinery.

Human Model Systems of DNA Modifications and Brain Diseases

Interestingly, several neurodevelopmental disorders have also been linked to proteins involved in the regulation of DNA modification emphasizing their relevance in cerebral cortex development. Rett syndrome, a rare X-linked postnatal neurological disorder, was the first among this group, when it was discovered in 1999 that it is caused by mutations in the DNA methylation binding protein MeCP2 (Amir et al., 1999). In the meantime many more diseases have been added: for

example, the *immunodeficiency, centromeric region instability, and facial anomalies syndrome* (ICF), caused by mutations in DNMT3B is often associated with mild cognitive and neurologic defects (Hagleitner et al., 2008). Similarly, childhood overgrowth syndrome, a developmental disorder resulting (amongst other phenotypes) in intellectual disabilities, is caused by mutations of the DNMT3A gene (Tatton-Brown et al., 2014). Moreover, it has been recently shown that brain tumors, use stem cell factors to interfere with astrocyte differentiation and the DNA methylation machinery (Bulstrode et al., 2017). Indeed some brain tumors are even driven by mutations in the isocitrate dehydrogenase 1 (IDH1). Mutations of this enzyme result in tumor cells which contain severely elevated global levels of DNA methylation. The reason for this is the abnormal accumulation of 2-hydroxyglutarate (2-HG), a powerful inhibitor of TET activity (Turcan et al., 2012). Thus, DNA methylation clearly also affects human NSC differentiation even though much more needs to be learnt about the exact mechanisms.

OUTLOOK

Taken together, the above mentioned experimental tests on the role of DNA methylation in cerebral cortex development do not lend much support to the model that it serves to repress alternative fates (**Table 2**). Besides GFAP up-regulation (Kim et al., 2016) there is not much evidence for aberrant glial fate instruction, including in genome-wide expression analysis (Hutnick et al., 2009; Ramesh et al., 2016), and no ectopic fate choices have been observed in any of the above mutants. Rather, a common theme is cell death, due to either the failure to fully differentiate and/or to repress repetitive elements (Ramesh et al., 2016), quite similar to what had been described in the postnatal retina (Rhee et al., 2012). Thus, the hypothesis that DNA methylation represses alternative fates has to be questioned, while the role in differentiation receives more support. Indeed, in the few studies of mouse mutants that examined the transcriptome, many aspects of immature cells, such as cell proliferation, fail to be repressed at later stages along with the failure to up-regulate genes involved in synaptic maturation. According to Wu et al., Prc2 mediated mechanisms could be involved in these processes as they showed that Dnmt3a-mediated DNA methylation adjacent to H3K4me3 high promoters interferes with Prc2 binding and H3K27me3 and thereby mediates up-regulation of neuronal progenitor genes (Wu et al., 2010). In addition or alternatively, Tet-mediated roles could be involved as described above from the Uhrf1 study (Ramesh et al., 2016). However, much remains to be understood about the repressive function of DNA-methylation in regard to differentiation and neuronal maturation. This is particularly evident from the poor correlation between changes in DNA-methylation and transcription. Further follow-up studies on the transcriptional changes that are crucial for the phenotypes aiming to correlate these to epigenetic mechanisms will hold the key to better mechanistic understanding of the mouse mutant phenotypes.

Indeed, so far virtually none of these phenotypes have been linked with precise sites in the genome being de-methylated, but always groups of sites, regions, or genes. This can be confounding as for example many methylation marks might have opposing roles in the body, such as maternal and paternal imprints that, respectively, reduce or activate growth (Barlow and Bartolomei, 2014). Thus, to elucidate which roles the epigenome plays in the brain, first we have to differentiate essential from specific and both from bystander marks, dissecting thereby secondary from causal DNA modifications. This can now be done by new options, which allow manipulating individual DNA modifications to evaluate their immediate causal effect on transcription and cell behavior. This new experimental field, collectively termed epigenome editing (Stricker et al., 2017), promises to deliver a better understanding of the role DNA modifications play during cortex development. Epigenome editing is mainly based on modified versions of the bacterial CRISPR system, allowing to precisely target any genomic locus in any cell. Fusing DNA modifying enzymes to dCas9 (the nuclease deficient targeting protein) has been proven to locally set or remove DNA modifications. So far, Tet1 and Dnmt3a have been used most prominently to show that DNA methylation on the accurate locus can indeed influence transcription of a gene close by (Amabile et al., 2016; Choudhury et al., 2016; Liu et al., 2016; Xu et al., 2016). However, we are still far from a comprehensive view about gene regulation by DNA modifications, especially during brain development, but studies successfully manipulating histone marks indicate this will be promising approach to study neurogenesis (Albert et al., 2017). Thus, tools of epigenetic engineering allowing methylating or demethylating specific genomic sites to investigate their function directly will help to causally link methylation of specific genes with functional phenotypes. This aim is more relevant than ever, as epigenome wide association studies (EWAS) suggest new targets for a variety of diseases on a regular basis (Stricker et al., 2017).

CONCLUSION

While much remains to be done, experimental tests propose already a revision of the concept that DNA methylation would repress alternative fates (**Tables 1**, **2**). Rather DNA methylation appears generally required for repression of ERVs, even though with striking cell type specificity. A further general concept that emerged is its role in orchestrating cell differentiation, but within a given lineage (neurons, oligodendrocyte progenitors, **Table 2**). The involvement of splicing as effector of changes in DNA methylation is an exciting new angle to pursue with more precise epigenetic engineering tools. Distinguishing essential from specific, and causal from secondary marks will be essential for neuro-epigenetics. New approaches promise to answer long outstanding questions and will likely facilitate the discovery that DNA modifications might have new unexpected roles in the brain.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Exploring the Complexity of Cortical Development Using Single-Cell Transcriptomics

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The developing neocortex in the mammalian brain is composed of multiple cell types including apical progenitors (AP), basal progenitors (BP), and neurons that populate three different layers, the ventricular zone (VZ), the subventricular zone (SVZ), and the cortical plate (CP). Despite recent advances, the diversity of the existing cell populations including those which are differentiating and mature, their biogenesis and the underlying gene regulatory mechanisms remain poorly known. Recent studies have taken advantage of the rapidly emerging single-cell technologies to decode the heterogeneity of cell populations at the transcriptome level during cortical development and their molecular details. Here we review these studies and provide an overview of the steps in single-cell transcriptomics including both experimental and computational analysis. We also discuss how single-cell genomics holds a big potential in future for brain research and discuss its possible applications and biological insights that can be achieved from these approaches. We conclude this review by discussing the current challenges in the implementation of single-cell techniques toward a comprehensive understanding of the genetic and epigenetic mechanisms underlying neocortex development.

Keywords: epigenetics, neurogenesis, development, neocortex, stem cells

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DECIPHERING THE GENE REGULATORY NETWORK UNDERLYING DEVELOPMENT OF NEOCORTEX USING SINGLE-CELL GENOMICS

The mammalian brain is one of the most complex organs in the body and plays a fundamental role in higher cognitive function (Striedter, 2005). During brain development, the transition of proliferative and multipotent neuroepithelial cells to fully differentiated neurons is called neurogenesis (Urban and Guillemot, 2014). The neurogenesis mainly occurs between embryonic day (E) 11–17 in mouse and gestational week (GW) 8–28 in human (Malik et al., 2013; Taverna et al., 2014; van den Ameele et al., 2014). During this period, neuroepithelium transforms into three different layers including the ventricular zone (VZ), the subventricular zone (SVZ), and the cortical plate (CP) by the sequential events of differentiation (Gotz and Huttner, 2005). Each of the germinal zones is known to be composed of distinct cell types such as apical progenitor cells (AP), basal progenitor cells (BP), and neurons, whose location of mitosis, polarity, and proliferative potential are different (Taverna et al., 2014). Especially, APs include three subtypes such as neuroepithelial cells, derivative apical radial glia (aRG) which express astroglial markers, and apical intermediate progenitors

(aIPs) which undergo one round of symmetric neurogenic division. BPs can be further categorized into proliferative basal radial glia (bRG) and neurogenic basal intermediate progenitors (bIPs) whose diversity and composition determine the rate of neuron production and cortical expansion across the species (Florio and Huttner, 2014; Taverna et al., 2014; Dehay et al., 2015). Those progenitor cells differentiate into neurons and constitute the diverse laminar (L1–L6) and areal identities in the cortical plate as a spatiotemporal manner to establish specialized function and neuronal circuit formation (Franco and Muller, 2013; Jabaudon, 2017). During this process, some of the neural progenitors in the germinal zones are differentiating and migrating into the CP in the early stage of neurogenesis, while some of them are still dividing and proliferating until the later time point of neurogenesis. In addition, while they are neurogenic at the early stages of cortical development, they gradually switch to astrogliogenesis in the later stages. This shows that the cell fate commitment of the progenitor cells is highly dynamic and tightly regulated. How many cell fates exist during neurogenesis and how such dynamic cell fate changes are programmed in the gene regulatory network within individual cells is not well-understood.

Current technological advances in single-cell genomics enabled us to isolate individual cells from complex tissues and explore their molecular profiles at the single cell level, which offers the possibility to characterize the cellular heterogeneity and subpopulations (Yuan et al., 2017). Recently, these technologies were implemented to investigate multiple cell types of neural progenitors and mature neurons generated during neurogenesis (Poulin et al., 2016; Telley et al., 2016). In this mini-review, we introduce current workflow in single-cell genomics, biological insights obtained by single-cell neurogenesis studies, and future challenges in the application of single-cell technologies toward a comprehensive understanding of the genetic and epigenetic mechanisms at the single-cell resolution.

CURRENT WORKFLOW OF SINGLE-CELL TECHNOLOGIES IMPLEMENTED IN THE NEUROGENESIS RESEARCH

Current workflow of single-cell genomics is organized in the set of steps: defining the biological system, appropriate isolation of relevant single cells, sequencing library preparation, high-throughput sequencing, and computational analysis (**Figure 1**).

The two very popular biological systems to investigate cortical development using single-cell genomics have been embryonic cortical tissues and brain organoids. (**Figure 1,** Step1) For example, single-cell studies have been performed in E13.5 and E14.5 cortex from mouse brain (Fan et al., 2016; Telley et al., 2016) and micro-dissected cortex from 14 to 16 GW and 16 to 18 GW from human fetal brain (Camp et al., 2015; Pollen et al., 2015; **Table 1**). As an alternative method to overcome the limited accessibility to the fetal human tissues, researchers have developed 3D *in vitro* culture "brain organoid" using human pluripotent stem cells, in which cells self-organize into complex structures. In this technology, inductive signaling molecules mimic endogenous patterning drive dorsal and ventral forebrain differentiation which generate proliferative ventricular-like zones containing neural stem cells that produce a multilayered cortical-like structure expressing markers of deep- and superficial-layer neurons (Di Lullo and Kriegstein, 2017). The brain organoid imitates the features of the developing human brain *in vivo* (Kelava and Lancaster, 2016), and it has been successfully used for single-cell transcriptome studies. For example, Camp et al. profiled single-cell transcriptome from 333 cells of human brain organoid and found that human cerebral organoids recapitulate gene expression programs of fetal neocortex development (Camp et al., 2015). Quadrato et al. profiled transcriptome from 80,000 single cells from 31 human brain organoids and showed that organoids could generate a broad diversity of cell types that reflect endogenous classes (Quadrato et al., 2017). Given their ability to recapitulate the cell diversity of the cortical development, the brain organoids in combination with single-cell techniques will continue to provide useful information on human neurogenesis and neurodevelopmental disorders (Bershteyn et al., 2017; **Table 1**).

To isolate individual cells (**Figure 1,** Step2), Fluorescence-activated cell sorting (FACS) (Fan et al., 2016; Telley et al., 2016) and microfluidic systems (Fluidigm C1) (Camp et al., 2015; Pollen et al., 2015; Mora-Bermudez et al., 2016; Bershteyn et al., 2017) have been most widely applied. FACS isolate cells of interest using the targeted cell-surface markers so that it provides the possibility to enrich for fluorescently-labeled cells of interest as described before (Telley et al., 2016). The microfluidic system such as Fluidigm C1 uses the microfabrication techniques and microfluidic chambers to isolate single-cells (Saliba et al., 2014). On the other hand, Drop-seq was currently developed as microdroplet system using microfluidic technologies to isolate single cells in aqueous droplets in a non-aqueous suspension which serve as individual nanoliter-scale aqueous reaction chambers for reverse transcription of PCR (Macosko et al., 2015; Poulin et al., 2016). Drop-seq was recently implemented for the study of 80,000 cells from human brain organoid (Quadrato et al., 2017). It seems that for hundreds to thousands of cells, FACS or microfluidic system (Fluidigm C1) is recommended for cell isolation, while to scale-up to thousands to tens of thousands of cells, Drop-seq technique is suitable (Poulin et al., 2016) though it has limitation of low gene-per-cell sensitivity compared to other scRNA-seq methods (Ziegenhain et al., 2017).

Following single cell isolation, cells are lysed and the RNA is captured for reverse transcription into cDNA to construct sequencing library. Previous single-cell genomics applied in the neurogenesis research mostly implemented template-switch-based protocols including Smart-seq and DROP-seq (**Figure 1,** Step3) (Camp et al., 2015; Pollen et al., 2015; Fan et al., 2016; Mora-Bermudez et al., 2016; Telley et al., 2016; Bershteyn et al., 2017; Quadrato et al., 2017). In case of Smart-seq, commercially available Smart-seq kit (Clontech) is used to generate full-length double-stranded cDNA which is converted into sequencing libraries by tagmentation (Nextera, Illumina) (Ziegenhain et al., 2017). Smart-seq2 protocol is similar to Smart-seq which generates full-length libraries, but it had improved reverse transcription, template switching, and pre-amplification

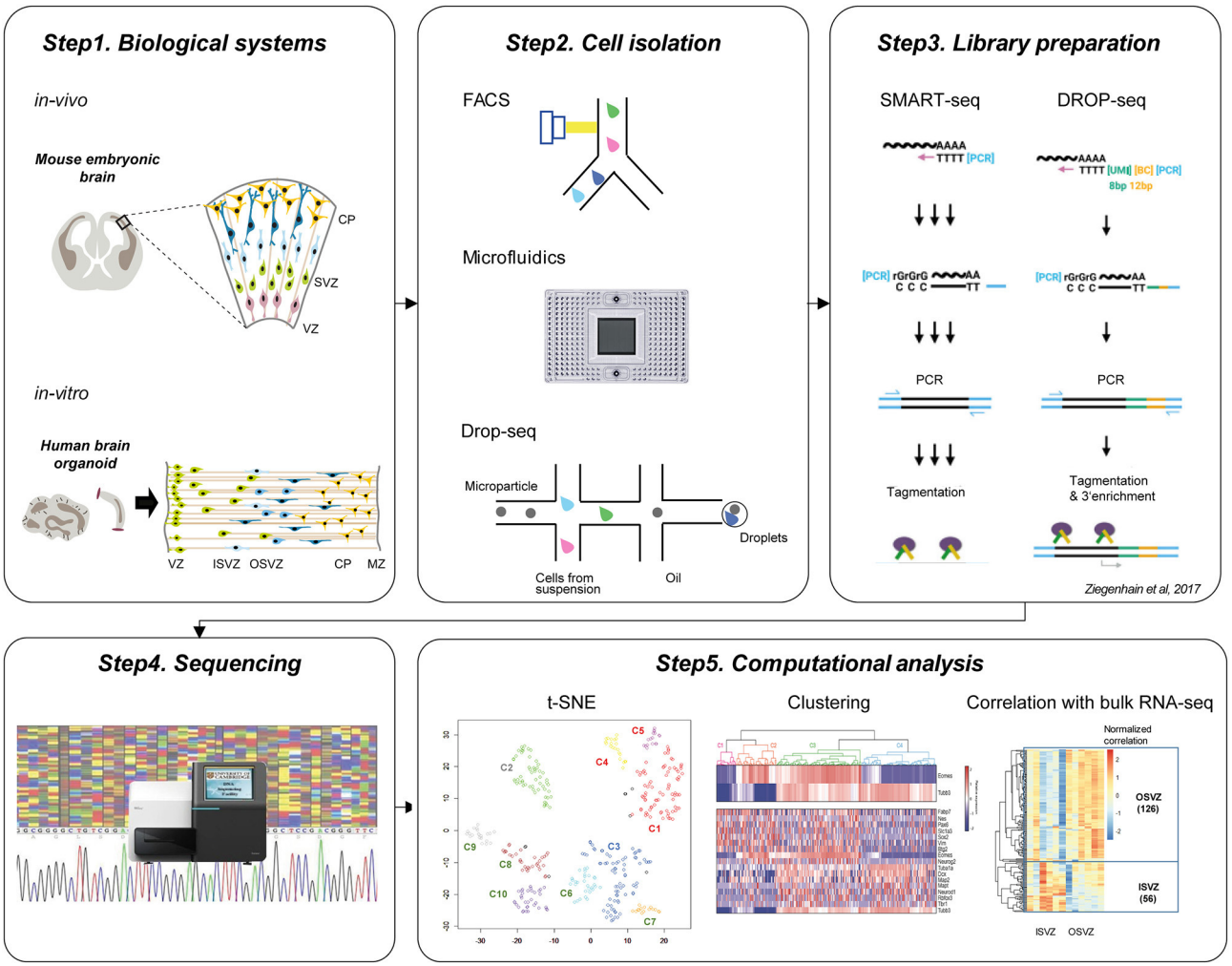

FIGURE 1 | Current workflow of single-cell technologies to study cortical development. Step1. Biological systems to study brain development. Upper panel shows *in vivo* mouse embryonic brain and below panel indicates *in vitro* human brain organoid which is commonly used for the single-cell neurogenesis studies. Step2. Cell isolation methods. Individual cells can be isolated using FACS, Microfluidic ChIP, or Drop-seq approaches. Step3. Library preparation. The common protocols include polyA+ mRNA capture, reverse transcription, cDNA amplification using PCR, and tagmentation. Step4. Sequencing of the library. Step5. Computational analysis. After the preprocessing of sequencing reads, visualization using t-SNE, unsupervised clustering, and correlation analysis with bulk RNA-seq is followed to identify subtypes of cells and characterize their identities.

to increase yield and length of cDNA libraries from single cells (Picelli et al., 2013; Ziegenhain et al., 2017). In Drop-seq, a flow of beads are suspended in lysis buffer and a flow of a single-cell suspension is brought together in a microfluidic chip, which generates nanoliter-sized emulsion droplets. Here each bead contains covalently bound oligo-dT primers carrying a unique molecular identifier (UMI) and a unique, bead-specific barcodes. UMI is a barcode of the individual molecule to estimate the number of transcribed molecules that is independent of amplification biases (Stegle et al., 2015), while bead-specific barcode provides the information of cell-of-origin (Macosko et al., 2015). Following cell lysis, their mRNA gets attached to the oligo-dT-carrying beads, and then as droplets are broken, cDNA and library are generated for all cells in parallel.

Prepared libraries undergo sequencing using next-generation sequencing platforms such as Illumina Hi-Seq and Nextseq (**Figure 1,** Step4). Both single-end (Chu et al., 2016; Xu et al., 2016) and paired-end (Telley et al., 2016) library preparation are used for the single-cell transcriptomic analysis. For the special purpose of investigation of transcript isoforms, paired-end sequencing is suitable to quantify multiple isoforms with high confidence. In terms of sequencing depth, the recent single-cell transcriptomics from the neurogenesis research sequenced 0.1–5 million reads per cell (**Table 1**). To get a saturated gene detection, 1 million reads per cell is generally recommended (Svensson et al., 2017). However, the sequencing depth has to be decided based on the purpose, as not all studies need to saturate detection but some of them more focus on the finding of the new cluster of cells which requires a large

TABLE 1 | Application of single-cell technology to neurogenesis research.

System	Species	Study	Age/Stage	Target layer	Cell isolation	Library generation	Number of cell	Reads per cells	Genes per cells	Computational analysis
In vivo neurogenesis	Mouse	Telley et al., 2016	E14.5	Isochronic cohorts of newborn VZ cells	FACS	SMARTer ultra low RNA kit for the C1 system (Takara Clontech, #634833)	272 cells	0.6 million	4,726	t-SNE, SCDE
	Mouse	Fan et al., 2016	E13.5	NPCs	FACS	SMARTer ultra low RNA kit for illumina sequencing (catalog no. 634936)	65 cells	20 million	5,909	PAGODA
	Human	Pollen et al., 2015	GW 16–18 (14–16 wpc)	VZ, SVZ	Microfluidic; Fluidigm C1	SMARTer ultra low RNA kit (catalog no. 63495, PT5163-1)	393 cells	2.5 million	3,100	t-SNE, ConsensusClusterPlus, EMCluster, DESeq2
	Human	Camp et al., 2015	12–3 wpc	neocortex	Microfluidic; Fluidigm C1	SMARTer ultra low RNA kit for Illumina (Clontech)	226 cells	2–5 million reads	2,744	Correlate with bulk RNA-seq, Monocle TF correlation network analysis
In vitro neurogenesis	Human	Camp et al., 2015	Days 33, 35, 37, 41, and 65, iPSC-derived	Cerebral organoid	Microfluidic; Fluidigm C1	SMARTer ultra low RNA kit for illumina sequencing (Clontech)	333 cells	2–5 million reads	4,218	t-SNE, correlate with bulk RNA-seq
	Chimpenzee	Mora-Bermudez et al., 2016	7 organoids (45–80 days)	Cerebral organoid	Microfluidic; Fluidigm C1	SMARTer ultra low RNA kit for the Fluidigm C1 system	344 cells	1 million	2,730	t-SNE, SCDE, correlate with bulk RNA-seq
	Human	Bershteyn et al., 2017	WT (2 individuals), MDS (3 individual), 5, 10, 15 weeks of differentiation	Cerebral organoid	C1 single-cell auto prep integrated fluidic circuit (IFC, Fluidigm)	SMARTer ultra low RNA kit	469 cells	–	–	PCA, ConsensusClusterPlus R
	Human	Quadrato et al., 2017	3–6 month	Cerebral organoid	Drop-seq	Drop-seq	82,291 cells	0.1 million	∼1,300	Seurat

number of cells rather than high sequencing depth. For example, Pollen et al. performed down-sampling analysis from the 301 single-cells of developing cerebral cortex and found that 0.05 million reads per cell is sufficient for unbiased cell-type classification and biomarker identification (Pollen et al., 2014).

Following sequencing, an extensive computational analysis is performed including read alignment, quantification, visualization of data, unsupervised clustering, and differential expression analysis to interpret these large-scale data sets (**Figure 1,** Step5). After the read alignment and quantification using Tophat (Kim et al., 2013), STAR (Dobin et al., 2013), Cufflinks (Trapnell et al., 2012), or Kallisto (Ntranos et al., 2016), the low-quality cells with small library size or high portion of mitochondrial reads need to be excluded from downstream analysis. Toward the visualization of single-cell transcriptomes at the collective level, most studies in past implemented Principal component analysis (PCA) and t-SNE to obtain the overview and structure of subpopulations (Poirion et al., 2016). For the unsupervised clustering, ConsensusClusterPlus R (Wilkerson and Hayes, 2010), EMCluster (Jung et al., 2014), SC3 (Kiselev et al., 2017), SNN-Cliq (Xu and Su, 2015), SCUBA (Marco et al., 2014), BackSPIN (Zeisel et al., 2015), and PAGODA (Fan et al., 2016) provide methods to identify the subpopulation from the single-cell transcriptome profiles. Following clustering, DESeq2 (Love et al., 2014), SCDE (Kharchenko et al., 2014), and MAST (Finak et al., 2015) are used to identify differentially expressed genes between clusters. Pseudotime is another important concept in the computational analysis of single-cell transcriptome, which estimates the cells' progress through the transition. The computational tools like TSCAN (Ji and Ji, 2016), Monocle (Trapnell et al., 2014), Waterfall (Shin et al., 2015), Sincell (Julia et al., 2015), Oscope (Leng et al., 2015), and Wanderlust (Bendall et al., 2014) provide *in silico* defined pseudotime for each single-cell during the cell fate transition.

To gain the first glimpse into the characteristic of single-cell clusters, typically the expression of marker genes such as proliferation, neuronal onset, and neuronal differentiation/maturation genes (Telley et al., 2016) and/or correlation with bulk-cell transcriptome profiles is integrated. For example, Camp et al. performed unsupervised clustering of 226 single-cells from human embryonic neocortex, and examined the characteristics of each clusters (Camp et al., 2015) using the correlation with existing bulk-cell RNA-seq profiled from cortical layers (VZ, ISVZ, OSVZ, and CP; Fietz et al., 2012) and FAC-sorted subpopulations (aRG, bRG, and N; Florio et al., 2015). Furthermore, Mora-Bermudez et al. performed single-cell RNA-seq from 344 cells of Chimpanzee cerebral organoids and compared each cell cluster with bulk-RNA-seq from germinal layers of the human embryonic brain (Fietz et al., 2012; Mora-Bermudez et al., 2016). These abovementioned steps are the most widely followed in the current single-cell studies to decode heterogeneity in cell populations during cortical development.

NOVEL BIOLOGICAL INSIGHTS INTO CORTICAL DEVELOPMENT USING SINGLE-CELL TECHNOLOGIES

Current single-cell genomics studies (**Table 1**) have provided unprecedented biological insights into the cellular diversity and its molecular code which was difficult to obtain using previous approaches. For example, a recent study performed single-cell RNA-seq of isochronic VZ cells after 6, 12, 24, and 48 h of birth (Telley et al., 2016). Following this, computational pseudotime modeling which projects each cell into the differentiation trajectory identified sequential waves of gene expression patterns, perturbation of which restricted formation of proper neuronal layers. Furthermore, epigenetic factors such as Kdm3a (lysine demethylase 3A) and MeCP2 (Methyl CpG binding protein-2) belonged to different sequential waves, suggesting that distinct epigenetic players contribute at defined steps of neurogenesis.

Interestingly further, single-cell transcriptome analysis in combination with an unsupervised clustering has not only been able to dissect cellular heterogeneity but also characterize molecular details of the identified subpopulations of cells. For example, a previous study revealed that the most significant aspect of heterogeneity was originating from genes associated with neuronal maturation and growth, which is closely tied to the spatial organization of their expression patterns across three layers (VZ, SVZ, and CP) of the developing cortex (Fan et al., 2016). In another study, two different radial glial cell populations oRG and vRG were separated based on the single-cell transcriptome profiles and it further allowed a thorough investigation of differences in the gene expression profiles between these two cell populations (Pollen et al., 2015). For example, the key regulators such as HOPX and PTPRZ1 were found to be differentially expressed between oRG and vRG and may guide future studies aimed to decipher the differential transcriptome underlying identity of oRG and vRG cells.

Another considerable point of single-cell RNA-seq analysis is that the identification of similarities and differences of cell populations between *in vivo* and *in vitro* neurogenesis, or between species. For instance, single-cell transcriptomes from *in vitro* human brain organoids could faithfully reconstruct genetic and cellular networks involved in germinal zone organization, neural progenitor cell (NPC) proliferation, and NPC-to-neuron differentiation *in vivo* (Camp et al., 2015). In this study, over 80% of genes that were differentially expressed across the fetal cortex lineages have similar expression profiles in organoid and fetal cerebral cortex (Camp et al., 2015). Furthermore, in a study comparing AP populations between species, about 12% of the genes specific to AP or neurons in both human and chimpanzee were found not specific to these cell types in the mouse, implying an involvement of certain specific developmental mechanisms during the development of the primate cerebral cortex (Mora-Bermudez et al., 2016). Altogether, these examples vouch for the strong and unprecedented discovery power that single-cell transcriptomics has conferred researchers in the field of cortical development.

CHALLENGES IN SINGLE-CELL TECHNOLOGIES FOR CORTICAL DEVELOPMENT RESEARCH

Despite exciting advances in single-cell genomics, there are several challenges toward deciphering the gene regulatory network and epigenetic mechanisms of cell fate specification during neurogenesis at the single cell level (Poulin et al., 2016). Current single-cell transcriptome studies in neurogenesis research implemented dissociation of cells from the tissue which involves external physical stress (Liu and Trapnell, 2016). In addition, this procedure requires the removal of cell-cell contacts. Since niche microenvironment and cell-cell adhesion are also means of signal transduction, it is not clear how much the loss of these properties influences the transcriptome at the single cell level. To reduce these issues, alternative single-cell transcriptome techniques such as *in situ* sequencing (Ke et al., 2013) and Fluorescent *in situ* sequencing (FISSEQ) (Lee et al., 2015) could be considered for future neurogenesis studies.

Furthermore, current single-cell transcriptome only gives a snap-shot of the analyzed cell at the time of capture. These transcriptome data also have a large sparsity with a very high proportion of genes that show zero read counts (Vallejos et al., 2017). This zero count can come from biological reasons (a transient state where a gene is not expressed) as well as technical reasons such as dropout events and read depth of sequencing. Therefore, it is not fully clear how much of the single-cell transcriptome data and resulting clusters are influenced by any of these variables. To reduce the bias from the technical issue, more effort to increase capture efficiency is needed for library preparation (Liu and Trapnell, 2016). In parallel, thorough normalization of data and quality control processes are needed to address the technical issues come from sparsity of the data or cell cycle phase transition (Vallejos et al., 2017). Also, it is essential to combine dual measurements from the same cell that allows transcriptome analysis simultaneous to another readout of the cellular state. Along these lines, new techniques combining live-cell imaging and single-cell sequencing (Lane et al., 2017), or electrophysiology and single-cell sequencing (Cadwell et al., 2016), which can track cellular state in parallel with genome-wide gene expression profiles are increasingly getting popular and should be applied to the studies of cortical development.

The recent decade has shown that epigenetic mechanisms are critical for gene regulatory programs underlying cell-fate changes during development. Recently, single-cell ATAC-seq (Buenrostro et al., 2015) was applied to neurogenesis study (Preissl et al., in review) to measure chromatin accessibility at the single cell level. However, many other single-cell epigenomics methods including DROP-ChIP (Rotem et al., 2015), scRRBS (Guo et al., 2013), and scHi-C (Ramani et al., 2017) to measure chromatin landscape, DNA methylatome and higher-order chromatin structures at the single cell level remained to be applied to study brain development. Furthermore, those protocol can be combined into single-cell multi-omics technique such as scMT-seq (Hu et al., 2016), scTrio-seq (Hou et al., 2016), and scNMT-seq (Clark et al., in review). Current single-cell epigenome technology has the limitation of low coverage of genome so that the clustering of cells are biased by easily profiled genomic regions. If this limitation is improved, these single-cell epigenomic technologies will enable us to decipher epigenetic control of cortical development and its contribution to the sequential waves of transcriptional changes that underlie neurogenesis. In addition, single-cell epigenomics also holds potential to identify new cell subpopulations during cortical development that were not detected by single-cell transcriptome analysis.

Given that the field of single-cell genomics is relatively new, the researchers also encountered challenges in having universally accepted and robust pipelines for the computational analysis of single-cell datasets. Compared to conventional bulk RNA-seq analysis, single-cell RNA-seq analysis requires more rigorous quality control and normalization to minimize the bias arising from low capture efficiencies and confounding factors like cell cycle state changes. Although individual tools specialized for the analysis of single-cell data are increasingly available (Poirion et al., 2016), a standard pipeline that includes quality controls, normalization, clustering, finding the identity of clusters and differential expression analysis should be established to provide robust and comparable results between different laboratories. Also, novel analysis ideas which can find new insight from the data, or improve the quality of unsupervised clustering need to be developed continuously.

Importantly further, it is also possible to use the existing single-cell transcriptome profiles from neurogenesis *in vivo* and *in vitro* to analyze splicing, non-coding RNA species, and intronic transcripts. While most of the single-cell transcriptome profiling protocols so far employed poly-A selection, a subset of the non-coding RNAs which contain poly-A tail can be assessed. The intronic reads from nascent RNAs can be measured from the single-cell transcriptome to study splicing and actual rates of transcription (Gaidatzis et al., 2015). Recently developed approaches including BRIE (Huang and Sanguinetti, 2017), WemIQ (Zhang et al., 2015), and SingleSplice (Welch et al., 2016) will help analyzing alternative splicing from the existing single-cell transcriptomes of neurogenesis. Given that alternative splicing (Vuong et al., 2016) and non-coding RNA-mediated gene regulation (Yao et al., 2016) are known to be important for neurogenesis, investigation of splicing regulation, non-coding RNA, and nascent RNA expression from the existing single-cell transcriptomes will provide novel insights into the heterogeneity of cell populations and molecular programs underlying cortical development.

CONCLUSIONS AND PERSPECTIVES

Recent single-cell transcriptome studies allowed novel discoveries on various aspects of cortical development including sequential waves of gene expression, cellular heterogeneity, and comparative analysis of cell populations across embryonic stages, species, and origins (*in vitro*/*in vivo*). Future studies should invest more effort to improve library preparation protocols to increase the molecular capture efficiency to reduce the bias from the technical issue. Also, simultaneous assessment of

cellular state such as live cell imaging and electrophysiology in addition to gene expression profiling at the single-cell level need to be considered. Moreover, efforts should be made to measure single-cell transcriptome without detachment of cells from cortex and organoids to allow proper assessment of cellular states and transcriptional programs underlying neurogenesis. These assessments will also remain incomplete unless complemented by a systematic investigation into the epigenetic landscape of single-cells using technologies such as DROP-ChIP, scMT-seq, and scTrio-seq. Those multi-omics approaches will enable the generation of mechanistic models relating genetic/epigenetic variation and transcript expression dynamics in neurogenesis (Macaulay et al., 2017). Additionally, development of robust and universally accepted computational pipelines is required to obtain more conclusive biological findings and their comparability across different laboratories. At the same time, existing single-cell genomics data can be further analyzed by novel computational methodologies to profile alternative splicing, non-coding transcripts, and nascent RNAs. Importantly, all of these comprehensive single-cell genomics analysis should be performed at various stages of cortical development for the comprehensive understanding of cellular subpopulations. Altogether, with these advances, we will get closer to decoding the complexity of cell types and underlying gene regulatory network during cortical development.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Epitranscriptomics: A New Regulatory Mechanism of Brain Development and Function

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Epigenetic modifications of DNA and chromatin are long known to control stem cell differentiation and organ function but the role of similar modifications at the level or regulatory RNAs is just beginning to emerge. Over 160 RNA modifications have been identified but their abundance, distribution and functional significance are not known. The few available maps of RNA modifications indicated their dynamic regulation during somatic stem cell differentiation, brain development and function in adulthood suggesting a hitherto unsuspected layer of regulation both at the level of RNA metabolism and post-transcriptional control of gene expression. The advent of programmable, RNA-specific CRISPR-Cas editing platforms together with the identification of RNA modifying enzymes now offers the opportunity to investigate the functional role of these elusive epitranscriptome changes. Here, we discuss recent insights in studying the most abundant modifications in functional mRNAs and lncRNAs, N6-methyladenosine and 5-(hydroxy-)methylcytosine, and their role in regulating somatic stem cell differentiation with particular attention to neural stem cells during mammalian corticogenesis. An outlook on novel CRISPR-Cas based systems that allow stem cell reprogramming by epitranscriptome-editing will also be discussed.

Keywords: epitranscriptomics, RNA-epigenetics, epitranscriptome-editing, N6-methyladenosine, 5-methylcytosine, 5-hydroxymethylcytosine, neural stem cells, brain development

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INTRODUCTION

During embryonic development, rapid changes in protein expression and their activity are required to initiate and promote the switch from proliferation to differentiation of stem cells. Historically, stem cell research has been primarily focused on understanding the control of gene expression at the transcriptional level by transcription factors or epigenetic modifications of DNA or histones (Atlasi and Stunnenberg, 2017). In addition, post-translational modifications are long known to influence protein stability and activity, which by definition has implications in all biological processes including in controlling the proliferation versus differentiation of somatic stem cells during development and adulthood. While modifications of both DNA and proteins have long been the focus of intensive research, very little is known about the modifications that may occur at the level of the molecules that transduce the genetic message from the DNA to the proteins: functional mRNAs.

Overall, mRNAs and protein levels fairly correlate but about half of the variation in the latter cannot be explained by mRNA concentrations alone (Vogel and Marcotte, 2012) implying that post-transcriptional regulation must also play critical roles in controlling protein abundance.

For instance, many aspects of mRNA metabolisms including, among others, splicing, capping, polyadenylation, nuclear export, and rates of translation versus degradation are regulated during brain development by RNA-binding proteins and/or microRNAs (Lennox et al., 2018). In addition to these classical mechanisms for post-transcriptional control of protein expression, over 150 chemical modification of nucleotides are being listed in a recently developed online database of RNA modifications (Boccaletto et al., 2017). However the abundance, distribution and function of essentially all of these RNA modifications have remained elusive.

Systematic mapping of RNA modifications across the transcriptome of different species and tissues by antibody pull-down or chemical labeling coupled to sequencing have just begun. These efforts revealed that RNA modifications are not only abundant in housekeeping, non-coding RNAs, such as tRNAs and rRNAs (He, 2010), but are also commonly found within functional mRNAs and lncRNAs (Boccaletto et al., 2017). Interestingly, some of the mapped modifications showed very dynamic patterns and tissue-specific distribution supporting the notion that they may harbor regulatory potential comparable to that of classical epigenetic marks, thus, opening up the new field of RNA-epigenetics (He, 2010) or epitranscriptomics (Saletore et al., 2012).

This field is still in its infancy and mapping the vast majority of the many RNA modifications is highly problematic due to the need of specific antibodies while lacking the possibility to validate any outcome by alternative methods. This can lead to contradicting results as for example in the case of N1-methyladenosine (m1A). Mapping of m1A by antibody pull-down and sequencing initially led to the conclusion that this modification is broadly abundant within mRNAs (Dominissini et al., 2016; Li et al., 2016), which was later confirmed by methods providing single-nucleotide resolution of m1A modifications (Li et al., 2017b). However, these results were contradicted by another study using a similar experimental approach but showing that m1A at mRNAs is rare and almost exclusively occurring within stem loops equivalent to those of tRNAs and that for this reason might be spuriously introduced by the tRNA m1A-methylation machinery (Safra et al., 2017).

Nevertheless, the rapidly advancing methodologies to characterize the epitranscriptome and the limited number of studies mapping these modifications within functional mRNAs and lncRNAs makes this a fast evolving field. Therefore, in this minireview we will only focus on the three most reproducibly mapped and intensely studied mRNA modifications known to date: N6-methyladenosine (m6A), 5-methylcytosine (5mC), and 5-hydroxymethylcytosine (5hmC). Their functions in different cell types will be discussed with particular attention to neural stem cell differentiation during mammalian corticogenesis and brain function in adulthood.

N6-METHYLADENOSINE

Methylation of adenine at the 6 position (m6A) is commonly found on DNA of prokaryotes but generally rare in eukaryotes and highly debated in mammals (Luo et al., 2015). In contrast, m6A in mRNAs and lncRNAs is frequently found in both prokaryotes and eukaryotes including mammals from rodents to humans (Desrosiers et al., 1975; Wei et al., 1975).

The synthesis of m6A requires the co-transcriptional addition of the methyl group of S-adenosylmethionine to adenine by the METTL3/METTL14/WTAP complex (Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014). In this complex, METTL3 exhibits the catalytic activity whereas METTL14 (Wang et al., 2016) and WTAP (Ping et al., 2014) provide the RNA binding scaffold. Additionally, FTO (Jia et al., 2011) and ALKBH5 (Zheng et al., 2013) have been identified as m6A demethylases allowing for a dynamic addition and erasure of this epitranscriptional mark. Specifically, FTO oxidizes m6A to the meta-stable N6-hydroxymethyladenosine and N6-formyladenosine that undergo spontaneously conversion to adenosine (Fu et al., 2013) while ALKBH5 directly catalyzes the demethylation of m6A (Zheng et al., 2013).

Transcriptome-wide mapping of m6A revealed that this modification is mainly deposited at the DRACH (where D=A, G or U; H=A, C or U) consensus motif (Dominissini et al., 2012; Meyer et al., 2012; Schwartz et al., 2014) displaying a conserved pattern across mRNAs and lncRNAs with the highest levels within long exons, transcription end sites, 3′ UTRs (Dominissini et al., 2012; Meyer et al., 2012) and to a lesser extend 5′ UTRs (Meyer et al., 2015; Zhang et al., 2017) (**Figure 1**, left). Levels of m6A varied across cell types (Chen et al., 2015) and displayed a high evolutionary conservation across mammalian species (Dominissini et al., 2012; Meyer et al., 2012; Batista et al., 2014; Schwartz et al., 2014). Furthermore, m6A levels revealed to be dynamic during embryonic stem cell differentiation (Batista et al., 2014; Schwartz et al., 2014; Chen et al., 2015; Geula et al., 2015) or environmental stimuli such as stress (Dominissini et al., 2012; Zhou et al., 2015). Interestingly, levels of m6A in the brain increase during embryonic and postnatal development and are the highest in the adult brain among all other tissues studied (Meyer et al., 2012).

The molecular function of m6A is just beginning to emerge and is subject of intense research. Several studies indicated roles in controlling various steps of mRNA metabolism including at the level of nuclear export (Zheng et al., 2013; Roundtree et al., 2017), microRNA mediated decay (Meyer et al., 2012), pre-microRNA processing (Alarcón et al., 2015) or polyadenylation (Ke et al., 2015). Furthermore, m6A promotes the binding of YTH or HNRNP protein families to RNA either directly or through m6A-induced changes in the RNA secondary structure, respectively (**Figure 1**, left) (Dominissini et al., 2012; Liu et al., 2015, 2017). Both YTH and HNRNP proteins are associated with alternative splicing suggesting a functional role of m6A in this process (Dominissini et al., 2012; Liu et al., 2015, 2017; Xiao et al., 2016). Specifically, recent studies suggested that m6A regulates alternative splicing only for a subset of mRNAs and lncRNAs rather than being general unspecific splicing factor (Bartosovic et al., 2017; Ke et al., 2017). Moreover, YTHDF1, 2 and 3 were found to be involved in translation (Meyer et al., 2015; Zhou et al., 2015; Shi et al., 2017; Slobodin et al., 2017) and RNA degradation (Wang et al., 2014a,b; Shi et al., 2017;

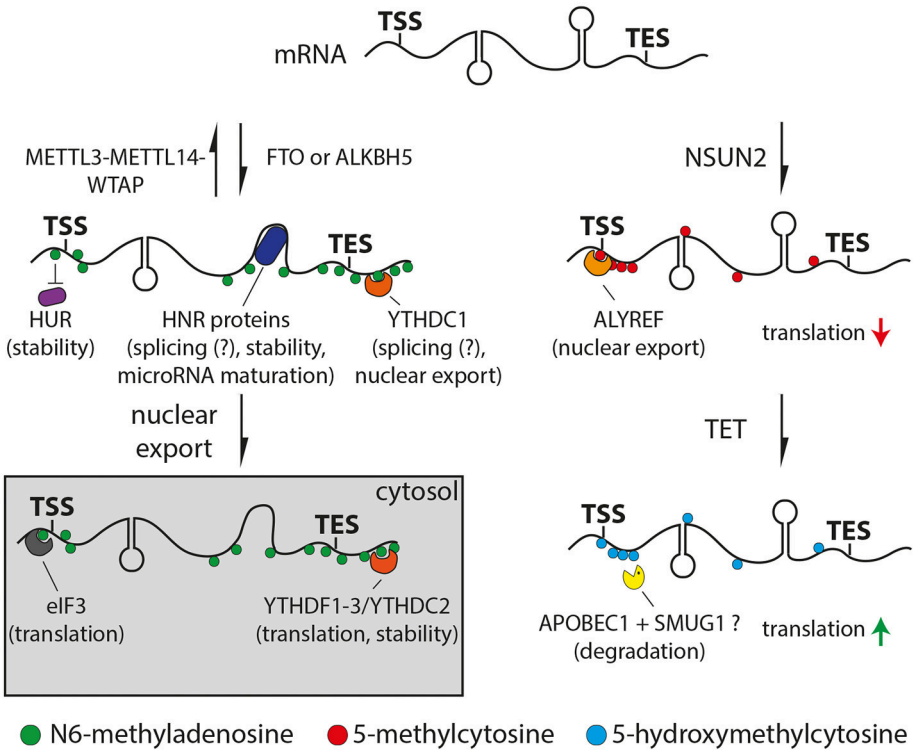

FIGURE 1 | Drawings of N6-methyladenosine (left) or 5-methylcytosine (right) pathways. **Left:** Adenosine is methylated (m6A, green) by the METTL3/METTL14/WTAP complex or removed by the FTO or ALKBH5 demethylases. Proteins can bind m6A directly (YTH and eIF3, orange and gray respectively), indirectly through changes in secondary structure (HNR, dark blue) or be repelled by m6A (HUR, purple). **Right:** Cytosine is methylated at the 5 position (5mC, red) by NSUN2 and oxidized to 5-hydroxymethyl- (5hmC) or 5-formylcytosine (light blue) by TET proteins. 5mC can recruit ALYREF (orange) decreasing translation efficiency, while 5hmC can enhance translation (red and green arrows, respectively). APOBEC1 and SMUG1 (yellow) may be involved in the removal of oxidized 5-methylcytosine resulting in the degradation of the cleaved mRNA. Potential functions of m6A or 5mC readers are indicated in brackets.

Zhang et al., 2017) via their combinatorial binding. For example, the binding of YTHDF1 promoted mRNA translation due to the recruitment of the eukaryotic initiation factor 3 (eIF3) (Wang et al., 2015), which can also directly interact with m6A (**Figure 1**, left) (Meyer et al., 2015). On the other hand, YTHDF2 has been reported to facilitate mRNA decay by recruiting deadenylases (Du et al., 2016). Finally, m6A can also inhibit RNA-protein interactions as shown for the well-established RNA stabilizer HuR, resulting in an increased RNA decay (**Figure 1**, left) (Wang et al., 2014b). Altogether, m6A can at the same time burst and sharpen the levels of critical proteins by promoting the rate of translation and a faster decay of functional RNAs, respectively. In this context it is interesting to note that transcription factors and genes required for cell-type specific processes show higher levels of m6A compared to housekeeping genes (Batista et al., 2014; Schwartz et al., 2014; Wang et al., 2014b; Chen et al., 2015; Yoon et al., 2017). Therefore, m6A seems to be ideally positioned for playing important roles during cell differentiation by modulating transcriptional networks that swiftly change during fate commitment.

A functional role of m6A in stem cell commitment is further supported by the observation that its ablation, for example by knock-down of METTL3 or METTL14, is compatible with naïve ESC survival but impairs their differentiation due to a higher stability of proliferation and pluripotency factors (Batista et al., 2014; Geula et al., 2015). Conversely, knock-down of ZFP217 led to a higher activity of METTL3, elevated levels of m6A in mRNAs encoding for pluripotency factors and resulting in their lower stability and faster degradation, thus, triggering ESC differentiation (Aguilo et al., 2015). Additionally, overexpression of METTL3 in iPSC promoted reprogramming whereas its knock-down had the opposite effect (Chen et al., 2015).

In animal models, decreasing the levels of m6A by ablation of METTL3 or METTL14 led to defects in (i) sex determination and neuronal function with impaired locomotion in flies (Lence et al., 2016), (ii) morphological and ectoderm and hematopoietic defects in zebrafish (Ping et al., 2014; Zhang et al., 2017) and (iii) embryonic lethality shortly after implantation in mice (Geula et al., 2015).

Moreover, conditional knock-out of METTL14 in mouse embryos resulted in reduced body size and postnatal lethality (Yoon et al., 2017) whereas ablation in the adult brain lead to impaired axonal regeneration (Weng et al., 2018). Concerning neural stem cells during corticogenesis, two recent studies showed that conditional knock-out of METTL14 resulted in aberrant cell cycles, particularly longer S and G2 phases, as

well as decreased generation of late-born neurons (Yoon et al., 2017; Wang et al., 2018). While it is unclear whether the causal link between cell cycle length and differentiation (Borrell and Calegari, 2014) applied in this context, these two studies provided different explanations for the observed phenotypes. Yoon et al. reported an impaired differentiation of neural stem cells due to an increased half-life of mRNAs enriched for cell fate determinants and cell cycle regulators upon reduction of m6A suggesting effects on priming and translation of such transcripts (Yoon et al., 2017). On the other hand, Wang et al. showed that the ablation of METTL14 increased differentiation by stabilizing mRNAs for histone modifying enzymes, leading to a decreased neural stem cell pool (Wang et al., 2018). In addition, it is reasonable to expect that also lncRNAs that are important during corticogenesis (Aprea and Calegari, 2015) were also affected by this reduction of m6A upon METTL14 deletion but lncRNAs were not assessed in neither of the two studies.

Additionally, ablation of the m6A eraser FTO in mice led to an increased level of m6A in a subset of mRNAs (Hess et al., 2013), postnatal growth retardation including microcephaly (Fischer et al., 2009; Li et al., 2017a) and impairments in adult neurogenesis (Li et al., 2017a).

In addition to neural stem cells and brain development, roles for m6A modifications were also found during adulthood in particular related to cognitive function such as learning and memory. For example, manipulating the levels of m6A in mouse resulted in changes in neuronal circuitry and activity (Hess et al., 2013) and while the levels of both m6A and FTO acutely changed in the prefrontal cortex or hippocampus of mice upon learning, ablation of FTO enhanced memory formation and consolidation of contextual fear conditioning (Widagdo et al., 2016; Walters et al., 2017). Interestingly, human mutations in FTO were associated with developmental failures specifically of the central nervous system (Boissel et al., 2009), brain atrophy (Ho et al., 2010) and psychological disorders in adulthood (Hess and Brüning, 2014).

Overall, several studies indicated that m6A plays several roles not only in neural stem cell differentiation during development but also in cognitive function and neurological disorders during adulthood, which is consistent with its effects in controlling the stability and expression of certain specific functional RNAs. Uncovering how this specificity is controlled for some, but not others, mRNAs or lncRNAs will be a challenge of future research.

5-METHYLCYTOSINE AND 5-HYDROXYMETHYLCYTOSINE

5-methylcytosine (5mC) and its oxidized form 5-hydroxymethylcytosine (5hmC) are widely found in eukaryotic DNA and are associated with transcriptional regulation and DNA stability (Li and Zhang, 2014). Four decades ago, 5mC was also described to occur in RNA (Desrosiers et al., 1975) and later found to be highly abundant particularly in tRNAs and rRNAs (Schaefer et al., 2009).

In mammals, 5mC can be catalyzed by DNMT2 (Goll et al., 2006; Tuorto et al., 2012; Khoddami and Cairns, 2013) and proteins of the NOP2/Sun domain RNA methyltransferase family (NSUN). These enzymes target tRNAs or rRNAs in a non-overlapping manner and levels of 5mC at these housekeeping RNAs is important for their stability, biogenesis and function (Motorin et al., 2010). NSUN2 displayed broader substrate specificity including functional mRNAs and lncRNAs (Squires et al., 2012; Hussain et al., 2013; Khoddami and Cairns, 2013; Yang et al., 2017).

Transcriptome-wide profiling of 5mC by bisulfite conversion-based approaches (Schaefer et al., 2009) revealed a high abundance of 5mC in mRNAs at CG dinucleotides around transcription initiation sites (**Figure 1**, right) (Squires et al., 2012; Hussain et al., 2013; Khoddami and Cairns, 2013; Yang et al., 2017), which also revealed to be evolutionary conserved (Yang et al., 2017). Additionally, the abundance of 5mC in mRNA was found to vary significantly across tissues and transcripts associated with both common metabolic processes and cell-type specific functions (Amort et al., 2017; Yang et al., 2017).

Loss of function of NSUN2 in mouse and human led to motor, neurodevelopmental and cognitive defects (Abbasi-Moheb et al., 2012; Khan et al., 2012; Martinez et al., 2012; Tuorto et al., 2012; Blanco et al., 2014; Komara et al., 2015; Flores et al., 2016). In particular, molecular analysis revealed that the ablation of NSUN2 in mouse caused an increase in angiogenin-induced cleavage of tRNAs, which resulted in a decreased global protein synthesis causing an inhibition of cell differentiation and migration, particularly in the brain (Tuorto et al., 2012; Blanco et al., 2014; Flores et al., 2016). However, these studies did not address additional mRNA-specific effects of NSUN2 ablation as potentially contributing factors to the observed phenotypes. For example, it has been shown that 5mC is required for ALYREF-mediated nuclear export of mRNAs (Yang et al., 2017) and negatively affects translation (**Figure 1**, right) (Delatte et al., 2016). Furthermore, 5mC might also play a role in microRNA meditated post-transcriptional regulation (Squires et al., 2012; Yang et al., 2017) although this is currently debated (Amort et al., 2017).

Similar to DNA, 5mC at RNA can be oxidized by enzymes of the ten-eleven translocator family (TET) to 5hmC (Fu et al., 2014) and further oxidized to 5-formylcytosine (Huber et al., 2015) and 5-carboxylcytosine (**Figure 1**, right) (Basanta-Sanchez et al., 2017). Whether or not this may be followed by the excision of the oxidized methylcytosine in RNA, as it is the case for methylation occurring on DNA, is not known. However, evidence for a potential mechanism comes from the observation that SMUG1, a key component of the base-excision repair machinery, can remove oxidized forms of 5-methyluracil (i.e., thymine) from RNA (Jobert et al., 2013). Given that cytosine to uracil conversions are common in RNA (Harjanto et al., 2016) it is tempting to speculate that a similar conversion of oxidized methylcytosine to oxidized 5-methyluracil may occur that would lead to its excision by SMUG1 and RNA degradation (**Figure 1**, right).

Transcriptome-wide mapping by antibody pull-down revealed low but significant levels of 5hmC in mRNA (Fu et al., 2014; Huber et al., 2015; Yang et al., 2017). Interestingly, the highest levels were found in the brain relative to other tissues

(Fu et al., 2014; Huber et al., 2015; Delatte et al., 2016), a specificity that is reminiscent of 5hmC levels in DNA (Lian et al., 2016). This suggests that the cellular environment or activity of TET enzymes may cause both hyper DNA and RNA hydroxymethylation in the brain compared to other tissues.

Studies addressing the role of 5hmC in mammalian mRNAs are lacking because, contrary to 5mC that is synthetized by mRNA-specific enzymes (NSUN2) that do not target DNA, synthesis of 5hmC is mediated by the very same TET enzymes that promiscuously target both RNA and DNA (Lian et al., 2016). For this reason, studies addressing RNA-specific roles of 5hmC are only available in flies that lack DNA methyltransferases and therefore have negligible levels of both 5mC and 5hmC in DNA while still showing abundant 5hmC in RNA. As the only study available to date showing the RNA-specific effects of TET manipulation, high levels of 5hmC in flies correlated with higher translation efficiency (**Figure 1**, right) and TET knock-down led to brain malformations in the larva and death during the pupal stage (Delatte et al., 2016). Given the current lack of mRNA-specific enzymes to target mammalian 5hmC, systems are needed that allow to overcome the use of conventional genetic deletion and knock-out lines.

EPITRANSCRIPTOME EDITING

The importance of RNA modifications for developmental processes has just begun to emerge and new studies will soon provide us with additional knowledge about their abundance, specificity and role. As a main limitation in this field, functional characterization of mRNA and lncRNAs modifications are so far restricted to the ablation of the enzymes acting as writers, readers or erasers. This has several intrinsic limitations such as that some of these enzymes are unknown, have overlapping or redundant functions or act on different substrates as shown in the case of TET enzymes. Furthermore, ablation of RNA modifying enzymes would still not resolve site-specific roles of such modifications and their impact on specific transcripts.

These limitations can be overcome by the development of site-specific manipulation of RNA modifications as a means to directly prove their functional implications in a way similar to what shown for recent advances in epigenome editing (Thakore et al., 2016). A potential platform for such approaches is provided by the PUF protein family in which a conserved pumilio homology domain (PUF) targets the protein toward a specific RNA sequence (Zamore et al., 1997; Zhang et al., 1997). Engineering of the PUF domain allowed its retargeting toward any 8 nucleotide sequence (Cheong and Hall, 2006; Dong et al., 2011), which was successfully used to track RNAs in living cells (Ozawa et al., 2007), manipulate alternative splicing (Wang et al., 2009, 2013) or translation (Cooke et al., 2011; Abil et al., 2014) and design costume-made RNA endonucleases (Choudhury et al., 2012). As a major drawback of this approach, the 8 nucleotide recognition sequence is typically too short to ensure transcript specificity and retargeting of the PUF domain is laborious and time consuming.

These challenges may be overcome by the recently characterized Class 2 subtype VI CRISPR-Cas effector Cas13 that has been used as a programmable endoribonuclease (Abudayyeh et al., 2016, 2017; East-Seletsky et al., 2016; Smargon et al., 2017). Pioneering work by Cox et al. has shown that fusion of a mutant, catalytically inactive Cas13 (dCas13) with the adenosine deaminase ADAR2 allows the site-specific deamination of adenosine to inosine (Cox et al., 2017) providing the first proof-of-principle that this system can be used to site-specifically manipulate mRNAs. Considering that conversion of adenosine to inosine seems to be particularly important for brain development and function (Hwang et al., 2016), this system may provide new avenues to study the role of this modification in neural stem cells and brain development. In addition, it is reasonable to conclude that this approach could readily be adapted to other RNA modifications by fusing the dCas13 with any other relevant RNA-modifying enzyme (**Figure 2**).

While the CRISPR-dCas13 system proved to be very specific, versatile and efficient it could still harbor potential drawbacks that need to be assessed. For example, the RNA secondary structure may alter binding recognition (Smargon et al., 2017) and therefore limit the available target sites within a transcript. On the other hand, dCas13 binding itself could influence RNA folding, which would be critical while assessing the role of RNA modifications on lncRNAs in which structure underlies function. Finally, although targeting of dCas13 to mRNA seems to not influence translation in general (Cox et al., 2017), it could still affect RNA-protein interactions particularly at regulatory regions or splice-sites resulting in unspecific side-effects. Nevertheless, despite these potential drawbacks, the CRISPR-dCas13 system seems to be broadly applicable to drive various RNA modifications, thus, providing a powerful new tool to filling the gap in knowledge about the molecular function on transcript- and site-specific modifications in functional mRNAs and lncRNAs.

CONCLUSIONS

Although identified decades ago (Desrosiers et al., 1975; Wei et al., 1975), number, abundance, specificity and role of chemical modifications on nucleotide residues of housekeeping and functional RNAs have since remained elusive. As often in science, opening up this new field of epitranscriptomics awaited the development of new methods and technologies that allowed the investigation, for at least a handful of these modifications, of their mechanism of action and physiological role. These breakthroughs led to a number of pioneering studies only in the last few years that clearly pointed toward a regulatory role of epitranscriptome modifications in controlling the stability and metabolism of specific functional RNAs predominantly, although not exclusively, involved in the control of cell fate change and cell type-specific functions.

Among different cell types and tissues, the developing and adult mammalian brain appears to be the organ system more vulnerable to manipulations of the epitranscriptome. For example, although individuals affected by mutations for

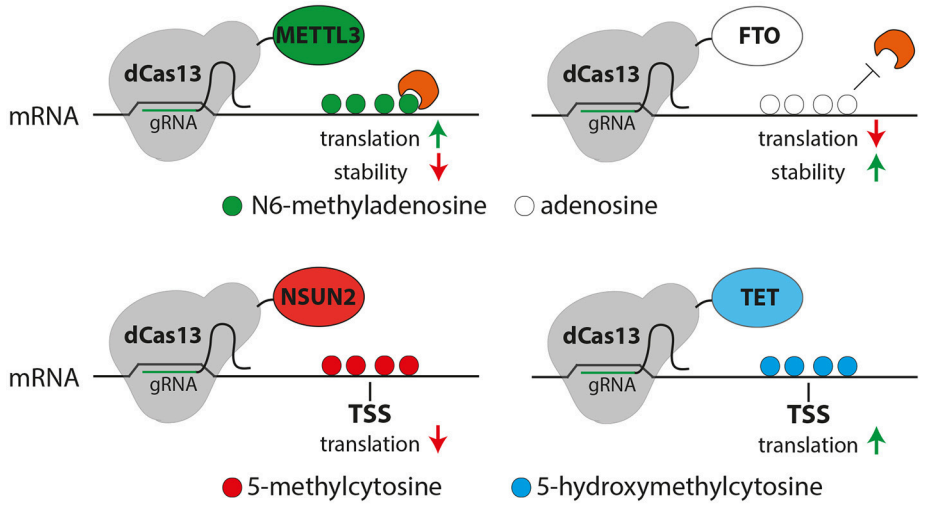

FIGURE 2 | Possible uses of the CRISPR-dCas13 (gray) system for epitranscriptome editing of N6-methyladensosine (m6A, top) or 5-methylcytosine (5mC, bottom). **Top:** Fusing dCas13 together with METTL3 (green) or FTO (white) may allow the site and transcript specific methylation (green) or demethylation (white) of mRNA, respectively resulting in m6A-mediated changes in translation or RNA stability (red or green arrows). **Bottom:** methylation of cytosine (red) or oxidation of 5mC (blue) of cytosine can be triggered by dCas13 fusion to NSUN2 (red) or TET (blue), respectively potentially resulting in a decreased (red arrow, left) or increased (green arrow, right) translation.

epitranscriptome writer or eraser genes showed different defects in various organ systems, they all share deficits in brain function including mental retardation and psychological disorders (Boissel et al., 2009; Abbasi-Moheb et al., 2012; Khan et al., 2012; Martinez et al., 2012; Komara et al., 2015). Whether an overall higher vulnerability to any mutation is a general feature of the brain or, alternatively, the epitranscriptome is a relatively late evolutionary addition to the cellular toolkit to attain higher cognitive functions is open to speculation.

With regard to evolution, in the Origin of Species Charles Darwin wrote that natural selection is constantly working to scrutinize *"…the slightest variations; rejecting those that are bad, preserving and adding up all that are good; silently and insensibly working, whenever and wherever opportunity offers, at the improvement of each organic being…"*. In light of this, it is not surprising that the mechanisms that allow the better tuning of gene expression by DNA modifications were revealed to be very similar to the ones used to better tune gene translation by RNA modifications. It is unclear whether during evolution the former were subsequently adapted to attain the latter but given life's origins from an "RNA World" the opposite possibility is also worth considering (Forterre and Grosjean, 2013). Quest for future research will be to decode the specificity and mechanisms underlying the control of RNA modifications and exploit this knowledge by epitranscriptome-editing for basic research and possible applications.

AUTHOR CONTRIBUTIONS

FN and FC contributed equally to the writing of this manuscript.

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SP8 Transcriptional Regulation of *Cyclin D1* During Mouse Early Corticogenesis

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Multiple signals control the balance between proliferation and differentiation of neural progenitor cells during corticogenesis. A key point of this regulation is the control of G1 phase length, which is regulated by the Cyclin/Cdks complexes. Using genome-wide chromatin immunoprecipitation assay and mouse genetics, we have explored the transcriptional regulation of *Cyclin D1* (*Ccnd1*) during the early developmental stages of the mouse cerebral cortex. We found evidence that SP8 binds to the *Ccnd1* locus on exon regions. *In vitro* experiments show SP8 binding activity on *Ccnd1* gene 3′-end, and point to a putative role for SP8 in modulating PAX6-mediated repression of *Ccnd1* along the dorso-ventral axis of the developing pallium, creating a medial^{Low}-lateral^{High} gradient of neuronal differentiation. Activation of *Ccnd1* through the promoter/5′-end of the gene does not depend on SP8, but on βcatenin (CTNNB1). Importantly, alteration of the *Sp8* level of expression *in vivo* affects *Ccnd1* expression during early corticogenesis. Our results indicate that *Ccnd1* regulation is the result of multiple signals and that SP8 is a player in this regulation, revealing an unexpected and potentially novel mechanism of transcriptional activation.

Keywords: corticogenesis, gene expression regulation, *Cyclin D1*, transcription factors, SP8, PAX6

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INTRODUCTION

The cerebral cortex is the most complex structure of the mammalian brain. It is the site of numerous higher-order sensory, motor, and cognitive functions. Cortical function relies on the proper formation of specialized cortical areas as well as on their sophisticated interconnections (Glasser et al., 2016).

During development, regionalization of the embryonic brain is achieved through multi-step processes. Sources of diffusible signaling molecules act as organizing centers and pattern neighboring domains through regulation of specific transcription factors expression, thereby creating molecular compartments that lead to the generation of distinct cortical fields (Rubenstein et al., 1998; Sur and Rubenstein, 2005; O'Leary et al., 2007).

Cortical projection neurons are generated in the germinal zones (GZ) of the dorsal telencephalon and, following cell-cycle exit, migrate radially to the cortical plate. Previous work has shown that regional differences in the proliferative programs in the GZ have far reaching consequences for histogenesis of cortical areas (Dehay et al., 1993; Polleux et al., 1997; Lukaszewicz et al., 2005).

Neuron number and types specific of each cortical layer and area are defined by the fine-tuned balance between proliferation and differentiation of cortical progenitor cells. While cell biology mechanisms underlying the switch from proliferative to differentiative divisions have been identified (Fish et al., 2006; Delaunay et al., 2014, 2017; Mora-Bermudez et al., 2014; Paridaen and Huttner, 2014; Matsuzaki and Shitamukai, 2015), it has been shown that the increasing fraction of progenitor cells that quit the cell cycle to embark on neuronal differentiation correlate with a lengthening of the G1 phase of the cell cycle (Takahashi et al., 1995; Calegari et al., 2005; Salomoni and Calegari, 2010; Arai et al., 2011). G1 phase is considered as a time window of susceptibility to differentiation signals (Mummery et al., 1987) and G1 phase lengthening increases the competence of a proliferating cell to withdraw from the cell cycle and to differentiate (Zetterberg et al., 1995).

During corticogenesis, proliferative and differentiative divisions are characterized by short and long G1 phases respectively (Dehay et al., 2001; Lukaszewicz et al., 2002, 2005; Calegari and Huttner, 2003; Dehay and Kennedy, 2007; Pilaz et al., 2009). Progression through G1 phase is regulated mainly by the kinase activity of Cyclin D/CDK4 and Cyclin E/CDK2 (Sherr and Roberts, 2004), both of which have been shown to play a key role in determining neuron number during mouse mid-corticogenesis (Lange et al., 2009; Pilaz et al., 2009). In particular, *Cyclin D1* (*Ccnd1*) dynamic expression levels have been shown to be at the heart of a regulatory network that control the balance between cortical progenitor proliferation and differentiation (Ghosh et al., 2014).

Here we have explored the transcriptional regulation of *Ccnd1* expression during early corticogenesis. Numerous transcriptional factors binding to the *Ccnd1* promoter have been identified (Klein and Assoian, 2008). It is targeted by TCF/βcatenin complex (Shtutman et al., 1999; Tetsu and McCormick, 1999), effector of the Wnt pathway, which plays a key role in regulating cortical expansion (Chenn and Walsh, 2003). More recently, it has been reported that the transcription factor PAX6, known to regulate proliferation and differentiation of cortical progenitors (Warren et al., 1999; Estivill-Torrus et al., 2002; Quinn et al., 2007; Sansom et al., 2009; Mi et al., 2013) binds to the *Ccnd1* locus (Sun et al., 2015).

PAX6 plays a key role in forebrain patterning and cortex arealization (Stoykova et al., 1997; Bishop et al., 2000, 2002; Muzio et al., 2002; Englund et al., 2005). Interestingly, *Pax6* shows a complementary expression pattern to the transcription factor *Sp8* in the developing pallium with a rostro-ventral^{High} gradient (Sahara et al., 2007; Borello et al., 2014). SP8 is a zinc finger transcription factor belonging to the Sp-family of transcription factors (Zhao and Meng, 2005). SP8 acts downstream of FGF8 signaling (Storm et al., 2006), regulates forebrain patterning and cortical arealization (Sahara et al., 2007; Zembrzycki et al., 2007; Borello et al., 2014), and regulates cortical progenitor cell differentiation (Borello et al., 2014). Interestingly, SP5/SP8 have been shown to act as co-activators of the Wnt pathway in mouse embryos and differentiating embryonic stem (ES) cells (Kennedy et al., 2016).

We have therefore sought to analyze the putative role of SP8, together with PAX6 and βcatenin, on the transcriptional control of *Ccnd1*. Our ChipSeq and mouse genetics analysis reveal that *Ccnd1* is a target gene of SP8. We show that SP8 is a critical player in the regulation of *Ccnd1* expression during *in vivo* mouse corticogenesis. SP8 is able to modulate the moderate repressive transcriptional activity exerted by PAX6 on the *Ccnd1* exon 1 region *in vitro*. By contrast, we did not observe cooperation between SP8 and βcatenin on *Ccnd1* activation from the promoter/5′end of the gene. Finally, we demonstrate that SP8 is able to specifically activate gene expression from the *Ccnd1* exon 5 fragment, containing part of the 3′UTR, suggesting that the 3′-end of the *Ccnd*1 gene may be target of gene regulation at multiple levels, including transcription.

MATERIALS AND METHODS

Animals

Foxg1$^{tTA+/-}$ and *tetO-Sp8-IE* mice (Waclaw et al., 2009), *Foxg1*^{cre} (Hebert and McConnell, 2000) and *Sp8*$^{fl/fl}$ (Waclaw et al., 2006) mice were maintained and genotyped as already described (Waclaw et al., 2006, 2009, 2010). Mouse colonies were maintained at the SBRI/INSERM U1208, in accordance with the European requirement for animal experimentation 2010/63/UE. The protocol APAFIS #4748 has been approved by the Animal Care and Use Committee CELYNE (C2EA #42).

Histology and *in Situ* RNA Hybridization (ISH)

Embryos were collected considering noon on the day of the vaginal plug as E0.5. The embryos were dissected and fixed overnight by immersion in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C. The tissue was cryoprotected by immersion in 30% sucrose/PBS, embedded in OCT (Tissue-Tek), and cryostat sectioned at 20 μm.

In situ RNA hybridization on cryostat sections was performed as previously described (Borello et al., 2008). cRNA probes used were: *Sp8* (K. Campbell, Cincinnati Children's Hospital, OH, USA), *Ccnd1* (A. Mallamaci, SISSA, Trieste, IT), *Axin2* (B. Cheyette, UCSF, USA), and *Pax6* (D. Price, University of Edinburgh).

Gene expression patterns were compared between brains of different genotypes by matching the plane of section according to multiple anatomical features. Whenever possible, this was performed for multiple planes of sections for each gene, and from at least three brains for each genotype.

Foxg1$^{tTA/+}$ and the *tetO-Sp8-IE* mice were used as control; differences in phenotype were not observed between these two lines or between *Foxg1*$^{tTA/+}$ and the *tetO-Sp8-IE* mice and the wild type embryos.

ChipSeq

Dorsal telenchephalon (pallium) was dissected from E12.5 CD1 mouse embryos. The cells were crosslinked with 1% formaldehyde for 10 min. The formaldehyde reaction was quenched by adding glycine to a final concentration of 0.125 M for 10 min. Cells were then pelleted, rinsed once in cold

phosphate-buffered saline (PBS) with 1 mM PMSF and once in cold lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40, and Roche Complete Protease Inhibitor Cocktail) to obtain nuclear pellets. Nuclei were sonicated in RIPA buffer (1X PBS, 1% NP-40 Substitute, 0.5% Sodium Deoxycholate, 0.1% SDS, and Roche Complete Protease Inhibitor Cocktail) at a concentration of 5×10^7 nuclei/mL using a diagenode sonicator (Bioruptor Plus). The DNA fragments bound by SP8 were isolated using a goat polyclonal anti-SP8 antibody (C-18, Santa Cruz Biotechnology), a rabbit polyclonal anti-SP8 antibody (ab739494, abcam), or rabbit polyclonal H3K27ac (ab4729, abcam) coupled to magnetic beads (Dynabeads, ThermoFisher). The beads were washed 5 times with LiCl Wash Buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate) and finally with TE buffer (10 mM Tris-HCl pH 7.5, 0.1 mM Na_2EDTA).

The DNA was incubated o/n at 65°C in elution buffer (1% SDS, 0.1 M $NaHCO_3$) to reverse the formaldehyde crosslink and was purified using a QIAquick PCR Purification Kit (Qiagen), following the manufacturer protocol. To check for fragment size distribution after sonication, a small fraction of the sample was reverse cross-linked for 2 h at 65°C, purified using DNA purification columns from Qiagen, then loaded onto a 2% agarose gel.

Sequence base calls were made using standard Illumina methods. Resulting 1×50 bp sequences were filtered to remove sequencing artifacts and adaptors and then mapped to the mouse genome (mm9) using the BWA algorithm (Li and Durbin, 2009). The resulting uniquely mapped reads were used for peak calling with MACS1.4 for SP8 and MACS2.1 for H3K27ac (Zhang et al., 2008; Feng et al., 2011), using recommended settings for transcriptional factor analysis and histone marks respectively. Called peaks were filtered to remove regions where a significant number of artifacts could originate (Consortium, 2012) (https://sites.google.com/site/anshulkundaje/projects/blacklists). Pearson's correlation on the two replicates calculated with a call to wigCorrelate (http://hgdownload.soe.ucsc.edu/admin/exe/macOSX.x86_64/) or Wiggletools (Zerbino et al., 2014) gave a value of 0.9. Peaks were annotated based on nearest transcription start site (TSS) using the Bioconductor package ChiPpeakAnno (Zhu et al., 2010) and ChiPseeker (Yu et al., 2015) and visualized using the Gviz package (Hahne and Ivanek, 2016).

The SP8 ChipSeq data presented in the "Results" section were obtained using the goat polyclonal anti-Sp8 antibody. These results were confirmed with a SP8 ChipSeq performed on two other independent biological replicates with the rabbit polyclonal anti-Sp8 antibody (**Table S1** and data not shown).

Cell Transfection and Luciferase Assay

P19 cells (ATCC number: CRL-1825) were maintained in growth medium: Alpha Minimum Essential Medium with ribonucleosides and deoxyribonucleosides (ThermoFisher) completed with 7.5% bovine calf serum and 2.5% fetal calf serum (ThermoFisher) (McBurney and Rogers, 1982; McBurney et al., 1982). The cells were transfected with the expression vector for the full-length cDNA of human βcatenin (gifts of Dr Grosschedl, Max Planck Institute of Immunology, Germany), or *Pax6* (D. Price, University of Edinburgh, UK), or *Sp8* (gift of K. Campbell, Cincinnati Children's Hospital, OH, USA), along with the different *Ccnd1* fragments identified by ChipSeq (**Table S1**), cloned in the pGL4.10[Luc2] vector (Promega) containing the human *βglobin* minimal promoter upstream of the luciferase gene (*Luc2*, *Photinus pyralis*). The fragment named *Ccnd1* exon 2.3 contains *Ccnd1* exons 2 and 3. The vector pG4.74[hRLuc/TK] (Promega), containing the Renilla luciferase gene, was co-transfected for normalization. The TK promoter of the pG4.74[hRLuc/TK] vector was substituted with the human *βglobin* minimal promoter (from vector BGZ40) (Yee and Rigby, 1993).

The cells were transfected with Lipofectamine 2000 (ThermoFisher) in OPTIMEM medium (ThermoFisher) following the manufacturer's instructions, and cultured after 6 h in growth medium. Twenty-four hours after the transfection the cells were harvested in lysis buffer (Promega), and the luciferase and renilla luciferase activities were measured using the Dual Luciferase Assay protocol (Promega). Each transfection experiment was performed in triplicate and repeated at least two times. Reporter gene activities shown in **Figures 3–5** represent the average of the three replicates obtained in one representative transfection experiment. Statistical analysis was performed using the statistical package R and ANOVA analysis was performed using the "aov" R function and Tukey multiple comparison test. $p < 0.05$ were considered statistical significant.

RESULTS

Cyclin D1 Is Expressed in the Developing Forebrain at E12.5 With a Rostro-Ventral^{High} Gradient

Cyclin D1 is a key regulator of G1 phase progression in neural progenitor cells. We analyzed the mRNA expression of *Cyclin D1* in the embryonic forebrain at E12.5. We found that while *Ccnd1* is strongly expressed in the ventricular zone (VZ) of the basal ganglia (**Figures 1A,B**, arrowheads), its expression in the pallial VZ follows a rostro-lateral^{High} gradient (**Figures 1A–C**). In particular, *Ccnd1* expression is low in the medial pallium compared to dorsal and lateral regions (**Figure 1A**, arrow), while it is not expressed caudally in the hem (**Figure 1B**, arrow). This shows that *Ccnd1* is not expressed in all pallial progenitor cells at the same level, suggesting that the complex *Ccnd1* expression pattern is regulated by different factors.

Sp8 is expressed in the pallium with a rostro-medial^{High} gradient (**Figures 1D–F**). *Sp8* is expressed in the pallial VZ, as *Ccnd1*. In the subpallium *Sp8* is expressed in the subventricular zone (SVZ) of the lateral ganglionic eminence (LGE) (**Figures 1D,E**, arrowheads), while *Ccnd1* is expressed in the subpallial VZ (**Figures 1A,B**, arrowheads).

Ccnd1 appears to be highly expressed in the dorsal pallium were *Sp8* expression is high (**Figures 1A,B,D,E**, asterisks); interestingly *Ccnd1* expression is lower in the medial pallium, a region of strong *Sp8* expression (**Figures 1A,B,D,E**, arrows).

In conclusion, the expression pattern of *Sp8* is compatible with the possibility that it contributes to the transcriptional regulation of *Ccnd1* in the dorso-medial pallium.

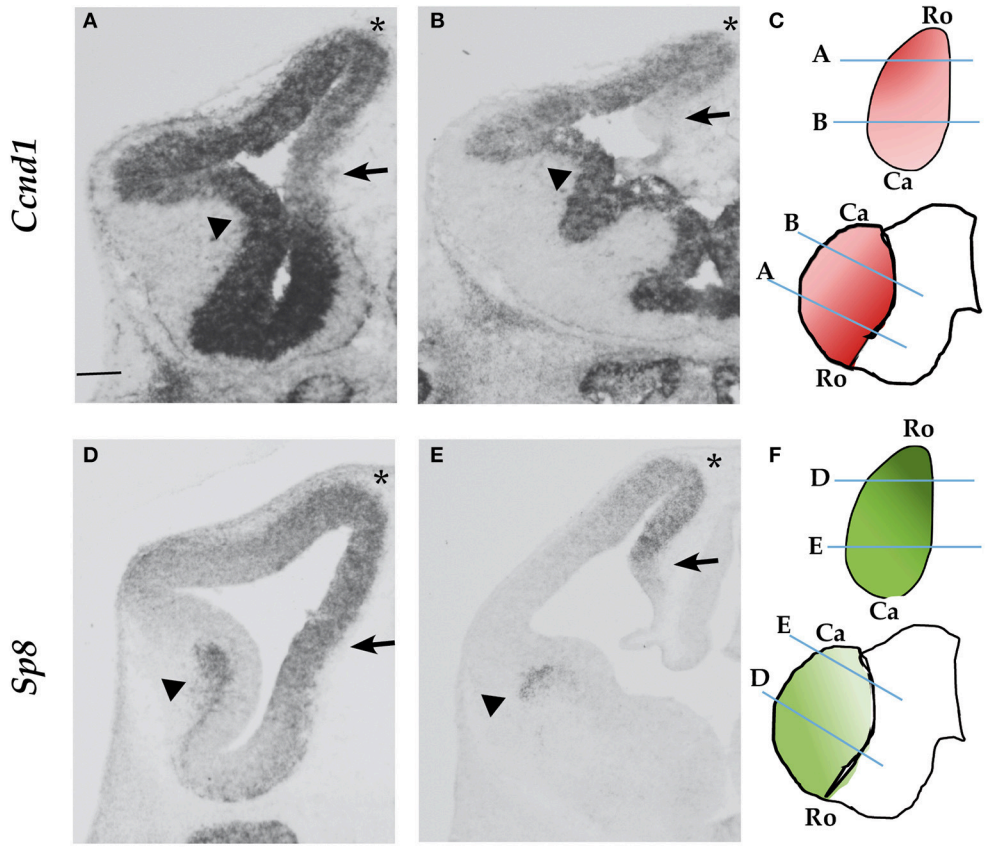

FIGURE 1 | *Ccnd1* and *Sp8* expression patterns at E12.5. ISH performed on E12.5 mouse forebrain coronal sections. Panels **(A,B)** show *Ccnd1* mRNA expression; Panels **(D,E)** indicate *Sp8* mRNA expression. Schematic of *Ccnd1* **(C)** and *Sp8* **(F)** gradients of expression are indicated along with the positions of sections shown in **(A–D)**. Panels **(A,D)** represent sections at the rostral level, while Panels **(B,E)** represent sections at the caudal level. Arrows point to the medial pallium; arrowheads indicate the VZ **(A,B)** or the SVZ of the LGE **(D,E)**. The asterisk indicates the dorsal pallium. Bar in panel **(A)** is 200 μm. Ro, rostral; Ca, Caudal.

The Zinc Finger Transcriptional Factor SP8 Binds to the *Ccnd1* Locus in Cortical Progenitor Cells

To test the hypothesis that SP8 regulates *Ccnd1* at the transcriptional level, we performed SP8 ChipSeq experiments using E12.5 mouse embryos pallial cells (manuscript in preparation). We found that SP8 binds the *Ccnd1* locus *in vivo* decorating *Ccnd1* exons (**Figures 2A,B**), with higher values for exon 1 containing the 5′UTR, exon 2, and exon 5 containing part of the 3′UTR.

The presence of acetylated histone H3 lysine 27 (H3K27ac) on exons 1 and 2 indicated that these regions correspond to active chromatin domains (**Figures 2C,D**). The *Ccnd1* exon 5 and exon 3 co-localize with the H3K27ac signal, even though it is of smaller intensity than in the exon 1 (**Figures 2C,D**). H3K27ac signals were obtained from ChipSeq experiments using, as for SP8, E12.5 mouse pallial cells (data not shown, manuscript in preparation).

The fact that *Ccnd1* 5′UTR showed H3K27ac signal and it contains a CpG island suggests a role for this region in the transcriptional regulation of *Ccnd1* in E12.5 cortical progenitor cells (**Figures 2C,D**). Moreover, *Ccnd1* promoter and the 3′UTR represent important regulative regions for the transcriptional regulation of this gene (Klein and Assoian, 2008; Deshpande et al., 2009; Guo et al., 2011). Together these data indicate that SP8 binds transcriptionally active regions in the *Ccnd1* locus *in vivo* in pallial progenitor cells.

SP8 Regulates Gene Expression Through *Ccnd1* Exon 5 Fragment *in Vitro*

The observation that SP8 binds mainly on *Ccnd1* exons is intriguing. It is generally assumed that the coding genome is physically distinct from the regulatory genome. Consequently, the binding of transcription factors to gene exons is considered generally non-functional (Li et al., 2008). Therefore, we evaluated the relevance of SP8 binding on *Ccnd1* exons observed in our ChipSeq experiments.

To test the transcriptional activity of SP8 on the different *Ccnd1* exons we performed a luciferase assay *in vitro*. We focused on the *Ccnd1* exons showing both SP8 ChipSeq peaks and active chromatin signature (i.e., H3K27ac signal) (**Figure 2**). The exon 1 (Ex1) fragment contained the last 293 bp of the *Ccnd1* promoter (Eto, 2000), the entire exon 1 (containing the 5′UTR) and the

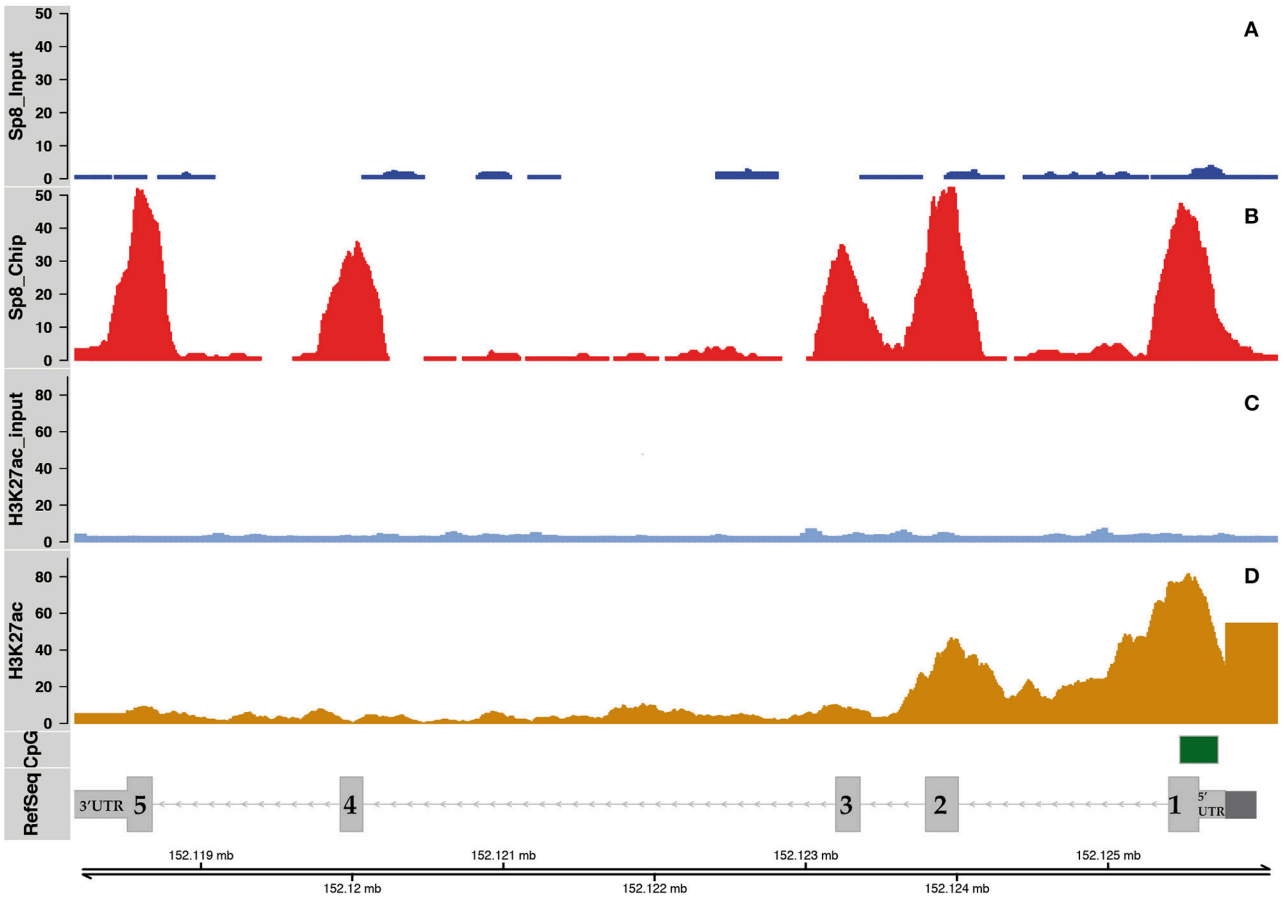

FIGURE 2 | SP8 binding regions on the *Ccnd1* locus. Panels **(A,B)** show SP8 ChipSeq peaks in input and SP8 antibody (Ab) treated samples respectively. Panel **(C)** shows normalized H3K27ac ChipSeq peaks. Panel **(D)** shows *Ccnd1* RefSeq gene model in light gray, the promoter (Eto, 2000) in dark gray, and the CpG islands in green. *Sp8* exons are numbered and the UTRs are indicated.

first 526 bp of intron 1 (**Figure 2** and **Figure S1**). The exon 5 (Ex5) fragment spanned from intron 4 (last 425 bp) to the coding region up to the first 299 bp of the 3′UTR (**Figure 2** and **Figure S2**).

The DNA region corresponding to the SP8 ChipSeq peaks were cloned upstream of the luciferase gene and tested in P19 cells in the presence of increasing levels of SP8. Surprisingly, we found that SP8 had no activity on the *Ccnd1* Ex1 fragment (**Figures 3A,B**), nor on exons 2 and 3 (Ex2.3) fragment (**Figures 3C,D**). However, increasing amounts of SP8 activated the luciferase gene through the *Ccnd1* Ex5 fragment (**Figures 3E,F**).

Bioinformatic analysis using the Jaspar software, indicated 7 putative SP8 binding sites located in the Ex5 fragment (**Table S2**); specifically, a cluster of 6 sites is located in the exon 5 ORF (**Figure S2**). This region contains the SP8 peak summit identified in our ChipSeq results (**Figure 5** and **Figure S2**). This unexpected result indicates that SP8 binds to the exonic region 5 of *Ccnd1*, thereby modulating its transcription.

βcatenin and PAX6 Regulate *Ccnd1* Exon1 Fragment Activity *in Vitro*

The Wnt/βcatenin pathway was demonstrated to be a major regulator of *Ccnd1* gene expression (Shtutman et al., 1999; Tetsu and McCormick, 1999). The Wnt pathway regulates gene expression by binding of the cofactor βcatenin to genomic regulative regions specifically recognized by TCF/LEF, the effectors of the Wnt pathway (Clevers, 2006; van Amerongen and Nusse, 2009).

Interestingly, the SP8 ChipSeq peak corresponding to exon 1 and containing the last 293 nucleotides of the mouse *Ccnd1* promoter (Eto, 2000), contains a highly conserved consensus for TCF/LEF transcriptional factors (Klein and Assoian, 2008) (**Figure 4A**). As in human, mouse *Ccnd1* promoter has no TATA or TATA-like sequence, and the TSS is determined by the Initiator sequence (Inr) (Eto, 2000). However, two possible Inr sequences are present in the mouse *Ccnd1* promoter, the second one located at nt +90 from the first Inr sequence, determining a TSS at nt +96 (Eto, 2000) (**Figure 4A**). According to the Inr site described by

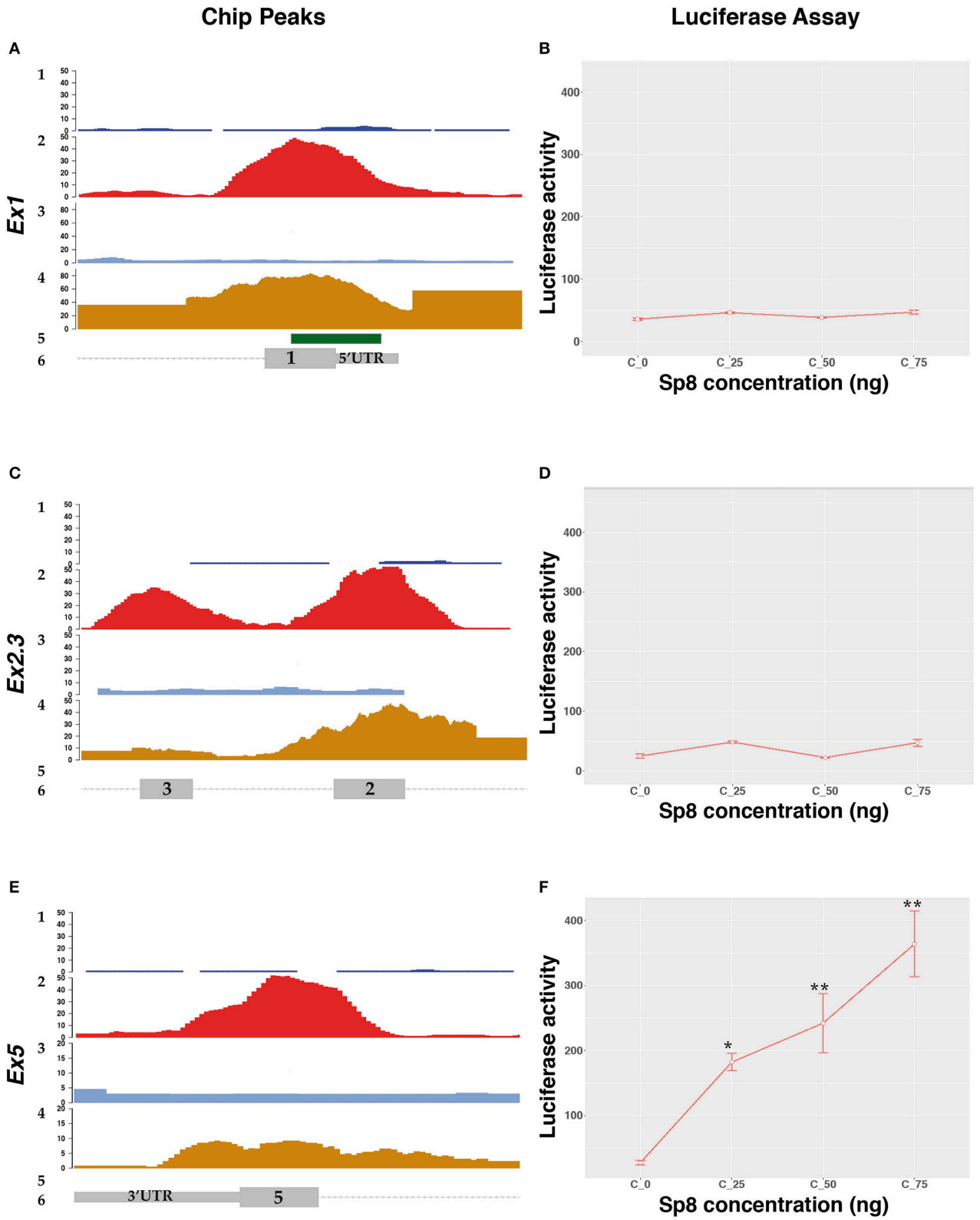

FIGURE 3 | SP8 transcriptional regulation of *Ccnd1* genomic regions bound by Sp8. Panels **(A,C,E)** show details of the *Ccnd1* locus where the SP8 ChipSeq peak have been detected: **(A)** SP8 peak at the *Ccnd1* Ex1 fragment; **(C)** SP8 peak at the *Ccnd1* Ex5 fragment; **(E)** Sp8 peak at the *Ccnd1* Ex2.3 fragment. 1: SP8 Chip

(Continued)

FIGURE 3 | input peaks, 2: SP8 Chip peaks, 3: H3K27ac Chip Input peaks, 4: H3K27ac Chip peaks, 5: CpG island, 6: *Ccnd1* RefSeq gene model. For details refer to the legend of **Figure 2**. Panels **(B,D,F)** show the results of the luciferase assays performed with *Ccnd1* Ex1 **(B)**, *Ccnd1* Ex2.3 **(F)**, and *Ccnd1* Ex5 **(D)** fragments. Statistical significance, ANOVA: $*p < 0.05$, $**p < 0.01$.

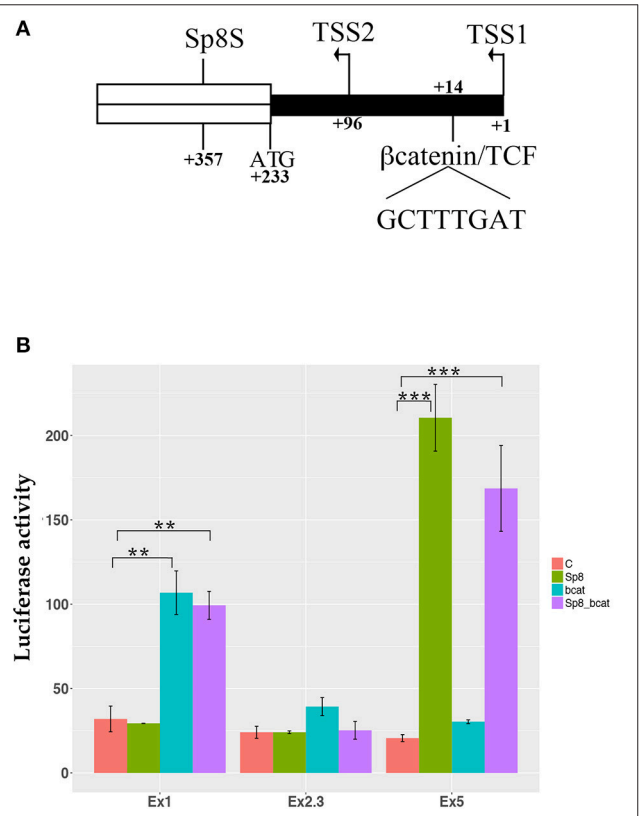

FIGURE 4 | βcatenin and PAX6 transcriptional regulation of *Ccnd1* regulative regions identified by SP8 ChipSeq. **(A)** represents the schema of the 5′ portion of the exon 1 fragment. The transcriptional start sites, TSS1 and TSS2, the first codon ATG, and the conserved βcatenin binding site (Klein and Assoian, 2008), and the SP8 summit (SP8S) positions are indicated. The 5′UTR region is depicted in black; the ORF of exon 1 is indicated in white. Panel **(B)** shows the results of the luciferase assay obtained from one representative experiment. P19 cells were transfected with the *Ccnd1* exon fragments identified by SP8 ChipSeq alone or in combination with βcatenin, SP8, or βcatenin together with SP8. Statistical significance, ANOVA: $**p < 0.01$, $***p < 0.001$.

Eto (2000), the conserved TCF/LEF site is located downstream to the first TSS, starting at nt + 14, in the *Ccnd1* 5′UTR (**Figure 4A**).

We tested βcatenin transcriptional activity in combination with SP8 on the above described *Ccnd1* fragments. We used a constitutively active form of βcatenin that is not degraded by the proteasome and accumulates into the nucleus (Hsu et al., 1998) in a luciferase assay *in vitro*. Our results showed that βcatenin activates luciferase transcription specifically through the *Ccnd1* Ex1 fragment, and that SP8 does not modulate this effect (**Figure 4B**). No effect was observed on *Ccnd1* Ex2.3 or Ex5 fragments (**Figure 4B**).

PAX6 is a transcription factor which regulates forebrain patterning and growth. It is expressed with a complementary gradient to that of *Sp8* (**Figures S3A,B**). PAX6 binds to the *Ccnd1* locus (Sun et al., 2015). When we compared the position of the PAX6 ChipSeq peak with that of SP8, we found that the two transcription factors bind to an overlapping region in the Ex1 fragment and that the SP8 peak summit was located near the PAX6 binding region (**Figures 5A,B** and **Figure S1**). Bioinformatics analysis using the Jaspar database showed a potential PAX6 binding site in the Ex1 fragment at position + 392 from the first TSS (**Figure 5C** and **Figure S1**); this predicted consensus sequence is near the summit of the PAX6 ChipSeq peak (**Figure S1**) (Sun et al., 2015).

We tested the effect of PAX6 on *Ccnd1* Ex1 fragment transcriptional activity and the effect of SP8 upon co-expression. Our results showed that PAX6 exerts a moderate repressive transcriptional activity on the *Ccnd1* exon 1 region, and that SP8 counteracts this repression when co-transfected with PAX6 (**Figure 5D**).

SP8 Regulates *Ccnd1* During *in Vivo* Corticogenesis

To further test the role of SP8 on *Ccnd1* gene regulation we analyzed the relevance of our *in vitro* results by altering the level of *Sp8* expression *in vivo* during corticogenesis. For this purpose, we took advantage of genetic systems in which *Sp8* was either overexpressed or absent. In the *Sp8* gain-of-function (GOF) transgenic mouse system, *Sp8* is over-expressed during forebrain development (Waclaw et al., 2009; Borello et al., 2014), while in the loss-of-function (LOF) transgenic mouse system (Waclaw et al., 2006; Borello et al., 2014), *Sp8* expression is eliminated (**Figure S4**).

When we analyzed *Ccnd1* expression during early corticogenesis using these genetic tools we found that *Ccnd1* expression was strongly increased after *Sp8* over-expression in the GOF mutant mice (**Figures 6A,B**), while it was strongly reduced in the LOF mutant mice in regions corresponding to the higher *Sp8* expression domain, i.e. the rostral dorso-medial pallium (**Figures 6A–C**).

These data were further confirmed by RNASeq experiments (data not shown) performed on E12.5 mouse pallial cells showing an increase of *Ccnd1* expression in the *Sp8* GOF mutants of 3 folds (FDR adjusted $p < 0.001$) and a reduction of 0.8 folds in the *Sp8* LOF mutants (FDR adjusted *p*-value 0.09) (**Table S3**).

These findings indicate that SP8 is a critical player in the regulation of *Ccnd1* expression during mouse corticogenesis *in vivo*.

DISCUSSION

Cyclin D1 is a major cell-cycle regulator (Ekholm and Reed, 2000; Sherr and Roberts, 2004) and has been shown to be at the heart of a regulatory network controlling the balance between

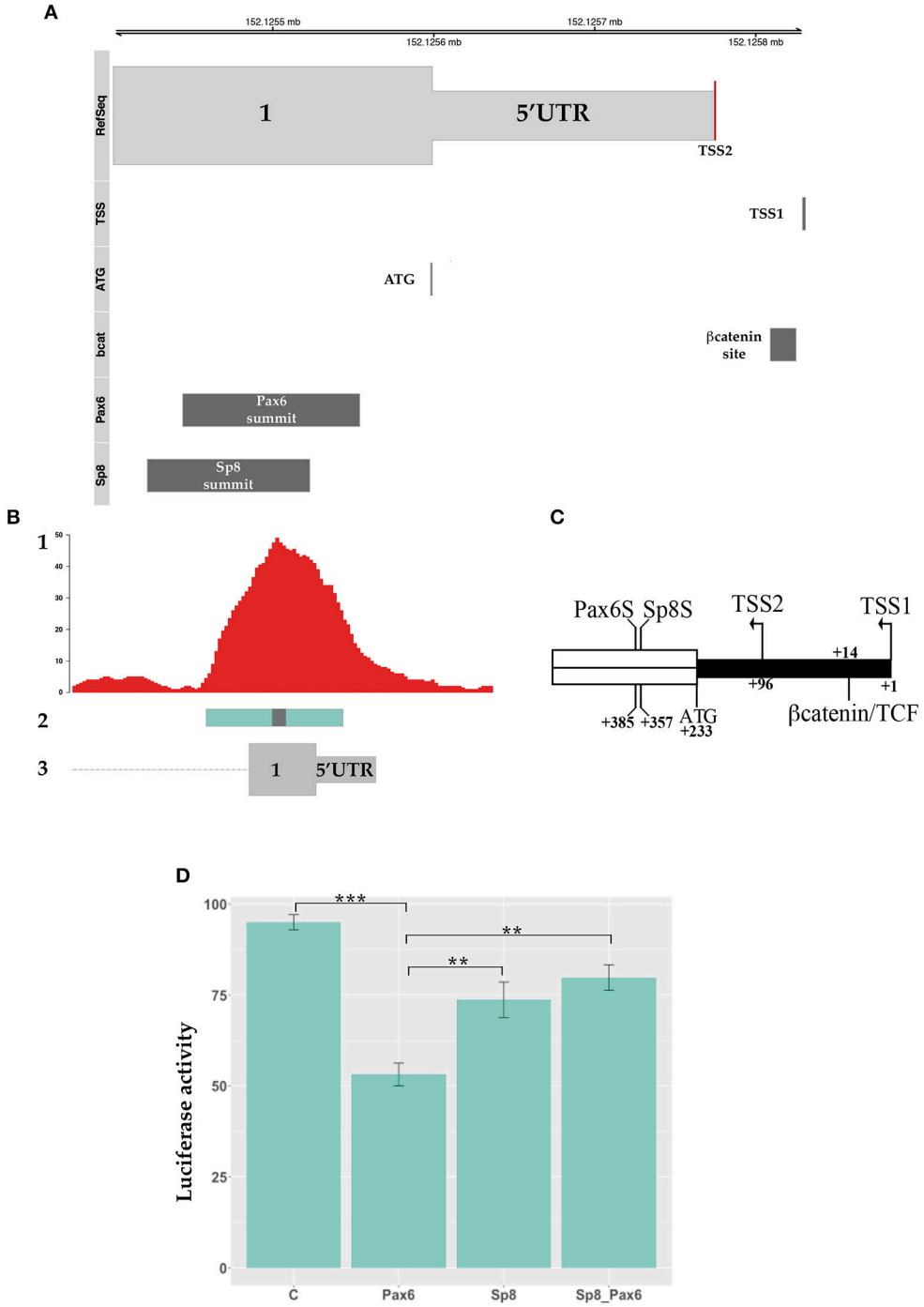

FIGURE 5 | PAX6 transcriptional regulation of *Ccnd1* regulative regions. Panel **(A)** shows the schema of *Ccnd1* locus. PAX6 [Chip peak fragment indicated in Sun et al. (2015)], SP8 (Chip peak summit ±50 bp), βcatenin binding sites, and the TSS (named TSS1) described in Eto (2000) are depicted together with *Ccnd1* exon 1 first codon (ATG) and the TSS reported by the RefSeq gene model (TSS2, red line on the RefSeq track). Panel **(B)** represents the schema of the SP8 and PAX6 peaks positions on the *Ccnd1* locus, showing the overlap between the SP8 (this work) and PAX6 ChipSeq peaks (Sun et al., 2015) on *Ccnd1* Ex1 fragment. 1: SP8 Chip peaks, 2: PAX6 chipped fragment, 3: *Ccnd1* RefSeq gene model. The gray box on the *Pax6* fragment is the PAX6 binding summit shown in **(A)**. Panel **(C)** shows a schematic of βcatenin, SP8 and PAX6 sites position on the 5′ portion of the exon 1 fragment. The PAX6 (PAX6S) and SP8 (SP8S) summit positions [indicated as the central nt of the Chip-qPCR fragment indicated in Sun et al. (2015) and the calculated ChipSeq summit respectively] are indicated. The TSS described in Eto (2000) (TSS1), the *Ccnd1* first codon (ATG), and the TSS reported by the RefSeq gene model (TSS2) are also shown. The 5′UTR is depicted in black. Panel **(D)** shows the luciferase assay results obtained from one representative experiment. *Ccnd1* exon 1 was transfected in P19 cells alone or in combination with PAX6, SP8, and PAX6 together with SP8 (SP8_PAX6). Statistical significance, ANOVA: $^{***}p < 0.001$, $^{**}p < 0.01$.

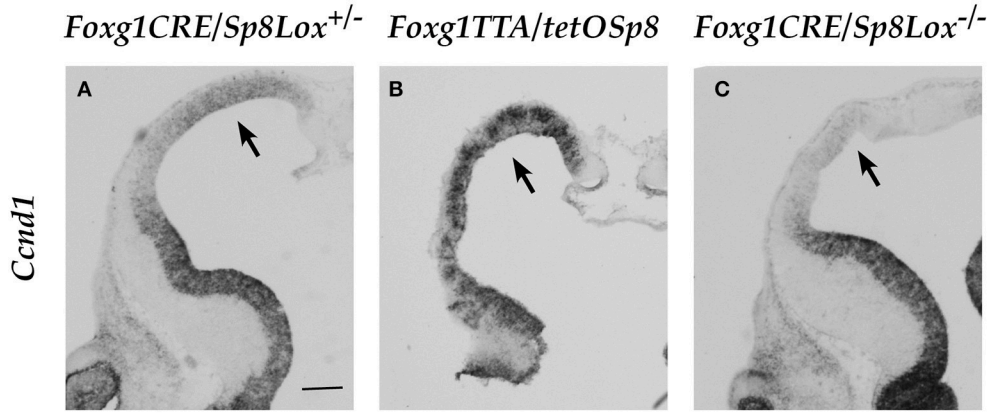

FIGURE 6 | Expression of *Ccnd1* in embryonic forebrain after *Sp8* misexpression *in vivo*. ISH on E12.5 mouse forebrain coronal sections. Arrow in Panel **(A)** shows *Ccnd1* expression in the control pallial VZ. Arrows in Panels **(B,C)** show *Ccnd1* expression in the *Sp8* GOF and *Sp8* LOF pallial VZ respectively. Bar in **(A)**: 200 μm.

proliferation and differentiation in the cerebral cortex (Ghosh et al., 2014).

βcatenin is one of the main regulators of *Ccnd1* expression (Shtutman et al., 1999; Tetsu and McCormick, 1999; Klein and Assoian, 2008). Our observations that *Ccnd1* expression signal does not necessarily correlate to regions of high Wnt/βcatenin activity during early *in vivo* corticogenesis is consistent with the idea that activation of the *Ccnd1* gene might be modulated by cooperation with other transcription factors. Indeed, *Axin2*, a direct target and recognized proxy of the Wnt/βcatenin pathway activity (Yan et al., 2001; Jho et al., 2002; Lustig et al., 2002; Kim et al., 2007; Al Alam et al., 2011; van Amerongen et al., 2012; Bowman et al., 2013), is strongly localized in the medial pallium where *Ccnd1* expression is low or absent and weakly expressed in the dorsal pallium where *Ccnd1* is highly expressed (**Figures 1A,B** and **Figures S3C,D**). Altogether these data suggest that while βcatenin regulates *Ccnd1* expression during corticogenesis *in vivo*, other transcription factors are also at work to produce the observed *Ccnd1* expression pattern in the dorso-medial pallium.

Sp8 and *Ccnd1* expression patterns in the early mouse corticogenesis *in vivo* are consistent with a potential role of SP8 on *Ccnd1* gene regulation. The present data confirm this hypothesis and show the identification of *Ccnd1* as the first SP8 target gene.

SP8 binds on the *Ccnd1* locus on regions of active chromatin, as indicated by the H3K27ac ChipSeq results. Interestingly, peaks with higher intensity were positioned at the promoter/exon1 region, and in exon 5, containing also the first 299 bp of the 3′UTR. Our findings were further confirmed by results from SP8 ChipSeq experiments using a second SP8 antibody (**Table S1** and data not shown). When we tested the responsiveness of these regions to SP8 we found that SP8 was able to activate gene expression from the *Ccnd1* Ex5 but not from the Ex1 fragment, containing the last 293 nucleotides of the *Ccnd1* promoter.

These results are unexpected. The regulatory regions of the genome are generally considered to localize outside of the coding sequences to keep the regulatory and the coding codes separated. However, a theoretical study predicts that the human genome, compared to a synthetic string of DNA letters, could accommodate short functional regulatory motifs in the protein coding regions (Itzkovitz and Alon, 2007). In addition, different studies aimed at identifying regulatory regions in the genome found that a small percentage of these regulatory domains are located in the coding sequences (Cawley et al., 2004; Visel et al., 2009) and that they are functional (Ritter et al., 2012). Recently, a comprehensive study mapping transcription factor binding on human genome exons in many cells lines found that ∼15% of human codons specify both amino acids and transcription factor binding sites (Stergachis et al., 2013). Stergachis and colleagues suggest the fascinating hypothesis that the transcription factors binding to conserved sequences inside a gene exons have a role in codon choice and protein evolution. Numerous studies report that intergenic regulative regions like enhancers are sites of active transcription (De Santa et al., 2010; Kim et al., 2010; Natoli and Andrau, 2012; Shlyueva et al., 2014; Kim and Shiekhattar, 2015; Li et al., 2016), blurring the distinction between transcribed gene regions and regulative domains.

The Sp-family transcription factors bind preferentially GC and/or GT-reach regions in TATA-containing and TATA-less promoters and stimulate transcription by associating with the basal transcription complex and other transcription factors (Lania et al., 1997; Philipsen and Suske, 1999; Zhao and Meng, 2005). Consistently, the SP8 ChipSeq experiments showed that 78% of SP8 peaks correspond to gene promoters while genome wide SP8 binds only ∼2% of gene exons and UTRs (see **Figure S5** for details on the genome-wide SP8 binding localization). Interestingly, while our bioinformatics analysis identified several SP8 binding sites in the *Ccnd1* promoter contained in the Ex1 fragment (**Table S4** and **Figure S1**), the ChipSeq experiment indicates that the SP8 summit is located in the coding region. We hypothesize that SP8 binding on *Ccnd1* exons is related to the fine-tuned regulation of *Ccnd1* transcription, probably through a precise chromatin 3D structure, as well as to *Ccnd1* mRNA maturation. There is also the possibility that SP8 is part of an epigenetic complex regulation of *Ccnd1* locus replication

and transcription. These questions will require further investigations.

Of interest, PAX6, which generally colocalizes with enhancers, binds *Ccnd1* exon 1 (Sun et al., 2015). The predicted PAX6 binding site starts at position + 392 from the TSS (166 nucleotides downstream to the ATG), (**Figures 2**, **5A–C** and **Figure S1**). Interestingly, while SP8 failed to directly regulate *Ccnd1* Ex1 fragment expression, we show that SP8 was able to counteract the repressive activity exerted by PAX6 on the *Ccnd1* Ex1 fragment *in vitro*. Moreover, the repressive activity we observed with PAX6 is consistent with the moderate increase of *Ccnd1* mRNA observed in the *Pax6* LOF E12.5 mutant forebrain (Mi et al., 2013; Sun et al., 2015).

SP8 could, therefore, interfere with PAX6 effect on *Ccnd1* expression. It is possible that, due to the close proximity of the SP8 and PAX6 consensus, the two transcription factors compete for the binding on *Ccnd1* exon 1. As mentioned above, *Sp8* and *Pax6* show opposite gradient of expression during early corticogenesis. At mid-gestation when *Pax6* expression becomes homogeneous in the pallium, *Sp8* expression is expressed at low levels. These data suggest that *Ccnd1* is activated differentially by *Sp8* and *Pax6* in opposite domains of the pallium and is modulated by PAX6 and SP8 dosages along the neurogenic gradient.

SOX2 activates *Ccnd1* in a dose-dependent manner during corticogenesis (Hagey and Muhr, 2014). SOX2, binding on different sites on the *Ccnd1* locus and interacting with the TCF/βcatenin complex, regulates *Ccnd1* expression and cortical progenitor cell mode of division and rate of differentiation (Hagey and Muhr, 2014). Considering the *Sp8* graded expression in the pallial VZ and the strong *Sp8* expression in the subpallial SVZ (Waclaw et al., 2006; Borello et al., 2014), one can hypothesize that a dose-dependent differential transcriptional regulation is also operant for SP8 (**Figure 7**).

In contrast to PAX6, βcatenin was able to activate transcription from the *Ccnd1* 5′UTR, and this activation was not dependent or modulated by SP8. The mouse *Ccnd1* Ex1 fragment described here contains a TCF/LEF consensus that is conserved among different species, including human (Klein and Assoian, 2008), suggesting a critical and fundamental role for this site in *Ccnd1* regulation. In addition, Tetsu and colleagues showed that activation of the *CCND1* human minimal promoter,−962CD1 (Albanese et al., 1995), by βcatenin depends on the presence of TCF binding sites but not of other transcription factors (Tetsu and McCormick, 1999). These observations are in agreement with our results showing that the exon 1 fragment, containing only the last 293 nucleotides of the mouse *Ccnd1* promoter, was sufficient to support βcatenin activity. The fact that βcatenin activity was independent of SP8 indicates that these two transcription factors do not cooperate by binding the *Ccnd1* exon 1 region. However, a potential cooperation between βcatenin and SP8 binding to different *Ccnd1* exon fragments (i.e., exon 5) needs further investigation.

Our results show that SP8 is able to specifically activate gene expression from the *Ccnd1* Ex5 fragment. Consistently with our luciferase results, we found a cluster of putative SP8 binding sites at the end of the ORF in Ex5 fragment; this cluster overlapped with the SP8 summit identified in our ChipSeq experiments (**Table S1** and **Figure S2**). These findings are very interesting as they rise the possibility that SP8 might control gene expression from binding to regions located at the 3′ end of the *Ccnd1* gene in addition to the classical enhancer/promoter regulative domains located upstream of the target genes.

Human *CCND1* 3′UTR region has been shown to act as a critical regulatory element. Different miRNAs are predicted to bind human and mouse *Ccnd1* 3′UTR and regulate the level of *Cyclin D1* expression (Deshpande et al., 2009; Ghosh et al., 2014); truncation or mutation of human *CCND1* 3′UTR alter the stability of the *CCND1* transcript activating its oncogenic potential (Lebwohl et al., 1994; Molenaar et al., 2003; Wiestner et al., 2007; Deshpande et al., 2009; Ghosh et al., 2014). In addition, different Snps are present in the 3′UTR of mouse and human *CCND1*: Snp rs7178, localized on *CCND1* 3′UTR, is involved in neuroblastoma (Wang et al., 2011), and Snp rs7177, localized on *CCND1* 3′UTR, is involved in cognitive behavior (Rietveld et al., 2013). Considering that there is a 78.1% identity between human and mouse *Ccnd1* 3′UTR (as revealed using the ECR Browser Ovcharenko et al., 2004), these observations suggest a similar role in gene regulation and neurogenesis for the mouse *Ccnd1* 3′UTR,

Our data, showing that SP8 binds and specifically regulates *Ccnd1* transcription from a region located at the end of the ORF in exon 5 and close to the 3′UTR, suggest that the 3′-end of the *Ccnd1* gene may be a target of gene regulation at multiple levels, including the transcriptional one. The *in vitro* validation of the activity of SP8, as well as the interaction with PAX6 and βcatenin on the *Ccnd1* locus, is based on an assay commonly used to screen the activity of genomic regulative regions. In addition, we provide further evidence based on manipulation of SP8 levels of expression *in vivo* in GOF and LOF transgenic mice as well as on RNAseq data that both clearly show a role for SP8 for *Ccnd1* expression regulation at early stages of pallium development.

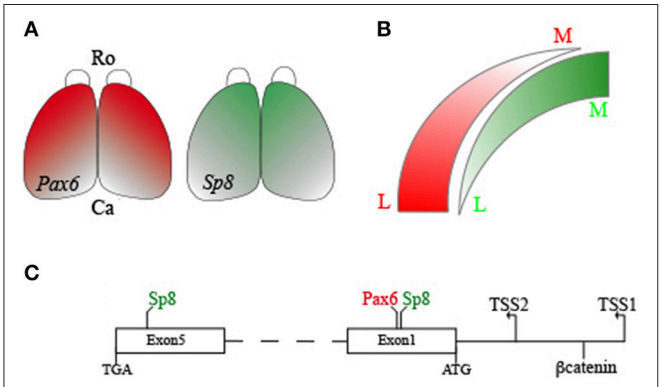

FIGURE 7 | Schema of the SP8, PAX6, and βcatenin interactions on the mouse *Ccnd1* gene. Panel **(A)** shows *Sp8* and *Pax6* expression gradients in the developing mouse cortex. Panel **(B)** summarizes differences in *Sp8* and *Pax6* expression levels along the medio-lateral axis of the pallium. Panel **(C)** is a graphical representation of SP8, PAX6, and βcatenin binding positions on *Ccnd1* locus. R, Rostral; C, Caudal; M, Medial; L, Lateral.

In summary, multiple signals regulate *Ccnd1* transcription in mouse pallium during corticogenesis, resulting in a complex pattern of *Ccnd1* expression. SP8 appears as a major player in this regulation, uncovering a potential novel role of the Sp-transcription factor family in transcription regulation, which awaits further analysis.

AUTHOR CONTRIBUTIONS

UB: Conceived the work; UB, BB, and ED: Collected and analyzed the data; UB, BB, DP, and CD: Wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00119/full#supplementary-material

Figure S1 | Predicted SP8 binding sites on *Ccnd1* Ex1 fragment. The predicted SP8 sites from **Table S4 (A–G)**, the SP8 summit (SP8S), the PAX6 summit (PAX6S), the predicted PAX6 binding site, and the ATG are indicated. The nt positions refer to the *Ccnd1* TSS1; SP8 **(G)** site position is indicated as 164 nt downstream of the exon 1 ORF. The SP8 A-E sites are located in the promoter region; SP8 **(F,G)** sites are the closest to the SP8 summit. Positions of the SP8S and PAX6S are indicated in bold and underlined.

Figure S2 | Predicted SP8 binding sites on *Ccnd1* Ex5 fragment. The 7 predicted SP8 sites from **Table S2 (A–G)**, the SP8 summit (SP8S), and the stop codon (TGA) are indicated. The nt positions refer to the *Ccnd1* TSS1; SP8 **(A,B)** position is indicated as 125 nt upstream of the exon 5 ORF. Position of the SP8S is indicated in bold and underlined.

Figure S3 | *Pax6* and *Axin2* expression at E12.5. ISH on E12.5 mouse forebrain coronal sections. Panel **(A)** shows *Pax6* expression in the rostral forebrain and panel **(B)** shows *Pax6* expression in a more caudal section, panel **(C)** shows *Axin2* expression, as proxy of the Wnt pathway activity, in the rostral forebrain and panel **(D)** shows *Axin2* expression in a more caudal section. Bar in **(A)**: 200 μm.

Figure S4 | *Sp8* expression analysis in the *Sp8* LOF and GOF mutants at E12.5. E12.5 mouse forebrain coronal sections. *Sp8* mRNA expression levels are shown in the control **(A)** and *Sp8* LOF mutant **(C)**, immunofluorescence of EGFP **(B)** is shown as a proxy of *Sp8* overexpression in the GOF mutant (Borello et al., 2014). Bar in **(A)**: 200 μm.

Figure S5 | Genome-wide distribution of the SP8 binding sites on gene features. Plot showing the percentage of the SP8 binding sites distributed genome-wide on gene features.

Table S1 | SP8 and H3K27ac ChipSeq fragments identified on the *Ccnd1* locus. MACS results of the SP8 and H3K27ac ChipSeq peak calling. Position of the peaks summits is indicated. The column "name" indicates the genomic fragment names used in this study. The fragment named Ex1.2.3 in the H3K27ac ChipSeq dataset contains *Ccnd1* promoter, 5′UTR, and exons 1–3; fragment Ex5 contains *Ccnd1* exon 5 and 3′U TR.

Table S2 | Bioinformatic analysis using the Jaspar software (Mathelier et al., 2016) of the *Ccnd1* Ex5 fragment. Position of the predicted SP8 sites refers to the Ex5 fragment full sequence, nt 1-889.

Table S3 | *Ccnd1* expression levels in the *Sp8* GOF and LOF mutants. Results of RNASeq analysis on *Sp8* mutants obtained with DESeq2 (Love et al., 2014).

Table S4 | Bioinformatic analysis of the *Ccnd1* Ex1 fragment using the Jaspar software (Mathelier et al., 2016). Position of the predicted SP8 sites refers to the Ex1 fragment full sequence, nt 1-1249. In bold are indicated the SP8 predicted sites in the *Ccnd1* promoter (SP8 **A–E)** showing the highest score values, and the two SP8 sites **(F,G)** close to the position of the SP8 summit. The position of the predicted PAX6 site is indicated.

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REVIEW
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ATP-Dependent Chromatin Remodeling During Cortical Neurogenesis

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The generation of individual neurons (neurogenesis) during cortical development occurs in discrete steps that are subtly regulated and orchestrated to ensure normal histogenesis and function of the cortex. Notably, various gene expression programs are known to critically drive many facets of neurogenesis with a high level of specificity during brain development. Typically, precise regulation of gene expression patterns ensures that key events like proliferation and differentiation of neural progenitors, specification of neuronal subtypes, as well as migration and maturation of neurons in the developing cortex occur properly. ATP-dependent chromatin remodeling complexes regulate gene expression through utilization of energy from ATP hydrolysis to reorganize chromatin structure. These chromatin remodeling complexes are characteristically multimeric, with some capable of adopting functionally distinct conformations via subunit reconstitution to perform specific roles in major aspects of cortical neurogenesis. In this review, we highlight the functions of such chromatin remodelers during cortical development. We also bring together various proposed mechanisms by which ATP-dependent chromatin remodelers function individually or in concert, to specifically modulate vital steps in cortical neurogenesis.

Keywords: chromatin remodeling, BAF (mSWI/SNF) complex, ISWI complex, CHD complex, INO80 complex, neurogenesis, neocortex

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INTRODUCTION

Development of the cortex (corticogenesis) is marked by coordination of many key molecular and cellular processes that afford proper brain structure and function. Neurogenesis, one of such cellular events, involves the generation of neurons from neural progenitor cells (NPCs). Embryonic cortical neurogenesis is thus the prenatal aspect of corticogenesis, at which stage the bulk of excitatory (neo)cortical neurons are generated by distinct types of NPCs. Different NPCs can be identified based on their molecular characteristics, morphology, cell lineage commitment, and their site of cell division (Lui et al., 2011; Florio and Huttner, 2014; Taverna et al., 2014; Tuoc et al., 2014; Dehay et al., 2015; Fernández et al., 2016). Apical (APs) and basal progenitors (BPs) constitute the two broad categories of NPCs in the developing cortex. APs include neuroepithelial cells (NEs), apical/ventricular radial glia cells (a/vRGs), apical intermediate progenitors (aIPs) that divide at the apical ventricular zone (VZ) surface. BPs are derived from of APs and include basal/outer radial glia (b/oRG) and basal intermediate progenitors (bIPs). All bRGs lack apical contact, and some lack basal contact. BPs have mitotic figures in the inner/outer subventricular zones (i/oSVZ) (Lui et al., 2011; Dehay et al., 2015).

Very early in development of the nervous system, the neural plate and tube are made up of a monolayer of NEs that together form a pseudostratified neuroepithelium and are able to undergo several symmetric divisions to expand their pool. In the part of the neural tube designated to become the telencephalon, commencement of cortical neurogenesis is indicated by the transformation of NEs to aRG and concomitant production of pioneer neurons through asymmetric cell division within a short developmental time window (**Figure 1**; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009; Martínez-Cerdeño and Noctor, 2016). The NE-aRG cell transition is hallmarked by reduction in some epithelial features of NEs such as loss of tight junction complexes and acquisition of astroglial characteristics (Mollgøard and Saunders, 1975; Aaku-Saraste et al., 1996; Hartfuss et al., 2001; Malatesta et al., 2003). By mouse embryonic day 12.5 (E12.5) and gestational week 7 of human development, most NEs are exhaustively converted to aRG cells in the developing cortex (Aaku-Saraste et al., 1996; Hartfuss et al., 2001; Noctor et al., 2002, 2004; Haubensak et al., 2004; Götz and Huttner, 2005; Bystron et al., 2006; Kriegstein and Alvarez-Buylla, 2009; Sahara and O'Leary, 2009).

aRGs are considered as the main cortical NPCs that give rise to the bulk of neurons in the cortical plate (Noctor et al., 2001; Campbell and Götz, 2002; Kriegstein and Götz, 2003; Malatesta et al., 2003; Haubensak et al., 2004; Miyata et al., 2004). This has led to the redefinition aRGs to include their originally perceived

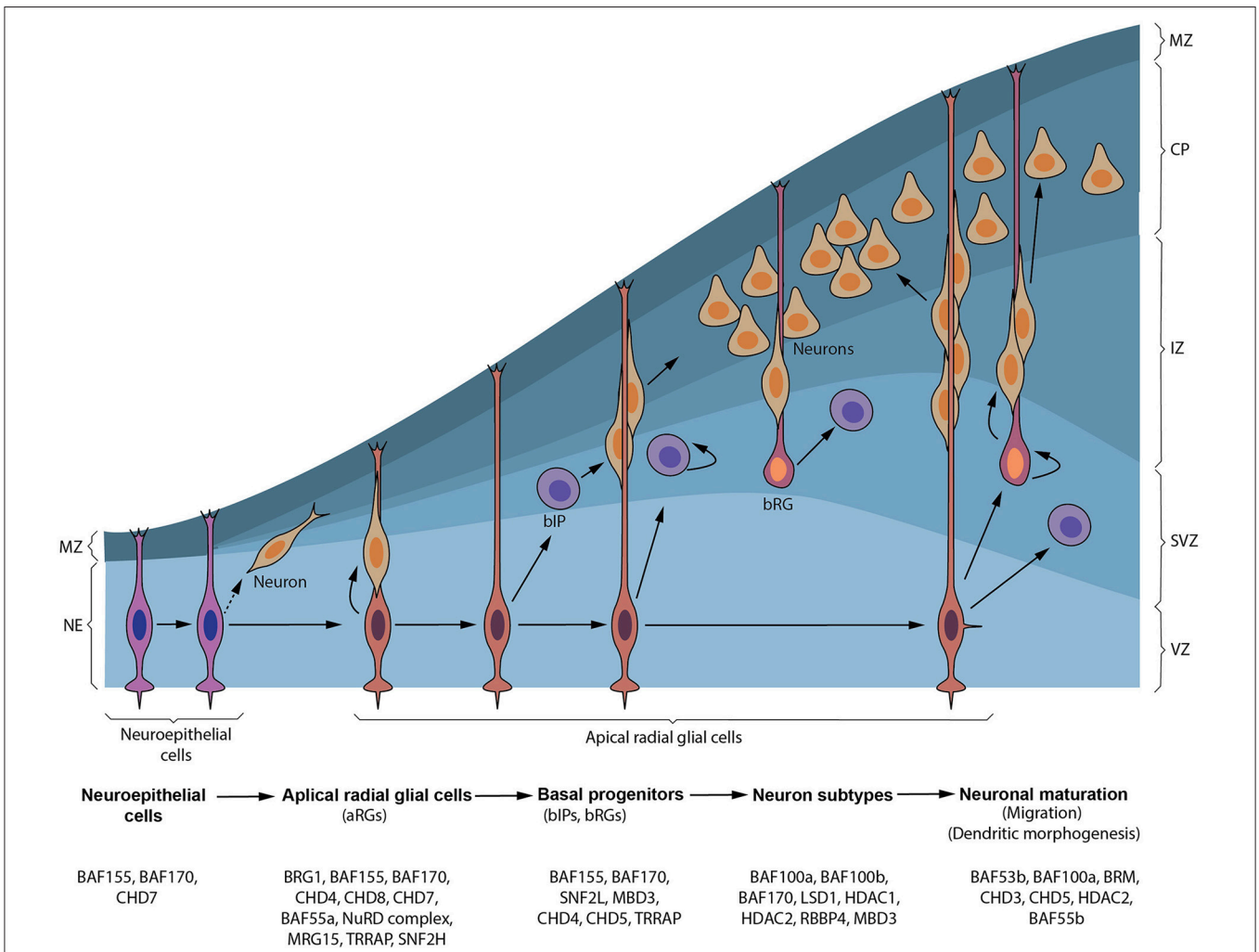

FIGURE 1 | Synopsis of cortical neurogenesis and ATP-dependent chromatin factors involved. Early in cortical neurogenesis, neuroepithelial cells (NEs) divide symmetrically to expand their pool and later divide asymmetrically to give apical NPCs including radial glial cells (RGs) in the ventricular zone (VZ) and pioneer neurons. These apical progenitors proliferate to increase their pool and later divide to give rise to neurons that form the cortical plate (CP) and/or basal progenitors, including basal intermediate precursor (bIP) and basal radial glial (bRG) cells, in the subventricular zone (SVZ). bIP and bRG cells can also self-renew alongside producing neurons before terminally differentiating. Neurons generated from apical progenitors or their progenitor derivatives, migrate predominately to a specified layer in the CP using the long fibers of apical and some basal RG cells as guidance. The marginal zone (MZ) is populated by reelin-producing neurons (Cajal–Retzius [CR] cells) that do not originate from cortical progenitor cells. Chromatin remodeling factors implicated in formation and transition of NEs to apical NPCs and production of basal progenitors through to generation and maturation of neurons during cortical neurogenesis are depicted.

limited function as scaffolds for migrating neurons (Levitt and Rakic, 1980; Rakic, 1988; Hatten, 2002). Characteristically, the somas of aRGs reside in the VZ of the developing neocortex, albeit they can undergo what is known as interkinetic nuclear migration therein (reviewed in Taverna and Huttner, 2010). They have short apical and long basal/pial anchorage (**Figure 1**), display astroglial characteristics, and exhibit increased expression of neuronal genes (Cameron and Rakic, 1991; Bentivoglio and Mazzarello, 1999; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). aRGs can self-renew and/or differentiate directly into neurons (direct neurogenesis) or into BPs which lead to indirect neurogenesis (**Figure 1**; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009; Wilsch-Brauninger et al., 2016). Other structurally and molecularly distinct derivatives of aRGs (i.e., aIPs) collectively called short neural precursors (SNPs) have been found to coexist with aRGs in the proliferative VZ (Gal et al., 2006; Kowalczyk et al., 2009; Stancik et al., 2010). While some aRGs exhaustively convert to neurons after several rounds of cell divisions, others progressively acquire glial progenitor fate and eventually generate cortical glia; thus constituting their developmental switch from neurogenesis to gliogenesis during cortical development (Qian et al., 2000; Costa et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Ge et al., 2012; Magavi et al., 2012).

Normally, after BPs are generated from aRGs via asymmetric division in the VZ, they move to locate in the SVZ (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Kowalczyk et al., 2009). In the developing mouse cortex, a small population of BPs (bIPs) can also self-renew through one or two proliferative division(s), while most of them undergo terminal differentiation to become post-mitotic neurons (**Figure 1**; Haubensak et al., 2004; Noctor et al., 2004). BPs thus function to increase the neuronal pool in the neocortex since they mediate amplification of neuronal output from individual aRGs. The relative amount of BPs in SVZ varies amongst species, with increasing numbers as the brain gains evolutionary complexity (Martínez-Cerdeño et al., 2006; Arnold et al., 2008; Pontious et al., 2008; Sessa et al., 2008; Nonaka-Kinoshita et al., 2013; Tuoc et al., 2013a).

Particularly, in the ferret and primate neocortices, a highly heterogeneous and dynamic population of basal progenitor (i.e., bRG and bIP) cells are resident in the SVZ or the oSVZ and afford another avenue for increasing neuron output in the developing cortex (Fietz et al., 2010; Hansen et al., 2010; Kelava et al., 2012; Betizeau et al., 2013). Across species, the abundance of basal progenitors, notably bRG, is highly variable and an increased abundance of basal progenitor generation and proliferation during corticogenesis is thought to correlate with increased neuronal numbers, neocortex size and cortical folding during evolution (Lewitus et al., 2013; Borrell and Götz, 2014; de Juan Romero and Borrell, 2017).

When cortical neurons are born in the V/SVZ, they switch on various instructive molecular programs that allow them to mainly migrate by locomotion in a radial orientation using fibers of RG cells for support and guidance (**Figure 1**). Some early born neurons (Nadarajah et al., 2001; Hawthorne et al., 2010) and bRG cells (Ostrem et al., 2017) have however been reported to radially migrate via somal translocation. A critical step during migration (locomotion) of cortical neurons is the switch in morphology from multipolar to bipolar shape in the intermediate zone (IZ) before finally migrating to their home lamina in the cortical plate (CP) (Noctor et al., 2004; Heng et al., 2008; Evsyukova et al., 2013). Classically, early born cortical neurons migrate to form lower layers whereas late born cortical neurons move to form upper cortical layers. Normally, neurons in the lower cortical layers make extra cortical connections whereas upper layer neurons form connections within the cortex. It is however interesting that the same cohort of primary NPCs generate distinct classes of neurons with upper and lower layer designations. It is becoming increasingly comprehensible that some spatiotemporal factors, including transcriptional and epigenetic factors, play key roles in such subtype specification of cortical neurons (Guillemot, 2007a; Yoo and Crabtree, 2009; Hirabayashi and Gotoh, 2010; Sokpor et al., 2017).

Finally in embryonic cortical neurogenesis, subtype, and areal differentiation processes ensure maturation of neurons so that they can functionally integrate into various cortical circuits in the brain. Usually as part of terminal differentiation and maturation of neurons, there is rapid spouting, pruning and specification of neurites to form either dendrites or a central axon that permit formation of input and output synapses needed for functional development and plasticity of the cortex. As it applies to other discrete steps in cortical neurogenesis, specific molecular factors are known to regulate maturation of neurons during neocortical development (Jan and Jan, 2003; Wu et al., 2007; Chen et al., 2016).

This review essentially gives an overview of important roles of ATP-chromatin remodeling factors during cortical neurogenesis. Detailed information on vital steps of mammalian corticogenesis can be found in other excellent reviews.

Chromatin remodeling complexes are made up of multiple subunits that are assembled in a combinatorial manner to tailor their function to regulating specific developmental events (reviewed in Ho and Crabtree, 2010). They have emerged over the past couple of decades as powerful regulators of many biological processes, including neural development (Yoo and Crabtree, 2009; Hirabayashi and Gotoh, 2010; Narayanan and Tuoc, 2014; Yao et al., 2016; Albert et al., 2017; Sokpor et al., 2017). Accordingly, many genes which encode for chromatin remodelers are found in the developing cortex (**Table 1**), offering an explanation why their entire ablation or specific subunit inactivation lead to diverse aberrant phenotypes during cortical development (**Table 2**).

As modulators of chromatin structure, chromatin remodelers exert their effect by influencing gene expression through altering the accessibility of specific DNA regions to transcriptional machinery, and other DNA-binding molecules. Chromatin remodeling subfamilies fall into 3 categories with respect to regulatory strategies they use, namely: nucleosome organization and assembly, chromatin access, and nucleosome editing (**Figure 2**). Although the modes of chromatin remodeling differ amongst remodeling complexes, there seems to be a common mechanism underlying all chromatin remodeling strategies: DNA translocation (Clapier et al., 2017).

TABLE 1 | Expression of genes, encoding for subunits of chromatin remodeling complexes in the developing cortex.

Subunit	Gene	E14.5 cortex		
		VZ/SVZ	IZ	CP
BAF (SWI/SNF) COMPLEX				
BAF250a	ARID1A	+++	++	+++
BAF250b	ARID1B	++	+	+++
BAF200	ARID2	+	–	+
BRG1	SMARCA4	+++	+++	++++++
BAF170	SMARCC2	+++++	+++	++
BAF155	SMARCC1	+++	++	+++
BAF180	PBRM1	++	+	–
BAF60a	SMARCD1	+	++	+
BAF60b	SMARCD2	–	–	–
BAF60c	SMARCD3	+++	++	++++
BAF53a	ACTL6A	++	+	–
BAF53b	ACTL6B	–	–	++++
BCL7a	BCL7A	+	+++	+++++
BCL7b	BCL7B	+	–	–
BCL7c	BCL7C		Not found	
BCL11a	BCL11A	+	+++	++++++
BCL11b	BCL11B	–	+	++++
BRD7	BRD7	+++	+++	++
BRD9	BRD9		Not found	
GLTSCR	BICRA		Not found	
GLTSCRL1	BICRAL		Not found	
BAF57	SMARCE1	+	+	+
BAF45a	PHF10	++++	++	++++
BAF45b	DPF1	+	+	++++
BAF45c	DPF3	++	+	++++
BAF45d	DPF2	+++	+	++
SS18	SS18	+	–	–
CREST	SS18L1	++++	+++	++
BAF47	SMARCB1	+++	++	+++++
BRM	SMARCA2	+	–	+++
β-actin	ACTB	++	+	+++
ISWI COMPLEX				
CHRAC15	CHRAC1	+	+	–
CHRAC17	POLE3	++	–	+
ACF1	BAZ1A	+++	++	+
SNF2H	SMARCA5	++	+	–
WSTF	BAZ1B	++++	++	++
RSF1	RSF1	+	+	–
TIP5	BAZ2A	+++	+++	+
BPTF	BPTF, FALZ	++	–	–
SNF2L	SMARCA1	–	–	+
RBAP46	RBBP7	+++	+	+
RBAP48	RBBP4	+	–	+
CECR2	*CECR2*	+	–	–
CHD (NuRD) COMPLEX				
CHD3	CHD3	+++	+++	+++++
CHD4	CHD4	+++	+	+++
HDAC1	HDAC1	+++	+	++
HDAC2	HDAC2	++	+	+++
MBD2	MBD2	+	–	+
MBD3	MBD3	++	+	++
MTA1	MTA1	++	+	++
MTA2	MTA2	+++	+	++
MTA3	MTA3	+++	+	+++
RBAP46	RBPP7	++	+++	+
RBAP48	RBBP4	+	–	+
INO80 COMPLEX				
FLJ20309	INO80D		Not found	
FLJ90652	INO80E		Not found	
MCRS1	MCRS1	+++	–	+
NFRKB	NFRKB	+++++	++++	+++++
UCH37	UCHL5	++	+++	–
AMIDA	TFPT	+	–	+
IES6	INO80C		Not found	
IES2	INO80B		Not found	
ARP5	ACTR5	++	–	–
INO80	INO80	++	–	+
ARP8	ACTR8	+++	+	++
ARP4	ACTL6A	+++	++	+
YY1	YY1	+	–	–
RVB1	RUVBL1	++++	++	+++
RVB2	RUVBL2	+++	++	+++
BRD8	*BRD8*	+	+	++
GAS41	YEATS4	++	+	++
YL1	VPS72	+	–	–
ARP6	ACTR6	–	–	+
ZNHIT1	ZNHIT1	+	–	+++
DMAP1	DMAP1	++	+	++
H2AZ	H2AFZ	+++	+++++	++
SRCAP	*SRCAP*	++	+	++
ING3	ING3		Not found	
EAF6	EAF6		Not found	
MRG15	MORF4L1	+	–	++
β-actin	ACTB	++	+	+++
MRGBP	*MRGBP*		*NA*	
BRD8	*BRD8*	++	+	+++
TIP60	KAT5	++++	++	++++

VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate progenitor; CP, cortical plate. –, +, ++, +++, ++++, +++++ indicate relative expression levels (none, weak, moderate, strong, very strong). The expression pattern of most genes was obtained from http://www.genepaint.org (Visel et al., 2004), except ACF1 (Gray et al., 2004), HDAC2 (MacDonald and Roskams, 2008), TIP60 (Thomas et al., 2007).

Characteristically, ATP-dependent chromatin remodeling complexes possess ATPase domains that make it possible for them to harness energy from ATP hydrolysis with which chromatin structure reorganization is effected to increase access to DNA. The ATP-dependent mobilization of DNA and its

TABLE 2 | Function of chromatin remodeling factors during cortical neurogenesis.

Subunit	Mutant	Cofactor/target	Phenotype	References
BAF (mSWI/SNF) COMPLEX				
BAF190a/ BRG1	$BRG^{fl/fl}$; Nestin-Cre		Defect in self-renewal and maintenance of murine NPCs	Matsumoto et al., 2006; Lessard et al., 2007; Zhan et al., 2011; Ninkovic et al., 2013
BRM	*BRM*$^{-/-}$	Hdac2, No	Impaired radial migration	Nott et al., 2013
BAF170	*BAF170*$^{f/f}$; *Emx1-Cre*	Pax6	Increased genesis of IPs, enhanced cortical volume, surface area and thickness	Tuoc et al., 2013a
	BAF170^{OE}	Pax6	Decreased genesis of IPs, diminished cortical volume, surface area and thickness	Tuoc et al., 2013a
BAF155	*BAF155*$^{-/-}$	Pax6	Abnormal proliferation and differentiation in heterozygotes	Kim et al., 2001
BAF155 BAF170	$BAF155^{f/f}$, $BAF170^{f/f}$ (dcKO); *FoxG1-Cre*	Pax6, Kdm6a/b	Telencephalon is not specified	Narayanan et al., 2015
	$BAF155^{f/f}$, $BAF170^{f/f}$ (dcKO); *Emx1-Cre*		Drastic reduction in cortical thickness	Narayanan et al., 2015
BAF100a	*CTIP1*$^{fl/fl}$; *Emx1-Cre; Nex1-Cre*		Specification of subcerebral PNs, reduced Tbr1 and Ctip2 expression, disrupted cortical PN pathfinding	Woodworth et al., 2016
	Bcl11a$^{fl/fl}$; *Emx1-Cre; Nex-Cre*	Sema3c	Impaired radial migration due to defective multipolar to bipolar morphology, cell accumulation in IZ transition; dysplasia of upper cortical layers	Wiegreffe et al., 2015
BAF100b	*CTIP2*$^{-/-}$		Specification of subcerebral PNs	Arlotta et al., 2005, 2008
BAF55a/ SS18	*SS18*$^{-/-}$,*SS18*^{kd}		Defect in closure of neural tube, NPC proliferation, dendritic outgrowth	de Bruijn et al., 2006; Staahl et al., 2013
BAF55b/CREST	*CREST*$^{-/-}$		Defects in dendrite development	Aizawa et al., 2004; Qiu and Ghosh, 2008
BAF53a	*BAF53a*^{kd}		Impaired neural stem/progenitor proliferation	Lessard et al., 2007
BAF53b	*BAF53b*$^{-/-}$		Defects in dendrite development	Wu et al., 2007
BAF45a	*BAF45a*^{kd}		Impaired neural stem/progenitor proliferation	Lessard et al., 2007
	BAF45a^{OE}		Extended proliferative phase of cortical neural stem/progenitor cells	Lessard et al., 2007
ISWI COMPLEX				
CECR2	*CECR2*$^{-/-}$		Neural tube defects	Banting et al., 2005
SNF2H	*SNF2H*$^{-/-}$		NPC proliferation and differentiation	Alvarez-Saavedra et al., 2014
SNF2L	*SNF2L*$^{-/-}$	FoxG1	Increased cortical progenitor proliferation, more IPs, bigger brain	Yip et al., 2012
NuRD/CHD COMPLEX				
CHD3	*CHD3*^{kd}		Impaired neuronal migration, cell accumulation in lower CP	Nitarska et al., 2016
CHD4	$CHD4^{fl/fl}$; Nestin-Cre		Reduced proliferation of NPCs, increased apoptosis of NPCs, decreased IPs	Nitarska et al., 2016
CHD5	*CHD5*^{kd}		Impaired neuronal migration, cell accumulation in IZ	Nitarska et al., 2016
		H3K27me3	Accumulation of undifferentiated BPs	Egan et al., 2013
CHD8	*CHD8*^{kd}	β-catenin	Reduction in NPC self-renewal	Durak et al., 2016
	CHD8$^{+/del5}$		Increased NPC proliferation	Gompers et al., 2017
HDAC1	*SATB2*$^{-/-}$	Ctip2, Ski1	Specifying the upper layer callosal projection neuron fate over subcerebral projection neuron fate	Alcamo et al., 2008; Britanova et al., 2008
	SKI$^{-/-}$	Ctip2, Satb2, Ski1	Specifying upper layer callosal projection neuron fate over subcerebral projection neuron	Baranek et al., 2012

(Continued)

TABLE 2 | Continued

Subunit	Mutant	Cofactor/target	Phenotype	References
HDAC2	*HDAC2^{kd}*	Bdnf, No	Neuronal dendritic growth and branching	Nott et al., 2008
	HDAC2$^{-/-}$	Protein kinase C, delta	Reduced proliferation of neural progenitors, precocious neuronal differentiation	Hagelkruys et al., 2014
LSD1, HDAC2, RBBP4	LHX2$^{fl/fl}$; Emx1 Cre	Lhx2	Specifying layer 5 Fezf2 and CTIP2-expressing neurons	Muralidharan et al., 2017
MBD3	*MBD3$^{fl/fl}$; Nestin-Cre*	Smek	Reduced Tbr2+ IPs, reduced cortical thickness, defects in the proper specification of cortical PN subtypes	Knock et al., 2015; Moon et al., 2017
INO80 COMPLEX				
TRRAP	*TRRAP$^{fl/fl}$; Nestin-Cre*	E2f	Reduced apical NPC proliferation, premature production of IPs and neurons	Tapias et al., 2014
MRG15	*MRG15$^{-/-}$*	p21	Decline in neural progenitor cell proliferation and differentiation	Chen et al., 2009, 2011

NPC, neural progenitor cell; BPs, basal progenitors; IPs, intermediate progenitors; CP, cortical plate; kd, knock-down; del, deletion; dcKO, double conditional knockout, PN, projection neuron.

coupling to associated proteins within the remodeling complexes have thus been proposed as the common mechanism across this class of chromatin remodeling factors (Clapier et al., 2017).

Taxonomically, chromatin remodeling factors can be categorized into four subclasses: switch/sucrose non-fermentable (SWI/SNF) complexes, imitation switch (ISWI) complexes, chromodomain helicase DNA-binding (CHD)/Nucleosome Remodeling Deacetylase (NuRD) complexes, and INO80/SWR complexes; based on differences and similarities in their catalytic ATPase domains (**Figure 3**; Flaus et al., 2006) and associated subunits. The specific modes of action and functional diversity of these specific ATP-dependent chromatin remodeling complexes are discussed further in the next sections.

MECHANISMS OF ACTION OF CHROMATIN REMODELING COMPLEXES

Nucleosome Assembly

Remodeling factors within this category are responsible for the maturation of prenucleosomes (early histone-DNA complexes) into octameric mature nucleosomes, as well as the correct spacing of newly formed nucleosomes. This occurs immediately after replication and in association with the replication machinery (Udugama et al., 2011). In general, the assembly of evenly-spaced nucleosomes into heterochromatin silences gene expression (Boyer et al., 2005; Kadoch et al., 2017). Factors responsible for regulating the assembly of nucleosomes belong almost exclusively to the ISWI and CHD subfamilies of proteins. There are some exceptions, like INO80, which has been shown to modulate nucleosome spacing and sliding in an ATP-dependent manner, but not nucleosome disassembly (**Figure 2A**; Udugama et al., 2011).

Following replication, quickly forming prenucleosomes will provide protection and stability to the freshly synthesized DNA. These prenucleosomes are formed by octameric histone complexes which bind to shorter strands of DNA. These histone-DNA complexes then require the ATP-mediated activity of a motor protein (usually ISWI-related proteins) to produce mature nucleosomes with ∼147 bp of DNA associated to them (Becker and Workman, 2013; Fei et al., 2015).

It is believed that multiple factors bind to a region of DNA and promote the translocation of bound DNA along the nucleosomes, in an ATP-dependent manner. These factors move along the DNA strand, pulling neighboring nucleosomes closer to each other until tightly packed, equally distanced arrays are formed (Corona et al., 1999).

Chromatin Access

This strategy is used primarily by members of the BAF (SWI/SNF) subfamily. They make the DNA within the nucleosomes more accessible to other DNA-associated molecules—exposing sites for other proteins (e.g., transcription, repair, or recombination factors) to bind and affect gene expression. They can do so by sliding nucleosomes, evicting nucleosome components (like histone dimers) or completely ejecting full nucleosomes (**Figure 2B**; Clapier and Cairns, 2014).

Through DNA translocation, this type of remodelers allow the release of longer stretches of linker DNA to be exposed to DNA-binding machinery. By doing so, nucleosomes can translocate near one another, invading the neighboring DNA territory and thus promoting the removal of histone dimers and eventually the expulsion of the complete histone octamer, resulting in nucleosome disassembly (Engeholm et al., 2009) and creating euchromatin state that supports gene expression (Hara and Sancar, 2002; Gong et al., 2006; Ho et al., 2009a; Hu et al., 2011; Tolstorukov et al., 2013). SWI/SNF complexes are known to promote nucleosome eviction in this way, resulting in the removal of a H2A/H2B histone dimer and the further loss of the remaining octamer (Dechassa et al., 2010).

The combinatorial effect of linker DNA translocation and nucleosome disassembly results in a strong increase in DNA availability in the regions targeted by SWI/SNF remodelers. These stretches of newly available DNA are then primed to be targeted by transcriptional machinery, consisting of activators, repressors, or other DNA-binding molecules.

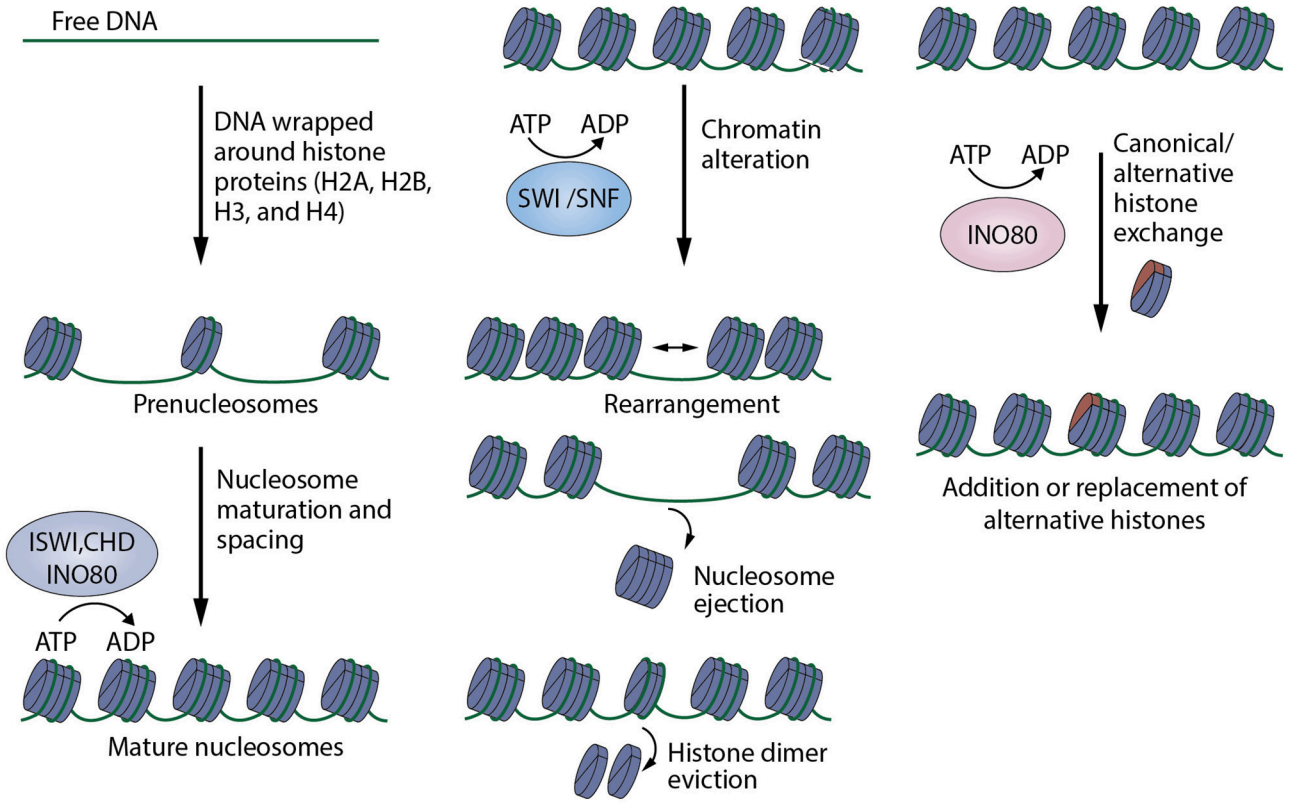

FIGURE 2 | Summary of mechanisms of action of chromatin remodelers. **(A)** Nucleosome organization and assembly: factors, especially those belonging to the ISWI, CHD, INO80 families, are responsible for the random distribution of newly formed nucleosomes, as well as their maturation and arrangement into regularly-spaced chromatin structures. **(B)** Chromatin access: factors that mainly belong to the SWI/SNF family mediate DNA accessibility by nucleosome rearrangement, nucleosome ejection or histone eviction. **(C)** Nucleosome editing: factors of the INO80 family are able to carry out nucleosome editing by promoting the exchange of canonical and variant histones (like H2AZ, shown in red) in chromatin (Adapted from Clapier et al., 2017).

Nucleosome Editing

In particular, nucleosome editing is undertaken by members of the INO80 family of chromatin remodelers (**Figure 2C**). They mediate the substitution of canonical histones (H2A, H2B, H3, and H4) within an existing nucleosome with alternative histones, in a replication-independent manner. The most prevalent histone variant is H2AZ, which substitutes H2A in H2A/H2B dimers; but other alternative histones exist, like the H3 variants H3.1, H3.2, or H3.3. ATP-dependent remodeling factors mastermind this exchange between canonical and alternative histones. It has been shown that DNA translocation induces mechanical stress within the nucleosome structure, which facilitates the expulsion of classical histone dimers and can promote the incorporation of alternative variants (Clapier et al., 2017).

The presence of alternative variants of H2 or H3 histones is generally associated with nucleosome instability, and nucleosomes containing both of these modifications (H2AZ/H3.3) are enriched in nucleosome-free regions of active promoters in the genome. H2AZ and H3.3 are associated with facilitated access to transcription factors and increased transcriptional activity (Jin et al., 2009).

Although H2AZ is localized all throughout the genome in repressed sites, given its diminished stability, it poises the locus for activation when the associated promoter gets targeted by a transcription factor (Zhang H. et al., 2005). The exact mechanism of alternative histone-dependent transcriptional regulation is not yet clear, but it may involve the regulation of higher chromatin structures (Rege et al., 2015).

BIOCHEMICAL AND FUNCTIONAL DIVERSITY OF ATP-DEPENDENT CHROMATIN REMODELING COMPLEXES

SWI/SNF Complex

The SWI/SNF superfamily is a class of ATP-dependent chromatin remodelers with homologs in a wide variety of organisms, including mammals (**Figure 3A**; Ho et al., 2011; Narayanan and Tuoc, 2014; Sokpor et al., 2017). The presence

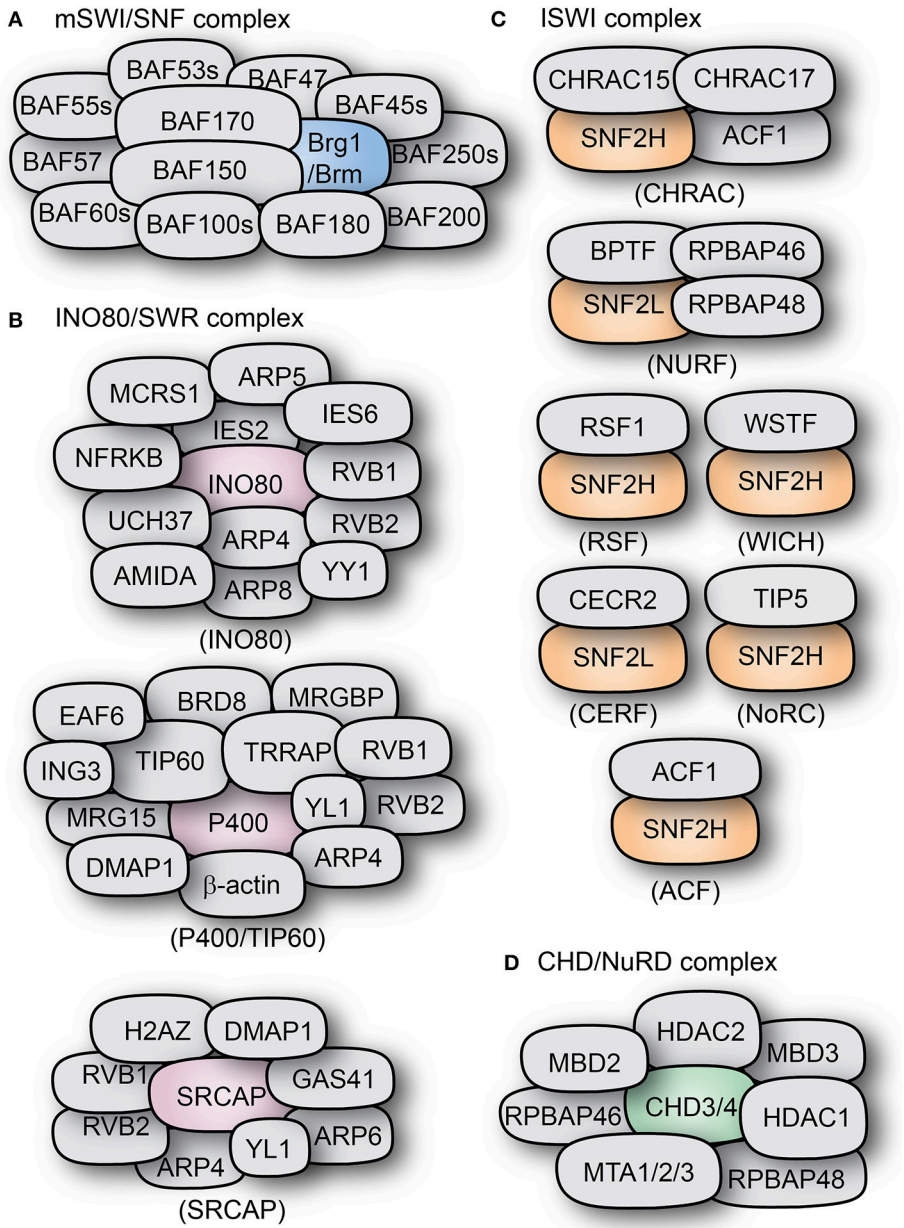

FIGURE 3 | Types and composition of chromatin remodeling complexes. The subunit compositions of some mammalian chromatin remodeling complexes are shown: **(A)** BAF complex, **(B)** INO80/SWR complexes, **(C)** ISWI complexes, and **(D)** the CHD-containing NuRD complex. The core ATPase subunits of the complexes are shown in distinct colors as compared to the other subunits shown in gray color.

of SWI/SNF remodelers is conserved throughout eukaryotic evolution.

As they are the case in yeast (ySWI/SNF, RSC complexes) and drosophila (BAP, PBAP complexes), the mammalian (m)SWI/SNF complexes are also present in two variants, namely homologous BAF (Brg1/BRM associated factor) and PBAF (Polybromo-associated BAF) complexes. The BAF and PBAF complexes have BRG1/BRM or only BRG1 as their catalytic subunit, respectively (Gangaraju and Bartholomew, 2007). They also differ from each other in the presence of the unique subunits BAF250 in BAF complex, and BAF180 (Polybromo homolog), BAF200 in PBAF complex; reflecting differences in their target specificity (Lemon et al., 2001; Leschziner et al., 2005).

Unlike in drosophila and yeast, mammalian BAF complexes are much more abundant than PBAF (Collins et al., 2002). Also, unlike their fly and yeast counterparts, mammalian BAFs have been found to have a high degree of tissue-specific variability in the subunits that conform the complex (Lessard et al., 2007; Ho et al., 2009a; Bachmann et al., 2016). The different variations of the BAF complex in mammals have been linked to the many

biological processes, especially during the development of brain (Matsumoto et al., 2006; Lessard et al., 2007; Wu et al., 2007; Qiu and Ghosh, 2008; Weider et al., 2012; Ninkovic et al., 2013; Tuoc et al., 2013a, 2017; Vogel-Ciernia et al., 2013; Yu et al., 2013; Bischof et al., 2015; Narayanan et al., 2015; Wiegreffe et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016).

SWI/SNF remodelers have been reported to interfere with the structure of chromatin, release of nucleosome-bound DNA, mobilization of DNA along nucleosomes, displacement of histone dimers promoting nucleosome disassembly, among other functions, that facilitate the binding of transcription factors to specific gene loci (Havas et al., 2000; Cairns, 2007; Gangaraju and Bartholomew, 2007). It does so in a step-wise manner, removing first H2A/H2B dimers and then the rest of the histones, releasing the naked DNA (Lorch et al., 2006). SWI/SNF can also remove histone dimers (H2A/H2B) from nucleosomes, independent of its DNA translocation functions (Yang et al., 2007). In addition, recent genome-wide studies indicated that there is enrichment of BAF complexes at promoters (Ho et al., 2009a,b, 2011), and also at super-enhancers (Bossen et al., 2015; Barutcu et al., 2016; Alver et al., 2017; Wang et al., 2017) of active genes involved in cellular processes such as cell proliferation and differentiation.

ISWI Complex

Like many chromatin remodelers, the ATPase ISWI is well conserved across species. ISWI complexes are known to play crucial roles in carrying out nucleosome assembly (**Figure 2**) as well as nucleosome remodeling and editing (Tsukiyama et al., 1995; Ito et al., 1997). Mechanistically, ISWI binds to the basic patch of the N-terminal tail of histone H4 and also to linker DNA, both of which positively regulate its activity. Binding to linker DNA occurs through the interaction of the C-terminal HAND-SANT-SLIDE (HSS) domain of ISWI. At a catalytic level, the drosophila ISWI is controlled by the action of 2 domains: AutoN and NegC, which negatively regulate ATP hydrolysis and DNA translocation, respectively. ISWI regulation is dependent on the basic patch and linker DNA interaction to AutoN and NegC to remove the catalytic inhibition on the complex, thus promoting DNA translocation and chromatin remodeling (Clapier and Cairns, 2012; Yan et al., 2016).

In mammals, one of the two homologs of ISWI (SNF2H and SNF2L) acts as the catalytic subunit for at least 7 complexes, amongst them are CHRAC, ACF, WICH, RSF, CERF, NoRC, and NURF (**Figure 3C**; Ito et al., 1997; Varga-Weisz et al., 1997; Strohner et al., 2001; Badenhorst et al., 2002; Cavellán et al., 2006).

ACF

In mammals, the basic ACF complex is formed by ACF1 and SNF2H (**Figure 3C**). ACF can act as a dimer to regulate nucleosome spacing, and does so bidirectionally from the longer to the shorter DNA strand (Racki et al., 2009). The ATPase subunit of ACF, SNF2H, binds as a dimer to both linker DNA and the nucleosome. It uses the HSS domain to sense linker DNA length and through conformational changes coordinates both activities in alternating units of the dimer (Leonard et al., 2015). ACF can also assemble periodic nucleosome arrays *in vitro* in the presence of a histone chaperone (like NAP1 or CAF1). It can also modulate the spacing between nucleosomes, thus changing chromatin accessibility (Ito et al., 1997). Additionally, ACF is also able to further affect chromatin structure by recruiting histone H1 (Lusser et al., 2005).

CHRAC

CHRAC is another remodeling complex containing the ATPase ISWI. In mammals, it contains the ISWI homolog SNF2H, as well as ACF1, which are the components of the ACF complex (Varga-Weisz et al., 1997). In addition to them, CHRAC contains two histone-binding proteins: CHRAC15 and CHRAC17 (**Figure 3B**; Poot et al., 2000). CHRAC is active during chromatin assembly and through its ATP-mediated activity it converts irregular chromatin into chromatin with regularly spaced nucleosomes (Varga-Weisz et al., 1997). Interaction with the tail of histone H4 is essential for CHRAC-mediated nucleosome sliding and ISWI-dependent regular chromosome spacing (Clapier et al., 2001).

WICH Complex

Functionally similar to ACF, the WICH complex has been associated to the regulation of replication and transcription, as well as the regulation of ribosomal genes. WICH is formed by the interaction of SNF2H and the Williams syndrome transcription factor (WSTF), a protein structurally similar to ACF1 (**Figure 3C**; Dirscherl and Krebs, 2004). Through WSTF, WICH is able to remodel chromatin at the sites of transcriptionally active rRNA genes promoting transcriptional activation and recruiting histone acetyltransferases like PCAF, p300, and GCN5 (Vintermist et al., 2011).

NoRC Complex

The complex termed NoRC (nucleolar remodeling complex) is formed by the interaction of the ISWI homolog SNF2H and the protein TIP5 (**Figure 3C**). It was shown to regulate nucleosome spacing in an ATP- and histone H4-dependent manner (Strohner et al., 2001; Santoro and Grummt, 2005). In addition to its intrinsic role in nucleosome spacing, NoRC can directly and specifically regulate the expression of ribosomal genes due to the interaction of TIP5 with the transcription termination factor TTF-I and the histone deacetylase HDAC1, resulting in transcriptional repression of target genes (Strohner et al., 2001; Zhou et al., 2002; Manelyte et al., 2014).

NURF/CERF Complex

The NURF complex has the ISWI homolog SNF2L as catalytic subunit, and is involved in transcriptional activation. Human NURF has been found in high levels in the brain, where it can regulate the transcription of genes like Engrailed, suggesting important roles during development (Barak et al., 2003; Li et al., 2016). It accomplishes this by promoting the sliding of histone octamers to release target DNA strands from the nucleosomes, thus increasing DNA availability (**Figures 2**, **3**; Hamiche et al., 1999).

Through the subunit BPTF, NURF can detect trimethylation marks in histone H3 (H3K4me3), targeting these sites for nucleosomal remodeling (Li et al., 2006; Hargreaves and

Crabtree, 2011). The N-terminal tail of H4 histone is also essential for the ATPase activity in the NURF complex and the N-terminal tail of H2B is important for the modulation of NURF-dependent nucleosome sliding (Hamiche et al., 2001).

The only other remodeler described so far to incorporate the ISWI homolog SNF2L is the CERF complex. Like NURF, CERF (CECR2 containing factor) is highly expressed in the nervous system and has been associated with regulation of neural development (Lazzaro and Picketts, 2001). The other component of the complex, CECR2 (cat eye syndrome chromosome region candidate 2) is associated to the human disorder cat eye syndrome, and its deletion causes exencephaly in mice (Footz et al., 2001). Together, this complex has nucleosome-dependent ATPase activity and remodels chromatin (Banting et al., 2005).

CHD/NURD Complex

The chromodomain-helicase-DNA binding (CHD) superfamily of proteins is a large class of DNA-binding proteins that can act as chromatin remodeling complexes, and thus regulate gene expression. The members of this class of ATP-dependent chromatin factors are diverse but share a few common characteristics, particularly in the presence of an N-terminal chromodomain, a central SNF2-like helicase motif and a C-terminal DNA-binding domain (Jones et al., 2000). So far, nine members of the CHD superfamily have been described in human (CHD1-9), and these are further classified in three subfamilies (subfamily I-III) according to their structural properties (Hall and Georgel, 2007).

Members of the subfamily I of CHD proteins (CHD1 and CHD2) can act as monomeric units to directly regulate chromatin and transcription (McDaniel et al., 2008; Gaspar-Maia et al., 2009). CHD1, is also directly involved in chromatin assembly and spacing. Monomeric CHD1 associates with histone chaperone NAP1 and catalyzes the addition of nucleosomes to DNA while promoting regular spacing of the resulting nucleosomes. CHD1 remodeling of the chromatin depends on its DNA-binding domains (DBD) to determine the direction of nucleosome sliding and the length of internucleosomal DNA (McKnight et al., 2011).

The subfamily II of CHD proteins (CHD3, CHD4) are core catalytic components (ATPases) of the nucleosome remodeling deacetylase NuRD complex (**Figure 3C**). They can directly bind to the histone deacetylases HDAC1 and HDAC2, as well as the DNA-binding proteins MDB2 and MDB3 and MTA1, MTA2, and MTA3 (Schmidt and Schreiber, 1999; Marhold et al., 2004; Le Guezennec et al., 2006). Like many chromatin remodeling complexes, NuRD can have a highly variable subunit composition which confers functional specificity to the complex in a tissue-dependent manner (Feng et al., 2002; Lai and Wade, 2011). NuRD activity has been mainly associated to transcriptional repression (Hirose et al., 2002; Srinivasan et al., 2006). It can bind directly to methylated DNA and to transcription factors (like the Polycomb group) and promote histone deacetylation in addition to its default chromatin remodeling activity; hence making NuRD a powerful regulator of chromatin structure and gene expression (Hendrich and Bird, 1998; Kehle et al., 1998; Wolffe et al., 1999; Zhang L. et al., 2005).

Subfamily III (CHD5-9) contains recently discovered members that have few known interaction partners. But like the other CHD proteins, they seem to be associated with chromatin structure and remodeling (Hall and Georgel, 2007).

INO80/SWR Complex

Although diverse, the members of the INO80 subfamily of chromatin remodelers are characterized by a central split ATPase domain subunit and the presence of two RuvB-like helicases, Rvb1 and Rvb2 (**Figure 3B**). Despite their core functional similarities, the incorporation of additional subunits to the INO80 complexes (most importantly INO80, SRCAP, and P400/TIP60) can confer very different roles in chromatin remodeling, nucleosome modification, and gene regulation.

INO80

By itself, the INO80 complex acts as a nucleosome spacing factor, promoting the generation of regularly spaced nucleosomal arrays (Yen et al., 2012; Gerhold and Gasser, 2014). This INO80-mediated chromatin remodeling modulates gene transcription, both as an activator and as a repressor (Morrison and Shen, 2009; Hogan et al., 2010). INO80 can also control the levels of H2AZ in transcriptionally active sites, by catalyzing the exchange of H2AZ/H2B dimers in the chromatin with free H2A/H2B. The interaction and recognition between INO80 and deacetylated H2AZ is essential for the maintenance of genome integrity (Papamichos-Chronakis et al., 2011). INO80's ATPase activity, DNA binding, and mobilization are dependent on regulation by associated ARPs, namely Arp8 and Arp5, which can also act as histone chaperones (Shen et al., 2003).

SWR1/SRCAP

In yeast, Swr1 is the catalytic subunit of the complex SWR-C that exchanges conventional H2A histones with H2AZ in specific locations in the genome, thus regulating gene expression. The exchange of histone varieties occurs between a H2A/H2B dimer and a H2AZ/H2B dimer with Swr1 acting as a histone chaperone (Mizuguchi et al., 2004; Hong et al., 2014). Swr1's closest ortholog in the human is the Snf-2 related CREB-binding protein activator protein (SRCAP) (**Figure 3B**) which acts as a coactivator for many transcription factors known to interact with CBP. As Swr1 does, SRCAP acts like the ATP-dependent catalytic subunit of its own complex which substitutes H2A-containing histone dimers with H2AZ variants, thus acting as coactivator (Ruhl et al., 2006).

H2AZ can be found all throughout the genome, flanking nucleosome-free regions. It is present at both, active and repressed genes. Addition of H2AZ is promoted by acetylation of the tails of histones H3 and H4 and the protein Bdf1, a component of the SWR1 complex (Raisner et al., 2005). Swr1's activity is positively regulated by the presence of H2A-containing nucleosomes, as well as by the presence of free H2AZ (Luk et al., 2010). Histone H3 has also variants (namely H3.1, H3.2, and H3.3) which differ slightly in amino acid sequence and regulation (Hake et al., 2006). H3.3 for instance is present in transcriptionally active genes and can be incorporated into nucleosomes in a replication-dependent or independent manner.

Interestingly, acetylation of histone H3 promotes H2AZ or H2A exchange from the nucleosomes (Watanabe et al., 2013).

TIP60–P400 Complex

TIP60 has been described as an acetyltransferase capable of acetylating core histones H2A, H3, and H4 as well as transcription factors and signaling molecules, regulating gene expression and modulating cellular responses (Halkidou et al., 2004; Sun et al., 2005; Sapountzi et al., 2006). In most of its biological roles, TIP60 can be found in association with various interaction partners forming transient complexes, but in cases of transcriptional regulation and DNA repair, it exists as a part of a stable multicomponent complex with at least 18 subunits (Sapountzi et al., 2006). Key components of this complex include the scaffolding protein TTRAP and the chromatin remodeling ATPase P400 (or Domino), as well as shared components with the SRCAP complex, like Rvb1 and 2, or Arps (**Figure 3B**; Ikura et al., 2000). The activity of the TIP60/P400 complex has been associated with many developmental processes (Ueda et al., 2007; Wu et al., 2007; Fazzio et al., 2008).

As a chromatin remodeler, TIP60/P400 complex acts mainly in response to DNA damage by detecting the affected sites and promoting the remodeling of neighboring chromatin into an "open" state, through the acetylation of histone H4 and the selective exchange of histone H2A variants (Ikura et al., 2000; Kusch et al., 2004; Tamburini and Tyler, 2005). This allows the repair machinery to efficiently access sites of double strand breaks in DNA and exert its function.

FUNCTION OF ATP-DEPENDENT CHROMATIN REMODELING COMPLEXES DURING CORTICAL NEUROGENESIS

Although most multimeric ATP-dependent chromatin remodeling factors are ubiquitously expressed, many specific functional variants of such complexes can be formed depending on the tissue- and/or cell-type involved. The functional plurality and specificity of such chromatin remodeling complexes can also be triggered by specific developmental cellular demands (e.g., DNA repair, proliferation, cell death, differentiation, maturation). It has been commonly proposed that some chromatin remodel complexes, such as the BAF and NuRD complexes, can be functionally specified by reconstituting or reshuffling some of their subunits to configure the entire complex toward specific ontogenetic functions. The existence of polymorphic or paralogous forms of the subunits of these ATP-dependent chromatin remodeling factors thus allow for some plasticity of their related complexes to customize their overall functional activity.

For example, as recently reviewed in Sokpor et al. (2017), the BAF complex, which is known to be composed of at least 15, varies in composition as pluripotent embryonic stem cells (ESCs) acquire multipotency to become NPCs that subsequently differentiate into neurons during neural tissue development. The embryonic stem cell BAF (esBAF) complex contains the following BAF complex subuints: BAF60a/b, BAF155, BAF250a, and BRG1 but not their polymorphic or paralogous forms: BAF60c, BAF170, and BAF250b and BRM, respectively.

The neural progenitor BAF (npBAF) complex formed in NPCs as ESCs acquire multipotent NPC fate, is distinctively composed of high amounts of BAF155, low levels of BAF170, BAF250a/b, and BRG1 or BRM ATPase. However, subunits like BAF45a/d, BAF53a, and BAF55a in the esBAF complex are maintained in the npBAF complex. On the other hand, the reconstitution of the npBAF complex to form the neuronal BAF (nBAF) complex during differentiation of NPCs into neurons entails substituting BAF45a, BAF53a, and BAF55a for BAF45b/c, BAF53b, and BAF55b, respectively, and alongside low levels of BAF155 and high amounts of BAF170. The combinatorial assembly of the BAF complex is also elegantly reviewed in Ho and Crabtree (2010) and that of the NuRD complex is described in Feng et al. (2002); Denslow and Wade (2007); Lai and Wade (2011).

As previously mentioned, neocortical development comprises specific developmental processes such as neural specification and patterning, establishment and subsequent transformation of NEs to RGs, proliferation and differentiation of neural progenitors, neuronal subtype specification and migration, neuronal maturation, and ultimate integration of neurons into maturating functional cortical circuits. By applying *in vivo* animal models and neural progenitor culture systems *in vitro*, these specific events have been investigated to elucidate various epigenetic mechanisms regulating them. In the following subsections, we put together various studies that implicate specific ATP-dependent chromatin remodeling factors in controlling the aforementioned aspects of cortical neurogenesis.

Neural Specification and Patterning

The induction and areal specification of neural tissue are fundamental processes responsible for the development of the nervous system. Through the orchestration of several morphogenetic elements, including signaling and transcriptional factors, specific aspects of the simple embryonic ectoderm receive instructions to progressively transform into more complex neural structures in the course of development (Muñoz-Sanjuán and Brivanlou, 2002; Schohl and Fagotto, 2002; De Robertis and Kuroda, 2004; Wilson and Houart, 2004; O'Leary et al., 2007).

The chromatin remodeling BAF complex appears to be an integral part of the regulatory cascade that determines specification and formation of the cortex and the entire nervous system (reviewed in Sokpor et al., 2017). This assertion is backed by experiments in which the entire BAF complex was conditionally ablated by knockout of the scaffolding subunits BAF155 and BAF170 under the control of the early acting (E8.5-9.0) Foxg1-Cre driver line in the emerging telencephalon (Narayanan et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016). Notably, the deletion of BAF complex from early telencephalic domains absolutely abolished specification of the cortex as well as other head structures (Narayanan et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016). The forebrain was however specified when the BAF complex functionality was lost conditionally at a later embryonic stage (∼E11.5) under the control of the Emx1-Cre promoter. However, such mutant mouse brains presented with extreme abnormalities that likely could

not support normal cortical functions (Narayanan et al., 2015; Nguyen et al., 2016).

SRG3 (SWI3-related gene product), a mouse homolog of the human BAF complex subunit BAF155, has been shown to be essential in the specification and spatial patterning of telencephalic regions of the developing brain. About 20% of mice heterozygous for SRG3 displayed abnormal location and formation of the forebrain, a condition called exencephaly (Kim et al., 2001), which is generally caused by failure in the elevation of the neural fold and subsequent expansion of neural tissue. Specifically, by performing *in situ* hybridization in E12.5 exencephalic SRG3 heterozygous embryos with BF-1 (brain factor-1)/Foxg1 probe, it was observed that the forebrain neuroepithelium was abnormally located underneath the thalamus. Examination of the SRG3 mutant head structures at E16.5 revealed several anomalies, including gross morphological malformation of the cerebral cortex and other forebrain structures. An elevated expression level of SRG3 protein early in the development of the telencephalon, that is during neural tube closure (E8.5–E9.5), and the sustenance of its constitutive high expression level in the central nervous system seem to be critical for the proper specification and development of the cortex (Kim et al., 2001).

To further support the role BAF complex in cortical specification and patterning, it has been shown in one study that the transcription factor Ctip1 (BAF100a/Bcl11a), which is also a variant subunit of the BAF complex, is a powerful morphogen in sensorimotor area specification and patterning during neocortical neurogenesis (Greig et al., 2016). Ctip1 was found to be an indispensable factor that operates in newly generated cortical neurons to control acquisition of sensory identity. This is mainly achieved through its role in establishing sensory-specific gene expression patterns for output circuitry, and also formation of sensory maps for thalamocortical inputs (Greig et al., 2016). Loss of Ctip1 function severely disrupted the molecular differentiation in primary cortical sensory areas likely due to downregulation of relevant gene expression programs and ectopic expression of motor cortex-specifying genes. This implies that, Ctip1 suppresses motor identity of projection neurons in primary sensory areas of the cortex, thereby contributing to creation of the molecular boundaries that parcel various functional cortical areas (Greig et al., 2016). The precise role of Ctip1 in the specification and connection of projection neurons will be subsequently discussed under the subheading "generation of neuronal subtype."

Put together, our knowledge of the involvement of epigenetic morphogens like ATP-dependent chromatin remodeling factors in determining the ultimate relative volume and location of functional domains of the neocortex during corticogenesis is expanding. More mechanistic details should be unraveled in further investigations to deepen our current understanding.

Expansion and Maintenance of Apical Neural Progenitor Pool

As previously discussed, the developmental transformation of NEs to RGs is a critical process that sets the stage for neurogenesis during corticogenesis. Although there is limited information on the involvement of ATP-dependent chromatin remodelers in this key transition process, there is compiling evidence indicating the importance of chromatin remodelers, especially the BAF complex, in directing proliferation, maintenance, and differentiation of primary NPCs, including aRGs. Hence, as parent cells and source of NPCs, NE cell proliferation and differentiation into aRGs may be regulated by such ATP-dependent chromatin factors during embryonic cortical neurogenesis.

As previously discussed, differential developmental demands during corticogenesis allow assembly of distinct BAF complexes: npBAF complex for progenitor proliferation and nBAF complex for neural progenitor differentiation (Lessard et al., 2007; Staahl et al., 2013; Bachmann et al., 2016). This suggests that, disruption of say key components of npBAF complex can interfere with its function and culminate in aberrant proliferation of NPCs. Indeed, heterozygous loss the SRG3, which is a subunit of the npBAF complex, in mouse was observed to cause abnormal brain development that was attributed to abnormal proliferation of NEs in the germinal zone of the telencephalon (Kim et al., 2001).

Lack of BRG1 in NPCs impaired their proliferation and self-renewal abilities leading to disturbance of neurosphere formation. To that end, brain size was reduced *in vivo* following Nestin-Cre mediated loss of BRG1 in apical NPCs of E10.5 mouse cortex. This phenotype was attributed to reduced proliferation of neural progenitors and diminished pool of neural precursors in such mutant brains (Matsumoto et al., 2006; Lessard et al., 2007). Furthermore, the phenotypic outcomes of knockdown of other BAF complex subunits like BAF45a/b, BAF53a, and BAF55a in NPCs indicate the importance of other BAF complex subunits in proliferation and expansion of progenitors during cortical neurogenesis (Lessard et al., 2007; Staahl et al., 2013).

Indeed, key signaling cascades such as sonic hedgehog, notch signaling, and Wnt-β catenin pathways that are known to regulate proliferation of progenitor cells during neural development have been shown to interact with the BAF complex (Zhan et al., 2011; Vasileiou et al., 2015). The notch signaling pathway, for instance, was observed to be activated by npBAF complex to cause proliferation of neural progenitors during neural patterning, whereas the sonic hedgehog pathway suppressed such proliferative activity under the influence of the BAF complex (Zhan et al., 2011). Also, promotion of telencephalic neural progenitor proliferation by Wnt-β catenin pathways seem to be modulated by BRG1-containing BAF complex (Vasileiou et al., 2015). This implies that manipulating such pivotal signaling pathways in the presence of npBAF complex functionality or vice versa, may provide corrective strategies that can rescue related aberrant cortical phenotypes.

The CHD complex and its close associate, the NuRD complex, appear to be essential in cortical development given the strong and specific expression of some of their subunits in the brain (Thompson et al., 2003; Miccio et al., 2010; Potts et al., 2011). As such, the subunit CHD4 in the NuRD complex

(**Figure 3C**) has been reported to be mostly expressed in neural progenitors during early cortical neurogenesis as opposed to its other family member CHD3, which rather provides ATPase function of the NuRD complex at differentiation stages (Nitarska et al., 2016). For this reason, deletion of CHD4 in apical NPCs, under the control of the Nestin promoter, highlighted its importance in the production and maintenance of apical neural progenitors, including Pax6+/Sox2+ aRGs in the VZ of the developing mouse cortex. In effect, loss of CHD4 in NPCs in the mutant mice ($CHD4^{fl/fl}$/Nestin-CRE) caused reduced proliferative capacity of the CHD4-deficient NPCs at late cortical neurogenesis stages. The decreased proliferation of such NPCs was linked to (i) their precocious exit from the cell cycle, (ii) failure to differentiate, and (iii) eventual cell death (Nitarska et al., 2016). This mainly formed the basis of the reduced cortical thickness observed in the CHD4 mutant mice.

It is therefore not surprising that proliferation of NPCs in the developing brain was also massively decreased when the NuRD complex function was indirectly ablated via disruption of its HDAC domains. Knockout of HDAC2 or chemical inhibition of HDAC in neural progenitors using TSA (Trichostatin A) resulted in blockage of proliferation (Liu et al., 2012; Hagelkruys et al., 2014), but not survival and migration of treated cells (Liu et al., 2012). This consolidates the significance of NuRD complex and/or its associated HDAC1/2 protein functions in finely regulating neural progenitor cell proliferation for proper late-stage differentiative schemes during cortical neurogenesis.

Nevertheless, the ATP-dependent chromatin remodeling factors CHD7 and CHD8 which can function independent of the NuRD complex, have also been implicated in controlling proliferation and maintenance of NEs or NPCs (Hurd et al., 2007; Gompers et al., 2017).

The overall forebrain size is reduced in mice heterozygous for CHD7 (Layman et al., 2011). The telencephalic neuroepithelium appeared dramatically hypoplastic when the developing (E10.5) brain of mice with homozygous loss of CHD7 were examined (Hurd et al., 2007). This suggests that CHD7 may play important role in cortical neurogenesis by exerting its effect early in brain development. Furthermore, several evidences indicating the role of CHD7 in regulating proliferation of olfactory neural stem/progenitor cells (Bosman et al., 2005; Hurd et al., 2007; Layman et al., 2009), neural progenitor maintenance or differentiation in the brain (Bergman et al., 2011; Feng et al., 2017a,b), and adult neurogenesis (Feng et al., 2013), corroborate the plausible role of CHD7 in controlling proliferation of NPCs during corticogenesis.

On the other hand, whereas loss (knockdown) of CHD8 resulted in premature reduction of neural progenitor pool (Durak et al., 2016), deletion of CHD8 in the mouse germline via heterozygous frameshift CHD8 mutation ($Chd8^{+/del5}$) caused increase in NPC proliferation in the developing mouse cortex (Gompers et al., 2017). Mechanistically, reduced expression (haploinsufficiency) of CHD8 in the brain led to aberrant activation of RE-1 silencing transcription factor (REST) which resulted in transcription repression of neuronal genes (Katayama et al., 2016). Chromatin remodeling activity of CHD8 can also regulate the expression of cell cycle genes, the polycomb repressor complex 2 (PRC2), RNA processing factors and inducers of the Wnt/β-catenin signaling pathway (Durak et al., 2016; Gompers et al., 2017). In effect, the delicate balance between the rate of cortical neural progenitor proliferation and differentiation was distorted as a result of CHD8 dysregulation during early corticogenesis; such defect was counterbalanced with β-catenin overexpression in the embryonic (E13–E16) mouse brain and in cultured N2a cells (Durak et al., 2016).

Given that both CHD7 and CHD8 are direct interaction partners and may also be indirectly connected via putative linker proteins (Batsukh et al., 2010), it will be interesting to investigate how their chromatin remodeling activities are coordinated *in vivo* and also the biological consequence of their interactive relationship during cortical development. Such a study promises to elucidate the apparent opposing effect of loss of CHD8 on NPC proliferation as observed in the study conducted by Durak et al. (2016) and Gompers et al. (2017).

MRG15, a stable subunit of the P400/Tip60 chromatin remodeling complex (**Figure 3B**) and component of the HAT (histone acetyltransferase) and HDAC complexes (Pardo et al., 2002), has been reported to be important in regulating NPC proliferation, maintenance and cell fate determination (Chen et al., 2009, 2011). This may partly be due to its role in regulating transcription, DNA repair, and apoptosis (Squatrito et al., 2006). Lack of MRG15 in the neuroepithelium of E10.5 embryonic brain (MRG15 null mice) rendered it thinner compared to wildtype neuroepithelium. Also, neurosphere formation by cultured NPCs isolated from MRG15-deleted embryonic brain was impaired. BrdU incorporation assay indicated that MRG15 mutagenesis decreased proliferative capacity of MRG15-deficient neural progenitors without affecting their rate of apoptosis *in vitro* (Chen et al., 2009).

Following the above study, the same research group consolidated their claim by showing that MRG15 regulates NPC proliferation by controlling the expression level of cyclin-dependent kinase (Cdk) inhibitor p21 (Chen et al., 2011). Specifically, they noticed that the expression of p21 was up-regulated in NPCs with truncated MRG15 function. For that reason, shRNA-expressing lentiviral plasmid-mediated knockdown of p21 in MRG15 null NPCs was sufficient to rescue their reduced proliferative capacity. As part of the underlying mechanisms, it was also found that activated p53 accumulated in MRG15-deficient NPCs, plausibly underpinning the elevated p21 expression, and making it logical that knockdown of p53 also resulted in restoration of cell proliferation in MRG15 mutant NPCs (Chen et al., 2011).

A cardinal component of the TIP60–p400 complex (**Figure 3D**) and cofactor of HAT, TRRAP (transformation/transcription domain-associated protein), is known to play specific roles in regulating programs involved in cell-cycle progression of cortical progenitors during neurogenesis. Nestin-Cre-mediated loss of TRRAP in the developing cortex disrupted transcription of E2F cell-cycle target genes through impairment of HAT recruitment and suppression of related transcriptional machinery. This caused cortical

NPCs to stay longer in the cell cycle with reduced proliferative capacity that resulted in their untimely differentiation in a cell-autonomous manner (Tapias et al., 2014).

Regulation of Basal Cortical Progenitor Generation and Differentiation

Deterministic developmental programs provided by extrinsic and intrinsic factors drive the decision of APs to either proliferate to increase their pool or differentiate into BPs or neurons (Guillemot, 2007a; Kriegstein and Alvarez-Buylla, 2009; Taverna et al., 2014; Tuoc et al., 2014). The role of transcription factors in regulating the generation of BPs during brain development has been extensively investigated. However, relatively little is known about specific epigenetic factors like chromatin remodelers in controlling neural BP generation and differentiation, although such factors are known to regulate chromatin fluidity to alter gene expression patterns. The outcomes of a few studies (see below) in that regard have provided strong evidence indicating important roles played by ATP-dependent chromatin remodeling factors in specifically regulating the genesis of basal neurogenic progenitors and their eventual differentiation during cortical development.

In one such key studies, when the ATP-binding motif of SNF2L (**Figure 3C**) was conditionally inactivated in mouse brain, it was observed that the head of the resultant mutant (Ex6DEL) was abnormally large. The expanded brain size phenotype was ascribed to excessive amount of cells produced in the Ex6DEL mutant brain. Distinctively, it was found that an unfettered proliferation rate resulted in aberrant increase in Tbr2+ bIP cells in the E15.5 Ex6DEL cortex without any abnormal alteration in the number of Pax6+ apical progenitors. Although neurogenesis in the SNF2L mutant neocortex was temporally disarrayed, the mutant cortex was thick and hypercellular (Yip et al., 2012).

Mechanistically, Yip et al. (2012) found that SNF2L binds to and regulates Foxg1, a transcription factor that regulates NPC self-renewal, basal progenitor expansion and temporal progress of neurogenesis (Siegenthaler and Miller, 2005; Shen et al., 2006; Siegenthaler et al., 2008; Fasano et al., 2009). This implies that dysfunction of Snf2l or related multimeric protein complexes like CERF (**Figure 3**; Banting et al., 2005) and NURF (Barak et al., 2003) may lead to deregulation of Foxg1 targeting, which further leads to distortion of progenitor cell cycle kinetics, proliferative decisions, and alteration in the timing of neurogenesis in the developing cortex. In an experimental phenotype rescue paradigm, Ex6DEL mutants were crossed with Foxg1 heterozygous mice to generate Ex6DEL:Foxg1$^{+/-}$ mutants with reduced Foxg1 expression in the presence of dysfunctional SNF2L. When E15.5 cortex was examined, it was found that the abnormal Tbr2+ progenitor proliferation phenotype was rescued as result of reduced Foxg1 dosage (Yip et al., 2012). Hence reinforcing the conclusion that SNF2L functions to maintain an appropriate Foxg1 expression level needed for proper basal progenitor generation during cortical neurogenesis.

The BAF complex subunit BAF170 has been reported as an intrinsic factor in regulating the number of basal progenitors in of the neocortex (Tuoc et al., 2013a,b). It was found that mouse cortex-restricted loss of BAF170 promotes Tbr2+ bIP-mediated generation of neurons. Overexpression of BAF170, however, resulted in diminished genesis of Tbr2+ bIP cells, hence promoted direct neurogenesis with associated reductive effect on cortical size due to reduced neuron number. A strong mechanistic detail underlining this phenotype includes the regulation of euchromatin structure due to dynamic competition between the incorporation of BAF170 or its counterpart, BAF155, in the BAF complex. The consequence of this competition dictates the binding effectiveness of Pax6/REST-corepressor regulatory complex to Pax6 gene targets that control the production of bIP cells and late neocortical progenitors. In other words, the genetic interaction between the chromatin remodeling protein BAF170 and Pax6 is critical in determining mouse cortical size via regulation of basal progenitor generation during development (Tuoc et al., 2013a).

Interestingly, deletion of the MBD3/NuRD (methyl binding domain 3/nucleosome remodeling and deacetylation) co-repressor complex also resulted in a reduction in Tbr2+ basal progenitors with attendant phenotype (cortical thickness reduction) quite reminiscent of BAF170 over-expression phenotype during embryonic corticogenesis. The MBD3/NuRD complex was however reported to be dispensable in the requirement of lineage commitment of Pax6+ apical progenitors such as aRGs (Knock et al., 2015).

In a recent study, Moon et al. (2017) showed that Suppressor of Mek null (Smek) interacts with MBD3 to form a critical epigenetic regulatory complex in determining the fate of neural precursor cells during cortical neurogenesis. Double knockout of Smek1 and 2 in mice perturbed cortical neurogenesis such that there was reduced generation Tuj1+/Tbr1+/MAP2+ neurons, whereas the number of Pax6+/Nestin+ progenitors was significantly increased in the early embryonic cortex (Moon et al., 2017). This implies that the increase in Pax6+/Nestin+ progenitors did not translate into increased neuronal output, probably because of inhibition of the production of bIPs that are known to amplify neuronal output from Pax6 or Nestin positive aRGs in the developing cortex. Moreover, even in its predominance, direct neurogenesis may be inadequate for generating enough neurons in the absence of bIP-mediated indirect neurogenesis. Mechanistically, it was reported that Smek facilitates polyubiquitylation and subsequent degradation of MBD3, thereby hampering the formation and recruitment of the MBD3/NuRD co-repressor complex to gene loci whose products are important drivers of neurogenesis (Moon et al., 2017). That implies that Smek functionality promotes acetyl histone H3 activity that in turn augments neuronal differentiation during cortical neurogenesis. As expected, overexpression of MBD3 noticeably stalls differentiation of neurogenic progenitor cells; hence neurogenesis defects consequent to Smek1/2 knockout in mice were significantly rescued by depletion of MBD3 proteins (Moon et al., 2017).

The role of CHD4, and by extension the NuRD complex, is not limited to regulating apical NPC proliferation and maintenance. It was observed that Tbr2+ basal progenitors were significantly reduced at E13.5 and E16.5 in CHD4 mutant

mouse brains (CHD4$^{fl/fl}$/Nestin-Cre), with striking reductive effects seen at later developmental stages (Nitarska et al., 2016). It is therefore not far-fetched to reason that such heavy loss of basal neural precursor cells may massively underscore the thin cortical phenotype impacted by CHD4 deletion. This was especially so because the upper cortical laminae, which are predominantly formed by SVZ basal progenitor-derived neurons (Satb2+/Cux1+), were conspicuously reduced as compared to an unchanged number of lower laminae neurons (Tbr1+/Ctip2+) in the cortex devoid of CHD4 expression (Nitarska et al., 2016).

According to Egan et al. (2013), loss of the chromatin remodeler CHD5 in the brain revealed its importance in regulating differentiation of cortical progenitors. Notably, they observed that CHD5 expression was activated in later-stage cortical progenitors and maintained in fully differentiated neurons. This makes it logical that knockdown of CHD5 in progenitors in the V/SVZ impaired neuronal differentiation and led to accumulation of undifferentiated neuronal precursors in the developing neocortex. CHD5 was identified to bind and activate considerable number of genes, including those that orchestrate neuronal differentiation. The study further revealed that neuronal differentiation is likely controlled by the direct interaction of CHD5 with H3K27me3 marks and other Polycomb targets via its chromodomain (Egan et al., 2013)

The importance of TRAPP in regulating cortical neurogenesis appears to include its ability to synchronize timing of cell cycle length of apical progenitors in the VZ of the developing cortex and their differentiation into BPs and neurons (Tapias et al., 2014). Deletion of TRRAP from apical NPCs in the early developing mouse cortex biased their fate toward neuronal and Tbr2+ basal progenitor identity. Interestingly, the unscheduled differentiation of TRRAP-deficient aRG cells to neurons and BPs was rescued by simultaneous gain-of-function of cyclin B1 and A2 (Tapias et al., 2014).

Overall, it is becoming clear that ATP-dependent chromatin remodeling factors and complexes establish regulatory axes together with other proteins to determine the production of basal progenitors through the regulation of their self-renewal and differentiative tendencies during cortical neurogenesis. Perhaps, the differences in the number of basal progenitors and their proliferative capacity in the mouse cortex compared to the primate cortex may be a clue to the existence of plausible differential evolutionary mechanisms or conditions giving rise to the inter-species variation thereof. For instance, it can be argued that the murine ATP-dependent chromatin remodeling factors may be functionally insufficient in causing transcriptional activation of bRG—expression genes like TNC, PTPRZ1, FAM107A, HOPX, and LIFR, which are found only in primate cortices (Fietz et al., 2010; Hansen et al., 2010; Lui et al., 2014; Florio et al., 2015; Pollen et al., 2015; Thomsen et al., 2016). There could also be among other reasons, evolutionary differences in the inheritability of the chromatin remodeling machinery that affords the pattern of BP generation from APs cross species.

One thing that remains unclear, however, is whether such epigenetic chromatin remodelers exclusively sculpt the epigenetic landscape in APs to influence their fate or that their activities linger and/or get modified in their derivatives (BPs) to exert later effects. If the latter is the case, at least as partly implicated in the recent work of Albert et al. (2017), then it would be interesting to investigate the effect of specific loss of such chromatin remodeling factors in specific basal progenitor cells during cortical development. That way, a more comprehensive understanding of how ATP-dependent chromatin remodeling factors epigenetically regulate the generation of various types of basal progenitors in the cortex. For now, the available evidence in that regard remain incomplete, as majority of previous studies only report generalized effects of ATP-dependent chromatin regulators on the generation of all basal progenitors in the telencephalon without specific mention of any subclass.

Genesis of Cortical Neuron Subtypes

During cortical development, a great number of neurons are generated from different progenitor populations. The vast number of neurons generated during cortical neurogenesis obtain various subtype identities, making it possible to generate (by definition) the six cellularly distinct laminae that typify the mature cortical plate (Molyneaux et al., 2007; Guy and Staiger, 2017).

For instance, the millions of projection neurons (PNs) that are born from progenitors in the germinative zones of the developing cortex are sorted out (molecularly, morphologically, and functionally) through differential activation and deactivations of batteries of developmental cell programs, including transcriptional and epigenetic mechanisms. These afford acquisition of specific identities to yield the typical PNs diversification in the neocortex (Guillemot, 2007b; Yoo and Crabtree, 2009; Hirabayashi and Gotoh, 2010; Narayanan and Tuoc, 2014; Yao et al., 2016; Albert et al., 2017; Sokpor et al., 2017). Thus, this subtype specification underlines the establishment of populations of PNs that specifically projects to subcerebral centers while others make ipsilateral or contralateral hemispheric intracortical connections (Custo Greig et al., 2013; Harb et al., 2016).

The BAF complex has been identified as one of the key molecular factors that regulate neuronal subtype specification during cortical neurogenesis. The BRM ATPase-containing BAF complex has been demonstrated *in vivo* to regulate the formation of upper layer neuronal population during cortical development (Tuoc et al., 2013a,b). Therefore, genes that are expressed by such upper layer neurons (Cux1 and Tle1) were identified to be regulated by the BAF complex in a time-dependent manner via recruitment of Pax6 to bind to such gene targets. As part of the mechanism(s) allowing binding of Pax6 to gene targets to specify upper layer neuronal identity (Tuoc et al., 2009; Georgala et al., 2011), it was reported that the BAF complex subunits BAF155 and BAF170 play important role(s) in the recruitment process (Tuoc et al., 2013a).

Ctip1 and its paralog Ctip2 (BAF100b/Bcl11b) have also been identified to play pivotal roles in neuronal subtype specification during corticogenesis. Whereas Ctip1 is distinctly expressed by post-mitotic cortical neurons that make callosal and corticothalamic connections, Ctip2 is strongly expressed by subcerebral cortical neurons that make corticofugal connections

with brainstem nuclei and the spinal cord. In other words, Ctip1 finely regulates cortical neurogenesis through modulation of pathways that lead to deep layer neuron generation, whereas Ctip2 expression or presence in the BAF complex orchestrates the establishment of neurons that make, for example, corticospinal projections (Arlotta et al., 2005; Woodworth et al., 2016).

These factors may however act together with others in determining cortical neuron subtype, as traditionally the case in most developmental pathways. At least it has been shown that the transcription factor Fezf2 critically controls specification of subcerebral PNs during cortical development through regulating the expression of Ctip2. However, both factors synergize functionally to repress the expression of genes like Sox5, Satb2, and Trb1 that lead to the specification of other neuronal subtypes (Arlotta et al., 2005; Chen et al., 2005, 2008; Molyneaux et al., 2005; Bedogni et al., 2010; Cánovas et al., 2015).

The converse also seems to be true. That is, in order to generate different neuronal subclasses during corticogenesis, at least in some instances, Ctip2 and its cofactors have to be co-repressed to allow the developmental of cortical layers as follows: (i) suppression by Tbr1 is needed for the formation of cortical layer 6 (Bedogni et al., 2010; Han et al., 2011), (ii) Sox5-mediated suppression promotes generation of neurons that make layer 5/6 (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012), and (iii) repression by Satb2 affords the formation of PN subtypes that will form upper cortical layers (Alcamo et al., 2008; Britanova et al., 2008).

Notably, there appears to be no obvious compensatory factor or mechanism that can substitute for the neuronal subclass specification function of Ctip1, and likely Ctip2, or probably the entire chromatin remodeling BAF complex. In line with that, it was observed that there was preponderance of subcerebral neuron generation in sensory areas of the developing cortex, as against specification of deep layer neurons, in the absence of Ctip1 expression, whereas overexpression of Ctip1 suppressed production of subcerebral neurons (Woodworth et al., 2016).

During very early stages of cortical development, the LIM homeodomain transcription factor LHX2 functions as a cortical selector gene to fundamentally specify the cerebral cortex (Mangale et al., 2008; Chou et al., 2009). However, in late embryonic corticogenesis, LHX2 acquires additional function in neuronal subtype identity establishment through the augmentation of the NuRD complex functionality (Muralidharan et al., 2017). The NuRD complex, as a proximal regulator of chromatin dynamics, is reported to interact with LHX2 via its component subunits LSD1, HDAC2, and RBBP4, in order to edit the epigenetic patterns at distal regulatory elements of its target loci: Fezf2 and Sox11, which are known determinants of subcerebral (deep layer) cortical PNs specification (Muralidharan et al., 2017). In the absence or excessive increase of LHX2-NuRD complex interaction, there is an abnormal increase or decrease, respectively, in the population of cortical layer 5 (Fezf2+/Ctip2+) neurons (Muralidharan et al., 2017). This suggests that the NuRD complex is able to alter epigenetic signatures of corticofugal neuron-specifying genes through targeting of Lhx2 to cause appropriate neuronal identity specification in the developing cortex.

To reinforce the essentiality of the NuRD complex in orchestrating specification of neuronal subtype identity, another research group (Knock et al., 2015) reported that deletion of the MBD3 component of the NuRD complex can interfere with proper specification of neocortical PN subtypes. In relation to Satb2+ upper layer neurons, normal proportions of Tbr1+ and Ctip2+ deep layer neurons were seen in MBD3-null cortices at E14.5, but from E16.5 onwards, these neuronal populations were out of proportion such that aberrantly more Tbr1 and Ctip2 expressing neurons compared with Satb2 expressing neurons were seen in the MBD3 cKO cortex (Knock et al., 2015). Cortical mislamination was hence evident in MBD3-deficient cortex since the classical cytoarchitectural layering of neuronal subtypes, as seen in the wildtype cortex, was demonstrably in disarray.

Strikingly, it was observed that cortical neural progenitors that have lost their MBD3/NuRD activity ambiguously express both deep- and upper-layer neuronal markers and hence reflective of some confusion in neuronal lineage selectivity programming during cortical neurogenesis (Knock et al., 2015). This is in consonance with earlier studies suggesting MBD3/NuRD complex as a decisive regulatory factor in the specification of Satb2+ upper layer neurons through the suppression of Ctip2 in Satb2 expressing neurons in the developing cortex (Britanova et al., 2008; Gyorgy et al., 2008). Therefore, the lack of MBD3/NuRD complex activity in mutant mice likely displayed an abnormal temporal extension of deep layer neuron differentiation at the expense of upper layer neurons generation (Knock et al., 2015).

Migration of Cortical Neurons

After principal neurons are generated from progenitors in both the VZ and SVZ, they migrate (move) out mainly radially from their birthplaces to their home layers in the cortical plate. Together with default dispositions such as specific time and place of birth, and type of parent progenitor involved, these newly born (immature) cortical neurons are able to collect many regulatory molecular cues in the microenvironment along their migratory trajectory (Evsyukova et al., 2013).

Amongst these regulatory factors, epigenetic regulators, including chromatin remodeling factors are emerging as prominent determinants in ensuring proper placement of neurons after they are born remote to their final position. Until now, one well-documented piece of evidence proving the plausible importance of ATP-dependent remodeling factors in neuronal migration during cortical neurogenesis is the one posited by Wiegreffe et al. (2015). In their study, they showed that Ctip1 is important in regulating how cortical neurons migrate radially during cortical neurogenesis.

It was previously reported that cells in the IZ of the developing cortex strongly express Ctip1 (Leid et al., 2004). Wiegreffe et al. (2015) then advanced the biological significance of the said expression pattern by deleting Ctip1 function via *in utero* electroporation of Cre-GFP plasmid into $Ctip1^{fl/fl}$ E14.5 mouse cortex. This resulted in the accumulation of Ctip1-deficient multipolar neurons in the IZ as compared to the corresponding control. Given that during radial migration multipolar neurons characteristically switch morphology to bipolar neurons so as to

migrate properly to their final destination in the CP, the observed stagnation of multipolar neurons in the IZ indicated disruption of the aforementioned critical morphological transition and hence the perturbation of neuronal migration (Wiegreffe et al., 2015). They concluded their investigation by mechanistically associating regulation of the polarity and orientation of radially migrating cortical neurons to Ctip1 and its downstream cofactor Sema3c, to permit normal radial migration known to be key for normal cortical lamination.

Cysteine nitrosylation (S-nitrosylation) of the NuRD complex subunit HDAC2 is known to control its association with chromatin (Nott et al., 2008). During cortical development, S-nitrosylation of HDAC2 at two cysteine residues (Cys262 and Cys274) in neurons is important for activation of specific gene expression programs that regulate radial migration of cortical neurons (Nott et al., 2013). To this end, cortical cells that were electroporated with a mutant form of HDAC2 ($HDAC2^{C262/274A}$), which cannot be nitrosylated at the said cysteine residues, could not migrate out of the IZ to reach the CP. Interestingly, by means of bead-array analysis of the developing cortex, it was observed that S-nitrosylation of HDAC2 activates the expression of the BRM component of the BAF (mSWI/SNF) complex (Nott et al., 2013). Knockout of BRM ($BRM^{-/-}$) caused disruption of radial migration of Cux1+ neurons in the developing cortex, which was a phenocopy of mouse cortical neurons lacking nitric oxide synthase ($nNOS^{-/-}$): the enzyme responsible for S-nitrosylation. Put together, NO signaling seem to cause HDAC2 nitrosylation which in turn regulates the levels of BRM to control radial migration of neurons in the developing cortex (Nott et al., 2013).

The versatility of the CHD/NuRD complex in cortical neurogenesis is again realized in its ability to orchestrate migration of newly born cortical projection neurons. Despite the general similarity in the expression pattern of CHD3 and CHD5, CHD3 is detected in neurons that have reached their home layer in the CP whereas CHD5 expression is observed in the SVZ of the developing cortex (Nitarska et al., 2016), thus possibly depicting their differential role in influencing neuronal differentiation (Egan et al., 2013) and/or migration during cortical neurogenesis.

Indeed, knockdown of CHD3 or CHD5 with short hairpin RNAs (shRNAs) electroporated into the E13.5 cortex affected radial cortical neuron migration when visualized at E18.5. Particularly, CHD3 knockdown caused delay in radial neuronal migration, with significant cell retention in the lower CP as compared with fewer numbers reaching the upper CP. Similarly, knockdown of CHD5 impaired neuronal migration such that many multipolar neurons abnormally accumulated in the IZ, likely reflecting defective multipolar-bipolar state transition, and their overall failure in reaching the CP. Interestingly or perhaps expectedly, loss of CHD4 using either shRNA or Cre-recombinase in $CHD4^{fl/fl}$ cortex did not perturb neuronal migration (Nitarska et al., 2016). This means that the CHD3 and CHD5 components of the NuRD complex are indispensable for proper neuronal migration during cortical neurogenesis, whereas CHD4 functional requirement appears to be reserved for their previously discussed role in neural progenitor genesis.

Terminal Differentiation and Maturation of Cortical Neurons

Following generation and migration of neurons, various differentiation and morphogenetic programs are turned on to ensure attainment of neuronal identity and maturity to permit correct functional neuronal circuitry in the cortex. The elaboration of dendrites (dendritogenesis) or extension of axons (axonogenesis) from neurites are major neuronal maturation events that ensure synapse formation needed for neuronal information processing.

Specific factors, including epigenetic chromatin regulators, have also been identified to play key roles in neuronal terminal differentiation and maturation during neural development (Whitford et al., 2002; Wu et al., 2007). As previous discussed, normally during neural development, npBAF complex respond to differentiation signals by means of subunit reconstitution to produce nBAF complex. Notably, together with other changes, the subunit BAF53a in npBAF complex is switched to BAF53b in the nBAF complex that is strictly functional in post-mitotic neurons (Olave et al., 2002; Lessard et al., 2007; Bachmann et al., 2016). During development of the telencephalon, expression of BAF53b subunit in post-mitotic neurons has been reported to be essential for dendritic arborization and synaptic plasticity (Wu et al., 2007; Vogel-Ciernia et al., 2013; Vogel-Ciernia and Wood, 2014; Choi et al., 2015). Furthermore, it was found that BAF53b-deficient ($BAF53b^{-/-}$) cultured cortical neurons are unable to undergo activity-dependent dendritic outgrowth. Such $BAF53b^{-/-}$ mutant cortical neurons were however able to elaborate dendrites only in the presence of BAF53b functional restoration, but not its homolog BAF53a (Wu et al., 2007). Interestingly, regulation of dendritogenesis during maturation of cortical neurons is not limited to the function of BAF53b but also other nBAF complex subunits like BRG1, BAF45b, and BAF57 (Lessard et al., 2007; Wu et al., 2007).

Ctip1 also plays a vital role in neuronal maturation during embryonic cortical neurogenesis which allows for the formation of thalamocortical axonal connections in the postnatal cortex. Its expression was identified as a regulator of layer 5 cortical neurons maturation needed for their correct integration into appropriate barrel-related column (Greig et al., 2016).

A component of the nBAF complex, BAF55b, also called CREST (calcium-responsive transactivator) or SS18-like protein 1 (SS18L1), has been shown to play an essential role in neuronal morphogenesis. CREST expression is detectable in the developing mouse cortex from E18.5, with peak expression level at P1 and minimal but constant levels from P10 onwards. Activation of CREST is suggested to be a mechanistic aspect of calcium signaling known to regulate development of dendrites during early cortical development (Aizawa et al., 2004). Targeted abolishment of CREST in mouse cortical neurons disrupted calcium-dependent dendritic growth, as revealed by Golgi staining. Such depolarization-induced dendritic elaboration impairment was rescued by overexpression of full-length CREST protein, indicating its cell autonomous function in regulating growth of dendrites during maturation of cortical neurons (Aizawa et al., 2004).

HDAC2 has also been reported to be critical for dendrite development of cortical neurons. The relatively high expression of HDAC2 and its nitrosylation in post-mitotic neurons, as compared to neural progenitors, has been argued to be of importance in regulating dendritic elaboration during neuronal maturation likely via activation of CREB (cyclic-AMP-responsive-element-binding protein)-dependent gene expression pathways (Nott et al., 2008). When S-nitrosylation of HDAC2 was inhibited in embryonic cortical neuron, it led to decrease in dendritic growth and branching. In particular, neurotrophins like brain-derived neurotrophic factor (BDNF) are reported to mediate nitric oxide (NO) signaling that leads to S-nitrosylation of HDAC2 and which ultimately can regulates neuronal dendritogenesis (Nott et al., 2008).

CONCLUSION AND FUTURE PERSPECTIVES

Neurogenesis in the cortex is a delicately organized developmental event that requires appropriate synchronization of molecular cues leading to proliferation, differentiation, migration, and the ultimate maturation of neurons. The developmental tendency of multipotent apical NPCs to self-renew or differentiate into more fate-restricted derivatives (basal progenitors and neurons), is critically regulated by external and inherent cellular programs that are mainly stimulated by neurogenic transcription and signaling factors. Epigenetic factors are known to implicitly contribute to such regulatory developmental decisions during cortical neurogenesis. Among such epigenetic programs, chromatin modification constitutes a formidable global mechanism used by NPCs to fundamentally adapt their transcriptional response to varying environmental conditions during corticogenesis. More so, extensive remodeling of chromatin architecture permits the sequential transformation of multipotent apical NPCs through specific intermediate precursor cell species into fully differentiated cortical neurons.

By using strategic regulatory mechanisms of action, ATP-dependent chromatin remodelers are able to modulate gene expression programs and other cofactors involved in specific aspects of neurogenic events leading to derivation of neurons from the simple neuroepithelium. The existence of diverse multi-subunit complexes that function as ATP-dependent chromatin remodeling factors may largely depict their unsubstituted requirement in regulating specific parts of cortical neurogenesis rather than providing compensatory functions in the absence or dysfunction of others. Classically, complete or partial inactivation of specific ATP-dependent chromatin remodelers in the developing brain elicit a range of abnormalities such as (i) compromise in neural specification, (ii) up or downregulation of proliferative capacity of apical and basal progenitors, (iii) precocious or delayed differentiation of apical/basal progenitor cells, and (iv) impaired migration and terminal differentiation of post-mitotic neurons. Such aberrant cortical developmental processes are known to culminate into various brain structure and function perturbations.

The increasing number of neurodevelopmental disorders linked to spontaneous or *de novo* mutations in genes coding for chromatin remodeling proteins gives compelling biological significance of stepping up investigative efforts into knowing how ATP-dependent chromatin remodelers regulate cortical neurogenesis. In that direction, applying state-of-the-art tools that can allow us to target and identify associated cofactors and mechanisms involved will help consolidate our understanding of chromatin regulation during brain development in health or disease (Sokpor et al., 2017). For instance, rather than studying the effects of loss of specific ATP-dependent remodeling factors on general population of NPCs (i.e., APs, BPs), it would be more enlightening to determine such consequences on specific progenitor cell types (NEs, aRGs, aIPs, bIPs, and oRGs) in the developing cortex.

The advent of new culture systems for neural cells and transgenic mouse models with cell type-specific reporters, coupled with recently developed proteomic approaches, can allow us determine the cell type-specific composition of each chromatin remodeling complex. Identification of species-specific genes that encode for chromatin remodelers can also be achieved via application of single cell (sc)RNA-seq technique. Furthermore, the newly developed super-resolution nanoscopy coupled with new-labeling methods will provide an additional insight into how chromatin-remodeling factors control chromatin dynamics during neural development. Finally, the application of a robust epigenome-editing technology can afford accurate targeting of chromatin remodeling factors at relevant gene loci to determine their inter- and intra-species gene expression regulatory patterns in the brain.

Altogether, these strategies can permit precise segregation of the heterogeneous cell populations in the developing cortex and identify their unique chromatin remodeling profiles and epigenetic landscapes that specifically contribute to cortical development and evolution.

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GS, RC-H, JR, JS, and TT all contributed to writing and editing the manuscript.

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DGCR8 Promotes Neural Progenitor Expansion and Represses Neurogenesis in the Mouse Embryonic Neocortex

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DGCR8 and DROSHA are the minimal functional core of the Microprocessor complex essential for biogenesis of canonical microRNAs and for the processing of other RNAs. Conditional deletion of *Dgcr8* and *Drosha* in the murine telencephalon indicated that these proteins exert crucial functions in corticogenesis. The identification of mechanisms of DGCR8- or DROSHA-dependent regulation of gene expression in conditional knockout mice are often complicated by massive apoptosis. Here, to investigate DGCR8 functions on amplification/differentiation of neural progenitors cells (NPCs) in corticogenesis, we overexpress *Dgcr8* in the mouse telencephalon, by *in utero* electroporation (*IU*Ep). We find that DGCR8 promotes the expansion of NPC pools and represses neurogenesis, in absence of apoptosis, thus overcoming the usual limitations of *Dgcr8* knockout-based approach. Interestingly, DGCR8 selectively promotes basal progenitor amplification at later developmental stages, entailing intriguing implications for neocortical expansion in evolution. Finally, despite a 3- to 5-fold increase of DGCR8 level in the mouse telencephalon, the composition, target preference and function of the DROSHA-dependent Microprocessor complex remain unaltered. Thus, we propose that DGCR8-dependent modulation of gene expression in corticogenesis is more complex than previously known, and possibly DROSHA-independent.

Keywords: corticogenesis, neurogenesis, DGCR8, DROSHA, microprocessor

INTRODUCTION

Corticogenesis is a complex neurodevelopmental process leading to the formation of the cerebral cortex, the outer-most horizontally six-layered structure of the mammalian brain. This process requires the precise coordination of neural progenitor cell (NPC) proliferation, differentiation and migration (Taverna et al., 2014). The evolutionary expansion of the neocortex is tightly connected with the development of higher cognitive functions and consciousness in humans (Sun and Hevner, 2014). Expansion of the neocortex occurs in both the radial and lateral dimensions and it is due to an increase in the number of neurons and glial cells (Martínez-Cerdeño et al., 2006; Rakic, 2009; Borrell and Götz, 2014). This process is determined during development and primarily reflects the increase in the number of NPCs in the germinative layers of the dorsal telencephalon, the foremost

region of the developing neural tube (Taverna et al., 2014). Thus, the number of NPC divisions and their switch from proliferative self-amplifying to neurogenic divisions is finely regulated in time and space, determining the size of the NPC pools during corticogenesis (Molyneaux et al., 2007; Taverna et al., 2014). Understanding the molecular mechanisms controlling the NPC pool size, temporal and spatial regulation of neurogenesis remain fundamental questions for developmental neurobiology, which entails important implications for neocortical expansion in evolution and for the pathophysiology of neurodevelopmental disorders.

The RNA binding protein DGCR8, encoded by the *DiGeorge syndrome critical region gene 8* (or *Pasha* in Drosophila), and type III ribonuclease (RNAse) protein DROSHA are the minimal functional core of the nuclear Microprocessor complex, essential for the biogenesis of canonical microRNAs (miRNA, Ha and Kim, 2014). In the last decade, conditional deletion of *Drosha*, *Dgcr8*, and other "miRNA-biogenesis" genes has been widely used to deplete mature miRNAs in corticogenesis *in vivo* (see for review Yang and Lai, 2011; Barca-Mayo and De Pietri Tonelli, 2014; Petri et al., 2014). This approach has contributed to elucidate the essential functions of these proteins during development of the central nervous system. However, it has also some disadvantages. For example, conditional knockout of *Drosha* or *Dgcr8*, in the developing nervous system often induces apoptosis entailing massive tissue derangement, complicating the interpretation of results (see for review Barca-Mayo and De Pietri Tonelli, 2014; Petri et al., 2014). Moreover, beside miRNA biogenesis, DROSHA and other "miRNA-pathway" proteins have additional RNA-processing functions (Burger and Gullerova, 2015). Indeed, DROSHA, DGCR8, and TAR DNA-binding protein 43 (TDP-43, another protein associated to the "Microprocessor" complex), also process messenger RNAs (mRNAs) encoding key transcription factors for neurogenesis, such as Neurogenin 2 (*Ngn2*), T-box brain 1 (*Tbr1*), and Nuclear factor 1 B (*NF1B*), silencing their expression independently of miRNAs (Knuckles et al., 2012; Di Carlo et al., 2013; Rolando et al., 2016; Marinaro et al., 2017). These alternative functions of miRNA-pathway proteins constitute a new post-transcriptional mechanism to control gene expression, which is still largely unexplored in neurogenesis.

We previously found, by phenotypic comparison of *Dgcr8* and *Dicer* conditional knockout mice, that miRNA-independent RNA processing functions of DGCR8 predominate over the miRNA-dependent ones in corticogenesis. In particular, *Dgcr8* deletion resulted in premature loss of NPCs, enhanced generation of TBR1+ neurons and induction of apoptosis leading to massive impairment of corticogenesis (Marinaro et al., 2017). However, the massive tissue derangement observed in the telencephalon of *Dgcr8* knockout mouse embryos, left unclear whether the premature neurogenesis observed in embryonic cortices of the mutants was due to DGCR8-dependent control of NPC fate, or a secondary effect due to loss of NPC polarity/delamination (Cappello et al., 2006; Arai and Taverna, 2017).

Here, to directly investigate DGCR8 functions on amplification/differentiation of NPCs in corticogenesis we overexpress *Dgcr8* in the mouse telencephalon, by *in utero* electroporation (*IU*Ep). Our results demonstrate that DGCR8 promotes the expansion of NPC pools and represses neurogenesis, possibly by promoting NPC proliferation. Moreover, we found that overexpression of DGCR8 in embryonic mouse neocortex does not alter the molecular composition of the "DROSHA-Microprocessor" complex or its preference for targets, suggesting the existence of multiple DGCR8-dependent mechanisms to regulate corticogenesis.

RESULTS

Overexpression of DGCR8 in the Mouse Telencephalon Alters the Relative Distribution of Cells Across the Cortical Wall in Absence of Apoptosis

To overexpress *Dcgr8* in NPCs and their differentiated progeny we used *in utero* electroporation (*IU*Ep, De Pietri Tonelli et al., 2006). By this means, we delivered pCAGGS-mCherry plasmid into the dorsal telencephalon of E12.5 wild-type (WT) mouse embryos (**Figure 1**, Control), or pCAGGS-mCherry along with a plasmid constitutively expressing mouse DGCR8 (pCAGGS-mmu-*Dgcr8*, **Figure 1** DGCR8 OE). Immunofluorescence analysis, performed at E14.5 (i.e., 48 h after co-electroporation), revealed that almost all the targeted cells ($97 \pm 0.2\%$; $n = 3$) overexpressed mCherry and DGCR8 proteins (when both plasmids were co-electroporated, **Figure 1A**), compared to the endogenous DGCR8 levels (Figure S1, control cortices and mCherry negative cells in DGCR8 OE cortices). Analysis of protein extracts from the electroporated cortices by western blotting confirmed a significant 5-fold increase of DGCR8 expression, compared to control cortices (**Figures 1B,C**, DGCR8 OE vs. Control, $n = 5$ independent experiments shown; Original Immunoblot in Figure S3).

To investigate effects of the DGCR8 overexpression on fate of the targeted cells, we analyzed the distribution of mCherry+ cells across the cortical wall at E14.5. Overexpression of DGCR8 led to a significant decrease in the proportion of targeted cells located in the neuronal layers (NL) and an increase in the proportion of targeted cells in the progenitor layers [i.e., the Ventricular Zone (VZ) and Subventricular Zone (SVZ)] compared to control cortices (**Figures 1D,E**, DGCR8 OE vs. Control). Whereas the proportion of targeted cells in the intermediate zone (IZ) remained unaltered in both conditions (**Figures 1D,E**).

We previously found that conditional deletion of *Dgcr8* during corticogenesis induces apoptosis leading to a massive disorganization of the developing cortex (Marinaro et al., 2017). Here, to ascertain whether the reduced proportion of cells in NL upon overexpression of DGCR8 (**Figure 1**) was due to cell loss, we analyzed electroporated cortices for apoptosis (**Figure 2** and Figure S2). Sections through cortices of E12.5 and E13.5 conditional *Dgcr8* knockout (*Dgcr8* cKO) mice (Marinaro et al., 2017) were used as positive control for apoptosis. As expected, apoptotic cells were observed in these cortices as revealed by pyknotic nuclei and by immunofluorescence staining for activated CASPASE-3 (**Figure 2** and Figures S2B,B', *Dgcr8* cKO), compared to cortices from WT littermates (**Figure 2**

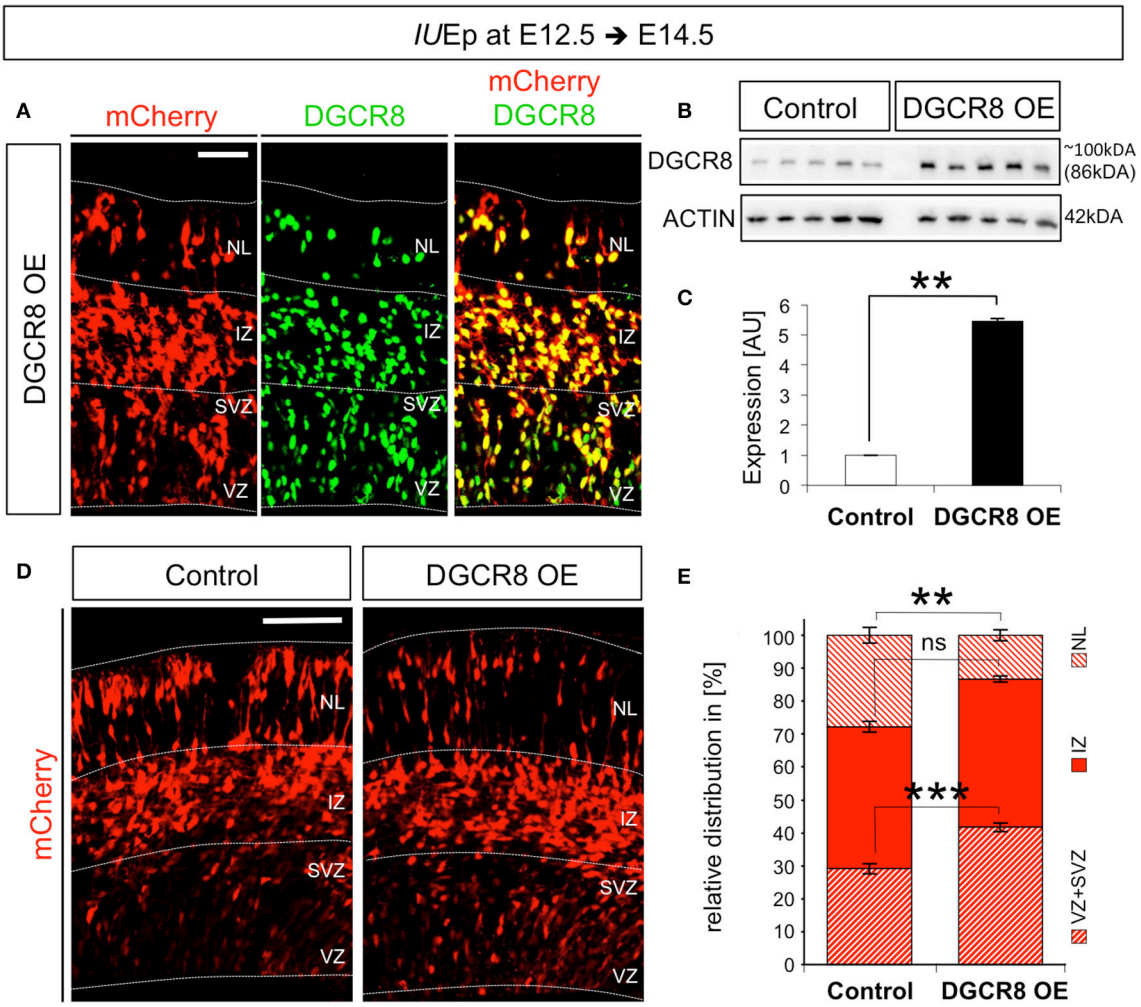

FIGURE 1 | Overexpression of DGCR8 in the mouse telencephalon alters the relative distribution of cells across the cortical wall **(A)** Immunofluorescence staining for DGCR8 and intrinsic mCherry fluorescence in coronal cryosections through the dorsal telencephalon of mouse embryos at E14.5 overexpressing DGCR8 **(B,C)**, after *IU*Ep at E12.5. **(B)** Western blot of DGCR8, and **(C)** Quantification of DGCR8 protein level in the telencephalon of E14.5 mice electroporated at E12.5 with pCAGGS-mCherry (Control, white bar, 5 independent pools shown) or pCAGGS-mCherry and pCAGGS-*Dgcr8* plasmids (DGCR8 OE, black bar, five independent pools shown). Values are normalized on ACTIN. Error bars indicate the variation of five Control and five DGCR8 OE independent pools (s.e.m.); each independent pool consists of four to five dissected electroporated cortical areas; unpaired Student's *t*-test. **(D)** Immunofluorescence microscopy of coronal cryosections through the telencephalon at E14.5 after *IU*Ep at E12.5 showing intrinsic mCherry fluorescence (red), as reporter of targeted cells. Dashed lines indicate borders of specific brain areas (from outside to inside: NL: neuronal layer, IZ: intermediate zone, SVZ: subventricular zone and VZ: ventricular zone), scale bar: 100 μm. **(E)** Quantification of the relative distribution of electroporated cells in NL, IZ, and SVZ+VZ expressed in % over total mCherry+ cells; Error bars indicate the variation of four Control and five DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. $^{**}p$-value < 0.01; $^{***}p$-value < 0.001.

and Figures S2A,A', *Dgcr8* WT). In contrast, overexpression of DGCR8 did not induce apoptosis either at E13.5 (i.e., 24 h after electroporation Figures S2C–D'), or at E14.5, (i.e., 48 h after electroporation, **Figures 2D,D'**, DGCR8 OE), compared to control-electroporated cortices (**Figures 2C,C'**, Control).

These results indicate that overexpression of DGCR8 impairs accumulation of cells in the NL in absence of cell death, while it promotes retention of cells in the VZ/SVZ. This suggests that DGCR8 function might promote self-renewal of NPCs and repress differentiation and/or migration of newborn cortical projection neurons.

Overexpression of DGCR8 Decreases the Generation of Deep-Layer Neurons

*IU*Ep has been previously used for birth-dating and fate analysis of newborn cells in the mouse neocortex, indicating that the majority of targeted NPCs at E12.5 give rise to neurons that populate cortical deep-layer VI (Langevin et al., 2007). To investigate whether the reduction of cells accumulating in the NL upon DGCR8 overexpression (**Figure 1**) was due to reduced generation and/or migration of deep-layer neurons, we quantified the proportion of targeted (mCherry+) cells that were also positive for TBR1, a transcription factor known to be expressed and to specify mostly deep-layer neurons

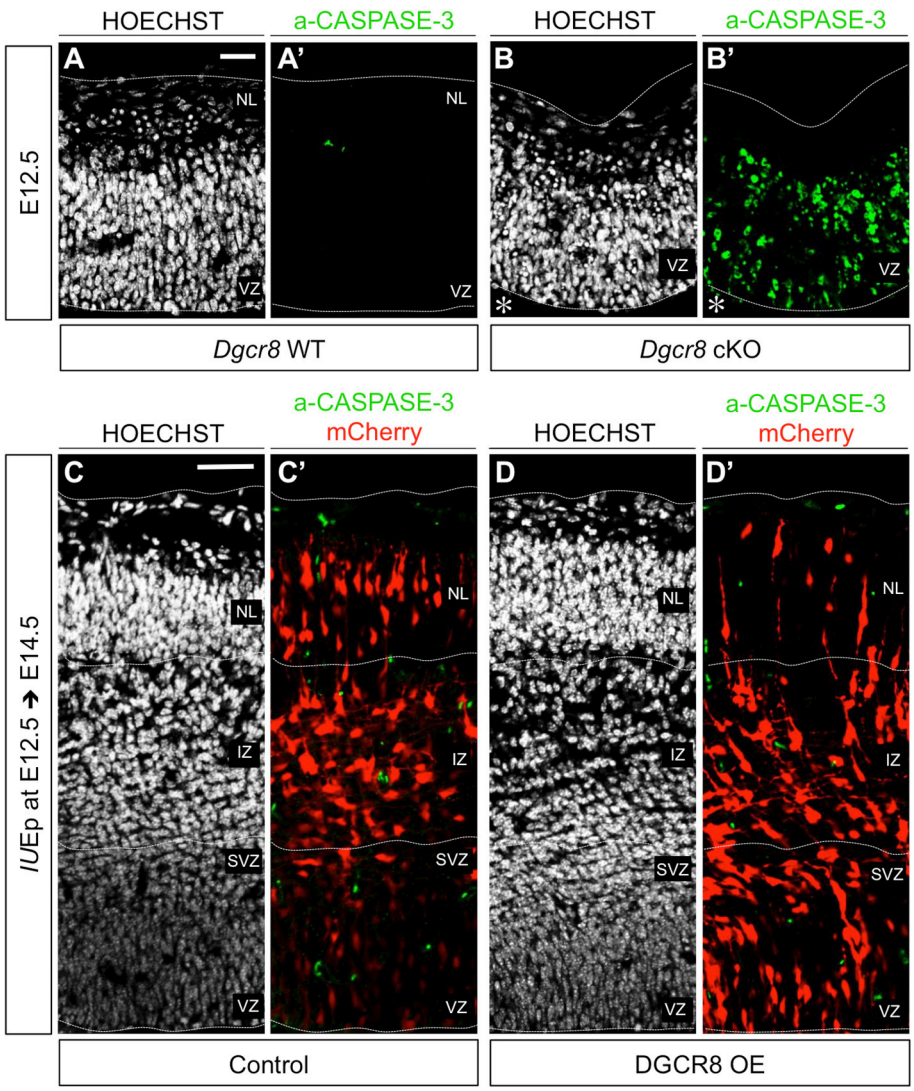

FIGURE 2 | Overexpression of DGCR8 does not lead to apoptosis at E14.5 **(A–D)** Hoechst staining on coronal cryosections through the dorsal telencephalon of *Dgcr8* WT **(A)** and *Dgcr8* conditional knockout (cKO) **(B)** mouse embryos at E12.5 or on coronal cryosections through the dorsal telencephalon of Control **(C)** and DGCR8 OE **(D)** mouse embryos at E14.5 after *IU*Ep at E12.5. **(A'–D')** Immunostaining for activated CASPASE-3 (green) on coronal cryosections through the dorsal telencephalon of *Dgcr8* WT **(A')** and *Dgcr8* cKO **(B')** mouse embryos at E12.5 or on coronal cryosections through the dorsal telencephalon of Control **(C')** and DGCR8 OE **(D')** mouse embryos at E14.5 after *IU*Ep at E12.5; electroporated cells (mCherry, red); dashed lines indicate limits of the cortical wall (from outside to inside: NL: neuronal layer, IZ: intermediate zone, SVZ: subventricular zone and VZ: ventricular zone); scale bars: 20 μm **(A–B')**; scale bar: 100 μm **(C–D')**. **p*-value < 0.05; ***p*-value < 0.01.

(Hevner et al., 2001), across the entire cortical wall at E14.5 (**Figure 3A**). Overexpression of DGCR8 reduced the proportion of TBR1+mCherry+ double-positive deep-layer neurons (**Figures 3A,B**, DGCR8 OE vs. Control).

We previously showed that TBR1 is post-transcriptionally repressed by DGCR8 (Marinaro et al., 2017), questioning its reliability as marker of deep-layer neurons upon DGCR8 overexpression. Thus, to ascertain whether the decrease in TBR1+mCherry+ double-positive cells reflected a reduction in TBR1 expression, or the generation of deep-layer VI neurons, we investigated by immunofluorescence staining the expression of another transcription factor, Sex Determining Region Y-Box 5 (SOX5), known to be involved in deep-layer VI neuron specification (Arlotta et al., 2005). Indeed, overexpression of DGCR8 also decreased the proportion of SOX5+mCherry+ double-positive deep-layer neurons compared to control (**Figures 3C,D**, DGCR8 OE vs. Control). Next, we corroborated these results by analysis of protein extracts from the electroporated cortices, confirming a significant decrease in the expression of SOX5 upon DGCR8 overexpression (**Figures 3E,F**, DGCR8 OE vs. Control, $n = 4$ (Control) and $n = 5$ (DGCR8 OE) independent experiments shown; Original Immunoblot in Figure S3). Given that DGCR8 overexpression reduces the generation of TBR1+ neurons (this study), while we

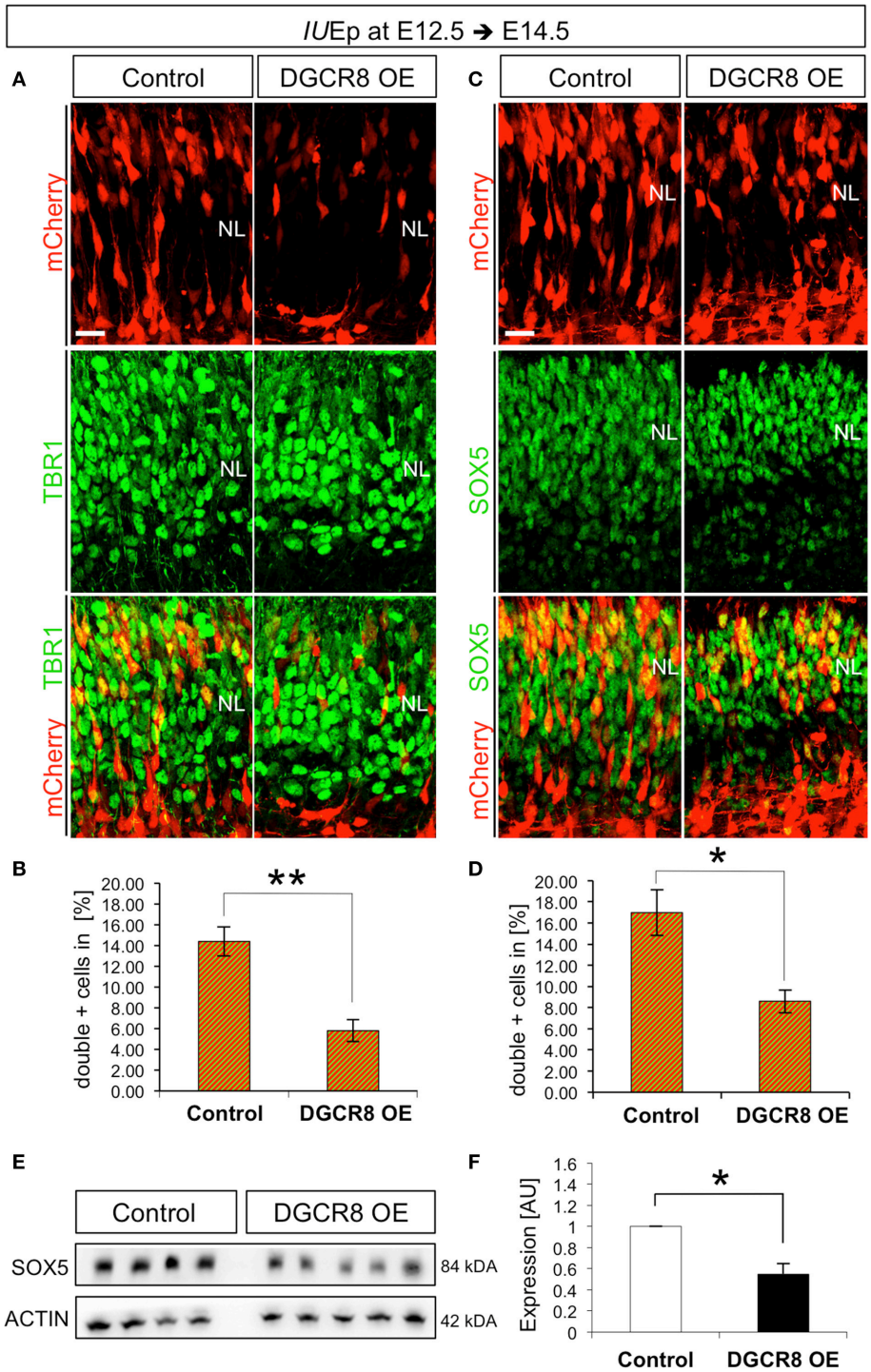

FIGURE 3 | Overexpression of DGCR8 decreases the generation of deep-layer neurons **(A–D)** Immunostaining for TBR1 (**A**, green) or SOX5 (**C**, green) and mCherry+ electroporated cells (**A,C**, red) and merged images on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E14.5 after *IU*Ep at E12.5, and quantification of the proportion of TBR1+mCherry+ **(B)** or SOX5+mCherry+ **(D)** cells expressed in % over total mCherry+ cells. NL: neuronal layer; scale bar: 20 μm. Error bars indicate the variation of four Control and five DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **E,F**) Western blot and quantification of SOX5 [four (Control) and five (DGCR8 OE) independent pools] in E14.5 Control (white bar) and DGCR8 OE (black bar) mouse dorsal telencephalon after *IU*Ep at E12.5. Values are normalized on ACTIN levels. Error bars indicate the variation of four Control and four DGCR8 OE independent pools (s.e.m.); each independent pool consists of four to five dissected electroporated cortical areas; unpaired Student's *t*-test.

previously found that depletion of *Dgcr8* increased it (Marinaro et al., 2017), collectively this evidence supports a function of DGCR8 to regulate neurogenesis in the embryonic mouse neocortex.

Overexpression of DGCR8 Promotes NPC Expansion

DGCR8 overexpression reduced the generation of neurons (**Figures 1**, **3**) while it increased the proportion of targeted cells retained in VZ/SVZ (**Figure 1**). Thus, we hypothesized that DGCR8 might decrease neurogenesis by promoting self-amplification of NPCs.

In the murine telencephalon, the two principal classes of NPCs can be identified by their location during mitosis and expression of specific markers (Taverna et al., 2014). In particular, neuroepithelial, radial glia cells and short neural precursors (from here collectively defined as "Apical Progenitors," APs) are elongated epithelial cells that divide at the ventricular surface, and express the transcription factor Paired Box gene 6 (PAX6) (Götz and Barde, 2005). APs generate other types of NPCs, such as basal intermediate progenitors (from here defined as "Basal Progenitors," BPs). BPs delaminate from the neuroepithelium, express the transcription factor T-Box Brain Protein 2 (TBR2, or Eomes, Englund et al., 2005) and divide at the basal side of the VZ and in the SVZ, becoming the predominant neurogenic type from E14.5 (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004).

To investigate the effects of DGCR8 manipulation in APs and BPs, we analyzed electroporated cortices (as in **Figure 1**) by immunofluorescence staining for PAX6 and TBR2 and quantified proportions of PAX6+mCherry+ APs and TBR2+mCherry+ BPs at E14.5 (**Figure 4**). DGCR8 overexpression led to a significant increase in the proportion of both PAX6+mCherry+ APs (**Figures 4A,B**, DGCR8 OE vs. Control) and TBR2+ mCherry+ BPs (**Figures 4C,D**, DGCR8 OE vs. Control) compared to control. These results indicate that DGCR8 promotes amplification of the two major subtypes of cortical NPCs, during early corticogenesis.

Overexpression of DGCR8 Promotes NPC Proliferation

Next, we asked whether DGCR8 overexpression promotes NPC expansion by stimulating their proliferation (**Figure 5**). Cell cycle is one of the key determinants of the NPC amplification and differentiation in corticogenesis (Dehay and Kennedy, 2007) and proliferating NPCs have a shorter cell cycle compared to neurogenic NPCs (Caviness et al., 1995; Takahashi et al., 1995). We electroporated mCherry (Control) and mCherry/*Dgcr8* (DGCR8 OE) at E12.5 and investigated proliferation of NPCs at E14.5, upon administration of Bromodeoxyuridine (BrdU) pulses (three, every 2 h) over 10 h (**Figure 5A**). Overexpression of DGCR8 increased BrdU incorporation in PAX6+mCherry+ APs (**Figures 5B,D**, DGCR8 OE vs. Control), compared to control. In contrast, analysis of BrdU+TBR2+mCherry+ BPs did not reveal significant differences between cortices electroporated with DGCR8 or control (**Figures 5C,E**, DGCR8 OE vs. Control).

Next, we investigated cell cycle re-entry and exit of NPCs at E14.5. After electroporation of mCherry (Control) or mCherry/*Dgcr8* (DGCR8 OE) at E12.5, we administered Bromodeoxyuridine (BrdU) pulses (five every 2 h, **Figure 5F**). Twenty-four hours later we repeated analysis of TBR2+ BPs for proportions of BrdU positive or negative staining and again we did not find differences between Control or DGCR8 OE cortices in this experimental setting (data not shown). Next, we immuno-stained sections from these electroporated cortices with antibodies anti-KI67 (a protein that is expressed in all phases of the cell cycle except G0 and early G1, Yu et al., 1992) and BrdU (**Figure 5G**, quantification in **Figure 5H**). In cortices overexpressing DGCR8, we found a ∼10% increase of mCherry and BrdU double-positive cells that were also KI67+ (cell cycle re-entry) and an equivalent decrease in mCherry and BrdU double-positive cells that were KI67– (cell cycle exit), compared to control cortices (**Figures 5G,H**; DGCR8 OE vs. Control). Importantly, the proportion of cell cycle re-entry and of exit in non-electroporated cells (mCherry–) remained similar in both conditions (**Figure 5G**, Hoechst+ mCherry– cells in both Control and DGCR8 OE cortices, quantification in **Figure 5I**). These results suggest that overexpression of DGCR8 cell-autonomously promotes the expansion of NPC pools by stimulating their proliferation. Our observations are consistent with evidence indicating that DGCR8 is required for normal proliferation and cell-cycle progression of embryonic stem cells (ESCs) (Wang et al., 2007) and with our previous data in NPCs of the *Dgcr8* cKO cortices (Marinaro et al., 2017).

Overexpression of DGCR8 Decreases the Generation of Upper-Layer Neurons and Promotes BP Expansion at E16.5

We aimed to investigate whether DGCR8 functions change at later stages of corticogenesis. Thus, we repeated the electroporation experiments at E14.5 and analyzed brains at E16.5 (i.e., 48 h after electroporation), a stage in which NPCs mostly generate neurons of cortical layers II to IV (Langevin et al., 2007). Layer II-IV neurons can be identified by immunofluorescence staining for Cut-Like Homeobox 1 (CUX1) a transcription factor that is already expressed by VZ/SVZ progenitors from which these neurons originate (Nieto et al., 2004). Quantification of CUX1 staining at E16.5 revealed that overexpression of DGCR8 led to a significant decrease in the proportion of targeted cells (mCherry+) that were also CUX1+ (**Figures 6A,A',B**, DGCR8 OE vs. Control), compared to control-electroporated cortices. Next, to investigate the effects of DGCR8 manipulation in APs and BPs at this stage of development we quantified proportions of PAX6+mCherry+ APs and TBR2+mCherry+ BPs (**Figures 6C–F**). This analysis revealed that overexpression of DGCR8 led to a selective increase in the proportion of TBR2+mCherry+ BPs (**Figures 6E,F**, DGCR8 OE vs. Control) but not of PAX6+mCherry+ APs (**Figures 6C,D**, DGCR8 OE vs. Control), compared to control.

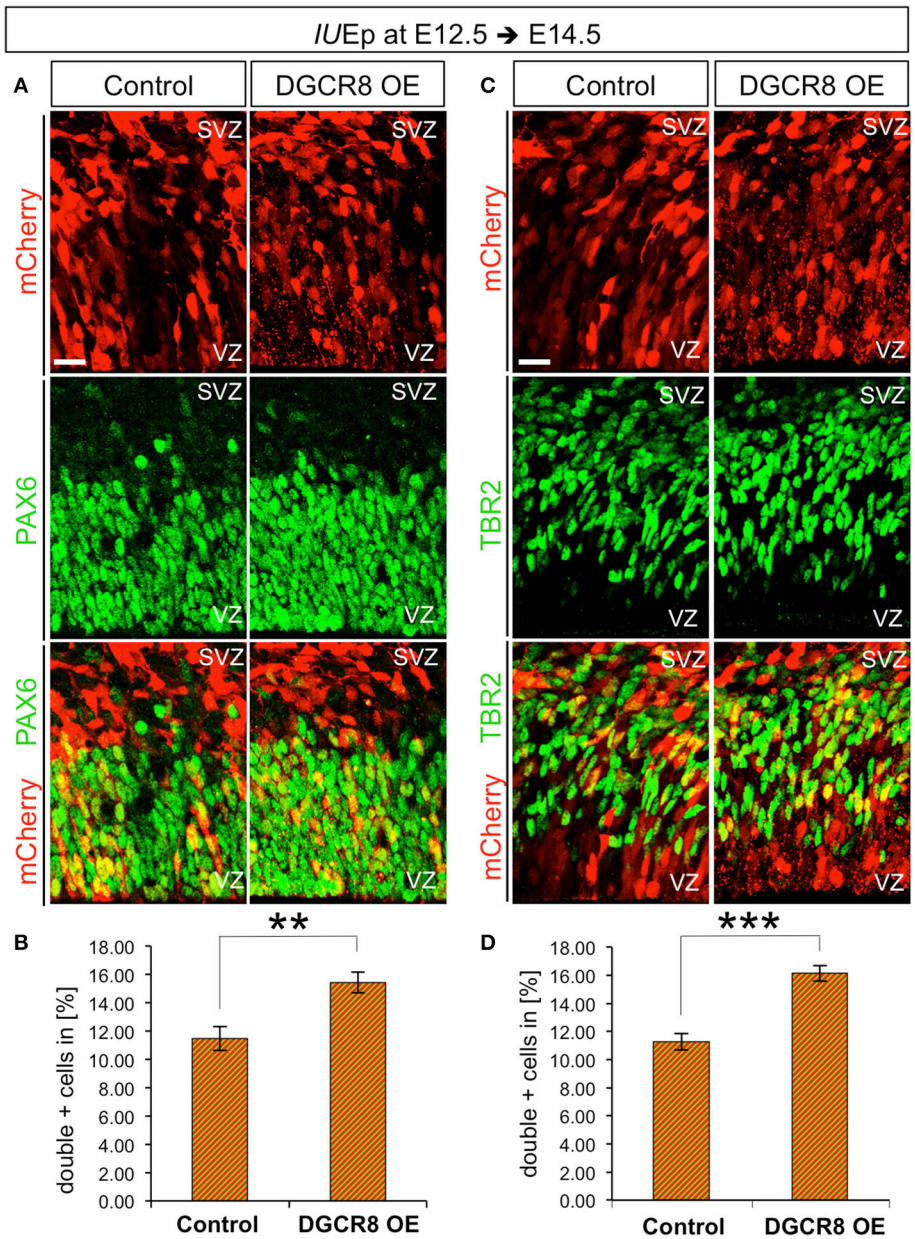

FIGURE 4 | Overexpression of DGCR8 promotes NPC expansion **(A–D)** Immunostaining for PAX6 (**A**, green) or TBR2 (**C**, green) and mCherry+ electroporated cells (**A,C**, red) and merged images on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E14.5 after *IU*Ep at E12.5, and quantification of the proportion of PAX6+ mCherry+ **(B)** or TBR2+ mCherry+ **(D)** cells expressed in % over total mCherry+ cells. SVZ: subventricular zone and VZ: ventricular zone: scale bar: 20 μm. Error bars indicate the variation of five Control and four DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. ***p*-value < 0.01; ****p*-value < 0.001.

Analysis of BrdU incorporation in TBR2+ BPs at E16.5 (five BrdU pulses every 2 h and analysis 11 h later) again did not reveal any difference upon overexpression of DGCR8 (data not shown). Together, these results are consistent with previous findings indicating BPs as major source of upper-layer neurons (Taverna et al., 2014), and remarkably, they suggest that DGCR8 function might differently affect AP and/or BP expansion in corticogenesis at specific developmental times.

Overexpression of DGCR8 Does Not Alter Composition or Functions of the "miRNA-Independent Microprocessor"

We previously found that miRNA-independent RNA processing functions of DGCR8 predominate over miRNA-dependent ones in corticogenesis (Marinaro et al., 2017). Indeed, DROSHA (Knuckles et al., 2012) and DGCR8 (Marinaro et al., 2017)

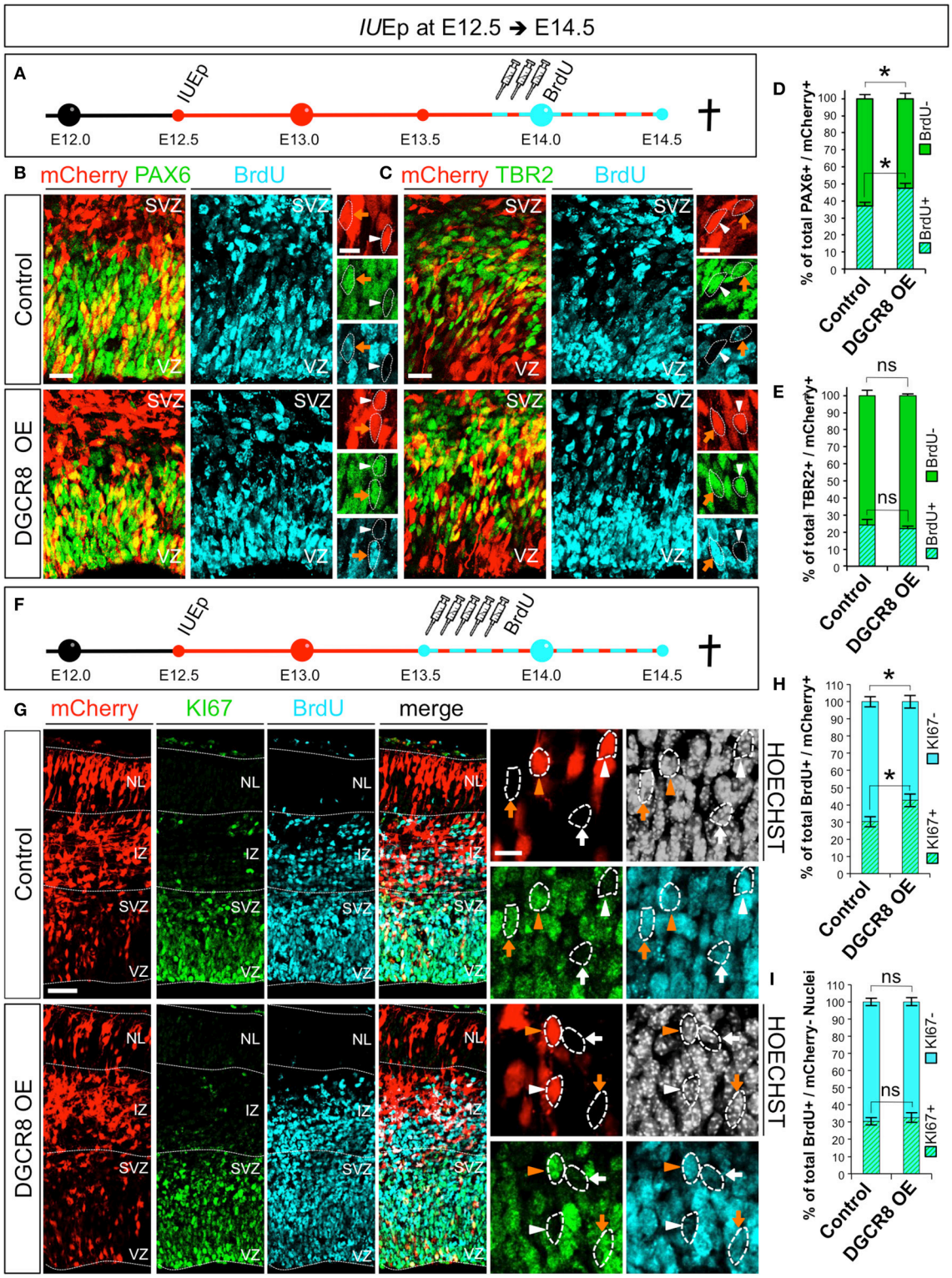

FIGURE 5 | Overexpression of DGCR8 stimulates NPC proliferation and cell cycle re-entry **(A)** Schematic representation of *in utero* electroporation and 10 h BrdU pulse/chase experiment. **B,C**) Co-Immunostaining for PAX6 (**B**, green) or TBR2 (**C**, green), BrdU (**B,C**, cyan) and mCherry+ electroporated cells (**B,C** red) on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E14.5 after *IU*Ep at E12.5. **D,E**) Quantification of the proportion of mCherry+PAX6+ that were BrdU+, or BrdU− **(D)**; or mCherry+TBR2+ that were BrdU+ or BrdU− **(E)** cells, expressed in % over total mCherry+PAX6+(or

(Continued)

FIGURE 5 | mCherry+TBR2+) cells in a selected area (i.e., VZ+SVZ); scale bar: 20 and 10 μm in high magnification images; white arrowheads: mCherry+ and PAX6+ or TBR2+ cells that are BrdU−, orange arrows: mCherry+ and PAX6+ or TBR2+ cells that are BrdU+. Error bars indicate the variation of five Control and six DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **(F)** Schematic representation of *in utero* electroporation and 24 h BrdU pulse/chase experiment. **(G)** Co-Immunostaining for KI67 (green), BrdU (cyan), mCherry+ electroporated cells (red), and Nuclei (Hoechst, gray) on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E14.5 after *IU*Ep at E12.5. **(H)** Quantification of the proportion of KI67+ (cell cycle re-entry) or KI67− (cell cycle exit) BrdU+ mCherry+ cells expressed in % over total BrdU+ mCherry+ cells across the whole cortical wall. **(I)** Quantification of the proportion of KI67+, or KI67−, BrdU+ mCherry− cells expressed in % over total BrdU+ mCherry– cells (identified by Hoechst) across the whole cortical wall (same ROI as in **H**); VZ: ventricular zone, SVZ: subventricular zone and NL: neuronal layer; scale bar: 100 and 10 μm in high magnification images; white arrowheads: mCherry+ and BrdU+ cells that are KI67−, orange arrowheads: mCherry+ and BrdU+ cells that are KI67+, white arrows: mCherry– BrdU+ cells that are KI67− and orange arrows: mCherry− BrdU+ cells that are KI67+. Error bars indicate the variation of five Control and five DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **p*-value < 0.05.

have been recently shown to regulate embryonic neurogenesis through miRNA-independent processing of *Ngn2* and *Tbr1* mRNAs. DROSHA and DGCR8 are essential components of the "miRNA-Microprocessor" complex (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004), but the molecular components of "miRNA-independent Microprocessor" in cortical NPCs and neurons are currently unknown. Indeed, the Microprocessor is a dynamic complex and several proteins have been found to associate with DROSHA and regulate its function, such as TDP-43 (Di Carlo et al., 2013), DEAD-box helicase 5 (DDX5) (Buratti et al., 2010; Kawahara and Mieda-Sato, 2012; Di Carlo et al., 2013; Dardenne et al., 2014; Jung et al., 2014), SMAD protein signal transducers of the TGFbeta/BMP pathways (Davis et al., 2010), TLX, homolog of the Drosophila tailless gene homolog of the Nuclear receptor subfamily 2 group E member 1 gene (Murai et al., 2016) and Forkhead box protein G1 (FOXG1, SCW and TV personal communication), a transcription factor critical for forebrain development (Siegenthaler et al., 2008) and several others (see also Shiohama et al., 2007).

We hypothesized that overexpression of DGCR8 might alter the molecular composition of the Microprocessor, thereby shifting preference/cleavage efficiency of this complex for target RNAs. Alternatively, as DGCR8 can bind RNA through its RNA-binding domains independently from DROSHA (Nguyen et al., 2015), another possibility is that overexpression of DGCR8 might sequester RNA targets preventing their cleavage by DROSHA-complex and/or eventually directly modulating target expression, independently of DROSHA-complex.

To discriminate between these possibilities, we investigated the composition of the DROSHA-Microprocessor complex *in vivo* upon overexpression of DGCR8 (as in **Figure 1**), by immunoprecipitation (IP) of DROSHA, followed by analysis of co-IP proteins in protein extracts from electroporated cortices (**Figure 7** and Original Immunoblot in Figure S4). Overexpression of DGCR8 did not alter the total levels of DROSHA, FOXG1, DDX5, and TDP-43 (**Figure 7A**, first two lanes, and **B**, INPUT, DGCR8 OE vs. Control), suggesting that the expression of these proteins is not controlled by DGCR8 in our condition. Surprisingly, overexpression of DGCR8 also did not alter levels of the proteins that co-precipitated with DROSHA (**Figures 7A,C**, DROSHA-IP, DGCR8 OE vs. Control), compared to control cortices, or mock IP (**Figure 7A**). This result suggests that the phenotypes observed upon DGCR8 overexpression in embryonic mouse neocortex are not due to changes in the molecular composition of the Microprocessor, with regard to the proteins considered in our analysis.

Ngn2 mRNA, encoding a transcription factor involved in a sequential transcriptional cascade during corticogenesis (PAX6>NGN2>TBR2>TBR1) (Englund et al., 2005), is repressed by DROSHA independent of miRNAs (Knuckles et al., 2012) and DGCR8 is dispensable for the DROSHA-dependent processing of *Ngn2* mRNA (Di Carlo et al., 2013; Marinaro et al., 2017). To investigate whether overexpression of DGCR8 alters the RNA target preference or cleavage efficiency of the "miRNA-independent Microprocessor," we analyzed the proportion of NGN2+ cells in the electroporated cortices by immunofluorescence staining. We found that overexpression of DGCR8 in the embryonic mouse neocortex does not alter NGN2 proportions (**Figures 7D,E**, DGCR8 OE vs. Control). This result indicates that overexpression of DGCR8 does not change target preference, or cleavage efficiency of the "miRNA-independent Microprocessor." Thereby, this evidence opens the possibility that DGCR8 might achieve a direct post-transcriptional control of its targets, as previously proposed for TDP-43—and DROSHA—mediated repression of *Ngn2* translation (Knuckles et al., 2012; Di Carlo et al., 2013).

DISCUSSION

By overexpression of DGCR8 in the embryonic mouse neocortex, our study demonstrates that DGCR8 promotes the expansion of NPC pools and represses neurogenesis, possibly by a cell-autonomous mechanism. Interestingly, DGCR8 selectively promotes BP expansion at later developmental stages. With regard to the proteins and developmental time considered in our study, composition, target preference and functions of the "miRNA-independent Microprocessor" complex remained unaltered upon DGCR8 overexpression, suggesting that DGCR8-dependent control of gene expression in corticogenesis is more complex than previously known.

Previous studies, where *Drosha* or *Dgcr8* were conditionally ablated in the embryonic mouse neocortex, reported phenotypes which were often dominated by apoptosis, and massive tissue disorganization already at early stages of development (see for review Yang and Lai, 2011; Barca-Mayo and De Pietri Tonelli, 2014; Petri et al., 2014). This left unclear which of the phenotypes observed were due to loss of gene function, or secondary effects due to massive derangement of VZ/SVZ structure and NPC polarity (Arai and Taverna, 2017).

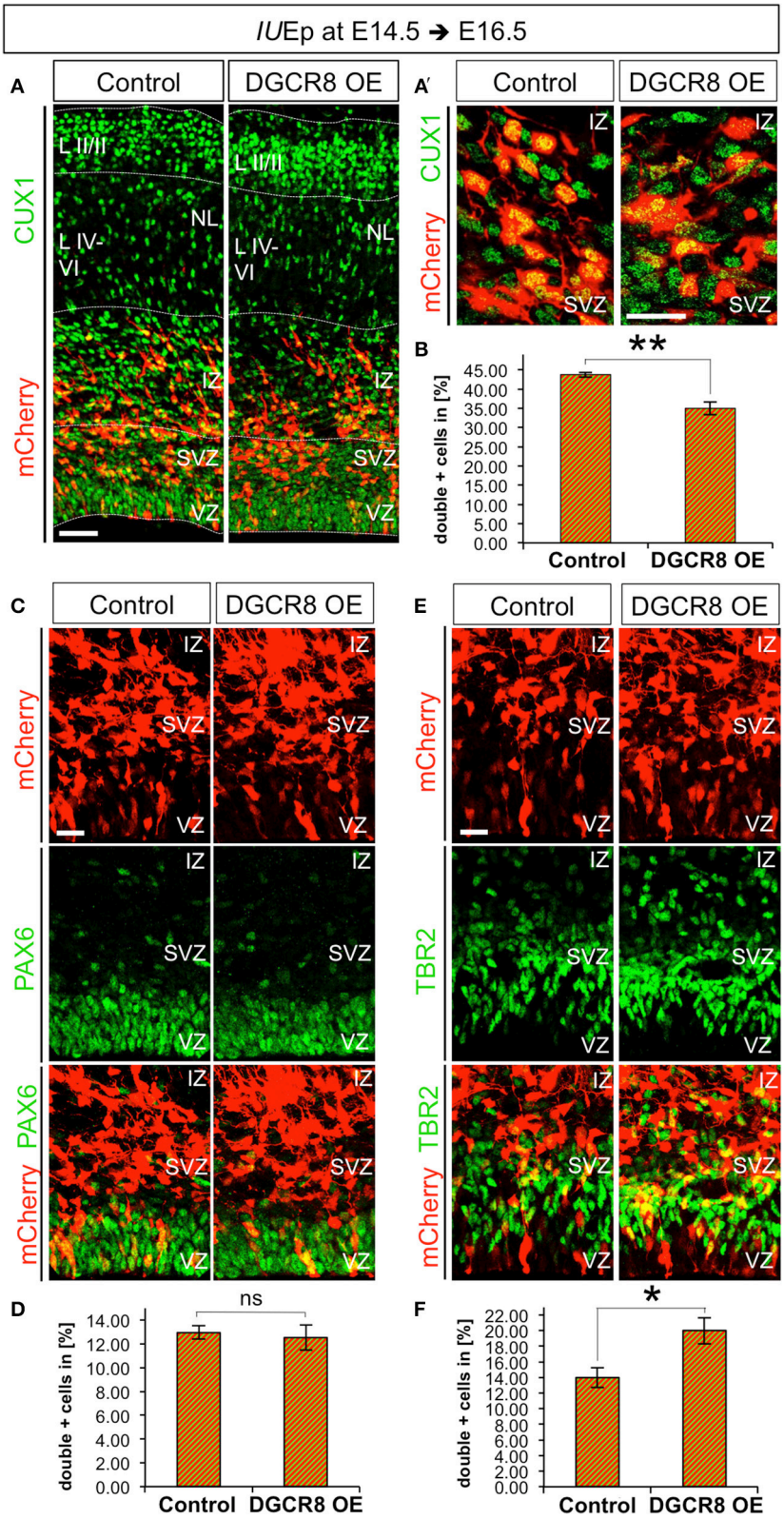

FIGURE 6 | Overexpression of DGCR8 decreases the generation of upper-layer neurons and selectively promotes BP expansion at E16.5 **(A,A')** Immunostaining for CUX1 (green) and mCherry+ electroporated cells (red) on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E16.5

(Continued)

FIGURE 6 | after *IU*Ep at E14.5. **(B)** Quantification of the proportion of CUX1+ mCherry+ **(A,A')** cells expressed in % over total mCherry+ cells; scale bar 50 μm **(A)** and 20 μm **(A')**. VZ: ventricular zone, SVZ: subventricular zone, IZ: intermediate zone, NL: neuronal layer, LII-III: cortical layer 2 and 3, LIV-VI: cortical layer 4-5. Error bars indicate the variation of four Control and four DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **(C,E)** Immunostaining for PAX6 (**C**, green) or TBR2 (**E**, green) and mCherry+ electroporated cells (**C,E**, red) and merged images on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E16.5 after *IU*Ep at E14.5. **D,F**) Quantification of the proportion of PAX6+ mCherry+ **(D)** or TBR2+ mCherry+ **(F)** cells expressed in % over total mCherry+ cells; scale bar: 20 μm. Error bars indicate the variation of five Control and five DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **p*-value < 0.05; ***p*-value < 0.01.

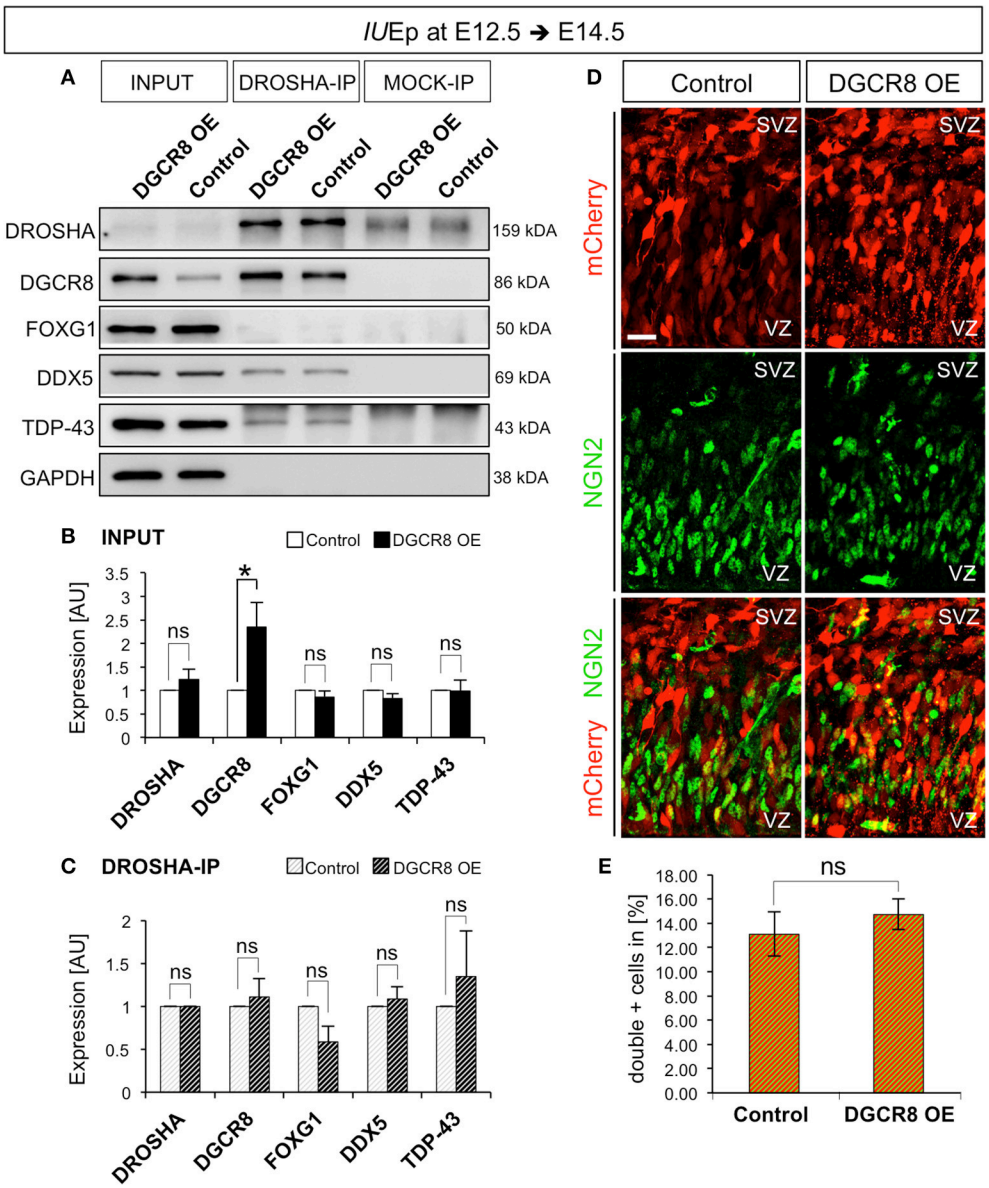

FIGURE 7 | Overexpression of DGCR8 does not change composition or functions of the "miRNA-independent Microprocessor" **(A–C)** Western blot **(A)** and quantification **(B,C)** of DROSHA, DGCR8, FOXG1, DDX5 and TDP-43, in lysate (INPUT) or lysate after co-immunoprecipitation (co-IP) with DROSHA, or MOCK immunoprecipitation, from E14.5 Control (white bars, in **B**, or white-gray-striped bars, in **C**) and DGCR8 OE (black bars, in **B**, or black-gray-striped bars, in **C**) mouse dorsal telencephalon after *IU*Ep at E12.5. Samples were normalized over GAPDH for input samples and normalized to DROSHA for co-immunoprecipitation, error bars indicate the variation of four Control and four DGCR8 OE independent pools (s.e.m.); each independent pool consists of five to six dissected electroporated cortical areas; unpaired Student's *t*-test. **(D)** Immunostaining for NGN2 (green) and mCherry+ electroporated cells (red) on coronal cryosections through the dorsal telencephalon of Control and DGCR8 OE mouse embryos at E14.5 after *IU*Ep at E12.5. SVZ: subventricular zone and VZ: ventricular zone; scale bar: 20 μm. **(E)** Quantification of the proportion of NGN2+mCherry+ cells expressed in % over total mCherry+ cells; Error bars indicate the variation of four Control and four DGCR8 OE electroporated cortices (s.e.m.); unpaired Student's *t*-test. **p*-value < 0.05.

Here, we aimed to understand whether the increased generation of TBR1+ neurons and premature consumption of NPC pools resulting after conditional ablation of *Dgcr8* in the embryonic mouse neocortex was a secondary or reflected a direct consequence of DGCR8 loss of function (Marinaro et al., 2017). For this purpose, we overexpressed DGCR8 in the embryonic mouse neocortex. This resulted in a mosaic model, in which we found largely complementary phenotypes compared to our previous study (Marinaro et al., 2017), in absence of apoptosis (**Figure 2** and Figure S2). Specifically, overexpression of DGCR8 in embryonic mouse neocortex reduces the generation of TBR1+ neurons and expands NPC pools (present study), while conditional knockout of *Dgcr8* increased generation of TBR1+ neurons and induced premature consumption of NPCs (Marinaro et al., 2017). Taken together, this evidence indicates that DGCR8 promotes cortical NPC self-renewal and represses their differentiation *in vivo*, possibly by a cell-autonomous function. Our results are consistent with previous observations in mouse ESCs (Wang et al., 2007; Cirera-Salinas et al., 2017a,b) and NPCs *in vitro* (Liu et al., 2017). Of note, overexpression of DGCR8 at later developmental stages (i.e., when upper cortical layer neurons are generated) selectively promotes expansion of BPs (**Figure 5**), opening intriguing perspectives for a DGCR8-dependent control in the radial neocortex enlargement in evolution, which reflects a striking increase in BP population and upper cortical layers size (Fietz and Huttner, 2011; Reillo et al., 2011; Shitamukai et al., 2011; Wang et al., 2011; Borrell and Reillo, 2012; Hevner and Haydar, 2012; Kelava et al., 2012; Betizeau et al., 2013; LaMonica et al., 2013).

On the other hand, the effects of DGCR8 overexpression on NPC proliferation (**Figure 5**) show distinct phenotypes compared to our previous study (Marinaro et al., 2017), and thus are less straight forward to interpret. For example, conditional deletion of *Dgcr8* led to decreased BrdU incorporation in BPs (Marinaro et al., 2017), while the proportion of BrdU+ BPs remained unaltered upon DGCR8 overexpression (present study). These differences could be due to the method used to label cell proliferation (BrdU incorporation), for instance BPs which undergo just one additional proliferative division might not be detected (**Figure 5**), or different developmental time dependent functions of DGCR8 (compare effects of NPC pools **Figures 4**, **6**). Another possibility is that DGCR8 might simply repress neurogenesis in BPs, so that more electroporated cells remain "progenitors," without changing proliferation index. Thus, despite our results support a model in which DGCR8 cell-autonomously promotes NPC expansion and represses neurogenesis, they did not provide conclusive evidence on the effect of DGCR8 on NPC proliferation.

Beside the well-known mechanism of DROSHA/DGCR8 Microprocessor complex in miRNA biogenesis *in vitro* (Ha and Kim, 2014) and *in vivo* (Yang and Lai, 2011; Barca-Mayo and De Pietri Tonelli, 2014; Petri et al., 2014), accumulating evidence indicates that these proteins also have alternative miRNA-independent functions (Burger and Gullerova, 2015). Indeed, DROSHA targets evolutionary conserved hairpin structures in mRNAs including *Dgcr8* itself, *Ngn2*, *Nf1a,* thereby regulating post-transcriptionally their expression independent of miRNAs (Han et al., 2009; Kadener et al., 2009; Shenoy and Blelloch, 2009; Karginov et al., 2010; Knuckles et al., 2012; Rolando et al., 2016; Kim et al., 2017; Marinaro et al., 2017). Similarly, we recently found that DGCR8 targets hairpins in *Tbr1* mRNAs. Thereby, DGCR8 represses *Tbr1* expression both at RNA and protein level (Marinaro et al., 2017). Other studies indicated that DGCR8 has also important functions in the regulation of splicing (Cirera-Salinas et al., 2017a,b). Thus DROSHA/DGCR8 alternative functions allow a fast regulation of the transcriptome and proteome, which might be crucially involved in the control of NPC maintenance and differentiation. However, the mechanisms and targets of DGCR8-dependent regulation in corticogenesis are still largely unknown. Here, we found that 3- to 5-fold increase of the DGCR8 level in electroporated cortices (**Figures 1**, **7**), does neither change total levels of DROSHA, TDP-43, FOXG1, and DDX5 (**Figure 7**), nor the composition of the Microprocessor complex, with regard to the proteins that co-immunoprecipitated with DROSHA (**Figure 7**), nor the "miRNA-independent Microprocessor" functions, as revealed by similar levels of NGN2 protein expression (**Figure 7**). These results therefore suggest that DGCR8 might not necessarily engage in the DROSHA-Microprocessor complex to exert its functions in cortical NPCs. This hypothesis is consistent with *in vitro* data showing that human DGCR8 controls the stability of small nucleolar RNA (snoRNA) and other transcripts independently of DROSHA (Macias et al., 2015)

In conclusion, our results demonstrate that DGCR8 is essential for proper cortical development, and indicate that DGCR8 functions control NPC pool maintenance and neurogenesis, independently of DROSHA-Microprocessor complex. This is also in agreement with a recent study showing that DGCR8 mediates repair of UV-induced DNA damage independently of RNA processing (Calses et al., 2017). Intriguingly, DNA repair has been previously proposed to be involved in the maintenance of NPC pools (Arai et al., 2011). Future studies will be needed to demonstrate whether DGCR8-DNA repair pathway is causally involved in the maintenance of the NPC pools in corticogenesis.

MATERIALS AND METHODS

Mouse Lines

Mice were housed under standard conditions at the animal facility of Istituto Italiano di Tecnologia (IIT), Genoa, Italy. All experiments and procedures were approved by the Italian Ministry of Health (Permits No. 057/2013; and 214/2015-PR – ref. IIT N° 071) and IIT Animal Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals of the European Community Council Directives. For *Dgcr8* cKO experiments *Emx1*-Cre$^{+/-}$ (Iwasato et al., 2000) and *Dgcr8*$^{flox/flox}$ (Yi et al., 2009) mice were crossed, genotyped and Cre-dependent *Dgcr8* deletion were ascertained as previously published (Marinaro et al., 2017). CD1 WT females and C57Bl6/J WT males were crossed, and embryos used for *in utero* electroporation experiments at the indicated days post coitum (dpc). For all time-mated animals vaginal plug day was defined as E 0.5.

Plasmid Cloning and *in Utero* Electroporation

Full length *Dgcr8* ORF (mmu-*Dgcr8* coding region NCBI Gene ID: 94223) was PCR amplified and cloned into pCAGGS vector (Niwa et al., 1991, modified in Clovis et al., 2012) with NheI and EcoRI. Primers used for *Dgcr8* amplification: forward: GGTCGGTGAGGGTCGACCGG and reverse: TTTATGTGTTCAGACCATCA.

In utero electroporation was performed as previously described (De Pietri Tonelli et al., 2006) with pCAGGS-mCherry/pCAGGS-mmu-*Dgcr8* (1:1 ratio, at 1 mg/ml, total concentration) or control pCAGGS-mCherry plasmids (at 1 mg/ml concentration). Cloning details for pCAGGS-mmu-DGCR8 plasmid are available upon request. Embryos were either immediately used (protein extraction) or fixed in 4% paraformaldehyde in Phosphate-buffered saline (PBS) at 4°C overnight (for immunofluorescence).

BrdU Labeling, Immunofluorescence and Imaging

BrdU labeling was carried out by 3 intraperitoneal injections, performed at 2-h intervals, of pregnant females at the indicated dpc (average mouse weight, 22–24 g), using 1 mg of BrdU (Sigma-Aldrich B5002-5G) in PBS, per injection. Mice were sacrificed 10 h after first BrdU injection [as previously performed (Marinaro et al., 2017)]. Coronal cryosections (20 μm) through brains (post-fixed in 4% PFA (paraformaldehyde; Sigma-Aldrich) and de-hydrated in 30% Sucrose) were prepared at the indicated ages, and processed for immunofluorescence. Immunofluorescence was performed as in Marinaro et al. (2017). Briefly, re-hydrated cryosections (subjected to antigen retrieval with 10 mM citric acid at pH 6.0 for 10 min at 95°C or 30 min at 80°C, if stained for BrdU), were permeabilized with progressive steps in 0.3 and 0.1% Triton X-100 in 1x PBS (PBST). For BrdU labeling 30 min incubation at 32°C in HCl 2N was performed prior to permeabilization, followed by blocking in 0.1% PBST + 5% normal goat serum for 1 h. Sections were afterwards incubated with primary antibodies: rabbit monoclonal anti-DGCR8 (Abcam, ab191875, 1:100), rabbit polyclonal anti-TBR1 (Abcam, ab31940, 1:200), rabbit polyclonal anti-TBR2 (Abcam, ab23345, 1:400), rabbit polyclonal anti-PAX6 (Covance, PRB2789, 1:500), mouse monoclonal anti-NGN2 (R&D, MAB3314, 1:500), rat monoclonal anti-BrdU (Abcam, ab6326, 1:200), rabbit polyclonal anti-KI67 (Abcam, ab15580, 1:250), rabbit monoclonal anti-CASPASE-3 (Cell Signaling, #9664, 1:400), rabbit polyclonal anti-SOX5 (Abcam, ab94396, 1:500), rabbit polyclonal anti-CUX1 (Santa Cruz, SC13024, 1:100) diluted in blocking solution overnight at 4°C in the darkness. Afterwards extensively washed in 0.1% PBST and incubated with secondary antibodies (Thermofisher: goat polyclonal anti-rabbit Alexa Fluor®488 (A-11034, 1:1000), goat polyclonal anti-rabbit Alexa Fluor®647 (A32733, 1:1000), goat polyclonal anti-mouse Alexa Fluor®488 (A32723, 1:1000) and goat polyclonal anti-rat Alexa Fluor®647 (A-21247, 1:1000), goat polyclonal anti-rabbit) diluted in blocking solution for 2 h at RT. Progressive washing steps in 0.1% PBST and then 1x PBS were performed, and sections were incubated with Hoechst (1:300 in 1x PBS from a stock solution of 1 mg/ml in dimethyl sulfoxide, DMSO, Sigma) for 30 min in the darkness, extensively washed in 1x PBS, mounted with ProLong Gold Antifade (Invitrogen), air-dried overnight in the darkness, and sealed with nail polish (Electron Microscopy Sciences). Fluorescent images were acquired with Nikon A1 using a 20x or 60x objective and analyzed with Nikon software version 4.11.0 (NIS Elements Viewer) and ImageJ version 1.48v (Wayne Rasband, National Institutes of Health, USA).

Analysis of Embryonic Dorsal Telencephalon Immunofluorescence Images

Immuno-positive cells for the indicated markers were counted through the depth of the telencephalic wall in the electroporated area and their numbers expressed as a proportion of total number of electroporated cells as indicated in figures and legends. For all the presented quantifications, all relevant sections containing electroporated cells from rostral to caudal were quantified upon DGCR8 overexpression and control conditions. Images represented in **Figures 3**, **4**, **5B**, **6C,E,** **7** show the maximum projection of 10 μm z-Stack acquisitions. Images represented in **Figures 1**, **2**, **5G**, **6A,A'** and Figures S1, S2 show single z-section acquisitions.

Western Blotting

For total protein extraction, electroporated areas of embryonic neocortices were homogenized in RIPA buffer (NaCl 3M, Triton X-100, Sodium Deoxycholate 0.5%, SDS 10%, TrisHCl 1M) supplemented with protease inhibitor (1 tablet protease inhibitor cocktail, 7x, after manufacture's instructions, Roche) and SOV (sodium orthovanadate, 1 mM, Sigma-Aldrich). Tissue was sonicated (10 short pulses, Branson Sonifier 150, Remote, Programm 1) and left on ice for 15 min. Lysate was clarified by centrifugation at 17949 × g for 30 min at 4°C. Protein concentration was determined by using the Bradford Assay kit (Bio-Rad) with a photospectrometer (Eppendorf; BioSpectrometer). For blot analysis, equal amounts of denatured protein (5 min at 100°C) were run on Mini-PROTEAN_TGXTM Precast Gels (Bio-Rad) and transferred on nitrocellulose membranes (GE Healthcare). Membranes were blocked in 5% milk powder in 0.2% PBS-Tween-20 for 1 h at RT, probed with rabbit polyclonal anti-DGCR8 (Proteintech, 10996-1-AP, 1:1000), rabbit polyclonal anti-SOX5 (Abcam, ab94396, 1:1000) and rabbit anti-ACTIN (Sigma, A2066; 1:5000) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody anti-rabbit (Invitrogen, A16104; 1:2000) for 2 h at RT. For all wash steps 0.2% PBS-Tween-20 was used. LAS 4000 Mini Imaging System (GE Healthcare, Little Chalfont, UK) was used for detection of chemiluminescence using SuperSignal® West Pico reagent (ThermoScientific). Band intensities were quantified using ImageJ.

Co-immunoprecipitation

For total protein extraction, electroporated areas of embryonic neocortices were lysed in Co-IP buffer (100 mM NaCl, 20 mM Tris, 1 mM EDTA, 0,5% NP-40) supplemented with complete Protease Inhibitor Cocktail (Roche-Diagnostics) for 30 min on ice and triturated with a 1 ml pipette every 10 min 20 times. Lysate was clarified by centrifugation at 17949 × g for 10 min at 4°C and the supernatant was collected. Protein concentration was determined by using the Bradford Assay kit (Bio-Rad) with a spectrophotometer (BioPhotometer, Eppendorf). Equal amounts of protein was used for all MOCK and Co-IPs, 5% was used for the input. Protein G Dynabeads (10004D, ThermoScientific) were coupled with rabbit polyclonal anti-DROSHA (Abcam, ab12286, 1:100) or rabbit-IgG (rabbit IgG kch-504-250, Diagenode, Seraing, Belgium) in Co-IP buffer under rotation for 1.5 h at RT and 1 h at 4°C. Tissue lysates were precleared with Protein G Dynabeads in Co-IP buffer under rotation for 1 h at 4°C. Subsequently tissue lysates were transferred to antibody-coupled beads and incubated while rotating at 4°C overnight. Beads were washed 3 times with 1 ml Co-IP buffer before they were re-suspended in 30 µl 1x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approximately 6.8). For immunoblotting equal amounts of denatured proteins (5% input and the complete Co-IP samples, 5 min at 95°C) were used. Protein and Co-IP samples were loaded on 10% SDS-polyacrylamide gels and run for 1.5 h at 120 V. Proteins were transferred to PVDF membranes (Trans-blot Turbo Transfer Pack) using the Trans-blot Turbo System (Bio-Rad) following the manufacturer's instructions. Membranes were blocked with 5% BSA or 5% milk powder in 0.1% TBS-Tween-20, probed with rabbit polyclonal anti-DDX5 (Abcam, ab126730, 1:2000), rabbit polyclonal anti-FOXG1 (Abcam, ab18259, 1:1000), rabbit monoclonal anti-DGCR8 (Abcam, ab191875, 1:1000), rabbit polyclonal anti-TDP-43 (Proteintech, 10782-2-AP, 1:5000) and mouse monoclonal anti-GAPDH (Abcam, ab8245, 1:3000). Followed by incubation with HRP-conjugated secondary antibody anti-rabbit (1:10000, donkey-anti-rabbit, 111-035-003, Dianova) or anti-mouse (1:10000, donkey-anti-mouse, 115-035-003, Dianova) for 1 h at RT. For all wash steps 0.1% TBS-Tween-20 was used. LAS 4000 Mini Imaging System (GE Healthcare, Little Chalfont, UK) was used for detection of chemiluminescence using Femto substrates (Thermo Scientific). Band intensities were quantified using ImageJ.

Statistical Analysis

Experimental numbers (n) in **Figure 1**, and **3–7** (immunostaining) are cortices from independent mouse embryos from at least 2 independent litters; while each "n" in **Figure 7** (Co-IP experiments) is a pool of 5–6 extracts from electroporated cortical areas. Data are expressed as standard error mean (s.e.m.) for all quantifications and assays. Differences between groups were tested for statistical significance, where appropriate using unpaired Student's *t*-test or two-way ANOVA followed by Tukey's *post hoc* testing. Significance was expressed as follows in all figures: $^{*}p$-value < 0.05; $^{**}p$-value < 0.01; $^{***}p$-value < 0.001; n.s.: not significant.

AUTHOR CONTRIBUTIONS

DD conceived, supervised and coordinated the project. FM co-supervised NH during initial experiments. Investigation: NH performed all the experiments and analyzed data. SW carried out the experiment in **Figures 7A–C** under TV supervision. Visualization: NH prepared all the figures. DD and NH co-wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00281/full#supplementary-material

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frontiers in Neuroscience

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ShinyCortex: Exploring Single-Cell Transcriptome Data From the Developing Human Cortex

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Single-cell mRNA sequencing (scRNA-seq) is a powerful method to identify and classify cell types and reconstruct differentiation trajectories within complex tissues, such as the developing human cortex. scRNA-seq data also enables the discovery of cell type-specific marker genes and genes that regulate developmental transitions. Here we provide a brief overview of how scRNA-seq has been shaping the study of human cortex development, and present ShinyCortex, a resource that brings together data from recent scRNA-seq studies of the developing cortex for further analysis. ShinyCortex is based in R and displays recently published scRNA-seq data from the human and mouse cortex in a comprehensible, dynamic and accessible way, suitable for data exploration by biologists.

Keywords: single cell RNA sequencing, cortex development, organoids, corticogenesis, single-cell transcriptomics

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New technologies to sequence the transcriptomes of single cells is having an enormous impact on developmental biology research (Tanay and Regev, 2017). This capacity to sequence single-cell transcriptomes allows the relatively unbiased analysis of diverse cell types within a complex tissue based on the abundance of messenger RNAs, an important component of a cell's state at any given time. Single-cell mRNA-seq (scRNA-seq) methods range from relatively low throughput, but high coverage across the full transcript (Picelli et al., 2013), to high throughput with coverage focusing on the 3' or 5' end of the transcript (Jaitin et al., 2014; Klein et al., 2015; Macosko et al., 2015), with most widely-used protocols selecting for poly-adenylated transcripts. Because scRNA-seq data are high-information content, new computational strategies have been developed to understand what the data represents. There are two general analytical methods that are typically used to analyze scRNA-seq data from developing tissues such as the cortex (**Figure 1**). First, cells can be clustered based on similar molecular signatures, which then allows for identification of discrete cellular populations and subpopulations that represent "cell types." Once clusters have been identified, it is possible to identify combinations of genes that are specifically enriched in particular clusters. Many of these marker genes have been previously described for different cell types in the cortex, however it is also possible to discover novel markers. Second, scRNA-seq experiments measure the transcriptome of individual cells that may be in the process of transitioning through various developmental states (e.g., from intermediate progenitor to early neuron). Since not all cells are in the exact same state of differentiation, the transcriptome of each cell can be thought as a representation of a single point of a developmental timeline. By linking the transcriptome from multiple individual cells following a similar developmental program, it is possible to determine the relative position of each cell across the reconstructed trajectory. In this way, entire differentiation

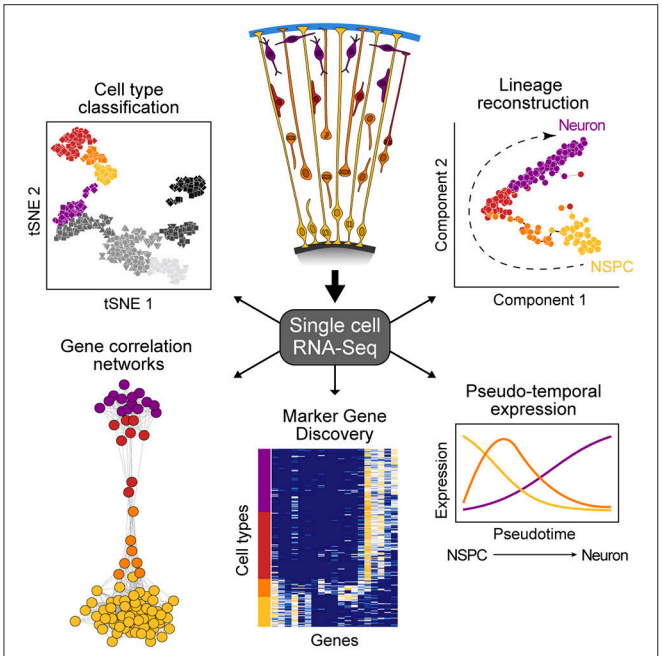

FIGURE 1 | Overview of single-cell RNA-seq data analysis**.** scRNA-seq allows for the study of heterogeneous tissue by avoiding the need to isolate specific cell types inherent to bulk functional genomic approaches. Such data can be used to classify cell types, elucidate gene networks, identify key genetic markers of cell populations, and delineate differentiation trajectories that describe the transcriptional changes cells undergo during development.

trajectories can be reconstructed based on the overlap of gene expression landscapes for cells in the developmental continuum (Haghverdi et al., 2015; Setty et al., 2016; Qiu et al., 2017). This so-called "pseudotime" ordering provides information about the dynamics of gene expression and the establishment of cellular identity in a developing tissue.

Recently, these methods have been applied to the developing human and mouse cortex, which has led to remarkable progress in understanding the molecular signatures that define cell states within these tissues (Camp et al., 2015; Pollen et al., 2015; Nowakowski et al., 2017). In addition, new protocols have been developed that generate three-dimensional human cortical tissue from induced pluripotent stem cells (Lancaster et al., 2013; Sasai, 2013; Qian et al., 2016; Birey et al., 2017). scRNA-seq dissections of engineered cortical tissues have revealed that the cell type-specific gene expression landscapes are very similar to fetal counterparts (Camp et al., 2015), making these excellent systems to study the genetic mechanisms underlying human-specific cortex development. These published studies have been essential to disentangle some of the developmental processes in cortex development and they provide a rich data resource for further studies. Here we briefly describe the data from four of these publications, and we compile the data into a browseable application called ShinyCortex (https://bioinf.eva.mpg.de/shiny/sample-apps/ShinyCortex/) that biologists can use to explore the gene expression patterns from these publications.

Pollen et al. used scRNA-seq (Fluidigm C1 microfluidic platform, SMART-seq, full length) to identify the molecular signatures that mark radial glia cells located in the outer sub-ventricular zone (known as outer or basal radial glial, oRG/bRG) (Pollen et al., 2015). The expansion of this particular population of RG cells in humans is thought to underlie the expansion of the neocortex on the human lineage (Lewitus et al., 2013). The authors microdissected the VZ and OSVZ and used scRNA-seq data from each location to classify and identify distinct RG populations (vRG and oRG). Their results shed light on the molecular characteristics that establishes the oRG identity, such as the production of trophic factors and extracellular matrix proteins, and the activation of the STAT3 signaling pathway.

Camp et al. (2015) used scRNA-seq (Fluidigm C1 microfluidic platform, SMART-seq, full length) to compare cerebral organoids to human fetal cortical tissue at 12–13 weeks post-conception. The authors first used scRNA-seq to establish a reference atlas of cell composition, progenitor-to-neuron differentiation trajectory, and gene expression networks in the early fetal human cortex at a time point comparable to cerebral organoid development. The authors used a cerebral organoid protocol (Lancaster protocol) (Lancaster and Knoblich, 2014) designed to mimic the early stages of brain development, which allows the organoid to self-organize into cerebral tissue containing multiple brain regions. The authors microdissected individual cortical regions and performed scRNA-seq on the dissociated tissue. The authors directly compared the fetal and organoid cortical cells and found that cellular subtypes in organoids and fetal tissue follow very similar gene expression programs.

Quadrato et al. (2017) used droplet microfluidics (Drop-seq, 3′ end counting, UMIs) to profile more than 80,000 individual cells derived from 31 brain organoids based on a modification of the Lancaster protocol. The organoids were analyzed at different times points (3–6 months), which revealed that neurons mature into potentially active neural networks within the organoid. Notably, the organoids contain multiple brain regions, and here we isolated the cortical cell clusters for compilation into the ShinyCortex application.

Birey et al. used signaling molecules to direct the development of 3D structures called spheroids that resemble two regions of the forebrain (the ventral forebrain and dorsal pallium). Spheroid fusion led to the formation of forebrain-like organoids and interneurons migrated from the ventral to the dorsal region, providing information on brain-region interactions. These authors also performed single-cell transcriptomics (BD Resolve microwell platform, 3' end counting, UMIs) on both ventral and dorsal forebrain spheroids individually and we compiled the dorsal forebrain data into the ShinyCortex application.

Telley et al. analyzed the transcriptome of single cells (Fluidigm C1 microfluidic platform, SMART-SEQ, full length) isolated from multiple time points from the developing mouse neocortex (Telley et al., 2016). The authors were able to bring temporal resolution into their scRNA-seq data by incorporating fluorescent tagging of newborn cells in the ventricular zone. This enabled the authors to identify early transcriptional waves that instruct the sequence and pace of neuronal differentiation events in the mouse cortex. We added this data set to the ShinyCortex

application to enable a comparison between human and mouse cell types in the developing neocortex.

Even though the raw sequencing data and processed gene expression matrices for these studies are publicly available online, it can be difficult for researchers to access and analyze this data. ShinyCortex consolidates the processed data into a web accessible Shiny application constructed using the R programming language and based on Plotly, which creates and displays interactive plots that are relatively intuitive and easy to manipulate (https://bioinf.eva.mpg.de/shiny/sample-apps/ShinyCortex/). ShinyCortex can be used to visualize the transcript level of any gene of interest as a function of cell types or over pseudotime, and it allows the user to explore the expression correlations between genes. Generally, the user of ShinyCortex first chooses the dataset that he/she wants to explore (i.e., Pollen, Camp fetal data, Camp organoid data, Quadrato, Birey, Telley). The application is then divided into four sections. The first panel plots the distribution of gene expression values of any gene of interest according to the cell type assignment from each study, and options include box, violin, or scatter plots (**Figure 2A**). The second panel identifies for a given gene of interest the top correlated or anti-correlated genes and visualizes the correlation coefficients between the genes in the form of correlograms (**Figure 2B**). Also, it allows the user

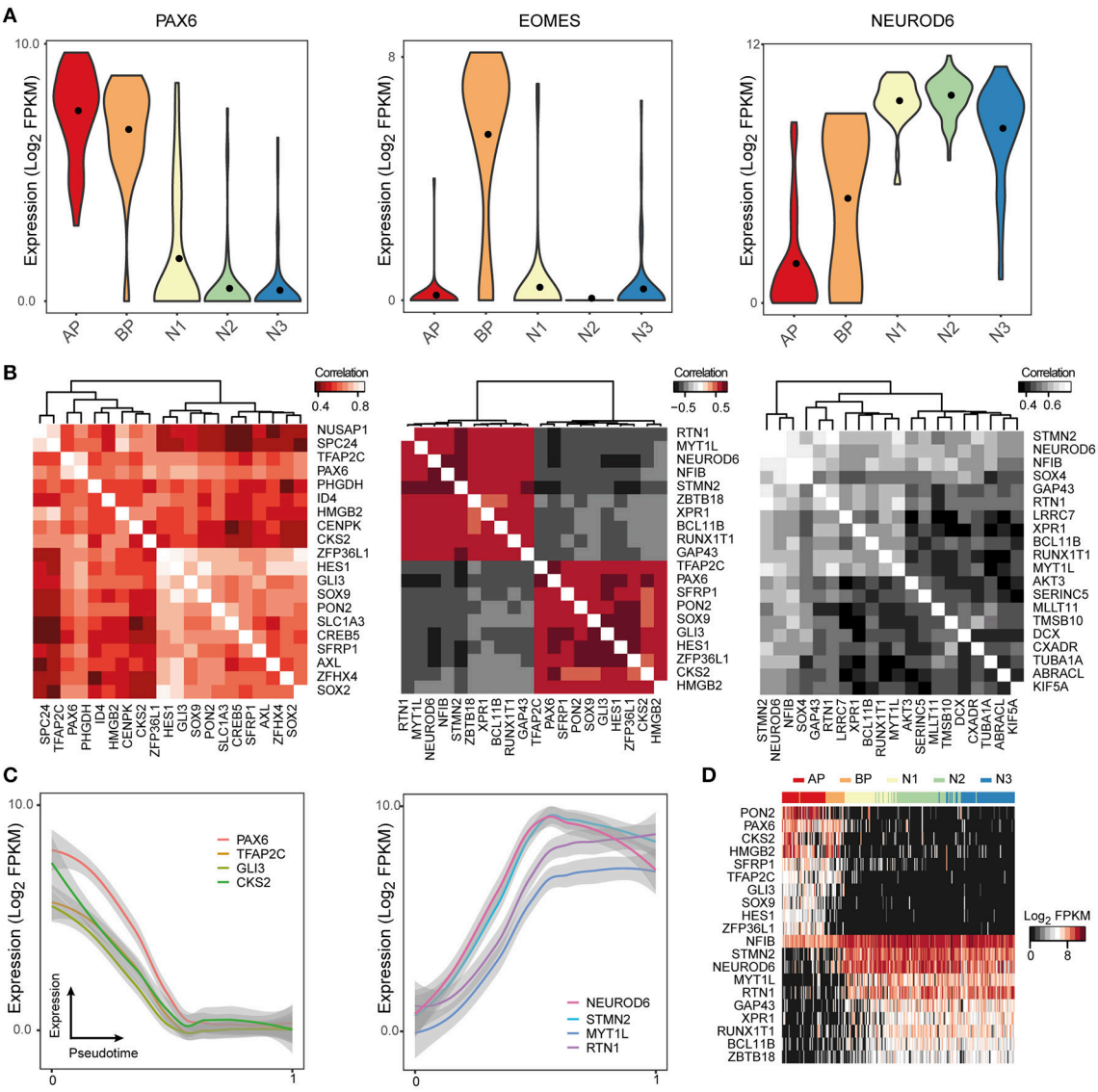

FIGURE 2 | ShinyCortex allows for interactive visualization of neocortex scRNA-seq data**.** The application can be used to plot and explore various analyses from each dataset starting from a user-defined gene. Representative visualizations are shown here for the fetal neocortex data from Camp et al. **(A)** Violin plots are dynamically generated showing the distribution of gene expression across cell types. **(B)** Heatmaps portray the highest correlated and anticorrelated genes to the selected gene of interest. **(C)** Line plots display the expression profile of genes of interest across developmental pseudotime with level of confidence interval shown (0.95 by default). **(D)** Heatmaps show the expression of selected genes across all cells of a given dataset.

to include additional genes that may be relevant for certain cellular processes, but are not among the highest correlated genes. The third section uses a generalized additive model to show how gene expression is changing according to pseudotime (**Figure 2C**), provided that pseudotime values were determined for individual cells in the chosen study. It allows for the display of multiple genes at the same time with visualization of both raw values and smoothened curves. Finally, the last panel uses a heatmap representation to display the expression of multiple genes of interest in individual cells that are ordered either based on their pseudotime (if available) or their cell type assignment (**Figure 2D**). All plots can be downloaded, and there are graphical parameters than can be modified in each individual plot, such as color palette and panel size. We believe that ShinyCortex will help the corticogenesis community access and explore cortical scRNA-seq datasets, which can lead to the identification of cell state-specific genes for functional analysis.

METHODS

All data was obtained through their different data repositories as described in the original papers and was used without any additional processing or filtering. Cell type classification (and pseudo-time if applicable) for each dataset was obtained directly through the authors. Person's correlation coefficients between genes were calculated with r base. All interactive plots were generated with plotly and ggplot2 R packages. Correlation heatmaps were generated with the gplot R package. The pseudotime plots use a generalized additive model (GAM) implemented with the mgcv R package and level of confidence interval (0.95 by default). Pseudotime heatmaps were produced with the stats R package.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Epigenetic and Transcriptional Pre-patterning—An Emerging Theme in Cortical Neurogenesis

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Neurogenesis is the process through which neural stem and progenitor cells generate neurons. During the development of the mouse neocortex, stem and progenitor cells sequentially give rise to neurons destined to different cortical layers and then switch to gliogenesis resulting in the generation of astrocytes and oligodendrocytes. Precise spatial and temporal regulation of neural progenitor differentiation is key for the proper formation of the complex structure of the neocortex. Dynamic changes in gene expression underlie the coordinated differentiation program, which enables the cells to generate the RNAs and proteins required at different stages of neurogenesis and across different cell types. Here, we review the contribution of epigenetic mechanisms, with a focus on Polycomb proteins, to the regulation of gene expression programs during mouse neocortical development. Moreover, we discuss the recent emerging concept of epigenetic and transcriptional pre-patterning in neocortical progenitor cells as well as post-transcriptional mechanisms for the fine-tuning of mRNA abundance.

Keywords: gene regulation, histone methylation, neocortical development, neural progenitor cell, Polycomb, epigenetics, chromatin, neurogenesis

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INTRODUCTION

The generation of neocortical neurons during mouse development is the result of balanced proliferative and differentiative divisions of neural stem and progenitor cells (Götz and Huttner, 2005; Lui et al., 2011; Florio and Huttner, 2014). In the early developing central nervous system, neuroepithelial cells (NECs) function as the primary neural stem cells which show apico-basal polarity and undergo symmetric proliferative divisions to expand the stem cell pool (**Figure 1**). With the onset of neurogenesis at around mouse embryonic day (E) 10, NECs transform into apical radial glia (aRG), which retain apico-basal polarity and become more elongated. Their cell bodies reside in the ventricular zone, whereas their long basal processes extend to the basal lamina and provide a scaffold for neuronal migration to the cortical plate. aRG are characterized by their ability to self-renew and to simultaneously give rise to neurons, mainly indirectly through basal intermediate progenitors (bIPs). bIPs delaminate from the ventricular surface and reside in the subventricular zone. They lack apico-basal polarity and in mouse typically divide symmetrically to produce two neurons. Neocortical neurons are organized into six horizontal layers, with the deep-layer neurons born first during neurogenesis followed by the generation of upper-layer neurons. At around E17, neurogenesis is terminated and the remaining neural stem and progenitor cells switch to gliogenesis. Thus, throughout mouse neocortical development, the potential of neural progenitor cells (NPCs) for proliferation and differentiation changes as NPCs pass through phases of expansion, deep- and upper-layer neurogenesis, and gliogenesis. In this review, we will discuss

the dynamic changes in transcriptional programs and epigenetic information that accompany and guide these transitions. We will mainly focus on post-translational modifications of histones, as the role of other epigenetic pathways, including DNA modifications and chromatin remodeling, in neocortex development are reviewed elsewhere (see Sokpor et al., 2018; Stricker and Gotz, 2018, in this Research Topic).

TRITHORAX AND POLYCOMB COMPLEXES

Epigenetic information, in concert with transcription factors, coordinates the instruction of specific cellular identities from the genomic DNA template, and as such plays an essential role in the transition of cell fates during development. Post-translational histone modifications represent one major epigenetic system, among others. In particular, chromatin modifiers of the Trithorax (TrxG) and Polycomb (PcG) groups were identified as part of an evolutionary conserved epigenetic memory system that acts antagonistically to maintain active and repressed gene expression states, important during stem cell differentiation and embryonic development (reviewed in Piunti and Shilatifard, 2016; Schuettengruber et al., 2017). PcG proteins assemble into two major complexes, PRC1 and PRC2 (**Figure 1**), which catalyze mono-ubiquitination of histone 2A lysine 119 (H2AK119ub1) and tri-methylation of histone 3 lysine 27 (H3K27me3), respectively. These complexes have also been shown to regulate gene expression during neocortical development, and importantly, are one of the major determinants of the ability of NPCs to either self-renew or to give rise to neurons or glial cells (Tyssowski et al., 2014; Mitrousis et al., 2015; Yao et al., 2016).

THE TRANSITION FROM EXPANSION TO NEUROGENESIS

During early development, the neural tube is formed by NECs that divide symmetrically to expand the neural stem cell pool. Following this initial expansion phase, NECs turn into neurogenic aRG, characterized by the appearance of glial hallmarks, a change in the mitotic behavior and a more restricted progenitor fate (Götz and Huttner, 2005; Taverna et al., 2014; Subramanian et al., 2017). This transition is accompanied by a major redistribution of the PcG-mediated H3K27me3 mark (Albert et al., 2017), which is associated with transcriptional gene silencing (Comet et al., 2016). Several tight junction-associated genes convert to a more repressive chromatin configuration, whereas the genes encoding the glial-specific glutamate transporter (*Slc1a3*/Glast) and the brain lipid-binding protein (*Fabp7*/Blbp) acquire H3K4me3 (Albert et al., 2017), a hallmark of TrxG-associated gene activation (Schuettengruber et al., 2017). Notably, in line with NECs representing the earliest and least committed neural stem cells of the developing neocortex, the majority of the genes marked by H3K27me3 in NECs carry H3K4me3 in addition (Albert et al., 2017), a configuration which has been termed "bivalent" (Bernstein et al., 2006). Such bivalent domains are abundant in embryonic and adult stem cells (Shema et al., 2016), where they decorate genes implicated in cell-fate determination and development (Schuettengruber et al., 2017). This has been hypothesized to keep future lineage choices open (Bernstein et al., 2006). With the transition of NECs to aRG, a large fraction of bivalent domains is resolved, either to H3K27me3 at promoters of genes involved in the development of other organs (**Figure 1**), or to H3K4me3 at genes involved in nervous system development, cell adhesion and cell surface signaling (Albert et al., 2017). Thus, the switch of NPCs from the initial expansion phase to the neurogenic phase is accompanied by major epigenetic changes.

THE NEUROGENIC PHASE

During the neurogenic phase, aRG have the potential to either proliferate or to self-renew and generate basal progenitors or, rarely, neurons. PcG complexes have been shown to contribute to the regulation of this balance between proliferation and differentiation. The PRC2 histone methyltransferase Ezh2, which generates H3K27me3, is highly expressed in NPCs of the mouse developing neocortex, particularly during early neurogenesis (Pereira et al., 2010; Piper et al., 2014). Specific deletion of *Ezh2* in the developing neocortex from E9.5 results in a loss of H3K27me3 and up-regulation of gene expression, consequently shifting aRG fate from self-renewal toward differentiation (Pereira et al., 2010). This shift results in an overproduction of bIPs and neurons at the expense of aRG, ultimately reducing the neuronal output and leading to a substantially smaller neocortex (Pereira et al., 2010). In light of this, it is interesting to note that the promoters of many transcription factors involved in bIP generation and neuronal differentiation (like *Insm1*, *Eomes*, *Neurog1/2,* and *Neurod1/2*) are H3K27me3-positive during the expansion phase of NPCs (Albert et al., 2017), and a loss of this repressive state might contribute to the precocious activation of these genes. In addition, the PRC1 component Bmi1 has been shown to regulate the self-renewal and differentiation of NPCs (Fasano et al., 2007, 2009; Yadirgi et al., 2011).

From these genetic studies, it is clear that PcG proteins contribute to the regulation of the balance between self-renewal vs. differentiation during neocortex development, but what are the underlying molecular mechanisms? Epigenome profiling in specific cell populations isolated at mid-neurogenesis (E14.5) has shown that H3K4me3 and H3K27me3 marks are highly dynamic during neocortical lineage progression (Albert et al., 2017). In particular, several transcription factors involved in cell fate commitment during neurogenesis display transient changes in histone methylation at their promoters, potentially involved in cell type-specific induction of gene expression. Notably, the promoter of the *Eomes* gene, which encodes the key transcription factor Tbr2 implicated in the generation of bIPs (Arnold et al., 2008; Sessa et al., 2008), changes from a repressive configuration marked by H3K27me3 in proliferative aRG to an active configuration marked by H3K4me3 in aRG undergoing neurogenic divisions (Albert et al., 2017). As these changes likely occur within one cell-cycle, it is conceivable

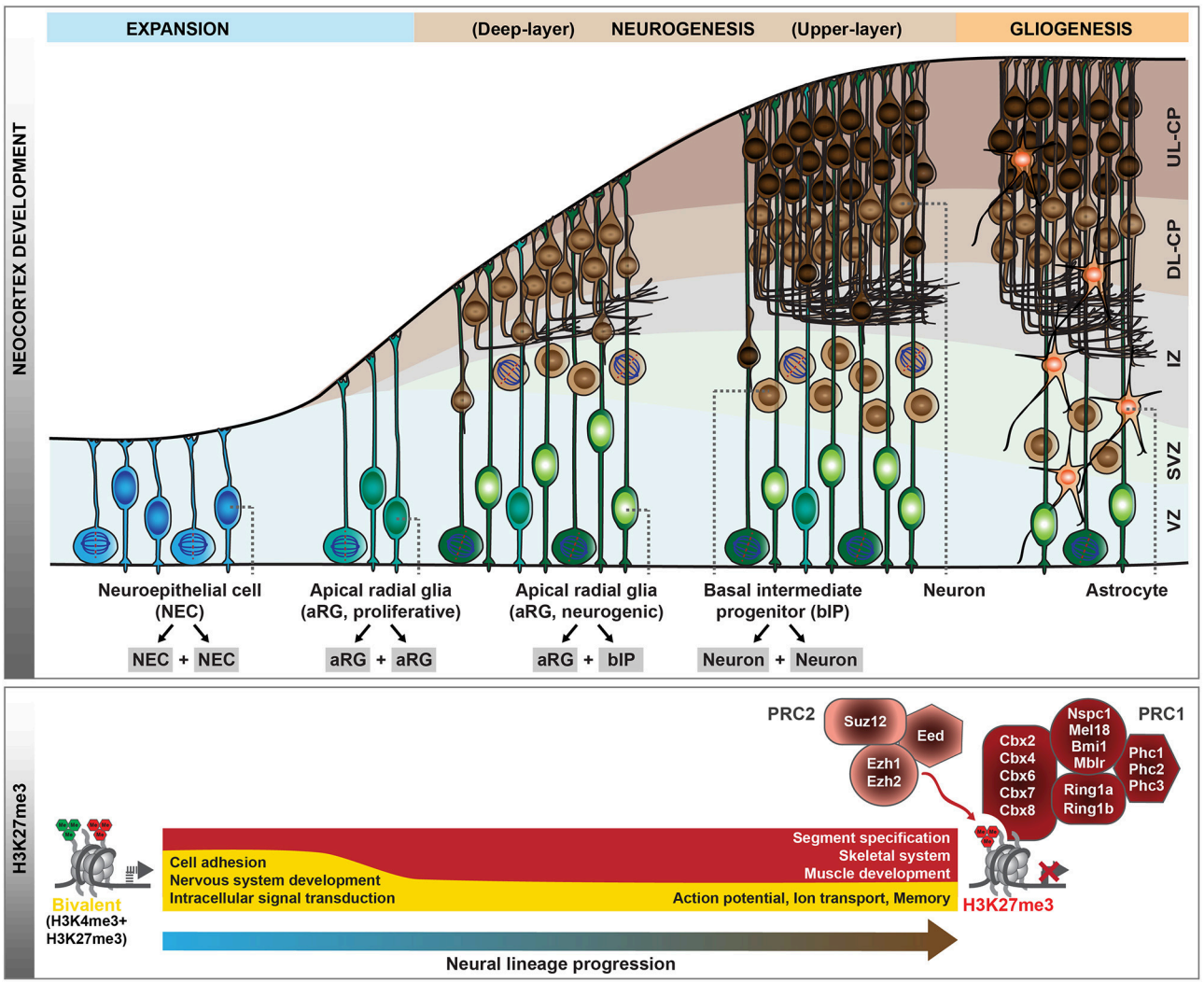

FIGURE 1 | Polycomb-mediated histone methylation during mouse neocortex development**.** During the development of the mouse neocortex, neural progenitor cells pass through consecutive stages of expansion, neurogenesis, and gliogenesis (top scheme). aRG undergoing neurogenic divisions give rise to bIPs, which are the main source of neurons in the mouse. As neural progenitor cells transition from proliferation to neurogenic divisions, their histone methylation profiles change dynamically. Whereas, many genes are in a bivalent configuration in early proliferative progenitor cells, many of these poised domains are resolved with progressive lineage commitment. The gene ontology categories characteristic of the genes marked by H3K27me3 (red) or bivalent modifications (yellow) during early neurogenesis and in neurons are indicated (bottom scheme). In addition, the core components of Polycomb repressive complex 1 (PRC1) and 2 (PRC2) are shown. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; DL, deep-layer; UL, upper-layer.

that the H3K27me3 mark is actively removed, most likely by the histone demethylase Jmjd3, which is expressed in the developing neocortex (Sessa et al., 2017) and has been shown to act on *Eomes* gene regulatory regions (Kartikasari et al., 2013). The active configuration of the *Eomes* promoter is largely maintained in bIPs, whereas H4K4me3 levels decline and H3K27me3 is re-established in neurons (Albert et al., 2017), in which *Eomes* is no longer expressed (Florio et al., 2015). Thus, *Eomes* is one example of a gene that undergoes dynamic changes in histone methylation during neocortical differentiation (**Figure 2**), and these changes correlate well with the gene expression dynamics. In addition, the regulation of other transcription factors that control progenitor proliferation or differentiation has been linked to various histone methylation states, including H3K4me3 and H3K79me3 (Büttner et al., 2010; Yang et al., 2012).

But are the histone methylation patterns instrumental for the correct expression of the related genes in the developing neocortex? Previous studies, which applied CRISPR/Cas9-based genome editing *in vivo* to disrupt *Eomes* expression in NPCs during neocortical development, showed that this acute targeting results in a reduction in bIPs and an increase in neuronal differentiation (Kalebic et al., 2016). Importantly, CRISPR/Cas9-based epigenome editing at the

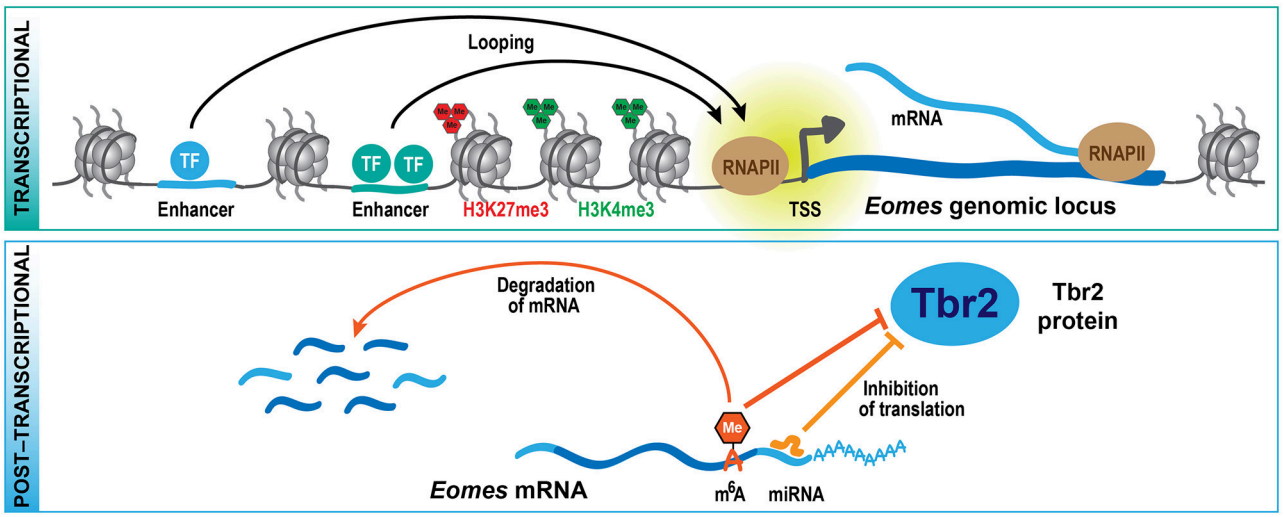

FIGURE 2 | Multi-layered regulation of gene expression. At the transcriptional level (top scheme), cell type-specific expression of genes is regulated by transcription factors that bind to regulatory sequences, including distal enhancers that contact their respective target genes by looping. Histone methylation, mediated by TrxG (H3K4me3) and PcG (H3K27me3) proteins, is part of an epigenetic system that also contributes to the regulation of specific gene expression. At the post-transcriptional level (bottom scheme), the translation and stability of mRNAs is regulated by miRNAs and epitranscriptomic mechanisms including m^6A, providing a multi-layered system for the control of protein expression during development. These mechanisms are exemplified here for the key transcription factor Tbr2 (*Eomes*), which regulates bIP generation during neocortex development.

Eomes locus in the developing neocortex has shown that the decrease in H3K27me3 in neurogenic NPCs is required for normal Tbr2 expression and bIP regulation (Albert et al., 2017). These results underscore the importance of epigenetic information in the regulation of specific gene expression and as facilitator of cell fate transitions during development.

The H3K27me3 mark is recognized by different "reader" proteins, one of which is the chromatin remodeler Chd5 expressed in neurons of the developing neocortex (Egan et al., 2013). Depletion of Chd5 during neurogenesis results in a block of neuronal differentiation, which can be rescued by Chd5 only if the latter contains an intact double chromodomain mediating H3K27me3 binding. In addition, components of the PRC1 complex can bind to H3K27me3, and at the majority of genomic target sites, H3K27me3 and PRC1 are found to co-localize, even though this traditional model of sequential binding of PRC2 followed by PRC1 complexes has been challenged by several studies (Puschendorf et al., 2008; Blackledge et al., 2015; Kloet et al., 2016). Deletion of Ring1b, an integral component of PRC1 (Leeb and Wutz, 2007), specifically in the mouse developing neocortex during the neurogenic phase results in altered neuronal subtype specification (Morimoto-Suzki et al., 2014). By mediating the timed termination of *Fezf2* expression, Ring1b regulates the number of subcerebral projection neurons produced. These data suggest that PcG complexes and associated proteins control several aspects of cortical neurogenesis, including the balance between self-renewal and differentiation of aRG as well as the switch from deep- to upper-layer neurogenesis in NPCs.

THE TRANSITION TO THE GLIOGENIC PHASE

In mouse, the neurogenic phase is followed by a period of gliogenesis, during which astrocytes and oligodendrocytes are generated. The timing of the switch from neurogenic to gliogenic fate of NPCs is critical for brain development, as it is one of the determinants of the final number of cortical neurons produced. In addition to extracellular cues, cell-intrinsic programs regulate NPC fate, to which epigenetic mechanisms are thought to contribute. The PcG proteins have been demonstrated to play an important role in the timing of the neurogenic to gliogenic transition. Depletion of PcG proteins during the neurogenic period leads to a prolonged neurogenic phase of NPCs and a delayed onset of astrogenesis (Hirabayashi et al., 2009; Sparmann et al., 2013). Toward the time when neurogenesis is normally terminated, several genes associated with the neurogenic lineage are selectively derepressed in PcG-mutant NPCs, including neurogenin 1 (*Neurog1*), a key proneural transcription factor that can suppress astrocytic differentiation (Hirabayashi et al., 2009).

Interestingly, deletion of Ezh2 before, or at, the onset of neurogenesis has the opposite effect, leading to a shorter neurogenic period and precocious astrocyte generation (Pereira et al., 2010; Sparmann et al., 2013). In NPCs *in vitro*, PcG proteins mediate the suppression of the key astrogenic marker *Gfap* (Mohn et al., 2008; Sparmann et al., 2013), which has been proposed to prevent the premature onset of gliogenesis (Sparmann et al., 2013). In the developing neocortex, however, the promoters of *Gfap* as well as of other genes characteristic of astrocytes are not marked by H3K27me3 at mid-neurogenesis

(ENCODE Project Consortium, 2012; Albert et al., 2017), which is in agreement with other reports suggesting a role for alternative repressive pathways, including DNA and H3K9 methylation, in the regulation of astrocyte-specific genes (Takizawa et al., 2001; Song and Ghosh, 2004; Fan et al., 2005; Hatada et al., 2008). Future research should be aimed at identifying PcG target genes underlying the context- and stage-dependent role of PcG proteins in NPCs during different phases of neocortex development, and should provide a more general view beyond the limited number of well-characterized known regulators.

CELL TYPE- AND STAGE-SPECIFIC ROLES OF POLYCOMB PROTEINS

Previous studies in mouse and human embryonic stem cells (Mikkelsen et al., 2007; Mohn et al., 2008; Burney et al., 2013; Ziller et al., 2015) and the developing mouse neocortex (Albert et al., 2017) have shown that H3K27me3 levels are highly dynamic at different NPC stages and during neuronal differentiation, raising the question of how PcG target gene specificity is achieved. One way to dynamically control PcG function is by altering the composition of PcG complexes, which in mammals, as opposed to flies, is highly diverse, enabling the assembly of various sub-complexes with different chromatin binding affinities and interaction partners (Piunti and Shilatifard, 2016; Schuettengruber et al., 2017).

In embryonic stem cells, the interchange of Chromobox (Cbx) family proteins, part of PRC1, has been reported to modulate the balance between self-renewal and lineage commitment (Morey et al., 2012; O'Loghlen et al., 2012; Santanach et al., 2017), and different Cbx paralogs are required for different cell lineages (Luis et al., 2011; Klauke et al., 2013). Of note, the Cbx paralogs are differentially expressed in neural sub-populations of the developing neocortex (Florio et al., 2015). Moreover, chromatin remodelers of the chromodomain helicase DNA-binding (Chd) family, which have been reported to interact with PcG complexes, also show differential expression during neocortex development. Whereas Chd5 is expressed in neurons and controls neuronal differentiation (Egan et al., 2013), Chd4 is expressed in NPCs during early neurogenesis where it has been proposed to function in PcG-mediated inhibition of astroglial differentiation (Sparmann et al., 2013). This switch in subunit composition may contribute to the re-targeting of PcG complexes during neocortex development.

PcG complexes themselves bind relatively unspecifically to CG-rich regions lacking DNA methylation (Schuettengruber et al., 2017). In addition, the chromatin targeting of PRC2 is stabilized by interactions with transcription factors, non-coding RNAs and other chromatin factors resulting in increased binding and H3K27me3 deposition at specific regions. The highly restricted expression pattern of many of these factors and RNAs during neocortex development (Aprea et al., 2013; Molyneaux et al., 2015; Liu et al., 2016) provides a potential mechanistic explanation for cell type-specific PcG targeting. Moreover, the H3K27me3-specific histone demethylase Jmjd3 has been implicated in the activation of neuronal gene expression (Jepsen et al., 2007; Park et al., 2014), and associates with the transcription factor Tbr2 in the developing neocortex (Sessa et al., 2017), further contributing to the dynamic regulation of H3K27me3.

TRANSCRIPTIONAL PRE-PATTERNING

During recent years, there have been massive efforts to characterize the transcriptomic signatures of the various NPC subtypes in the mouse developing neocortex, but also in other mammalian species including the ferret, macaque and human (Ayoub et al., 2011; Fietz et al., 2012; Aprea et al., 2013; Arcila et al., 2014; Miller et al., 2014; Pollen et al., 2014; Camp et al., 2015; De Juan Romero et al., 2015; Florio et al., 2015; Johnson et al., 2015; Liu et al., 2016; Telley et al., 2016; Nowakowski et al., 2017; Zhong et al., 2018). From these studies, a variety of gene expression differences have been uncovered that underlie specific cell biological features, proliferative capacities and differentiation potential of the distinct NPC types (reviewed in Silver, 2016; Florio et al., 2017). Interestingly, several of these studies described the expression of genes in aRG whose protein products are well-known to function only downstream in the lineage, in bIPs or neurons (Florio et al., 2015; Telley et al., 2016; Nowakowski et al., 2017), raising the possibility that there is a delay in translation for certain mRNAs.

One example of such a gene that is expressed already in aRGs, specifically those undergoing neurogenic divisions, is *Eomes* (Florio et al., 2015), which gives rise to the bIP transcription factor Tbr2 (Arnold et al., 2008; Sessa et al., 2008). What is it that keeps the *Eomes* mRNA from being translated in aRG? The Tbr2 protein has been shown to be repressed by the microRNAs (miRNAs) miR-92 and miR-92b, and both miRNAs regulate bIP specification in the developing neocortex (Bian et al., 2013; Nowakowski et al., 2013). Interestingly, miR-92 and miR-92b are specifically expressed in aRG undergoing neurogenic divisions, where the *Eomes* mRNA is highly expressed (Florio et al., 2015). In contrast, bIPs, which express Tbr2 protein, have low levels of both miRNAs (Nowakowski et al., 2013; Florio et al., 2015). Of note, many other miRNAs display unique profiles of expression in the developing neocortex (Barca-Mayo and De Pietri Tonelli, 2014; Rajman and Schratt, 2017), and among their validated target genes are several cell cycle and neurogenesis regulators (Arcila et al., 2014; Fei et al., 2014), indicating that miRNA-mediated control of RNA translation (**Figure 2**) may play a widespread role during neocortex development and also evolution. Moreover, two components of the miRNA microprocessor complex, Drosha and DGCR8, were shown to regulate gene expression in the developing neocortex in a miRNA-independent fashion (Knuckles et al., 2012; Marinaro et al., 2017), further adding to the complexity of post-transcriptional gene regulation.

In addition, recently a new epitranscriptomic mechanism has been identified that regulates the metabolism and translation of mRNAs, which involves the post-transcriptional modification of mRNAs by N^6-methyladenosine (m^6A) (reviewed in Zhao et al., 2017). Depletion of m^6A during neocortex development

leads to a prolonged cell cycle of aRGs and extends neuron production to postnatal stages, suggesting that m^6A regulates cortical neurogenesis (Yoon et al., 2017). Among the transcripts that are tagged by m^6A, several encode transcription factors regulating NPC fate, such as Pax6, Sox2, Neurog2, and Tbr2. The presence of m^6A on these transcripts promotes their rapid turnover, and in absence of the m^6A methyltransferase complex component *Mettl14*, several neuronal lineage proteins, like Neurod1 and Tbr2, are precociously expressed in aRG. This observation led to the proposal of the novel concept of transcriptional pre-patterning during cortical neurogenesis, whereby a subset of neuronal lineage genes is already expressed in aRG but their levels actively suppressed post-transcriptionally by m^6A-dependent decay (Yoon et al., 2017). A second study that analyzed the role of m^6A during neurogenesis found that *Mettl14* deletion results in decreased radial glia proliferation and premature differentiation (Wang et al., 2018). The authors of this study ascribed the observed phenotypes to genome-wide changes in histone methylation patterns which may result from the destabilization of transcripts that encode histone-modifying enzymes. While further mechanistic studies are required to dissect the role of m^6A in specific neural subpopulations, the two studies (Yoon et al., 2017; Wang et al., 2018) describe a novel post-transcriptional mechanism regulating protein expression during neurogenesis (**Figure 2**).

EPIGENETIC PRE-PATTERNING

Whereas, transcriptome analysis provides a snapshot of a cell's gene expression pattern at a specific point in time, the corresponding epigenetic information captures gene regulatory mechanisms, developmental origins, and potential future responses to developmental stimuli (Mo et al., 2015). Transcription factors, which are thought to be instrumental for the specification of cell type-specific gene expression programs, bind to DNA in the context of chromatin, which carries multiple post-translational modifications, and these affect transcription factor binding (Shlyueva et al., 2014; Yin et al., 2017). As such, the epigenetic landscape can permit the transcription of certain genes, while rendering others inaccessible to most transcription factors.

That said, the transition from "closed" to "open" chromatin, and vice versa, is determined by regulatory proteins, most prominently a special class of transcription factors, called pioneer factors (Shlyueva et al., 2014). These factors can bind to repressed chromatin and recruit chromatin remodelers to evict nucleosomes to open up the region, thereby making the DNA accessible to other transcription factors. During neural differentiation, such pioneer factors have been proposed to remodel the binding site repertoire for proneural factors at the NPC stage by changing the epigenetic landscape at their respective target sites (Ziller et al., 2015). This is also thought to ensure proper further lineage specification by restricting the binding capacity of proneural and other transcription factors toward appropriate sites.

Differential gene expression in specific cell types is mainly controlled by distal *cis*-regulatory elements, among which enhancers are the most abundant (Spitz and Furlong, 2012; de Laat and Duboule, 2013). Enhancer sequences contain short DNA motifs that serve as binding sites for sequence-specific transcription factors. In a given tissue, active enhancers are brought into spatial proximity of their respective target gene by looping (Shlyueva et al., 2014). Our understanding of how chromatin is organized and folded within the nucleus, and how this affects gene regulation and cell fate decisions, has greatly expanded during recent years, mainly due to technological advances in detecting chromatin contacts in 3D (Bonev and Cavalli, 2016; Franke et al., 2016).

During neural differentiation, both *in vitro* and in the mouse developing neocortex, chromatin interactions change dynamically, frequently related to neural transcription factors that contribute to chromosome reorganization (Bonev et al., 2017). In addition, PcG proteins have been proposed to facilitate neural induction by establishing physical interactions between poised enhancers and their target genes in embryonic stem cells (Cruz-Molina et al., 2017). These preformed contacts are thought to provide a permissive topology that facilitates the timely and robust induction of major neural genes upon differentiation. The importance of understanding chromosome conformation has been underscored by recent studies in the human developing neocortex, which have revealed regulatory relationships relevant to the evolution of human cognition but also to diseases (Won et al., 2016; de la Torre-Ubieta et al., 2018).

CONCLUSIONS

It is well-established that epigenetic mechanisms contribute to the regulation of gene expression during stem cell differentiation and development. In this review, we have summarized recent advances in our understanding of the role of Polycomb proteins during mouse neocortex development. In particular, recent epigenome profiling has shed further light on the context-dependent functions of Polycomb proteins during the proliferative and neurogenic phase of neocortex development. It remains to be shown on a genome-wide scale how PcG targets change with the transition to the gliogenic phase. Moreover, in the future, it will be interesting to apply the emerging CRISPR/Cas9-based epigenome editing tools (Pulecio et al., 2017) to dissect the role of epigenetic changes at gene regulatory regions of important regulators of neocortex development. In a proof of principle study, the role of H3K27me3 has been analyzed *in vivo* during neocortex development at the *Eomes* gene promoter (Albert et al., 2017). From such epigenome editing experiments, further functional insights into chromatin-mediated gene regulation can be expected. Importantly, such studies will allow to move the field forward beyond correlations of epigenetic information and gene expression to interrogating the functional relevance of histone modifications at regulatory regions in specific neural cell types and at various periods of neocortex development. Recent technological advances have revealed important insights into

the 3D genome organization during neocortex development and have led to the identification of distal regulatory elements. With CRISPR/Cas9-based genome and epigenome editing techniques at hand, the functional interplay of histone modifications, genome organization, and gene expression can now be unraveled.

The epigenetic landscape provides a framework within which many transcription factors operate, but which, in turn, is modulated by the action of transcription factors and gene expression itself. During development, epigenetic patterning is important for the correct spatio-temporal regulation of gene expression. In addition, the translation of expressed mRNAs is regulated by miRNAs and novel epitranscriptomic modifications, providing a multi-layered mechanism to precisely control the dynamic expression of genes, both at the mRNA and protein level. The challenge for the future will be to integrate the different layers of transcriptional and post-transcriptional gene regulation into a comprehensive framework that allows to link the different mechanisms and to understand the cross-talk between these systems.

AUTHOR CONTRIBUTIONS

MA designed the figures. MA and WH wrote the manuscript.

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The Epigenetic Factor Landscape of Developing Neocortex Is Regulated by Transcription Factors Pax6→ Tbr2→ Tbr1

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Epigenetic factors (EFs) regulate multiple aspects of cerebral cortex development, including proliferation, differentiation, laminar fate, and regional identity. The same neurodevelopmental processes are also regulated by transcription factors (TFs), notably the Pax6→ Tbr2→ Tbr1 cascade expressed sequentially in radial glial progenitors (RGPs), intermediate progenitors, and postmitotic projection neurons, respectively. Here, we studied the EF landscape and its regulation in embryonic mouse neocortex. Microarray and *in situ* hybridization assays revealed that many EF genes are expressed in specific cortical cell types, such as intermediate progenitors, or in rostrocaudal gradients. Furthermore, many EF genes are directly bound and transcriptionally regulated by Pax6, Tbr2, or Tbr1, as determined by chromatin immunoprecipitation-sequencing and gene expression analysis of TF mutant cortices. Our analysis demonstrated that Pax6, Tbr2, and Tbr1 form a direct feedforward genetic cascade, with direct feedback repression. Results also revealed that each TF regulates multiple EF genes that control DNA methylation, histone marks, chromatin remodeling, and non-coding RNA. For example, Tbr1 activates *Rybp* and *Auts2* to promote the formation of non-canonical Polycomb repressive complex 1 (PRC1). Also, Pax6, Tbr2, and Tbr1 collectively drive massive changes in the subunit isoform composition of BAF chromatin remodeling complexes during differentiation: for example, a novel switch from *Bcl7c* (Baf40c) to *Bcl7a* (Baf40a), the latter directly activated by Tbr2. Of 11 subunits predominantly in neuronal BAF, 7 were transcriptionally activated by Pax6, Tbr2, or Tbr1. Using EFs, Pax6→ Tbr2→ Tbr1 effect persistent changes of gene expression in cell lineages, to propagate features such as regional and laminar identity from progenitors to neurons.

Keywords: cortical development, polycomb, BAF, NuRD, histone acetylation, lncRNA, microRNA, trithorax group

INTRODUCTION

Development of the embryonic cerebral cortex is regulated by intrinsic genetic programs and signaling interactions that ultimately give rise to diverse cortical areas, layers, and neuron subtypes with distinct gene expression profiles (Sun and Hevner, 2014; Silbereis et al., 2016). In each cell type, the gene expression profile is determined by a combination of transcription factors (TFs) that bind specific DNA sequences to activate or repress transcription, and epigenetic factors (EFs) that control chromatin structure and accessibility for transcription (Bernstein et al., 2007; Allis and Jenuwein, 2016). Transcriptional activity thus depends on the epigenetic status of the chromatin, as well as the presence or absence of specific TFs that bind promoters, enhancers, and other *cis*-acting regulatory elements in the genome (Nord et al., 2015; Shibata et al., 2015).

Among many important TFs in cortical development, the Pax6→ Tbr2→ Tbr1 cascade is significant because these TFs are expressed sequentially in radial glial progenitors (RGPs), intermediate progenitors (IPs), and postmitotic projection neurons (PNs), respectively (Englund et al., 2005; Hevner et al., 2006). Furthermore, these three TFs regulate important features of cortical neurons, including rostrocaudal (area) identity, PN migration, and axon projections (reviewed by Georgala et al., 2011; Mihalas and Hevner, 2017). Significantly, all three TFs are expressed in high rostral-low caudal gradients, and parallel shifts of rostrocaudal identity are found in *Pax6*, *Tbr2* (MGI: *Eomes*), and *Tbr1* mutant mice (Bishop et al., 2000; Bedogni et al., 2010a; Elsen et al., 2013). To explain their sequential expression, we hypothesized that Pax6, Tbr2, and Tbr1 form a genetic cascade in cortical PN lineages.

Epigenetic mechanisms are prominently involved in the etiology of intellectual disability (Iwase et al., 2017). While definitions of "epigenetics" have changed over time (Deans and Maggert, 2015; Allis and Jenuwein, 2016), most current studies recognize four broad categories of epigenetic mechanisms (Hsieh and Zhao, 2016; Yao et al., 2016): (1) DNA methylation; (2) histone covalent modifications ("marks"), such as lysine acetylation and methylation; (3) ATPase-dependent chromatin remodeling, by complexes such as BAF and NuRD; and (4) effects of non-coding RNA (ncRNA), including microRNA (miR). These epigenetic mechanisms are broadly mediated by at least 800 protein-coding EF genes, and untold numbers of ncRNA species (Medvedeva et al., 2015; Silbereis et al., 2016). In the current project, we focused on EF genes that exhibit cell-type or region-specific expression; or that are dysregulated in the neocortex of *Pax6* (Holm et al., 2007), *Tbr2* (Elsen et al., 2013; Mihalas et al., 2016), *Tbr1* (Bedogni et al., 2010a), or *Tbr1* and *Tbr2* (*Tbr1/2*; present study) mutant neocortex.

Previous studies have demonstrated physical and genetic interactions between EFs and TFs during neurogenesis. In adult subependymal zone progenitors, Pax6 forms a complex with BAF, a large, multi-subunit ATPase-dependent chromatin remodeler, to activate neurogenic genes such as *Sox11* (Ninkovic et al., 2013). In developing neocortex, Tbr2 interacts with Jmjd3 (Gene: *Kdm6b*), a histone lysine demethylase that removes repressive trimethylation marks on histone H3 lysine 27 (H3K27me3) placed by Polycomb repressive complex 2 (PRC2), to thereby derepress transcription (Sessa et al., 2017). Such interactions illustrate that TFs sometimes function by physically recruiting and targeting EFs to specific genes.

Examples where TFs and EFs regulate each other at the transcriptional level are also known. In developing forebrain, Jarid1b (*Kdm5b*), a histone lysine demethylase that removes activating epigenetic marks (H3K4me2/3) placed by Trithorax-Group (TrxG) complexes, is required to deactivate and thus limit *Pax6* expression (Albert et al., 2013). Similarly, Af9 (*Mllt3*), a YEATS domain protein that binds acetylated lysine residues, negatively modulates transcription of *Tbr1* during genesis of upper cortical layers (Büttner et al., 2010).

Conversely, Pax6, Tbr2, and Tbr1 also regulate the expression of some EF genes, although in many cases it remains unclear whether such regulation is direct. For example, *Dnmt3a* (a DNA methyltransferase) is upregulated in *Pax6* null embryonic cortex (Holm et al., 2007), but it is unknown if Pax6 regulates *Dnmt3a* directly or indirectly (Ypsilanti and Rubenstein, 2016). A few EF genes are known targets of Tbr2, such as *Gadd45g*, important in DNA demethylation (Sessa et al., 2017). Tbr1 is known to activate *Auts2* (Bedogni et al., 2010a), a Polycomb repressive complex 1 (PRC1) non-canonical subunit (Gao et al., 2014); and *Arid1b*, an important BAF subunit (Notwell et al., 2016). Building on these few examples, one goal of the present study was to comprehensively identity EF genes that are directly bound and regulated by Pax6, Tbr2, and Tbr1.

In addition to studying regulation of EF genes, we also wished to characterize EF genes associated with cortical differentiation, comprising the "EF landscape." In embryonic neocortex, histological zones are correlated with cell identity and differentiation (Bystron et al., 2008), while rostrocaudal and mediolateral gradients of gene expression presage arealization (O'Leary et al., 2007). Indeed, zonal expression patterns can be used to infer specificity of gene expression in RGPs, apical IPs, basal IPs, and neurons (Kawaguchi et al., 2008). In the present study, by combining microarray analysis of RGP and IP transcriptomes (Nelson et al., 2013) with *in situ* hybridization (ISH) to define gene expression patterns, we find that dozens of EF genes exhibit cell-type or region-specific expression, and together constitute a rich EF landscape involving all categories of epigenetic mechanisms.

Our analysis depicts a new, comprehensive view of the EF landscape in developing neocortex, and its regulation by Pax6, Tbr2, and Tbr1. In addition, this approach yields an updated portrayal of the Pax6→ Tbr2→ Tbr1 cascade, including feedforward and feedback regulation. Importantly, the data indicate that Pax6 is not a specific marker of RGPs, but is also expressed in many Tbr2+ IPs, as we have noted (Englund et al., 2005). Other TFs, such as Sox9, are more specific RGP markers. Together, our results show how a cortical TF network implements cortical differentiation by controlling diverse EFs.

MATERIALS AND METHODS

Data Sources

To study gene expression and regulation in the context of cortical neurogenesis, we analyzed data from experiments using embryonic mouse cortex, in the age range from embryonic day (E) 13.5 to E15.5. For microarray and chromatin immunoprecipitation-sequencing (ChIP-seq) experiments, data were reanalyzed from previous studies, and from a new microarray dataset (**Supplementary Table S1**). For *in situ* hybridization (ISH), data were sourced from Genepaint (http://genepaint.org); the Allen Brain Atlas Developing Mouse Brain (http://developingmouse.brain-map.org/); the Brain Gene Expression Map (BGEM), hosted at Gensat (http://gensat.org); and previous literature.

Screen to Identify Cell-Type and Region-Specific Gene Expression

Previously, transcriptome profiling and unbiased cluster analysis of single cells indicated that the ventricular zone (VZ) and subventricular zone (SVZ) of E14.5 mouse neocortex contain four cell types: RGPs, apical IPs (aIPs), basal IPs (bIPs), and postmitotic projection neurons (PNs) (Kawaguchi et al., 2008). Each cell type occupies characteristic histological zones in developing neocortex: RGPs in VZ; aIPs in VZ; bIPs in SVZ; and PNs in SVZ, intermediate zone (IZ), and cortical plate (CP). Using this information, we screened the top 300 differentially expressed genes (up- and downregulated) from a previous microarray experiment comparing RGP and IP transcriptomes (Nelson et al., 2013). For the selected genes, we assessed histological expression patterns as revealed by ISH or microdissection (Ayoub et al., 2011). The primary goal was to identify RGP and IP genes, but as it happened, PN-specific genes were also enriched in *Tbr2*-GFP+ sorted cells, reflecting perdurance of GFP in daughter neurons of IPs (Nelson et al., 2013). Conversely, non-PN lineages (e.g., meninges) were highly enriched in *Tbr2*-GFP$^-$ sorted cells.

Cell-type specificity was determined using the following criteria. RGP genes were enriched in *Tbr2*-GFP$^-$ cells on microarray ($\log_2$FC < 0; $p < 0.05$), and expressed mainly in VZ; aIP genes were enriched in *Tbr2*-GFP+ cells ($\log_2$FC > 0; $p < 0.05$), and expressed mainly in VZ; bIP genes were enriched in *Tbr2*-GFP+ cells, and expressed mainly in SVZ; PN genes were enriched in *Tbr2*-GFP+ cells, and expressed in IZ/CP. Some neuronal differentiation genes were expressed by not only neurons, but also progenitor cells undergoing neuronal differentiation. Also, some neuronal genes were widely expressed in forebrain neurons, while others were restricted to cortical PNs. Thus, neuron-specific genes were further classified according to initial zone of expression (VZ earliest, CP latest), and specificity for cortical or general neurons. If different microarray probes for the same gene showed enrichment in *Tbr2*-GFP+ and *Tbr2*-GFP$^-$ cells ("conflicted" probes), the gene was not considered specific for cell type. Genes with rostrocaudal expression gradients were identified, and classified according to zone of expression, as previously described (Bedogni et al., 2010a; Elsen et al., 2013; Alfano et al., 2014). Further details of our approach, including analysis of gene expression in other cell types (such as GABAergic neurons), will be presented in a separate manuscript (in preparation).

By this approach, 52 EF genes with cell-type-specific expression in developing neocortex were ascertained (**Supplementary Table S2**), as were 11 EF genes with rostrocaudal gradients; 4 genes exhibited both cell-type and region-specific expression (**Supplementary Table S3**).

New Microarray Analyses of *Tbr1*, *Tbr2*, and *Tbr1/2* Deficient Cortex

Tbr1 knockout (KO), *Tbr2* conditional knockout (cKO), and *Tbr1/2* double KO/cKO (dKO) mouse embryos were produced as described (Bedogni et al., 2010a; Elsen et al., 2013). The *Tbr1/2* double mutants were generated by breeding to combine the necessary alleles ($Tbr1^{-/--}$;$Tbr2^{2F/2F}$;$Nes11^{Cre}$). On E14.5, embryos were harvested, and neocortex was immediately dissected and frozen as described (Elsen et al., 2013). Genotypes were determined by PCR of tail DNA. Controls were wild type (+/+) for Tbr1 and non-recombined for *Tbr2*. RNA was purified from neocortex, quality checked, and submitted for microarray analysis (Affymetrix Mouse Exon 1.0 ST). Each embryonic neocortex was an independent biological replicate. The number of samples (n) of each genotype was: 3 control, 4 *Tbr1* KO, 2 *Tbr2* cKO, and 3 *Tbr1/2* dKO. The microarray results were analyzed statistically as described (Elsen et al., 2013). In the current paper, we also analyzed previous microarray data from *Tbr1* KO (Bedogni et al., 2010a) and *Tbr2* cKO (Elsen et al., 2013) neocortices, designated microarray 1 (MA1); the new microarray data were designated microarray 2 (MA2). *Tbr1/2* dKO neocortex was analyzed only in MA2 (**Supplementary Table S1**). The new microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE115703).

Ethics Statement

This study was carried out in accordance with the recommendations of Guide for the Care and Use of Laboratory Animals, National Research Council. The protocol was approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute.

Analysis of ChIP-Seq and Other TF Binding Data

Previous ChIP-seq raw data were obtained and reanalyzed for Pax6 (Pattabiraman et al., 2014), Tbr2 (Sessa et al., 2017), and Tbr1 (Notwell et al., 2016). TF binding sites (peaks) were determined from BED files using the Bioconductor ChIPpeakAnno package (Zhu et al., 2010), as well as the TxDb.Mmusculus.UCSC.mm9.knownGene package, which is simply a re-packaging of the UCSC known gene table for the mm9 genome build (Rosenbloom et al., 2015). Peaks were annotated to the closest gene within 50 kilobases (kb) of the binding site. In the present analysis, TF binding was considered "positive" if the binding site was located anywhere in the transcribed sequence, or within 50 kb upstream or downstream.

The ChIP-seq data listed in **Supplementary Table S1** were our main sources, but TF binding was also evaluated by reference to previous literature. For Pax6, previous studies included genome-wide ChIP analyses of Pax6 binding in E12.5 neocortex (Sansom et al., 2009) and forebrain (Sun et al., 2015); as well as computational analysis and prediction of Pax6 binding sites (Coutinho et al., 2011). Results of all TF binding analyses for selected EF genes are included in **Supplementary Table S3**.

Defining Direct Target Genes Regulated by Transcription Factors

Genes were defined as direct targets of Pax6, Tbr2, or Tbr1 regulation if the gene showed both TF binding by ChIP-seq, and differential expression ($p < 0.05$) in TF mutant neocortex compared to control on microarray. For analysis of Tbr1 and Tbr2 direct target genes, differential expression ($p < 0.05$) on either MA1 or MA2 was accepted as evidence of regulation. Genes regulated synergistically by Tbr1 and Tbr2 were identified by the presence of both Tbr1 and Tbr2 binding sites, and significant differential expression ($p < 0.05$) in *Tbr1/2* dKO cortex, but not in *Tbr1* KO or *Tbr2* cKO cortex independently.

By this approach, 36 EF genes were identified as direct targets of transcriptional regulation by Pax6, Tbr2, and/or Tbr1; direct regulation was also assessed for the key TFs Pax6, Insm1, Tbr2, and Tbr1 (**Supplementary Table S4**).

RESULTS AND DISCUSSION

Cell-Type Specific Expression of *Pax6*, *Tbr2*, and *Tbr1*

Using the methods described above to evaluate cell-type-specific gene expression, we began by evaluating the expression of *Pax6*, *Tbr2*, *Tbr1*, and other selected TFs. As expected, *Tbr2* and *Tbr1* were highly enriched in the *Tbr2*-GFP+ lineage, and showed zonal expression patterns on ISH consistent with IPs (aIPs and bIPs) and PNs, respectively (**Figure 1A**). However, *Pax6* expression was not cell-type-specific: different probes for *Pax6* on the *Tbr2*-GFP microarray were enriched in different cell groups (conflicted probes), while ISH showed *Pax6* in both VZ and SVZ (**Figure 1A**). These results accord with our previous observations that Pax6 protein is expressed not only in RGPs, but also in some IPs (Englund et al., 2005). However, other TFs were identified as specific markers of RGPs, such as *Sox9* (**Figure 1A**). Immunohistochemistry and genetic lineage tracing have confirmed that *Sox9* is specifically expressed in RGPs (Kaplan et al., 2017).

Feedforward and Feedback Regulation in the Pax6→ Tbr2→ Tbr1 Cascade

Using an intersectional approach to identify genes that were both bound and regulated by each TF (details in section Materials and Methods), we first examined whether Pax6, Tbr2, and Tbr1 transcriptionally regulate each other and/or themselves.

Previous studies have found that Pax6 directly represses its own transcription (Manuel et al., 2007), and directly activates *Tbr2* expression (Sansom et al., 2009). Our analysis confirmed

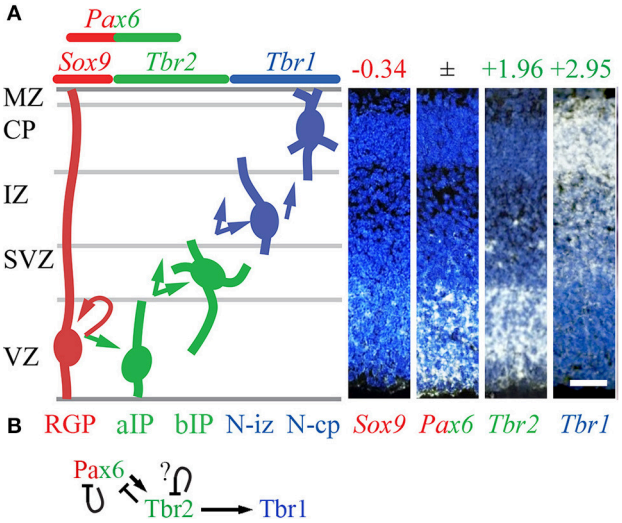

FIGURE 1 | Cell types, TF expression, and histological zones in E14.5 mouse neocortex. **(A)** Neurogenesis and cell-type-specific TF expression. Histological zones and cell types (left) are aligned with TF gene ISH (right; white ISH signal, blue nuclear counterstain). Arrows indicate common (but not exclusive) pathways of neurogenesis. Numbers above ISH panels indicate $\log_2$FC on *Tbr2*-GFP microarray (all $p < 0.05$). Abbreviations: see text. ISH: Allen Brain Atlas Developing Mouse Brain, E15.5 (colors inverted for figure). Scale bar: 50 μm. (**B**) The Pax6→ Tbr2→ Tbr1 cascade involves direct feedforward activation (arrows) and feedback repression (bars). The effect of Tbr2 binding at the *Tbr2* locus could not be determined from available data (see text), but could be feedback repression.

that both *Pax6* and *Tbr2* were bound and regulated by Pax6. In *Pax6* null (*Pax6*$^{Sey/Sey}$) neocortex, expression of *Pax6* (non-functional mRNA) was greatly increased ($\log_2$FC = +1.20; $p = 10^{-6}$), while *Tbr2* was greatly decreased ($\log_2$FC = −1.07; $p = 10^{-6}$).

Previous studies have also suggested that Tbr2 directly binds and activates *Tbr1* (Sessa et al., 2017). This was confirmed in the present analysis. Moreover, we found that Tbr2 binds and represses *Pax6*: in *Tbr2* cKO neocortex, *Pax6* was significantly upregulated ($\log_2$FC = +0.36, $p = 10^{-3}$ on MA1; $\log_2$FC = +0.49, $p = 10^{-3}$ on MA2). In contrast, *Tbr1* was downregulated in *Tbr2* cKO cortex. We also noted Tbr2 binding to its own gene (*Tbr2*), although the functional effects were uncertain: *Tbr2* mRNA expression is reduced due to *Tbr2* cKO (Elsen et al., 2013), so the effects of Tbr2 on its own transcription could not be evaluated. We speculate that, like Pax6, Tbr2 may repress its own transcription as a feedback mechanism (**Figure 1B**).

ChIP-seq analysis of Tbr1 showed that Tbr1 binds to the *Tbr2* locus, but not to *Pax6* or *Tbr1*. On microarray, however, *Tbr2* expression was not significantly changed in *Tbr1* null mice (**S3**). Thus, Tbr1 does not appear to directly regulate *Tbr2*, *Pax6*, or *Tbr1*.

Together, these data indicate that the Pax6→ Tbr2→ Tbr1 cascade operates as a positive feedforward cascade, but also self-regulates by direct negative feedback effects (**Figure 1B**).

Since Pax6, Tbr2, and Tbr1 are expressed in different cell types (differentiation stages in the same lineage)—except for

overlapping expression of Pax6 and Tbr2 in some IPs (Englund et al., 2005)—feedforward activation may involve epigenetic mechanisms. For example, Tbr2 and Tbr1 exhibit virtually no overlap of protein expression in developing neocortex, yet Tbr2 expression in IPs is essential for high levels of Tbr1 expression in postmitotic PNs (**S4**). One explanation is that Tbr2 may drive epigenetic changes at the *Tbr1* locus that persist in postmitotic neurons. For example, removal of repressive histone marks by Jmjd3, an interacting protein of Tbr2, may create a permissive chromatin environment for *Tbr1* transcription (Sessa et al., 2017).

Identification of EFs With Cellular, Regional, or TF-Regulated Expression

To identify genes with cell-type or region-specific expression in E14.5 mouse neocortex, we screened differentially expressed genes from a previous microarray experiment comparing RGP and IP transcriptomes (Nelson et al., 2013). We used ISH to characterize expression patterns in developing neocortex (**Supplementary Figure S1**; Section Materials and Methods). To identify EF genes regulated by Pax6, Tbr2, and Tbr1, we selected EF genes that were both bound by the TF per ChIP-seq, and significantly regulated in TF null neocortex per microarray. All EF genes that were evaluated are listed in **Supplementary Table S3**, which also includes results from microarrays, ISH, and ChIP-seq; annotations of cell-type and regional identity; and previous literature citations.

Of more than 350 EF genes evaluated, 52 exhibited cell-type-specific expression: 14 in RGPs, 2 in aIPs, 6 in bIPs, 9 in aIPs and bIPs, 18 in general neurons or precursors, and 3 in PNs or precursors (**Supplementary Table S2**). In addition, 11 EF genes exhibited rostrocaudal gradients: 4 high rostral, 7 high caudal (**Supplementary Table S3**). Furthermore, 36 EF genes were bound and regulated by Pax6, Tbr2, and/or Tbr1 (**Supplementary Table S4**). Of these, 9 were regulated by two TFs independently, but always in the same direction; and 2 EF genes were regulated only synergistically by Tbr2 and Tbr1. The effects of TFs on target gene expression were mixed: Pax6 activated 5 EF genes, and repressed 5; Tbr2 activated 8, and repressed 10; Tbr1 activated 13, and repressed 2; Tbr1 and Tbr2 (Tbr1/2) coordinately activated 2 EF genes. In sum, 73 EF genes showed cell-type or regional specificity, or were directly regulated by at least one of the TFs (Pax6, Tbr2, and Tbr1).

Results for each category of EFs are presented and discussed in the following sections. Neurodevelopmental implications are discussed in the final sections.

DNA Methylation and Demethylation

DNA methylation (5-methylcytosine on CpG) mediates chromatin compaction and gene silencing, and is actively regulated during neurogenesis (Moore et al., 2013; Sharma et al., 2016). DNA methylation is mediated by N-methyltransferases (*Dnmt1/3a/3b*), and can be reversed (erased) by pathways involving *Gadd45a/b/g*, *Tet*, and *Aicda* genes (Moore et al., 2013; Matsunaga et al., 2015). Dnmt1 is active on hemimethylated DNA in newly replicated cells, while Dnmt3a/3b catalyze targeted *de novo* methylation. Silencing of methylated DNA is mediated by "reader" proteins, such as methyl-binding domain proteins (*Mecp2* and *Mbd* genes), and zinc-finger proteins such as Kaiso (*Zbtb33*), Zbtb4, and Zbtb38. Dnmt activity can also be modulated by factors such as Np95 (*Uhrf1*), a histone reader that stabilizes and potentiates Dnmt1 (Murao et al., 2014).

In the present analysis, all three *Dnmt* genes (*Dnmt1/3a/3b*) were specifically enriched in RGPs (**Figure 2**). In addition, *Mbd2* and *Uhrf1* were enriched in Tbr2-GFP$^-$ cells, but they were not detected on ISH, and could not be assigned RGP identity with confidence. Downregulation of DNA methylation activity in IPs was directed in part by Tbr2, which directly repressed *Dnmt3a*. Also, *Mbd2* was directly repressed by Tbr2, consistent with the possibility that *Mbd2* is RGP-specific, and actively repressed upon IP differentiation.

Among DNA demethylation genes, *Gadd45g* was regionally enriched with a high caudal gradient in VZ/SVZ, and was directly repressed by Pax6 and Tbr2 (**Figure 2F**). *Gadd45a*, although not detected by ISH, was also directly repressed by Tbr2 (**Figure 2E**). *Tet1* was significantly enriched in *Tbr2*-GFP+ cells (although not detected on ISH), and was directly activated by Tbr1.

Mecp2, a methyl-cytosine reader linked to Rett syndrome (Qiu, 2017), was enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +0.72), but not in any specific cell type, as ISH showed high levels in multiple zones. During embryonic neurogenesis, Mecp2 is

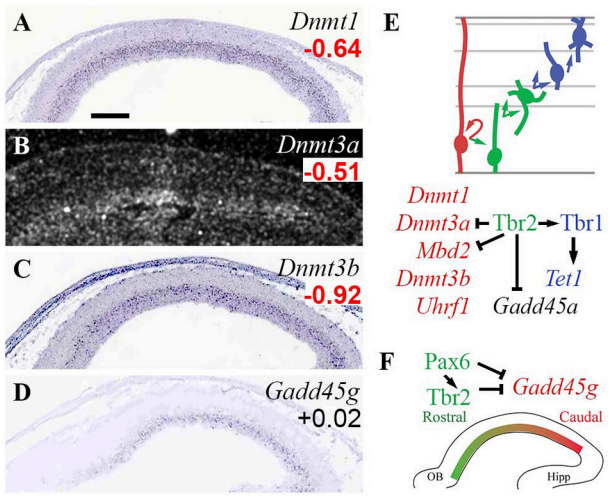

FIGURE 2 | Expression and regulation of DNA methylation/demethylation factors. **(A–D)** Expression of the indicated genes in E14.5 mouse neocortex. *Dnmt1* **(A)**, *Dnmt3a* **(B)**, and *Dnmt3b* **(C)** were expressed in VZ, and were significantly enriched in *Tbr2*-GFP$^-$ cells, defining them as RGP markers (**Supplementary Table S2**). *Gadd45g* **(D)**, part of a pathway for DNA demethylation, was expressed in a high caudal gradient in the VZ, but was not significantly enriched in RGPs or IPs on microarray. (Significant $\log_2$FC values are indicated by **bold** text, in red or green). Sagittal sections, rostral left, ventral down (see also **Supplementary Figure S1**). ISH: Genepaint **(A,C,D)** and BGEM (**B**; darkfield). Scale bar: 100 μm. **(E)** Cell-type-specific gene expression and regulation by TFs. Arrows, direct transcriptional activation; bars, direct repression. **(F)** Pax6 and Tbr2 may shape the *Gadd45g* gradient by direct repression.

necessary to limit Pax6 expression in Tbr2+ IPs, and to modulate the pace of PN maturation (Cobolli Gigli et al., 2018).

These results indicate that DNA methylation activity is mainly enriched in RGPs, and that PN differentiation is associated with reduced DNA methylation, and increased DNA demethylation. Also, the high caudal gradient of *Gadd45g* in progenitor zones implicates DNA demethylation in cortical regionalization. Pax6, Tbr2, and Tbr1 regulate this system by repressing and activating key genes, including repression of the caudal marker (*Gadd45g*) by Pax6 and Tbr2 (**Figure 2F**). Thus, DNA methylation and demethylation may regulate not only neuron differentiation (Sharma et al., 2016) and astrogenesis (Fan et al., 2005), but also cortical regionalization under the control of Pax6 and Tbr2.

Histone Marks

Histone marks are covalent modifications associated with regulation of chromatin structure and transcriptional activity (Allis and Jenuwein, 2016; Gates et al., 2017). Histone marks include acetylation, methylation, ubiquitylation, sumoylation, phosphorylation, and crotonylation. Generally, histone marks are placed by multisubunit enzyme complexes, are recognized by reader proteins, and are reversible by other enzyme complexes. Many EFs that place or remove histone marks have multiple subunit isoforms encoded by different genes, expressed in specific tissues or differentiation stages.

Histone Acetylation and Deacetylation

Histone lysine acetylation generally opens chromatin and activates transcription, while deacetylation represses transcription. Many families of histone acetyltransferases (HATs) and deacetylases (HDACs) mediate placement and reversal of the acetyl marks (Hodawadekar and Marmorstein, 2007; Bannister and Kouzarides, 2011; Sapountzi and Côté, 2011; Drazic et al., 2016). Type-A HATs, such as those in the MYST (e.g., Morf; *Kat6b*), GNAT (e.g., Gcn5; *Kat2a*), and Cbp/p300 (*Crebbp*/*Ep300*) families, regulate transcription and, in some cases, may also acetylate non-histone proteins. Some type-A HATs, such as p300, function as modular activating units that can be recruited by various EF/TF complexes, such as non-canonical PRC1-Auts2 (Gao et al., 2014). Type-B HATs (*Hat1*, *Hat2*) function in cytoplasmic nucleosome biogenesis. Likewise, some class I HDACs (*Hdac1/2*) serve as modular repressive units, in complexes such as NuRD and Rest/CoRest.

The present analysis identified several HATs and HDACs with cell-type-specific expression, and extensive regulation by Pax6, Tbr2, and Tbr1 (**Figure 3**). Among HATs, *Hat1* and *Kat7* were RGP-specific. *Hat1* encodes a type-B HAT important in cell proliferation, while *Kat7* (*Myst2*; HBO1), an H3K14 acetyltransferase, is required for general transcriptional activation, especially in progenitor cells during embryonic development (Kueh et al., 2011). *Hat1* was directly repressed by Pax6, and indeed was among the top 100 upregulated genes in *Pax6* null cortex ($\log_2$FC = +0.84; $p = 2 \times 10^{-4}$). Type-A HATs Cbp (*Crebbp*) and p300 (*Ep300*) were highly expressed in cortex, but without clear zonal specificity on ISH; nor were they directly regulated by Pax6, Tbr2, or Tbr1.

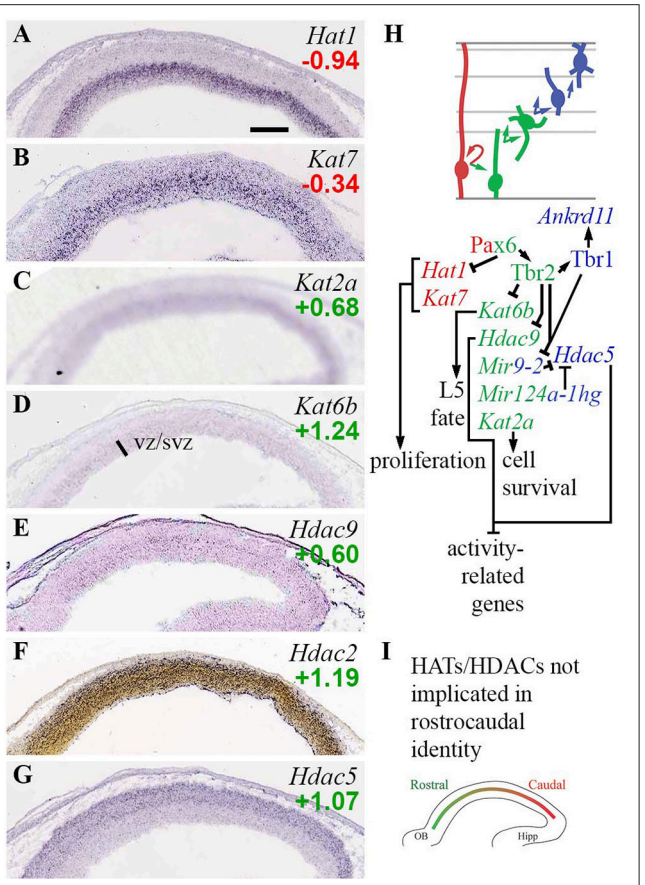

FIGURE 3 | Histone acetylases (HATs) and deacetylases (HDACs). **(A–G)** Expression of indicated genes. *Hat1* **(A)** and *Kat7* **(B)** were RGP-specific. Interestingly, *Hdac9* **(E)** and *Hdac5* **(G)** showed complementary zonal expression in VZ/SVZ and IZ/CP, respectively. ISH: Genepaint **(A–E,G)** and Allen Institute (**F**). Scale bar: 100 μm. **(H)** Cell-type-specific gene expression and regulation. Tbr2, Tbr1, and *Mir9-2* regulate a switch from *Hdac9* in progenitors, to *Hdac5* in PNs. **(I)** No HATs or HDACs exhibited regional expression gradients.

Two type-A HATs, *Kat2a* and *Kat6b*, were specifically enriched in aIPs and bIPs in the VZ/SVZ (**Figures 3C,D**). *Kat2a* (Gcn5) is required to prevent apoptosis (Wu et al., 2017). *Kat6b* (*Myst4*; querkopf, Morf), despite being an aIP and bIP marker (**Supplementary Table S2**), was directly repressed by Tbr2 (**Figure 3H**; **Supplementary Table S4**). Interestingly, *Kat6b* is essential for the differentiation of layer 5 neurons (Thomas et al., 2000), and *Tbr2* cKO cortex shows an expanded layer 5 (Mihalas et al., 2016). Also, mice lacking *Brpf1*, an activator of Morf (*Kat6b*), have thin cortex, especially layer 5, and reduced numbers of Tbr2+ IPs (You et al., 2015). Thus, layer 5 differentiation is regulated by a network that includes Tbr2, Morf (*Kat6b*), and Brpf1.

Among HDACs, *Hdac9* (Mitr; an *Hdac* family member without deacetylase activity) was specifically expressed in aIPs and bIPs (**Figure 3E**; **Supplementary Table S2**), and was potently repressed by Tbr2 (**Supplementary Table S4**). In *Tbr2* cKO mice, *Hdac9* was one of the top 100 upregulated genes

($\log_2$FC = +0.55, $p = 2 \times 10^{-4}$ on MA1; $\log_2$FC = +0.68, p = 0.008 on MA2). One function of Mitr (*Hdac9*) is to limit gene expression driven by Mef2 and physiological excitation (Méjat et al., 2005). In the context of IPs, we speculate that Mitr might negatively regulate HDAC signaling.

Another HDAC, *Hdac5*, was specifically expressed by PNs in IZ/CP. Recent studies suggest that Hdac5 limits the expression of Mef2c target genes, thus restraining neurite outgrowth (Gu et al., 2018). In turn, *Hdac5* has been identified as a target of miR-124 and miR-9 (Gu et al., 2018), elements of the ncRNA system in developing neocortex (described below). This is noteworthy because both Tbr1 and Tbr2 directly repress *Mir9-2* (host gene of miR-9), and thus indirectly potentiate *Hdac5* expression. *Hdac3* was moderately enriched in *Tbr2*-GFP+ cells, and widely expressed on ISH.

Of the class I HDACs, *Hdac2* was enriched in *Tbr2*-GFP+ cells, and was expressed predominantly by differentiating neurons in the IZ/CP of cortex (**Figure 3F**), and other forebrain regions (not shown). Thus, *Hdac2* was classified as a marker of general neuronal differentiation starting in the IZ (N-iz; **Supplementary Table S2**). In contrast, *Hdac1* showed no lineage bias on *Tbr2*-GFP microarray, and was widely expressed with highest levels in the VZ (see the section on Rest/CoRest complexes, below). In sum, *Hdac1* and *Hdac2* showed complementary enrichment in progenitors and neurons, respectively.

Among related factors in histone acetylation, *Uhrf1*, which recruits Dnmt1 and HATs to chromatin during proliferation (Murao et al., 2014), was RGP-specific, as noted (**Figure 2E**). *Ankrd11*, a scaffolding molecule that potentiates Hdac3 signaling (Gallagher et al., 2015), was significantly enriched in the neuronal lineage, and was activated by Tbr1.

Together, these results reveal an important genetic circuit in IPs that regulates layer 5 differentiation. Also, *Hdac9* and *Hdac5* seem to play similar roles limiting Mef2- and activity-driven gene expression in mature cells, but their expression and regulation in IPs and new PNs suggest they may possibly have distinct functions during neurogenesis. During the IP-PN transition, both Tbr2 and Tbr1 promote the shift from *Hdac9* to *Hdac5* expression. Tbr2 directly represses *Hdac9*, while Tbr2 and Tbr1 indirectly potentiate *Hdac5* expression, by directly repressing *MiR9-2* and thus limiting targeted degradation of *Hdac5* by miR-9 (**Figure 3H**). These findings support our view that Tbr2 drives the transition from IP to PN, while Tbr1 drives PN differentiation (Mihalas et al., 2016; Mihalas and Hevner, 2017).

Trithorax/COMPASS Activating Complexes

Another important category of histone marks consists of lysine methylation (mono-, di-, and trimethylation) and demethylation. The best-known epigenetic systems using these marks are Trithorax/COMPASS complexes, which place H3K4 trimethyl (H3K4me3) and other marks at active promoters; and PRC2, which places repressive H3K27me3 marks that silence chromatin. The PRC2 system is furthermore connected to PRC1, which places another silencing histone mark—monoubiquitylation of H2A on K119 (H2AK119u1)—and functions synergistically with PRC2. In *Drosophila*, TrxG and Polycomb group (PcG) systems are considered antagonistic; genes marked with both H3K4me3 (activating) and H3K27me3 (repressive) are considered to be in a "bivalent" state, poised for long-term repression or activation. In mammals, the Trithorax and Polycomb systems have become more complex and diverse, with many tissue-specific isoforms and non-canonical subunits. While TrxG genes (as defined by PcG antagonism) also encompass other classes of molecules, such as chromatin remodelers (Schuettengruber et al., 2011; Moccia and Martin, 2018), those other molecules are classified separately for purposes of this article.

Mammalian TrxG H3K4 methyltransferases form complexes known as COMPASS and COMPASS-like, which include core WRAD proteins (*Wdr5, Rbbp5, Ash2l, Dpy30*) and other subunits (Schuettengruber et al., 2011; Piunti and Shilatifard, 2016). Other TrxG proteins are not H3K4 methyltransferases, but have related functions such as H3K36 methylation (Ash1l), chromatin remodeling, modulation of HATs, and general transcriptional regulation (Schuettengruber et al., 2011). Activating marks placed by TrxG complexes can be reversed by demethylation, for example, by Jarid1b (*Kdm5b*) and Lsd1 (*Kdm1a*)—both markers of neuronal differentiation beginning in progenitor zones (**Supplementary Table S2**).

In the present analysis, both H3K4 methylase and demethylase genes were expressed predominantly in *Tbr2*-GFP+ cells; none were specifically enriched in RGPs (**Figure 4**). Among H3K4 methyltransferases, *Setd1b* was enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +0.88), and was expressed at highest levels in CP (**Figure 4A**). *Kmt2a* (Mll1) was also enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.36), but was not detected on ISH. Likewise, *Kmt2c* (Mll3) was enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.22), but not detected on ISH. Notably, *Kmt2c* was directly activated by Tbr1 (**Supplementary Table S4**), suggesting that *Kmt2c* (Mll3) is important for PN differentiation. Indeed, mutations in human *KMT2C* have been linked to intellectual disability (Koemans et al., 2017). Interestingly, Mll3 (*Kmt2c*) forms COMPASS-like complexes with Utx (*Kdm6a*), a demethylase that removes repressive H3K27me3 marks placed by PRC2 (Schuettengruber et al., 2011). By directly activating *Kmt2c* (Mll3) expression, Tbr1 may orchestrate not only the placement of activating H3K4me3 marks by Mll3, but also removal of repressive H3K27me3 marks by Utx.

Among H3K4 demethylases, *Kdm1a* (Lsd1) was enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.14), and was directly activated by Tbr2 (**Supplementary Tables S2**, **S4**). Functionally, previous studies have found that Lsd1 interacts with CoRest (*Rcor1/2*), a repressor scaffold protein enriched in aIPs and bIPs (see section Rest and CoRest Complexes, below), to promote a shift from multipolar to bipolar migration (Fuentes et al., 2012). By activating *Kdm1a* (Lsd1) expression, Tbr2 may drive this change of migration mode. *Kdm1b* (Lsd2; an H3K4 demethylase) was similarly enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.03), but its expression was not zone-specific on ISH (**Figure 4C**). Another H3K4 demethylase, *Kdm5b* (Jarid1b), was enriched in neuronal lineages, and was directly activated by Tbr1 (**Figures 4D,F**). Thus, Tbr1 drives both deposition and removal of H3K4me3 marks, by activating *Kmt2c* (Mll3) and *Kdm5b*

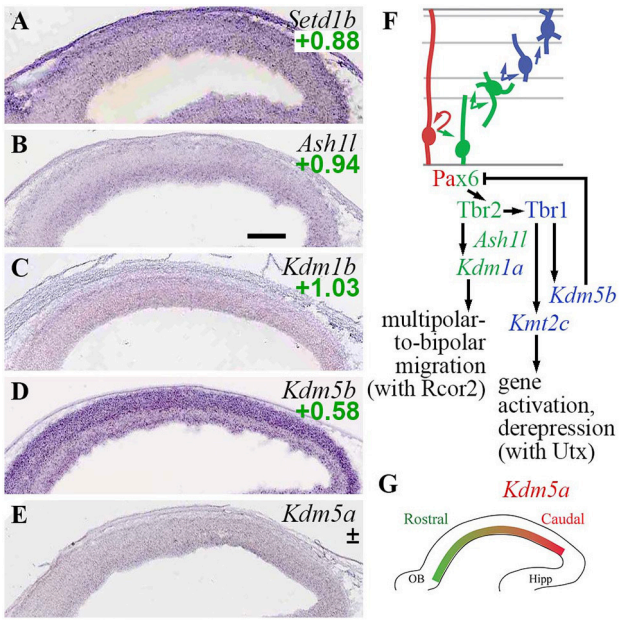

FIGURE 4 | TrxG histone methylation/demethylation EFs. **(A–E)** Expression of indicated genes. The plus/minus symbol (±) indicates that different probes for the same gene, in this example *Kdm5a* **(E)**, showed enrichment in both *Tbr2*-GFP+ and *Tbr2*-GFP– cells on microarray (conflicted). ISH: Genepaint. Scale bar: 100 μm. **(F)** Summary of gene expression and regulation. Interestingly, Tbr1 activated both H3K4 methyltransferase (*Kmt2c*; Mll3) and H3K4 demethylase (*Kdm5b*; Jarid1b) genes. **(G)** Expression of *Kdm5a* (high caudal) was not directly regulated by Pax6, Tbr2, or Tbr1.

(Jarid1b) respectively, to reconfigure the landscape of active promoters in differentiating PNs.

Functionally, Jarid1b (*Kdm5b*) is necessary to remove inappropriate H3K4me3 marks during development, and thereby deactivate neural progenitor genes such as *Pax6* (Albert et al., 2013). Thus, Tbr1-mediated activation of *Kdm5b* may help block inappropriate *Pax6* expression in neurons (**Figure 4F**). Indeed, *Pax6* was upregulated in *Tbr1* KO cortex, but not quite significantly (*Pax6* $\log_2$FC = +1.05, $p = 0.18$ on *Tbr1* KO MA1; $\log_2$FC = +0.20, $p = 0.054$ on *Tbr1* KO MA2).

Kdm5a (Jarid1a), another H3K4me3 demethylase, was expressed in a regional gradient (high caudal) in the VZ/SVZ (**Figure 4E**). On microarray, different *Kdm5a* probes were enriched in *Tbr2*-GFP+ and GFP$^-$ cells (conflicted), so expression of *Kdm5a* could not be specifically assigned to RGPs or IPs.

Ash1l, an H3K36 methylase that may activate or repress transcription in different contexts (Schuettengruber et al., 2011; Zhu et al., 2016), was highly enriched in aIPs and bIPs (**Figure 4B**; **Supplementary Table S2**), but was not regulated by Pax6, Tbr2, or Tbr1.

These results indicate that deposition and removal of TrxG marks are actively regulated by Tbr2 and Tbr1 during neuronal differentiation (**Figure 4F**). Also, cortical regionalization may be influenced by Jarid1a (*Kdm5a*), without direct regulation by Pax6, Tbr2, or Tbr1 (**Figure 4G**).

Polycomb Repressive Complex 2

PcG proteins include components of two distinct complexes, PRC1 and PRC2, which deposit different repressive marks on chromatin (Schuettengruber et al., 2007; Simon and Kingston, 2009; Di Croce and Helin, 2013; Schwartz and Pirrotta, 2013). The marks placed by PRC2 can recruit PRC1, although non-canonical forms of PRC1 also function independently of PRC2 or H3K27me3 (Tavares et al., 2012).

In mammals, a variety of PRC2 complexes with different subunit or isoform composition have been identified (Margueron and Reinberg, 2011). Core PRC2 components include Ezh1 or Ezh2 (methyltransferases), Eed, and Suz12. Canonical PRC2 complexes also contain Rbbp4 or Rbbp7 scaffold proteins. Non-canonical subunits (not found in all PRC2 complexes) can include PCL1-3 proteins (*Phf1*, *Mtf2*, *Phf19*, respectively), and Aebp2 or Jarid2. PRC2 also interacts with or is regulated by other EFs, such as Chd4 (Sparmann et al., 2013) and Chd5 (Egan et al., 2013). The repressive H3K27me3 marks placed by PRC2 can be erased by demethylases Utx (*Kdm6a*) and Jmjd3 (*Kdm6b*).

Previously, the PRC2 system has been shown to regulate the timing of neurogenesis in developing neocortex. RGPs lacking Ezh2 undergo accelerated differentiation to produce IPs and neurons, followed by precocious gliogenesis (Pereira et al., 2010). Moreover, *Tbr2* and other key IP-genic or neurogenic genes are marked by high levels of H3K27me3 in RGPs, but these repressive PRC2 marks are removed during IP or neuron differentiation (Albert et al., 2017). PRC2 also regulates rostrocaudal patterning of cortex, as *Suz12* heterozygous null mice have reduced occipital cortex (Miró et al., 2009).

In the present study (**Figure 5**), analysis of core PRC2 subunits showed that *Ezh2* was widely expressed in developing neocortex, with slight enrichment in *Tbr2*-GFP+ cells; while *Ezh1* was not detectable. In contrast to the widespread expression of *Ezh2*, the other core PRC2 subunits *Suz12* and *Eed* were expressed almost exclusively in VZ/SVZ, although neither was specifically enriched in *Tbr2*-GFP+ or GFP– cells. Moreover, both *Suz12* (Miró et al., 2009) and *Eed* (**Figure 5G**) exhibited high caudal to low rostral gradients within VZ/SVZ.

The gradient of *Suz12* expression has previously been linked to cortical regionalization. In *Suz12* heterozygous null mice, occipital cortex was greatly reduced, suggesting that high PRC2 activity instructs occipital identity (Miró et al., 2009). With parallel gradients of core *Suz12* and *Eed* subunit genes, overall PRC2 activity may be steeply graded within the VZ/SVZ. Also, the low levels of *Suz12* and *Eed* expression outside progenitor compartments suggest that PRC2 activity may be essentially limited to the VZ and SVZ.

Other canonical and non-canonical subunits of PRC2 also displayed cell-type-specific or regional expression patterns. *Rbbp7* was specifically expressed in RGPs (**Figure 5B**), while *Rbbp4* was enriched in *Tbr2*-GFP+ cells. *Aebp2*, encoding a protein that enhances PRC2 activity on PRC1-marked chromatin, was also specifically expressed in RGPs (**Figure 5A**). In contrast, *Jarid2* (jumonji), a non-canonical PRC2 subunit that may inhibit PRC2 activity (Shen et al., 2009), was specifically enriched in bIPs (**Figure 5C**), and was directly activated by Tbr2 (**Supplementary Table S4**). *Mtf2* (PCL2) was highly enriched in

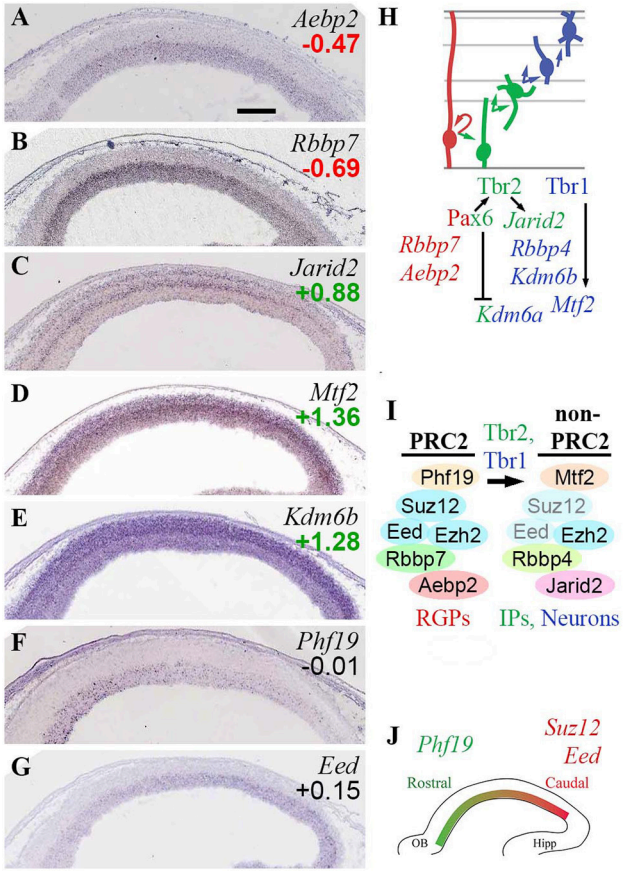

FIGURE 5 | PRC2 complexes. **(A–G)** Expression of indicated genes. ISH: Genepaint. Scale bar: 100 μm. **(H)** Summary of subunit gene expression and regulation. **(I)** Changes in PRC2 subunit expression were associated with PN differentiation, and were regulated by Tbr1/2. Eed and Suz12 subunits were downregulated in differentiating cells (transparent subunits), potentially leading to formation of "non-PRC2" Ezh2 complexes. **(J)** Graded expression of PRC2 subunits is important in cortex regionalization, but these genes are not under direct control of Pax6, Tbr2, or Tbr1.

the neuronal lineage (**Figure 5D**), and was directly activated by Tbr1. *Phf19* (PCL3), which targets PRC2 to H3K36me3-marked chromatin, was expressed in a high rostral gradient in VZ/SVZ (counter to *Suz12* and *Eed*). The *Phf19* (PCL3) countergradient suggests that not only the abundance of PRC2 complexes, but also the formation of non-canonical PRC2 complexes, are regionally modulated within VZ/SVZ.

H3K27me3 demethylases Utx (*Kdm6a*; $\log_2$FC = +0.93) and Jmjd3 (*Kdm6b*; $\log_2$FC = +1.28) were both enriched in the *Tbr2*-GFP+ PN lineage, but ISH was not available for *Kdm6a*, and *Kdm6b* did not exhibit strict zonal expression (**Figure 5E**). Importantly, Jmjd3 (*Kdm6b*) interacts with Tbr2 in IPs to derepress neuronal differentiation genes, such as *Tbr1* (Sessa et al., 2017). *Kdm6a* (Utx) was directly repressed by Pax6.

These results suggest that PRC2 complexes undergo extensive subunit switching during differentiation, with overall reduction or loss of canonical PRC2 activity in IPs and neurons (**Figures 5H,I**). In RGPs, PRC2 likely contains Rbbp7, Aebp2, and PCL3 (*Phf19*) in addition to core subunits. Outside the proliferative zones, *Suz12* and *Eed* are expressed very little, and PCL2 (*Mtf2*) is upregulated in neurons by Tbr1, leaving Ezh2 to potentially form non-PRC2 complexes (Schwartz and Pirrotta, 2013). In IPs, PRC2 activity may be actively suppressed by Tbr2-driven expression of *Jarid2*, an inhibitory subunit (Shen et al., 2009).

Previously, Jarid2 has been associated with Aebp2-containing PRC2 complexes (Schwartz and Pirrotta, 2013; Grijzenhout et al., 2016), but in E14.5 neocortex, *Aebp2* and *Jarid2* showed virtually non-overlapping expression in RGPs and IPs, respectively (**Figures 5A,C**). Without Aebp2, Jarid2 can nevertheless form alternative PRC2 complexes (Grijzenhout et al., 2016).

Overall, differentiation of IPs and neurons was associated with upregulation of *Kdm6a* (Utx) and *Kdm6b* (Jmjd3), which "unlock" chromatin by remove the H3K27me3 marks placed by PRC2. For regionalization, high canonical PRC2 activity is necessary in caudal VZ/SVZ for occipital cortex identity (Miró et al., 2009), but non-canonical PRC2 is also implicated in regionalization, by the high rostral gradient of *Phf19* (PCL3). Despite the important role of PRC2 in regionalization, the subunits with graded expression are not directly regulated by Pax6, Tbr2, or Tbr1 (**Figure 5J**).

Polycomb Repressive Complex 1

PRC1 catalyzes monoubiquitylation of H2A lysine 119 (H2AK119u1), and drives chromatin compaction (Schuettengruber et al., 2007; Simon and Kingston, 2009; Di Croce and Helin, 2013; Schwartz and Pirrotta, 2013). Core subunits of canonical PRC1 include: Ring1a (*Ring1*) or Ring1b (*Rnf2*) E3 ligase; PcG ring finger (Pcgf) 2 or Pcgf4 (*Bmi1*); chromobox1-8 (Cbx1-8); Hph1-3 (*Phc1-3*); and substoichiometric amounts of Scm (*Scmh1/2*) (Margueron and Reinberg, 2011; Gao et al., 2012; Tavares et al., 2012; Di Croce and Helin, 2013; Schwartz and Pirrotta, 2013). The multiple isoforms of each subunit produce diverse canonical PRC1 complexes.

Non-canonical PRC1 complexes contain Rybp or Yaf2 instead of Cbx, and may contain canonical (Pcgf2/4) or non-canonical (Pcgf1/3/5/6) Pcgf proteins (Gao et al., 2012; Gil and O'Loghlen, 2014; Almeida et al., 2017). In developing cortex, a non-canonical PRC1-Auts2 complex has been described (Gao et al., 2014). Composed of Auts2, Ring1b, Pcgf3/5, Rybp, and casein kinase 2 (CK2), PRC1-Auts2 recruits p300 (*Ep300*), a type-A HAT, to activate (not repress, as usual for PRC1) transcription.

In developing neocortex, PRC1 is thought to regulate the tempo of differentiation, and the balance of neuron subtypes. In Ring1b (*Rnf2*)-deficient RGPs, neurogenesis is prolonged (Hirabayashi et al., 2009), and Ctip2+ layer 5 neurons are increased at the expense of upper layer neurons due to impaired repression of *Fezf2* (Morimoto-Suzki et al., 2014). Non-canonical PRC1-Auts2 complexes are implicated in mouse behavioral development (Gao et al., 2014). In humans, *AUTS2* is an important intellectual disability and autism gene (Beunders et al., 2016).

In the present analysis, *Rnf2* (Ring1b) appeared to be the predominant E3 ligase in developing neocortex. *Rnf2* was enriched in *Tbr2*-GFP+ cells, and was seen in all zones by

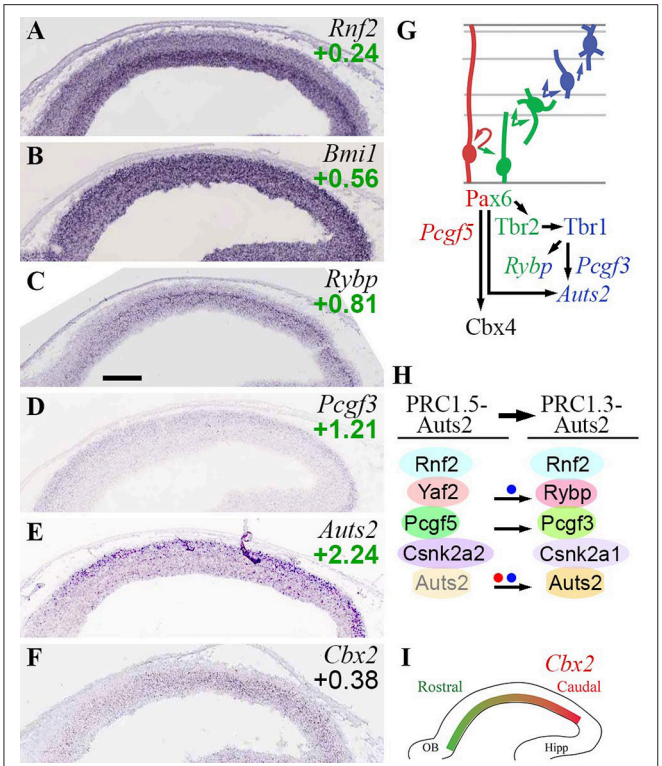

FIGURE 6 | PRC1 complexes. **(A–F)** Expression of indicated genes. ISH: Genepaint. Scale bar: 100 μm. **(G)** Summary of gene expression and regulation. **(H)** Changes in PRC1 subunit expression, and formation of non-canonical PRC1, were associated with PN differentiation, and were regulated by Pax6 (red dot) and Tbr1 (blue dots). *Auts2* was expressed at low levels in VZ/SVZ (transparent Auts2 subunit). **(I)** Graded expression of *Cbx2* (high caudal) was not regulated by Pax6, Tbr2, or Tbr1.

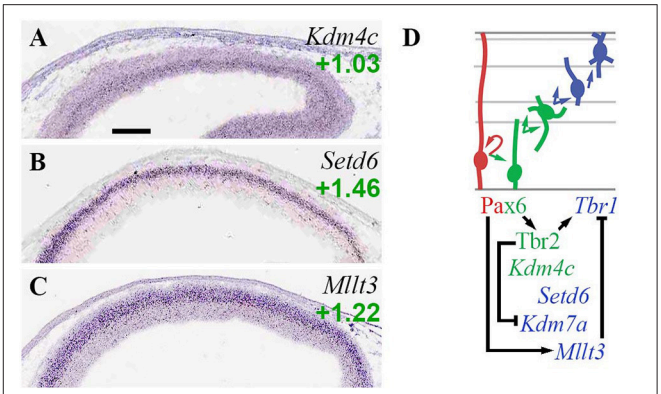

FIGURE 7 | Other histone marks and EFs. **(A–C)** Expression of indicated genes. Interestingly, *Set6d* **(B)** was specifically and exclusively expressed by PNs in developing forebrain. ISH: Genepaint. Scale bar: 100 μm. **(D)** Gene expression and regulation. Notably, Pax6 activated *Mllt3* to indirectly repress *Tbr1*.

ISH, though highest in the VZ (**Figure 6A**). In contrast, *Ring1* (Ring1a) was barely detectable on microarrays and ISH.

Canonical PRC1 subunits were, for the most part, widely expressed and little regulated by Pax6, Tbr2, or Tbr1. *Bmi1* (Pcgf4; **Figure 6B**) and *Pcgf2* were both detected in all zones of neocortex, but highest in VZ. Also, *Bmi1* (Pcgf4) was moderately enriched in *Tbr2*-GFP+ cells, and more highly expressed than *Pcgf2*. Multiple *Cbx* genes were expressed in developing neocortex, but none exhibited cell-type specificity. However, *Cbx4* was directly activated by Pax6. Since Cbx4 promotes sumoylation of Dnmt3a (Li et al., 2007), the upregulation of *Cbx4* by Pax6 may suppress *de novo* DNA methylation during IP genesis. *Cbx2* was expressed in a high caudal gradient in VZ/SVZ (**Figures 6F,I**). *Phc1-3* were enriched in *Tbr2*-GFP+ cells, but none showed cell-type specificity by ISH. Overall, these findings are consistent with previous studies of PRC1 gene expression in embryonic mouse cortex (Vogel et al., 2006).

Several non-canonical PRC1 subunits exhibited cell-type-specific expression. *Pcgf5* was specifically enriched in RGPs (**Supplementary Table S2**). In contrast, *Pcgf3* was expressed mainly in the *Tbr2*-GFP+ lineage, especially new neurons (**Figure 6D**). Similarly, the CK2 alpha isoform switched from alpha-2 (*Csnk2a2*) in progenitors, to alpha-1 in neurons (*Csnk2a1*). *Rybp* was highly enriched in aIPs and bIPs ($\log_2$FC = +0.81), and was expressed at lower levels in neurons (**Figure 6C**). *Rybp* was also identified as an IP-specific gene in a previous study (Telley et al., 2016). Significantly, *Rybp* was directly activated by Tbr1 (**Supplementary Table S4**). *Auts2* was enriched in CP neurons (**Figure 6E**), but was also expressed at lower levels in VZ/SVZ progenitors (Bedogni et al., 2010b). *Auts2* was directly activated by Tbr1 and Pax6 (**Supplementary Table S4**; see also Bedogni et al., 2010a).

These data suggest that canonical PRC1 complexes are present in all types of cortical cells (although most abundant in progenitors), and are minimally regulated by Pax6→ Tbr2→ Tbr1. In contrast, non-canonical PRC1 complexes exhibit differentiation-related changes, such as upregulation of *Rybp* in IPs and new PNs. Notably, Tbr1 directly activated two non-canonical PRC1 subunits (*Rybp*, *Auts2*) implicated in brain development (Gao et al., 2014).

Other Histone Marks and Factors

Kdm4c (Jmjd2c), which encodes an enhancer-associated H3K9 demethylase and scaffold that primes cells for differentiation (Tomaz et al., 2017), was specifically enriched in aIPs and bIPs (**Figure 7A**; **Supplementary Table S2**). *Setd6*, an H2AZK7 methyltransferase that confers repressive histone marks, was specifically enriched in migrating PNs in IZ/CP (**Figure 7B**; see also **Supplementary Figure S1F**). *Kdm7a* (ISH not available) was enriched in *Tbr2*-GFP+ lineages ($\log_2$FC = +0.55), but was repressed by Tbr2 (**Figure 7D**; **Supplementary Table S4**).

Mllt3 (Af9), a histone H3K9ac reader, was enriched in neurons of the IZ and CP (**Figure 7C**). Previously, Af9 has been reported to inhibit deep layer identity by repressing *Tbr1* transcription (Büttner et al., 2010). In the present study, we found that Pax6 directly activated *Mllt3* (**Supplementary Table S4**). Since previous studies have also found that Pax6 drives upper layer identity (Schuurmans et al., 2004), it seems plausible that Pax6 indirectly represses *Tbr1* by activating high expression of *Mllt3* in precursors of upper layer neurons. Thus, Pax6 indirectly

activates *Tbr1* via *Tbr2*, and indirectly represses *Tbr1* via *Mllt3* (**Figure 7D**).

ATP-Dependent Chromatin Remodeling Complexes

Chromatin remodeling complexes use ATP to modify the positioning, conformation, and isoform composition of histones in nucleosomes—and thereby alter the availability of genes for TF binding (reviewed by López and Wood, 2015; Hota and Bruneau, 2016). These types of complexes contain an Snf2-domain ATPase, along with other proteins that modulate the ATPase activity and confer chromatin target specificity.

In mammals, four main types of chromatin remodeling complexes have been identified: BAF (Brm/Brg1-associated factor), ISWI (Imitation Switch), CHD (chromodomain helicase DNA-binding), and INO80 (inositol auxotrophy 80). The complexes are defined by their ATPase subunits: Brm (*Smarca2*) or Brg1 (*Smarca4*) in BAF (Son and Crabtree, 2014; Sokpor et al., 2017); Snf2h (*Smarca5*) or Snf2l (*Smarca1*) in ACF/CHRAC and NuRF types of ISWI complexes, respectively (Bao and Shen, 2007; Yadon and Tsukiyama, 2011); Chd1-9 alone or in CHD complexes, such as Chd3/4/5 in NuRD (Sims and Wade, 2011; Basta and Rauchman, 2015); and Ino80, Srcap, or p400 (*Ep400*) in INO80 complexes (Gerhold and Gasser, 2014; Hota and Bruneau, 2016).

Most chromatin remodeling complexes contain multiple subunits: up to 16 in BAF, 4 in ISWI, 7 in CHD (NuRD), and 15 in INO80 complexes (Hota and Bruneau, 2016). Some subunit isoforms exhibit tissue-specific or differentiation-related expression. For example, BAF complex subunits are extensively switched in cortical differentiation (Son and Crabtree, 2014).

Besides these large complexes, other ATP-dependent chromatin remodelers, such as Atrx (a Snf2-type ATPase and histone reader protein that places H3.3 in heterochromatin) are also implicated in epigenetic regulation of neurodevelopment (Iwase et al., 2017).

ISWI Chromatin Remodeling Complexes

At least eight ISWI complexes have been described in mammals (Goodwin and Picketts, 2017). Furthermore, the ATPase core subunits of ISWI complexes (Snf2h/l) have been shown to be important in brain development. *Smarca1* (Snf2l) mutant mice exhibit excessive, prolonged proliferation of cortical progenitors, especially IPs (Yip et al., 2012); while *Smarca5* (Snf2h) mutant mice exhibit reduced proliferation, at least in cerebellum (Alvarez-Saavedra et al., 2014).

In the present analysis, of the ATPase subunits, *Smarca5* (Snf2h) was expressed in all zones of developing neocortex, with highest levels in VZ/SVZ (**Figure 8A**), and was overall enriched in neuronal lineages ($\log_2$FC = +0.46). *Smarca1* (Snf2l) was expressed in multiple zones, and did not show differential expression on *Tbr2*-GFP microarray. Thus, both ISWI ATPases were widely expressed in developing neocortex, although *Smarca5* (Snf2h) was somewhat higher in progenitors. This interpretation matches a previous description (Lazzaro and Picketts, 2001).

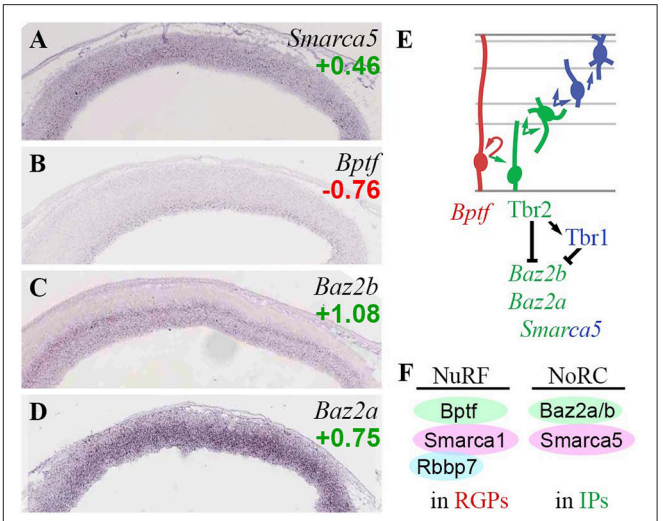

FIGURE 8 | ISWI chromatin remodeling complexes. **(A–D)** Expression of indicated genes. The bilaminar expression of *Baz2b* (**C**) in VZ and SVZ is typical of aIP- and bIP-specific genes (Kawaguchi et al., 2008). ISH: Genepaint. Scale bar: 100 μm. **(E)** Gene expression and regulation. Although *Baz2b* **(C)** is an IP marker, it was directly repressed by Tbr2 and Tbr1 (**Supplementary Tables S2**, **S4**). **(F)** NuRF complexes are enriched in RGPs, and NoRC complexes in IPs.

Bptf, an essential core subunit of NuRF (nucleosome-remodeling factor) complexes, was specifically enriched in RGPs (**Figure 8B**). In addition to Bptf, NuRF contains not only Snf2l (*Smarca1*), but also either RbAP48 (*Rbbp4*) or RbAP46 (*Rbbp7*) (Qiu et al., 2015). Like *Bptf*, *Rbbp7* was specifically expressed in RGPs (**Figure 5B**). In contrast, *Rbbp4* was highly enriched in *Tbr2*-GFP+ lineages ($\log_2$FC = +1.58). These data suggest that NuRF complexes are restricted to RGPs, and are comprised of Bptf/Snf2l/RbAP46 (**Figure 8F**). Bptf also interacts with Myc to promote cell cycle progression (Richart et al., 2016).

Baz2b, a reader that binds H3K14ac as part of an unknown ISWI complex (Bortoluzzi et al., 2017), was specifically expressed in aIPs and bIPs, and was directly repressed by Tbr2 and Tbr1 (**Figures 8C,E**). *Baz2a* (Tip5), a component of NoRC (nucleolar remodeling complex) in the Snf2h-containing ACF/CHRAC group of ISWI remodelers, was also highly enriched in IPs (**Figure 8D**). Similarly, *Baz1b* (Wstf) was expressed at high levels in VZ, and was moderately enriched in the *Tbr2*-GFP+ lineage ($\log_2$FC = +0.85); thus, WICH complexes (Wstf/Snf2h) may be enriched in progenitors, especially IPs.

Overall, the present analysis suggests that NuRF complexes are specifically present in RGPs, while NoRC complexes are particularly abundant in IPs (**Figure 8F**). The direct repression of *Baz2b* by Tbr2 and Tbr1 suggests that downregulation of some ISWI complexes (possibly a Baz2b-containing NoRC variant) is important for differentiation from IPs to PNs.

INO80 Chromatin Remodeling Complexes

Among ATPase subunit genes, *Ino80* was detected primarily in VZ, but was not enriched in *Tbr2*-GFP$^-$ or GFP+ lineages (**Supplementary Table S3**). *Ino80b* (Ies2), which activates the

ATPase activity of Ino80, was specifically expressed in RGPs ($log_2FC = -0.45$), suggesting that Ino80-containing complexes are enriched and activated in RGPs. The INO80 remodelers are important in DNA replication and repair, as well as transcriptional regulation (Poli et al., 2017), so the enrichment of Ino80 activity in RGPs may be related to high proliferative activity in this cell type.

Srcap and *Ep400* (p400) were detected in multiple zones, and were moderately enriched in *Tbr2*-GFP+ cells ($log_2FC = +0.28$ for *Srcap*; +0.76 for *Ep400*). Most Srcap complex subunits were widely expressed, while several p400 complex subunits, such as *Kat5* (Tip60), were relatively enriched in neurons. Pax6, Tbr2, and Tbr1 were not implicated in the regulation of INO80 complex subunits.

Together, these findings suggest that Ino80-containing complexes are specifically active in RGPs, while p400/Tip60 complexes are most active in postmigratory CP neurons. The functions of INO80 complexes in cortical development are unknown.

CHD Chromatin Remodeling Complexes

Among *Chd* ATPase genes, only *Chd7* exhibited cell-type or region-specific expression—indeed, both. *Chd7* was enriched in *Tbr2*-GFP+ cells ($log_2FC = +0.80$) on microarray, and was expressed specifically in VZ on ISH, identifying *Chd7* as a specific marker of aIPs. Within VZ, *Chd7* exhibited high caudal expression (**Figure 9A**), suggesting its involvement in regionalization. Consistent with this possibility, we also found that *Chd7* was directly bound and repressed by Pax6 and Tbr2 (**Figure 9I**; **Supplementary Table S4**), both of which promote rostral identity. Previous studies suggest that *Chd7* binds mainly to enhancers and active transcription start sites, and is essential for activation of neuronal differentiation genes (Moccia and Martin, 2018). Mutations in human *CHD7* cause CHARGE syndrome, a complex disorder with significant brain and somatic anomalies (Feng et al., 2017; Moccia and Martin, 2018).

Other *Chd* genes regulated by TFs included *Chd1*, repressed by Pax6; and *Chd3*, jointly activated by Tbr1 and Tbr2. *Chd1* was not specifically enriched in *Tbr2*-GFP+ or GFP– lineages, nor was ISH available, so the topography of *Chd1* expression is unknown. Chd1 protein recognizes H3K4me3 marks (active promoters) and globally activates transcription (Guzman-Ayala et al., 2015). Also, Chd1 interacts with FACT complex (Ssrp1 and Supt16) at centromeres to facilitate histone exchange (Okada et al., 2009). Of the FACT subunits, *Ssrp1* was RGP-specific (**Figure 9B**), while *Supt16* was widely expressed. These data suggest that FACT-Chd1 complexes may be abundant in RGPs, but downregulated in IPs, in part by Pax6 repression of *Chd1* (**Figures 9G,H**).

Chd3 (**Figure 9C**), directly activated by Tbr1 and Tbr2, encodes a core component of NuRD (nucleosome remodeling deacetylase) complexes. Other core Chd subunits in NuRD include *Chd4* (**Figure 9D**) or *Chd5* (mutually exclusive alternatives). NuRD complexes have at least six subunits, each of which has multiple alternatives or isoforms: Chd3-5, Hdac1/2; Mbd2/3; Mta1-3; Gatad2a/b; and Rbbp4/7 (Basta and Rauchman, 2015). Recent studies suggest that NuRD complexes are comprised of different Chd proteins during different stages of differentiation (Nitarska et al., 2016). In RGPs, NuRD was found to contain Chd4, Mta2, and Hdac2; in neurons, Chd4 was replaced by Chd3 and Chd5 (Nitarska et al., 2016). Also, NuRD was recently found to interact with Lhx2 to repress layer 5 genes (Muralidharan et al., 2017). Functionally, loss of NuRD components *Mbd3* (Knock et al., 2015) or *Chd4* (Nitarska et al., 2016) cause similar defects of RGP proliferation, leading to reduced IP genesis and thinner cortex. Such phenotypes are consistent with the general function of NuRD complexes in cell cycle progression (Basta and Rauchman, 2015), but much remains to be learned about the control of PN differentiation by NuRD.

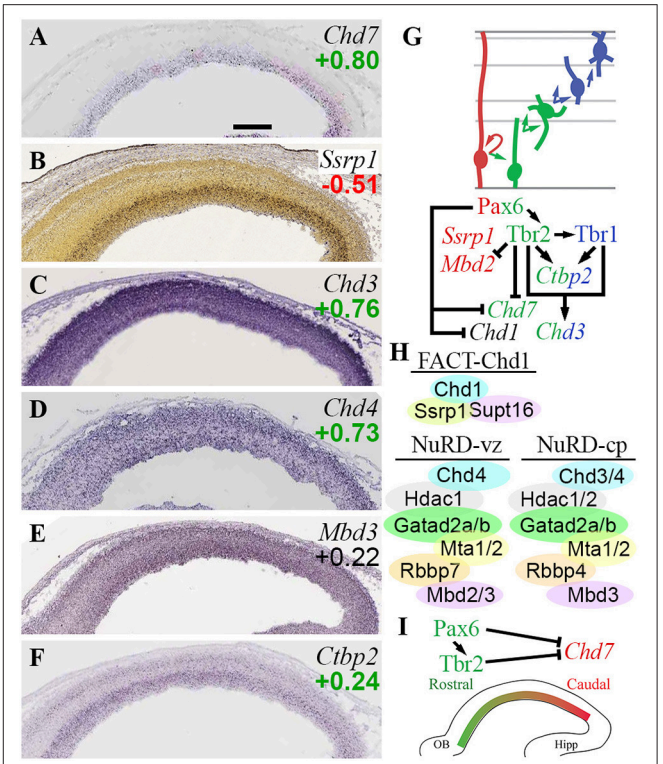

FIGURE 9 | CHD chromatin remodeling complexes. **(A–F)** Expression of indicated genes. ISH: Genepaint **(A,C–F)** and Allen Brain Atlas Developing Mouse Brain **(B)**. Scale bar: 100 μm. **(G)** Gene expression and regulation. Notably, Tbr1 and Tbr2 synergistically activate *Chd3*, a NuRD subunit. **(H)** CHD complexes in E14.5 neocortex include FACT-Chd1 in RGPs, and NuRD in progenitors (Chd4-containing) and neurons (Chd3/4-containing). **(I)** Gradient of *Chd7* expression (high caudal) is shaped by Pax6 and Tbr2.

Direct activation of *Chd3* by Tbr2 and Tbr1 supports the conclusion that *Chd3* expression increases with neuronal differentiation. In the present analysis, *Chd4* was not, however, specifically enriched in RGPs as previously suggested (Nitarska et al., 2016). Rather, *Chd4* exhibited widespread expression in cortical zones, and *Chd4* was (like *Chd3*) enriched in *Tbr2*-GFP+ cells on microarray (**Figures 9C,D**), while *Chd5* was essentially undetectable. These data suggest that in RGPs, NuRD complexes contain mainly Chd4, while in neurons, NuRD complexes contain both Chd3 and Chd4 (**Figure 9H**).

Most other NuRD subunits did not exhibit cell-type-specific expression, but a few did. As noted above, *Mbd2* was specifically enriched in *Tbr2*-GFP$^-$ cells (likely RGPs; ISH not

informative), and was directly repressed by Tbr2 (**Figure 2E**; **Supplementary Tables S3**, **S4**). In contrast, *Mbd3* was widely expressed (**Figure 9E**). *Rbbp7* was specifically expressed in RGPs (**Figure 5B**), while *Rbbp4* was primarily enriched in neuron lineages (see also sections on *Rbbp4/7* in PRC2 and NuRF complexes). *Hdac1* was expressed in all zones but enriched in VZ/SVZ, while *Hdac2* was moderately enriched in neurons (**Figure 3F**). *Mta1/2* were widely expressed, while *Mta3* was essentially undetectable. *Gatad2a/b* were both enriched in *Tbr2*-GFP+ cells, and *Gatad2a* was widely expressed on ISH, but *Gatad2b* ISH was not available. *Ctbp2*, a NuRD partner that targets it to active genes that require silencing during differentiation (Kim et al., 2015), was directly activated by Tbr2 and Tbr1 (**Figures 9F,G**).

Overall, these findings suggest that NuRD subunit composition and silencing activity are modulated during differentiation from RGPs to neurons. These changes are driven in part by Tbr2 and Tbr1 (**Figures 9G,H**). Also, the graded expression of *Chd7*, and its repression by Pax6 and Tbr2, implicate *Chd7* in cortical regionalization (**Figure 9I**), although further studies will be necessary to substantiate this role.

BAF Chromatin Remodeling Complexes

Among EFs with documented importance in cortical development, the BAF chromatin remodeling complex plays a well-established role in regulating cerebral cortex size and function (Narayanan et al., 2015; Sokpor et al., 2017). Moreover, BAF subunit switching occurs at specific stages of neuronal differentiation (Son and Crabtree, 2014). The BAF complex is important for human brain development, as genetic defects of BAF subunits, such as Baf250b (*Arid1b*), cause Coffin-Siris syndrome, a microcephaly disorder with intellectual disability (Son and Crabtree, 2014).

Conserved subunits of BAF between yeast and mice include a core ATPase, consisting of either Brm (*Smarca2*) or Brg1 (*Smarca4*); Baf155/170 (*Smarcc1/2*); Baf60a-c (*Smarcd1-3*); Baf53a/b (*Actl6a/b*); and Baf47 (*Smarcb1*). In addition, mammalian BAF complexes contain ≥10 other subunits, such as Baf250a/b (*Arid1a/b*) (Son and Crabtree, 2014; Hota and Bruneau, 2016).

In cortical development, BAF has been shown to exchange four subunits during differentiation from progenitors to neurons (Son and Crabtree, 2014). Neural progenitor BAF (npBAF) contains Baf53a (*Actl6a*), Ss18, and Baf45a/d (*Phf10*/*Dpf2*); in neuronal BAF (nBAF), these subunits are replaced with Baf53b (*Actl6b*), Crest (*Ss18l1*), and Baf45b/c (*Dpf1/3*), respectively. Interestingly, the shift from Baf53a (*Actl6a*) to Baf53b (*Actl6b*) is driven by microRNA (miR)-9* and miR-124, which target *Actl6a* (Baf53a) for degradation (Son and Crabtree, 2014). In the section on ncRNA, we show that *Mir9-2* (encoding miR-9*) is directly repressed by Tbr2 and Tbr1.

The present analysis confirmed previously described BAF subunit switching, and found multiple additional subunits that switch during differentiation (**Figure 10**). Of the core ATPase subunits, *Smarca4* (Brg1) was ubiquitous, but *Smarca2* (Brm) was specifically expressed by postmigratory PNs (**Figures 10A,B**). *Smarca2* also displayed a high rostral gradient, and was directly activated by Pax6. Among the other core subunits, *Smarcd1* (Baf60a) was ubiquitously expressed, while *Smarcd3* (Baf60c) was enriched in the CP (**Figure 10C**), and was directly activated by Tbr2 (**Figure 10J**). Similarly, *Smarcc1* (Baf155) was ubiquitously expressed, while *Smarcc2* (Baf170) was abundant in CP (**Supplementary Table S3**). Previously, Baf170 (*Smarcc2*) has been linked to repression of IP genesis and neurogenesis (Tuoc et al., 2013).

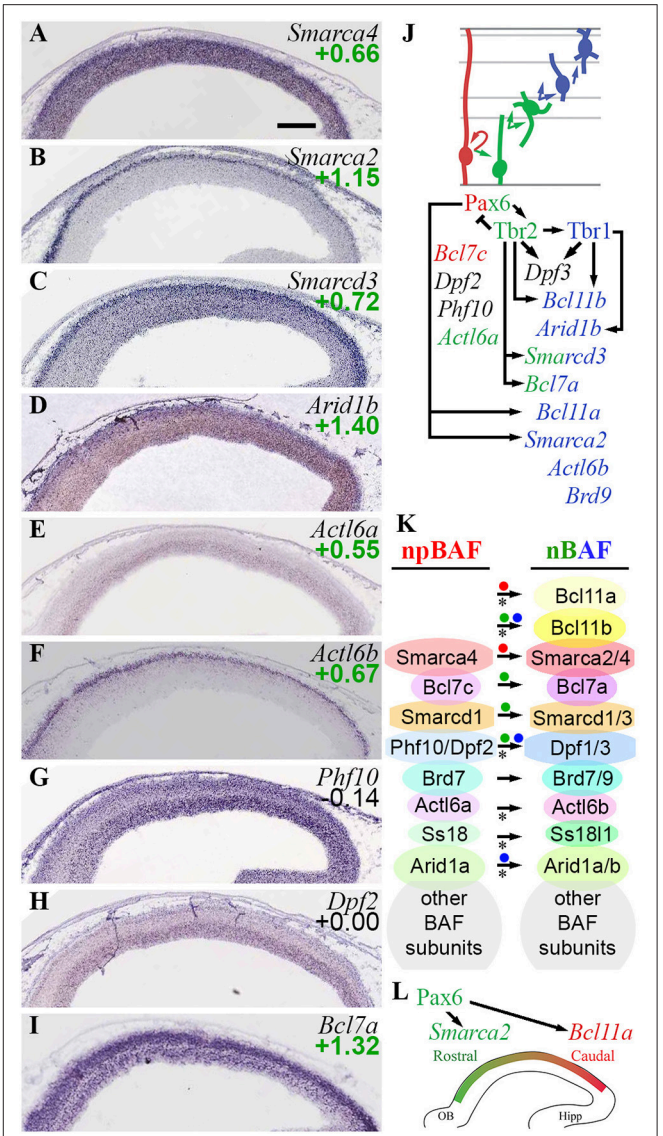

FIGURE 10 | BAF chromatin remodeling complexes. **(A–I)** Expression of indicated genes. Remarkably, *Smarca2* (Brm; **B**) was specifically expressed by postmitotic PNs in the CP, with a high rostral gradient. ISH: Genepaint. Scale bar: 100 μm. **(J)** Summary of gene expression and regulation. **(K)** BAF subunit switching was controlled by Pax6 (red dots), Tbr2 (green dots), and Tbr1 (blue dots). Asterisks: previously described switches in BAF subunit composition, confirmed here. **(L)** *Smarca2* (high rostral in CP) and *Bcl11a* (high caudal in IZ/CP) were both directly activated by Pax6, reflecting multiple functions of Pax6 in cortical development (see text for details).

The previously described (Son and Crabtree, 2014) shift from *Actl6a* (Baf53a) to *Actl6b* (Baf53b) was confirmed on ISH (**Figures 10E,F**), although enrichment of *Actl6a* in *Tbr2*-GFP+ cells ($\log_2$FC = +0.55) suggested that *Actl6a* was expressed in not only RGPs, but also IPs. Likewise, npBAF subunits *Phf10* (Baf45a) and *Dpf2* (Baf45d) were highly expressed in VZ/SVZ (**Figures 10G,H**), while nBAF subunits *Dpf1/3* (Baf45b/c) were highly expressed in IZ/CP. Upregulation of *Dpf3* (Baf45c) in differentiating neurons was directly activated by Tbr1 and Tbr2.

Among the newly observed subunit exchanges, *Bcl7c* (Baf40c) was specifically expressed in RGPs ($\log_2$FC = −1.07), while *Bcl7a* (Baf40a) was enriched in IPs and neurons ($\log_2$FC = +1.32). These findings define Baf40c and Baf40a as components of npBAF and nBAF, respectively (**Figure 10I**; **Supplementary Table S2**). Moreover, *Bcl7a* expression was directly activated by Tbr2.

Mammalian BAF complexes are sometimes categorized by Baf250 isoform, as Baf250a- (BAF-A) and BAF250b-containing (BAF-B) complexes (Hota and Bruneau, 2016). We observed that *Arid1a* (Baf250a) was ubiquitously expressed, while *Arid1b* (Baf250b) was enriched in the CP (**Figure 10D**), and was directly activated by Tbr1 (**Supplementary Table S4**). These results suggest that BAF-A predominates in progenitors, while cortical PNs express BAF-A and BAF-B complexes, the latter driven by Tbr1-mediated activation of *Arid1b*.

A special type of BAF complex, called Polybromo-associated BAF (PBAF), is formed by the incorporation of four specific subunits in Brg1 (*Smarca4*)-containing BAF: Baf180 (*Pbrm1*), Baf200 (*Arid2*), Baf45a (*Phf10*), and Brd7 (St. Pierre and Kadoch, 2017). These genes were generally enriched in progenitor zones (VZ/SVZ) relative to IZ/CP, and were moderately enriched in *Tbr2*-GFP+ cells (**Supplementary Table S3**). Thus, PBAF may be most abundant in progenitor cells, and decline with PN differentiation. The upregulation of *Smarca2* (Brm) in PNs (**Figure 10B**) may further diminish the overall formation of PBAF complexes.

Ctip1/Baf100a (*Bcl11a*) and Ctip2/Baf100b (*Bcl11b*) are BAF subunit TFs with major roles in PN differentiation and regionalization (Arlotta et al., 2005; Wiegreffe et al., 2015; Greig et al., 2016; Woodworth et al., 2016). Both *Bcl11a* ($\log_2$FC = +1.50) and *Bcl11b* ($\log_2$FC = +1.75) were highly enriched in the *Tbr2*-GFP+ lineage, and both were expressed predominantly in neuronal differentiation zones. Additionally, *Bcl11a* was expressed in a high caudal gradient, as described (Greig et al., 2016). We found that Pax6 directly activated expression of *Bcl11a*, while Tbr2 and Tbr1 directly activated *Bcl11b* (**Figures 10J,K**). The activation of *Bcl11a* by Pax6 suggests that Pax6 drives *Bcl11a* as part of the programs for neuron migration (Wiegreffe et al., 2015) and subtype specification (Woodworth et al., 2016); the high-caudal *Bcl11a* gradient runs counter to *Pax6* and is presumably shaped by other TFs.

The present results indicate that the subunit composition of BAF complexes is highly regulated in cortical PN differentiation; and that the Pax6→ Tbr2→ Tbr1 cascade is responsible for activation of many BAF subunit genes in IPs and neurons, as well as the activation of *Smarca2* in a high rostral gradient (**Figures 10J–L**). Interestingly, Pax6, Tbr2, and Tbr1 did not directly repress any npBAF subunit genes. Recently, BAF complexes were reported to interact with Utx (*Kdm6a*) and Jmjd3 (*Kdm6b*), and potentiate their H3K27me3 demethylase activity (Narayanan et al., 2015). Thus, the Pax6→ Tbr2→ Tbr1 cascade drives the formation of two complexes that recruit H3K27me3 demethylases: BAF (Narayanan et al., 2015) and Mll3/COMPASS-like (Schuettengruber et al., 2011).

Rest and CoRest Complexes

A longstanding paradigm of TF-EF interactions is the recruitment of Hdac1/2 by Rest (repressor element-1 silencing TF) to prevent neuronal differentiation (Qureshi et al., 2010). Seminal research showed that Rest binds specific DNA sequences, and recruits corepressor scaffold proteins (CoRest, Sin3) that also bind class I HDACs (*Hdac1/2*), to silence neuronal genes (Ballas et al., 2001; Lunyak et al., 2002). Complicating the picture, two isoforms of CoRest (*Rcor1/2*) have been distinguished, and other CoRest interactions and functions have been discovered (Ooi and Wood, 2007; Qureshi et al., 2010). In developing neocortex, *Rcor1/2* have been implicated in neuron subtype specification (Abrajano et al., 2009) and migration (Fuentes et al., 2012). Some functions of CoRest appear to be mediated by novel complexes with Lsd1 (*Kdm1a*; Fuentes et al., 2012) and Insm1 (Monaghan et al., 2017). The Rcor/Insm1 complex promotes neuronal differentiation, and immature progenitors accumulate in the absence of *Rcor1/2* (Monaghan et al., 2017).

In the present analysis (**Figure 11**), *Rest* was specifically expressed in RGPs (**Figure 11A**), consistent with its established function of suppressing neuronal differentiation. Of corepressors, *Sin3a* and *Rcor1* were expressed mainly in VZ (and *Rcor1* was enriched in *Tbr2*-GFP+ cells), while *Rcor2* was expressed mainly in SVZ/IZ and inner VZ (**Figures 11B,D,E**). The enrichment of *Rcor2* in *Tbr2*-GFP+ cells ($\log_2$FC = +1.94), together with its bilaminar expression pattern in VZ and SVZ (**Figure 11E**), indicated specific enrichment in aIPs and bIPs (**Supplementary Table S2**). Of the interacting HDACs, *Hdac1* was expressed at highest levels in the VZ (**Figure 11C**), while *Hdac2* was expressed mainly in IZ/CP, and was enriched in *Tbr2*-GFP+ cells (**Figure 3F**). Thus, Rest/CoRest complexes form predominantly in RGPs, where Rest recruits mainly Sin3a and Hdac1, and possibly Rcor1 (**Figure 11H**). Interestingly, one function of Rest is to repress miR-9* and miR-124 (Yoo et al., 2009); as shown below in the section on ncRNA, miR-9* is also repressed by Tbr1 and Tbr2.

Of other proposed Rcor1/2-interacting factors, *Kdm1a* (Lsd1) was ubiquitously expressed (Fuentes et al., 2012) and was enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.14). Also, *Kdm1a* (Lsd1) was directly bound and activated byTbr2 (**Figure 11G**). *Insm1* was expressed mainly in VZ and SVZ (**Figure 11F**), and was also highly enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.16). In contrast to *Kdm1a* (Lsd1), which was activated by Tbr2, *Insm1* was repressed by both Tbr2 and Pax6 (**Figure 11G**; **Supplementary Table S4**). These results suggest that Pax6 and Tbr2 promote the formation of Rcor/Lsd1 complexes regulating PN migration, but suppress IP-genic Rcor/Insm1 complexes (**Figures 11G,H**).

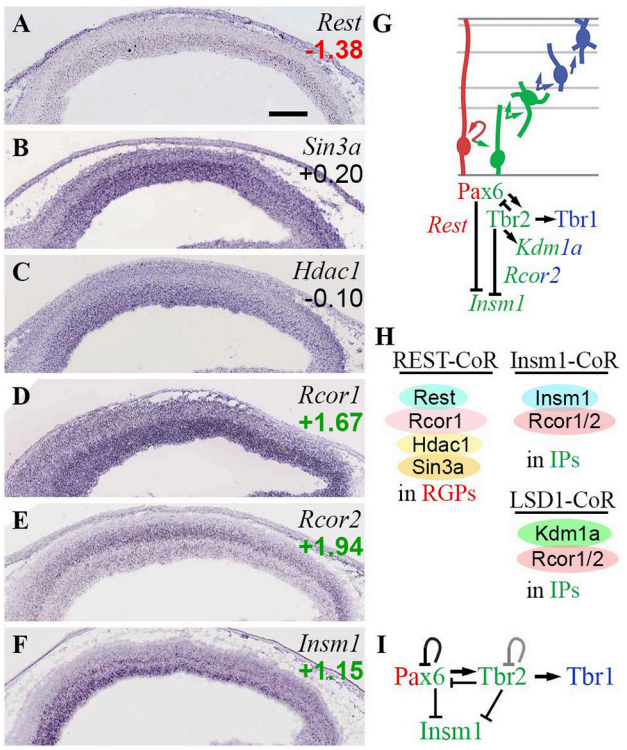

FIGURE 11 | Rest and CoRest complexes. **(A–F)** Expression of indicated genes. While *Rest* **(A)** was specifically expressed in RGPs, CoRest genes *Rcor1* **(D)** and *Rcor2* **(E)** were enriched in IPs, as was *Insm1* **(F)**. ISH: Genepaint. Scale bar: 100 μm. **(G)** Summary of gene expression and regulation. Interestingly, Tbr2 activated *Kdm1a* (Lsd1) but repressed *Insm1*; both are CoRest (*Rcor1/2*) binding partners. Pax6 also repressed *Insm1*. **(H)** Rest/CoRest complexes form in RGPs, while Insm1/CoRest and Lsd1/CoRest complexes form primarily in IPs. **(I)** Repression of *Insm1* by the Pax6→ Tbr2→ Tbr1 cascade. Like Pax6 and Tbr2, Insm1 is a key regulator of IPs (Farkas et al., 2008). The *Tbr2* loop is shown in gray to reflect unknown effect of Tbr2 on its own transcription.

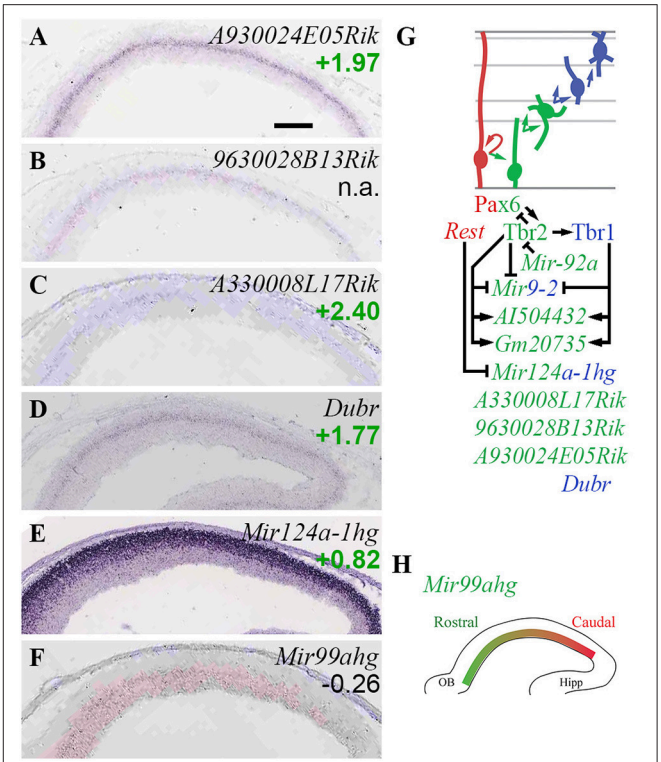

FIGURE 12 | Non-coding RNA. **(A–F)** Expression of indicated genes. Remarkably, several lncRNA genes **(A–D)** showed similar expression patterns in bIPs and/or new neurons in SVZ/IZ. ISH: Genepaint. Scale bar: 100 μm. **(G)** Summary of ncRNA gene expression and regulation. **(H)** Expression of *Mir99ahg* (high rostral) was not regulated by Pax6, Tbr2, or Tbr1; but may shape the high caudal gradient of its target, *Fgfr3* (not shown). See text for details.

Importantly, *Insm1* has previously been implicated in the genesis of IPs: *Insm1* null mice have decreased IP abundance, and reduced *Tbr2* expression (Farkas et al., 2008). One function of Insm1 is to promote the delamination of cortical progenitors, by directly repressing *Plekha7* (Tavano et al., 2018). Since Insm1 is thought to be a transcriptional repressor, and directly represses *Rest* (Monaghan et al., 2017), it seems unlikely that Insm1 directly activates *Tbr2*. Nevertheless, Insm1 is an integral component of the TF network regulated by Pax6→ Tbr2→ Tbr1 (**Figure 11I**).

Non-coding RNA-Mediated Epigenetic Regulation

Many ncRNA species regulate the expression of target genes at transcriptional or post-transcriptional levels. One well-known example of the former is *Xist*, a long (>200 nt) ncRNA (lncRNA) that binds chromatin to mediate X-inactivation (Almeida et al., 2017). Typically, microRNAs (miRs) target specific mRNAs for degradation (Hsieh and Zhao, 2016; Yao et al., 2016).

Previous studies of developing neocortex have shown that miRs in the miR-17-92 cluster prevent the transition from RGPs to IPs, in part by targeting *Tbr2* and *Cdkn1a* (p21) (Bian et al., 2013; Chen et al., 2014). Within the cluster, miR-92a was found to target *Tbr2* (Bian et al., 2013). Genesis of IPs was likewise found to be limited by miR-92b (Nowakowski et al., 2013). Conversely, miR-7 promotes IP genesis (Pollock et al., 2014). As noted above, miR-9* and miR-124 target *Actl6a* (Baf53a) to promote BAF subunit switching, and are themselves repressed by Rest (**Figure 12G**; Son and Crabtree, 2014). Additionally, miR-9 and miR-124 target *Hdac5* for degradation (**Figure 3H**), and thus control neuritogenesis (Gu et al., 2018).

In the present analysis (**Figure 12**), three lncRNAs showed zonal expression restricted to the SVZ, and enrichment in *Tbr2*-GFP+ cells consistent with specific expression in bIPs: *A330008L17Rik* ($\log_2$FC = +2.40), *9630028B13Rik* ($\log_2$FC n.a.), and *A930024E05Rik* ($\log_2$FC = +1.97) (**Figures 12A–C**; **Supplementary Table S2**). An additional lncRNA, *Dubr* ($\log_2$FC = +1.77), was similarly expressed in SVZ/IZ, consistent with bIPs and new PNs (**Figure 12D**). *AI504432* ($\log_2$FC = +0.91), a lncRNA expressed specifically in bIPs with a high lateral gradient (Kawaguchi et al., 2008), was directly activated by Tbr2 and Tbr1. Similarly, lncRNA *4833418N02Rik* was significantly enriched in the Tbr2-GFP+ lineage, and was directly activated by Tbr1.

TABLE 1 | Summary of differentiation-related EF gene expression and regulation by TFs.

EF pathway/complex	Identity: identity-specific *genes*	TFs and regulated genes
DNA CpG methylation (repression)	RGP: *Dnmt1,-3a,-3b, Mbd2, Uhrf1*	Tbr2 represses *Dnmt3a, Mbd2*
DNA CpG demethylation (activation)	Caudal VZ/SVZ: *Gadd45g* PN lineage: *Tet1*	Pax6, Tbr2 repress *Gadd45g* Tbr1 activates *Tet1*
Histone acetylation (activation)	RGP: *Hat1, Kat7 (HBO1)*	Pax6 represses *Hat1*
	aIP and bIP: *Kat2a, Kat6b*	Tbr2 represses *Kat6b*
Histone deacetylation (repression)	aIP and bIP: *Hdac9*	Tbr2 represses *Hdac9*
	N-iz: *Hdac2, Mir9-2*, *Mir124a-1hg*	Tbr1, Tbr2 repress *Mir9-2*
	PN-iz: *Hdac5*	
	mixed: *Ankrd11*	Tbr1 activates *Ankrd11*
Trx H3K4 methylation (activation)	aIP and bIP: *Ash1l*	
	PN lineage: *Kmt2c*	Tbr1 activates *Kmt2c*
Trx H3K4 demethylation (repression)	N-vz: *Kdm1a*	Tbr2 activates *Kdm1a*
	N-svz: *Kdm5b*	Tbr1 activates *Kdm5b*
	Caudal VZ/SVZ: *Kdm5a*	
PRC2 H3K27 methylation (repression)	RGP: *Rbbp7, Aebp2*	
	PN lineage: *Rbbp4, Mtf2*	Tbr1 activates *Mtf2*
	Rostral VZ/SVZ: *Phf19* Caudal VZ/SVZ: *Suz12, Eed*	
PRC2 H3K27 demethylation (activation)	N-vz: *Kdm6b (Jmjd3)*	
	bIP: *Jarid2 (inhibits PRC2)*	Tbr2 activates *Jarid2*
	PN lineage: *Kdm6a (Utx)*	Pax6 represses *Kdm6a (Utx)*
PRC1 H2AK119 ubiquityl (repression)	RGP: *Pcgf5*	
	aIP and bIP: *Rybp (non-canonical)*	Tbr1 activates *Rybp*
	N-cp: *Pcgf3, Auts2 (non-canonical)*	Pax6, Tbr1 activate *Auts2*
	Caudal VZ/SVZ: *Cbx2*	
Other histone methylation or demethylation	aIP and bIP: *Kdm4c (GASC1)* PN-iz: *Setd6*	
	PN lineage: *Kdm7a*	Tbr2 represses *Kdm7a*
	N-iz: *Mllt3 (Af9)*	Pax6 activates *Mllt3*
ISWI chromatin remodeling	RGP: *Bptf, Rbbp7 (NuRF)*	
	aIP and bIP: *Baz2a,-2b (NoRC)*	Tbr2, Tbr1 repress *Baz2b*
	PN lineage: *Smarca5 (NoRC)*	
INO80 chromatin remodeling	RGP: *Ino80b* (INO80) PN lineage: *Srcap, Ep400, Kat5*	
CHD chromatin remodeling	RGP: *Ssrp1* (FACT), *Mbd2* (NuRD)	Tbr2 represses *Mbd2*
	aIP, caudal VZ: *Chd7*	Pax6, Tbr2 repress *Chd7*
	N-iz: *Chd3* (NuRD), *Hdac2* (NuRD)	Tbr2/Tbr1 activate *Chd3*
	PN lineage: Ctbp2 (NuRD related)	Tbr2, Tbr1 activate *Ctbp2*
	mixed: *Chd1* (FACT)	Pax6 represses *Chd1*
BAF chromatin remodeling	RGP: *Bcl7c*	
	N-vz: *Arid1b, Smarcd3, Bcl7a*	Tbr2 activ. *Smarcd3, Bcl7a*; Tbr1 activates *Arid1b*
	N-iz: *Actl6b, Bcl11b*	Tbr2, Tbr1 activate *Bcl11b*
	N-iz, caudal IZ/CP: *Bcl11a*	Pax6 activates *Bcl11a*
	PN-cp: *Brd9*	
	PN-cp, rostral CP: *Smarca2*	Pax6 activates *Smarca2*
	mixed: *Dpf3*	Tbr2, Tbr1 activate *Dpf3*
Rest and CoRest complexes (repression)	RGP: *Rest*	
	aIP and bIP: *Insm1, Rcor2*	Pax6, Tbr2 repress *Insm1*
	N-vz: *Kdm1a* (LSD1)	Tbr2 activates *Kdm1a*
ncRNA	bIP: *AI504432,A330008L17Rik 9630028B13Rik, A930024E05Rik Dubr*	Tbr2, Tbr1 activate *AI504432*
	N-iz: *Mir124a-1hg*	Tbr2, Tbr1 repress *Mir9-2*
	N: *Mir9-2*	Tbr2, Tbr1 repress *Mir9-2*
	unknown: *Gm20735*	Tbr2/Tbr1 activate *Gm20735*
	Rostral VZ/SVZ: *Mir99ahg*	

Expression of lncRNA *Gm20735* was jointly activated by Tbr2 and Tbr1 (**Figure 12G**; **Supplementary Table S4**). Functions of these lncRNAs in cortical development are unknown, although some have been associated with different cortical neuron subtype fates, such as *A330008L17Rik* in PNs projecting axons to subcortical targets (Molyneaux et al., 2015).

Among miR genes, *Mir17hg* was highly enriched in *Tbr2*-GFP+ cells ($\log_2$FC = +1.96), and was localized in the inner VZ (Bian et al., 2013), suggesting that *Mir17hg* is specifically expressed by aIPs (**Supplementary Table S2**). Possibly, miR-17 expression in aIPs limits IP proliferation (Bian et al., 2013). *Mir9-2*, encoding miR-9/9*, was directly repressed by Tbr2 and Tbr1, suggesting that downregulation of these miRs may be important for PN differentiation (**Figure 12G**). In contrast, *Mir124a-1hg* ($\log_2$FC = +0.82) was highly expressed in new neurons of the IZ and CP (**Figure 12E**), suggesting it is necessary for neuron differentiation. One intriguing novel observation was a high rostral gradient of *Mir99ahg* in VZ/SVZ (**Figure 12F**). Significantly, miR-99 has been reported to target *Fgfr3* (Jiang et al., 2014), which is expressed in a high caudal gradient and regulates growth of occipitotemporal cortex (Hevner, 2005; Thomson et al., 2009). Thus, miR-99 may shape the *Fgfr3* gradient, and thereby regulate regional identity.

Together, these findings indicate that several lncRNAs are specifically expressed at high levels in IPs and new PNs, and that several miR genes are expressed with cellular or regional specificity. The gradient of *Mir99ahg*, and its possible targeting *Fgfr3*, suggest a new role for miR in cortical patterning. Finally, their direct regulation by Tbr2 and Tbr1 suggests that lncRNA and miR genes have significant functions in cortical development (**Figure 12G**).

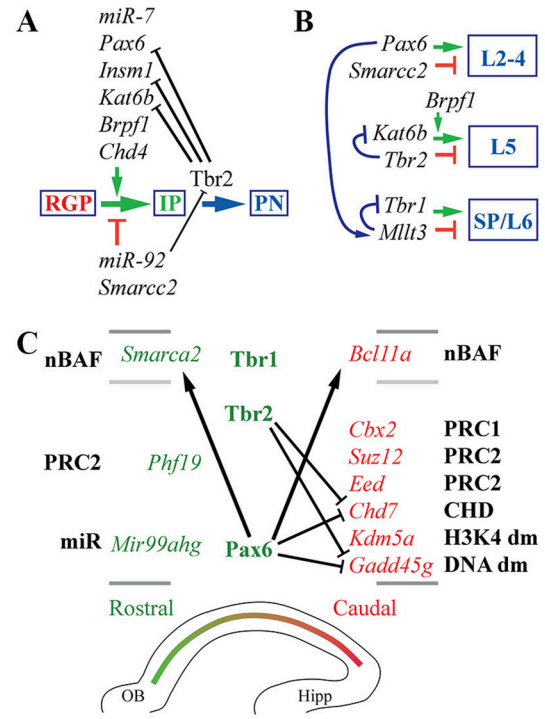

FIGURE 13 | Cortex-specific neurodevelopmental processes regulated by EFs under the control of Pax6, Tbr2, and Tbr1. **(A)** IP genesis is reportedly regulated by multiple EFs, as well as TFs such as Pax6 and Insm1. Tbr2 directly represses IP-genic factors. Arrows: **(B)** Differentiation of all cortical layers is regulated by interacting EFs and TFs. SP: subplate. **(C)** Rostrocaudal regionalization is extensively regulated by TFs and EFs in an expansive gene regulatory network. Pax6 and Tbr2 regulate several regionally graded EF genes (italic), which are components of several different epigenetic complexes or systems (bold). Abbreviations: dm, demethylation; others as in text. Lines indicate zonal separation of IZ/CP above, and VZ/SVZ below. Arrows indicate direct transcriptional activation; bars, repression.

Neurodevelopmental Processes Controlled by EFs and Regulated by Pax6, Tbr2, and Tbr1

The major findings from our analysis, summarized in **Table 1**, indicate that all kinds of EFs exhibit cell type-specific expression, and many EFs are regulated by Pax6, Tbr2, and/or Tbr1. These results implicate EFs in regulating cortical development at every stage of differentiation. Together with available functional information, our findings show that Pax6, Tbr2, and Tbr1 use transcriptional regulation of EF genes to modulate many important processes, notably IP genesis, laminar identity, and rostrocaudal regionalization of neocortex.

Regulation of IP Genesis

Previous studies have found that Pax6, Insm1, and Tbr2 each play distinct roles in IP genesis (**Figure 13A**). In *Pax6* null embryos, basal progenitors divide in the SVZ but do not express Tbr2, because Pax6 is required for *Tbr2* activation (Quinn et al., 2007). *Insm1* mutants exhibit severe reduction (∼50%) of basal IPs with proportionately decreased *Tbr2* expression (Farkas et al., 2008).

In *Tbr2* cKO embryos, conflicting phenotypes have been reported. In studies using *Foxg1*-Cre recombinase, *Tbr2* inactivation caused ∼75% reduction of basal IPs (Sessa et al., 2008). However, *Foxg1*-Cre heterozygosity itself causes ∼38% IP deficiency (Siegenthaler et al., 2008), making *Foxg1*-Cre a sensitized, anomalous background. In contrast, *Tbr2* cKO mice produced with *Nes11*-Cre have normal or increased numbers of bIPs, which migrate into the IZ and divide ectopically (Mihalas et al., 2016). Importantly, *Nes11*-Cre is a transgene that does not interfere with cortical development. Thus, the data suggest that Insm1 and Pax6 promote IP genesis and differentiation, respectively; while Tbr2 promotes the transition from IP to PN, in part by repressing IP genes (**Figure 11I**).

Previously, many EFs have also been implicated in controlling IP genesis (**Figure 13A**). Among these, *Kat6b* (Morf, querkopf) was directly repressed by Tbr2 (**Figure 3H**; **Supplementary Table S4**). Morf (*Kat6b*) is a MYST family HAT that activates gene expression, and is required for forebrain growth (Thomas et al., 2000). It is unknown if IPs are reduced in *Kat6b* (Morf) deficient embryos, but deficiency of the MYST coactivator, Brpf1, has been found to reduce IP genesis and cortical growth (You et al., 2015). These findings indicate that Tbr2 is required to repress IP-genic EF (*Kat6b*) and TF (*Pax6*, *Insm1*) genes in IPs (**Figure 13A**).

Laminar Fate

Previous studies have suggested that Pax6 promotes upper layer identity (Schuurmans et al., 2004); Tbr2 suppresses layer 5

identity (Mihalas et al., 2016); and Tbr1 promotes subplate and layer 6 identity (Hevner et al., 2001). Many EFs are also known to regulate laminar identity, and some are regulated by the Pax6→ Tbr2→ Tbr1 cascade (**Figure 13B**).

The present analysis found that Pax6 directly activated *Mllt3* (Af9), a YEATS domain acetylation reader that directly mediates *Tbr1* repression for upper layer identity (Büttner et al., 2010). Thus, Pax6 may promote upper layer identity in part by repressing lower layer identity. Paradoxically, Pax6 activates *Tbr1* indirectly (via *Tbr2*) to promote PN differentiation (**Figure 11I**), but also represses *Tbr1* indirectly (via *Mllt3*) to control laminar identity (**Figure 13B**).

Tbr2 may suppress layer 5 differentiation in part by directly repressing expression of *Kat6b* (Morf), a MYST family HAT that promotes layer 5 differentiation, as well as cortical growth (Thomas et al., 2000). In *Tbr2* cKO cortex, upregulation of *Kat6b* ($\log_2$FC $= +0.18$; $p = 0.005$) was associated with increased abundance of layer 5 neurons (Mihalas et al., 2016). The involvement of Morf (*Kat6b*) in layer 5 differentiation is supported by the phenotype of *Brpf1* mutant mice: Brpf1 is an activator of Morf (*Kat6b*), and *Brpf1* mutants have prominent layer 5 defects (You et al., 2015).

Rostrocaudal Regionalization

The cerebral cortex is patterned by molecular expression gradients that confer different properties on cortical cells, according to their rostrocaudal and mediolateral coordinates (O'Leary et al., 2007). As part of this system, Pax6, Tbr2, and Tbr1 regulate molecular gradients at each stage of differentiation from RGPs→ IPs→ PNs (Bishop et al., 2000; Bedogni et al., 2010a; Elsen et al., 2013; Mihalas and Hevner, 2017). In the present study, many EFs that are expressed in rostrocaudal gradients were identified, including some that are directly regulated by Pax6 and Tbr2 (**Figure 13C**).

Both Pax6 and Tbr2 directly repressed two EF genes with high caudal gradients in VZ/SVZ: *Gadd45g* and *Chd7* (**Figure 13C**). These findings suggest that Pax6 and Tbr2 shape the *Gadd45g* and *Chd7* gradients. However, the roles of *Gadd45g* and *Chd7* in cortical regionalization remain unknown.

Interestingly, Pax6 directly activated the expression of BAF subunits *Smarca2* (Brm) and *Bcl11a* (Ctip1), in CP and IZ/CP respectively (**Figure 13C**). Since Pax6 is not expressed in IZ/CP, its ability to activate *Smarca2* and *Bcl11a* may depend on epigenetic mechanisms, such that Pax6 "unlocks" these genes in neurogenic progenitors, making them available for activation in PNs. The dependence of *Bcl11a*, a caudal enriched gene, on Pax6, a rostral enriched TF, suggests that while Pax6 may be necessary to unlock *Bcl11a*, Pax6 probably does not drive the *Bcl11a* gradient. While *Smarca2* has no known role in cortical regionalization, *Bcl11a* has been implicated in the acquisition of sensory cortex identity (Greig et al., 2016).

Although *Mir99ahg* was not directly regulated by Pax6→ Tbr2→ Tbr1, its high rostral expression gradient in the VZ (**Figure 12F**) was noteworthy because miR-99 targets *Fgfr3* (Jiang et al., 2014), which is expressed in a high caudal gradient and promotes growth of occipitotemporal cortex (Hevner, 2005; Thomson et al., 2009). Also, canonical PRC2 complexes play an important role in promoting occipital identity with high caudal gradients of *Suz12* and *Eed* (**Figure 5J**), but these PRC2 core genes were, in our analysis, not directly regulated by Pax6→ Tbr2→ Tbr1 (**Figure 13C**).

Coordinate Regulation of Cortical Development by TFs and EFs

The present study demonstrates that many types of EFs are direct targets of gene activation or repression by Pax6, Tbr2, or Tbr1 (**Table 1**). In many examples, the regulation of EFs by TFs was robust and affected multiple elements in an epigenetic system or signaling pathway. For example, Pax6, Tbr2, and Tbr1 activated multiple BAF subunit genes, to effect subunit switching and neuronal differentiation (**Figure 10**). In another example, Tbr1 activated non-canonical PRC1 subunits (*Rybp*, *Auts2*) in PNs (**Figure 6**). Also, many HATs and HDACs were regulated by this TF cascade (**Figure 3**). Overall, our results indicate that Pax6, Tbr2, and Tbr1 utilize EFs to modulate neurodevelopmental processes such as IP genesis, laminar fate acquisition, and regional identity (**Figure 13**). The Pax6→ Tbr2→ Tbr1 cascade itself emerges as a complex network with feedforward and feedback regulation (**Figure 1B**).

Epigenetic mechanisms appear well-suited to regulation of regional and laminar identity, persistent phenotypes that are initially determined in progenitor cells, then propagated into IPs and finally, new PNs. For example, the cortical "protomap" is initially specified in RGPs, then propagated into IPs and PNs, where regional identity continues to be refined (Bedogni et al., 2010a; Elsen et al., 2013; Alfano et al., 2014).

Besides EFs, other target genes regulated by Pax6, Tbr2, and Tbr1 can be identified using the same approach, and are currently under analysis. Through these studies, it will be possible to comprehensively profile gene expression by RGPs, IPs, and PNs; and to better understand how Pax6, Tbr2, and Tbr1 control the genesis of cortical PNs.

AUTHOR CONTRIBUTIONS

GE designed and conducted experiments, produced new microarray data from Tbr1/2 mutant embryos, analyzed results, and wrote the manuscript. FB and RH produced microarray data. TB and JM analyzed data from microarray and ChIP-seq experiments. SL and JR produced and analyzed Pax6 ChIP-seq data. RH designed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00571/full#supplementary-material

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REVIEW
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Regulation of Chromatin Structure During Neural Development

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The regulation of genome architecture is a key determinant of gene transcription patterns and neural development. Advances in methodologies based on chromatin conformation capture (3C) have shed light on the genome-wide organization of chromatin in developmental processes. Here, we review recent discoveries regarding the regulation of three-dimensional (3D) chromatin conformation, including promoter–enhancer looping, and the dynamics of large chromatin domains such as topologically associated domains (TADs) and A/B compartments. We conclude with perspectives on how these conformational changes govern neural development and may go awry in disease states.

Keywords: neural development, chromatin, chromatin conformation capture (3C), topologically associated domain (TAD), A/B compartments

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INTRODUCTION

The human and mouse genomes consist of ∼6 and ∼5 billion base pairs, respectively, and are packaged in chromosomes that are contained within a nucleus with a diameter of only ∼5 μm. Chromosomes possess multilayered structures that can be broadly classified on the basis of classical cytological and biochemical analyses either as euchromatin, an open chromatin state characteristic of gene-rich regions, or as heterochromatin, a closed chromatin state characteristic of gene-poor regions. At a higher level of resolution, local associations between gene promoters and other regulatory elements, such as enhancers, define the structural relations within active transcriptional domains (Vernimmen and Bickmore, 2015).

High-throughput chromatin conformation capture (3C) techniques have recently allowed the categorization of chromosomal domains into two major classes (**Figure 1**; van de Werken et al., 2012; Bonev and Cavalli, 2016; Dekker and Mirny, 2016; Dixon et al., 2016; Hansen et al., 2018). In this review, we first briefly summarize advances in our understanding of the molecular mechanisms that regulate the formation of TADs and A/B compartments. We then address recent studies that have examined changes in genomic interactions and three-dimensional (3D) genome organization including TADs and A/B compartments during mammalian neural development, and we discuss how these chromosomal changes regulate this process.

FORMATION OF TADS AND A/B COMPARTMENTS

Recent studies have revealed some molecular mechanisms underlying the formation of TADs. The zinc-finger DNA-binding protein CTCF and the ring-shaped cohesin complex bind to many boundaries between TADs (Dixon et al., 2012; Nora et al., 2012; Rao et al., 2014), and some studies have proposed that "loop extrusion" mediated by the cohesin complex and the convergent

orientation of CTCF binding play a role in TAD formation (Sanborn et al., 2015; Fudenberg et al., 2016). Real-time imaging revealed that the condensin complex, which belongs to the same Smc family as the cohesin complex, indeed induced DNA loop extrusion *in vitro* (Ganji et al., 2018). Importantly, forced degradation of CTCF or Rad21, an essential component of the cohesin complex, with the use of the auxin-induced rapid degradation system, resulted in the almost complete elimination of TADs (Nora et al., 2017; Rao et al., 2017). Conditional knockout of the cohesin-loading factor Nipbl or Scc4 also induced deformation of TADs (Haarhuis et al., 2017; Schwarzer et al., 2017). These observations have suggested that CTCF and the cohesin complex are essential for the establishment of TADs. However, even though TADs were essentially eliminated in cells depleted of CTCF or Rad21, A/B compartments were largely unaffected (Nora et al., 2017; Rao et al., 2017). This finding indicates that A/B compartmentalization of mammalian chromosomes emerges independently of proper insulation of TADs, even though TADs serve as units of A/B compartments. Interestingly, acute loss of cohesin had only limited effects on gene expression and the distribution of various histone modifications (Rao et al., 2017; Schwarzer et al., 2017), which may suggest that regulatory interactions are somewhat preserved after the loss of TADs.

With regard to A/B compartments, heterochromatin has been proposed to serve as a driver of compartmentalization. Lamina-associated domains (LADs), defined as genomic regions that contact the nuclear lamina, constitute heterochromatin at the nuclear periphery (van Steensel and Belmont, 2017). LADs revealed by a method known as DamID (DNA adenine methyltransferase identification) analysis showed cell-to-cell heterogeneity and a strong correlation with the B compartment (Rao et al., 2014; Kind et al., 2015). Given that the nuclear lamina provides a platform for chromatin reassembly during the M-to-G_1 phase transition of the cell cycle (Güttinger et al., 2009), LAD formation may underlie compartmentalization of heterochromatin domains and the B compartment, although this is still under debate (Falk et al., 2018). Another emerging feature of heterochromatin domains is phase separation into liquid droplets mediated by heterochromatin protein 1 (HP1) (Larson et al., 2017; Strom et al., 2017). Liquid phase separation is thought to provide a basis for the formation of membrane-less structures (Boeynaems et al., 2018). The B compartment can be considered as such a membrane-less structure given the enrichment of histone H3 methylated at lysine-9 (H3K9) in this compartment (Rao et al., 2014), which provides a platform for HP1 binding and oligomerization required for liquid phase separation, as supported by a recent modeling experiment (Falk et al., 2018).

Although these various studies have elucidated the framework for 3D organization of the genome, many questions regarding TAD formation – including the role of transcription, whether loop extrusion is asymmetric, and the relevance of DNA replication – remain unanswered. In addition, the mechanisms underlying A/B compartmentalization remain largely elusive. A key unanswered question regarding genome architecture is, how do local and global-scale associations, including those mediated by A/B compartments and TADs, govern changes in transcription and cell fate during development. In this review, we focus on studies on neural development in an attempt to tackle this question.

GLOBAL CHANGES IN 3D GENOME ORGANIZATION DURING NEURAL DIFFERENTIATION

Global Compaction During Neural Differentiation

The 3D architecture of chromatin changes markedly during the neural development of pluripotent stem cells. Assays based on micrococcal nuclease (MNase) or DNase I accessibility or on histone extraction have revealed that the chromatin state is globally open in embryonic stem cells (ESCs) and becomes condensed during differentiation into neural progenitor cells (NPCs) (Meshorer et al., 2006). Even among NPCs, the loss of neurogenic potential during neocortical development is associated with chromatin condensation on a large scale (Kishi et al., 2012a; Tyssowski et al., 2014). The "openness" of chromatin may be related to differentiation potential ("stemness") in these cells, given that the factors responsible for global chromatin accessibility – Chd1 in ESCs and Hmga in NPCs – are also required for differentiation potential (Gaspar-Maia et al., 2009; Kishi et al., 2012a). Chromatin state also undergoes pronounced changes during neuronal differentiation of NPCs. For example, the number and shape of chromocenters – heterochromatin foci strongly stained with DNA-intercalating dyes – change during neuronal differentiation (Billia et al., 1992; Solovei et al., 2004, 2009; Clowney et al., 2012; Le Gros et al., 2016). Likewise, an increase in the deposition of the active histone mark H3K4me3 (trimethylated lysine-4 of histone H3) at chromocenters, accompanied by an increase in transcription of major satellites, is also observed during neuronal differentiation in the neocortex (Kishi et al., 2012b). Recent examinations of chromatin accessibility by the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), DNase-seq, and formaldehyde-assisted isolation of regulatory elements (FAIRE)-seq have revealed progressive changes in chromatin openness during neuronal differentiation processes (Frank et al., 2015; Thakurela et al., 2015; de la Torre-Ubieta et al., 2018; Preissl et al., 2018), which would link chromatin accessibility to the genome architecture associated with these processes.

Loss of Active-Domain and Increase in Inactive-Domain Interactions During Neural Differentiation

So, how are TADs and A/B compartments regulated during neural development? TADs are structurally dynamic overall (Hansen et al., 2018), but TAD boundaries, on the other hand, are stable for many cell divisions and invariant across diverse cell types or lineages (Nora et al., 2012; Rao et al., 2014; Dixon et al., 2015, 2016). Indeed, differentiation of ESCs into NPCs

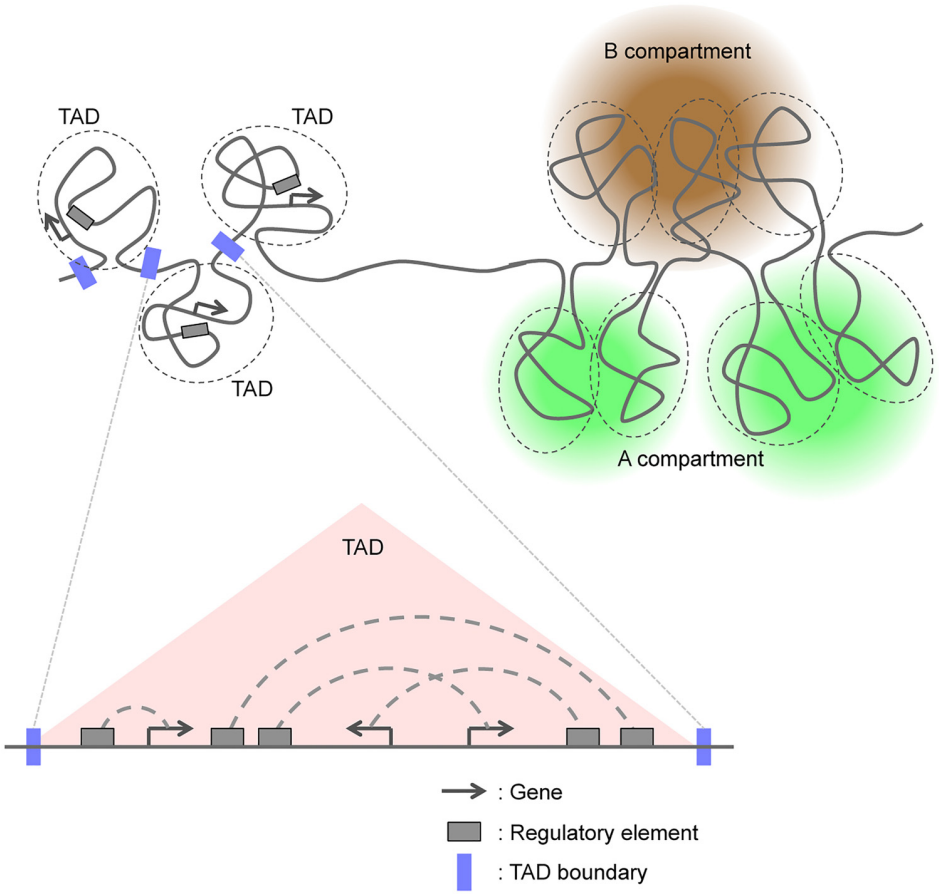

FIGURE 1 | Three-dimensional genome organization based on TADs and A/B compartments.

and then into neurons is not accompanied by changes in the boundaries of most TADs (Fraser et al., 2015). Rather, inter-TAD interactions as well as chromatin interactions within TADs (sub-TAD or intra-TAD level, including chromatin looping) change during differentiation (Fraser et al., 2015; Dixon et al., 2016, 2015). Fraser et al. (2015) proposed that TADs are organized into meta-TADs in a hierarchical manner, and that neural differentiation of ESCs is accompanied by the rearrangement of meta-TAD components (**Figure 2A**). A fraction of inter-TAD rearrangement is associated with changes in gene expression within TADs (Fraser et al., 2015), and TAD allocation to A/B compartments changes during differentiation (Dixon et al., 2015).

In contrast to TADs, A/B compartments are differentially regulated during neural development. Recent studies have examined and compared genome-wide 3D chromatin organization during neural differentiation from ESCs (Dixon et al., 2015; Bonev et al., 2017) by Hi-C analysis, which allows the detection of complete "all versus all" long-distance chromatin interactions across the entire genome (Lieberman-Aiden et al., 2009). One study (Dixon et al., 2015) found that the total size of the A compartment in differentiated cells including NPCs was reduced by 5% compared with that in ESCs (**Figure 2B**). This finding appears to be consistent with the global condensation of the chromatin state observed when ESCs differentiate into neural cells mentioned above. Another study (Bonev et al., 2017) based on higher-resolution Hi-C analysis (maximum of 750 bp) found that interactions within the A compartment decreased during the ESC-to-NPC transition, interactions between A and B compartments transiently increased in NPCs, and interactions within the B compartment increased during the NPC-to-neuron transition, supporting the notion that chromatin undergoes global compaction in association with differentiation (**Figure 2B**). Also consistent with this idea, the positive correlation between active histone marks [H3K4me1, H3K27ac (acetylated lysine-27 of histone H3), and H3K36me3] and the A compartment became weaker, whereas that between the inactive mark H3K9me3 and the B compartment became stronger, during neural (ESC-NPC-neuron) differentiation (Bonev et al., 2017). Regarding the inactive (B) compartment, as extreme cases, rod photoreceptor cells manifest heterochromatin aggregation in the center of the nucleus (Solovei et al., 2009), and postmitotic olfactory sensory neurons show pronounced compaction of olfactory receptor gene loci (Clowney et al., 2012; Le Gros et al., 2016). However, Hi-C results suggest that the compaction of heterochromatin

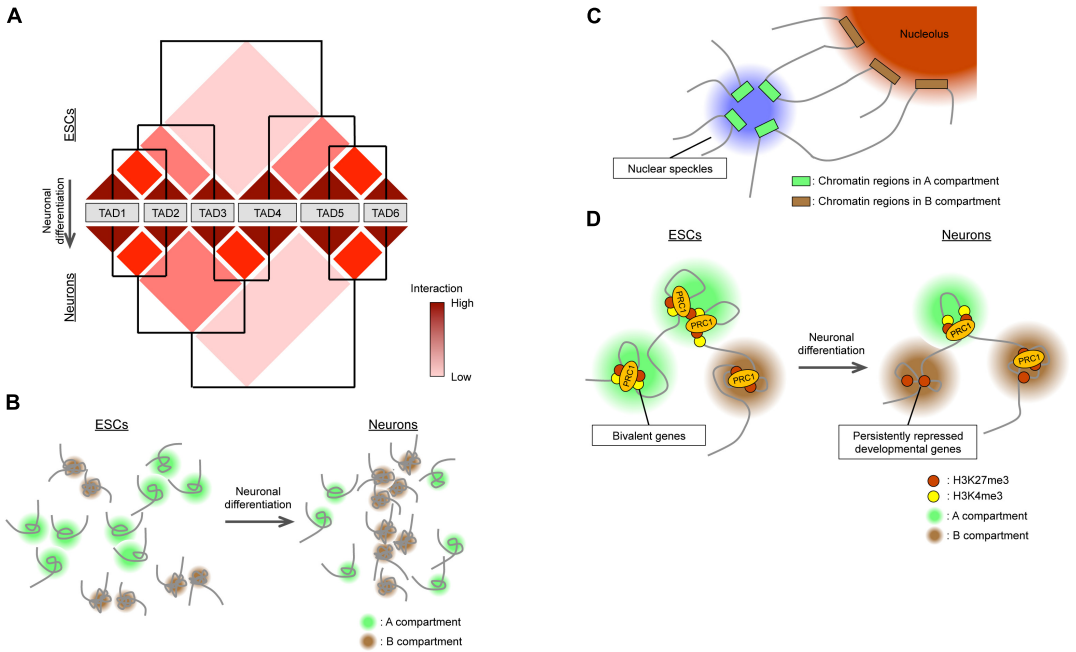

FIGURE 2 | Global changes in 3D genome organization during neural differentiation. **(A)** TADs are organized in a hierarchical manner, and their reorganization accompanies neural differentiation. **(B)** During neural differentiation from ESCs, interactions within the A compartment decrease while those within the B compartment increase. The size of the A compartment also decreases during neural differentiation. **(C)** Nuclear speckles and nucleoli act as hubs for interactions within A and B compartments, respectively. **(D)** In ESCs, bivalent genes interact with each other *via* PRC1 in the A compartment. Neural differentiation is accompanied by the loss of PRC1-mediated interactions between bivalent genes, with the genes becoming persistently repressed and relocating to the B compartment.

domains may be a general feature of differentiating neurons and contribute to the stable silencing of unnecessary genes for differentiated neurons (Solovei et al., 2009; Clowney et al., 2012; Bonev et al., 2017). Given the changes in LADs during neural development (Peric-Hupkes et al., 2010), the downregulation of a lamin B receptor apparent during neuronal differentiation provides a possible common mechanism for this heterochromatin reorganization (Clowney et al., 2012; Solovei et al., 2013).

How then are regions in the A compartment regulated? High-level interactions within the A compartment in ESCs can be explained in part by long-range (>30 Mb) associations between active promoters, enhancers, and actively transcribed genes both *in cis* and *in trans* (Li et al., 2012; Schoenfelder et al., 2015; Tang et al., 2015; Bonev et al., 2017). In addition to 3C-based methods, a technique known as genome architecture mapping (GAM) can determine the proximity of genomic loci without cross-linking by ultrathin cryosectioning of nuclei followed by laser microdissection and DNA sequencing (Beagrie et al., 2017). GAM confirmed an abundance of long-range interactions, especially between "super-enhancers" [which are marked by extremely high levels of H3K27ac (Hnisz et al., 2013; Parker et al., 2013)] in ESCs. Super-enhancers are cell type specific and play key roles in cell fate determination (Hnisz et al., 2013). Given that they are enriched in binding elements for cell type-specific transcription factors (Hnisz et al., 2013), it is possible that homotypic interactions between these factors can induce the aggregation (high-density interaction) of super-enhancers. Moreover, whereas high-density contacts between active promoters were found to be independent of CTCF (Bonev et al., 2017), degradation of the cohesin component Rad21 resulted in an increase in the number of long-range interactions between super-enhancers (Rao et al., 2017), suggesting that the cohesin complex insulates long-range interactions between super-enhancers and thereby ensures the fidelity of cell type-specific gene expression patterns.

On the basis of classical immunocytochemical analyses, nuclear bodies, which are subcompartments within the nucleus, were hypothesized to serve as hubs for active or inactive gene loci (Rino et al., 2007; Sutherland and Bickmore, 2009; Padeken and Heun, 2014), although there was no genome-wide evidence to support this notion. A ligation-independent method known as split-pool recognition of interactions by tag extension (SPRITE) that relies on uniquely tagged cross-linked chromatin fragments to determine the proximity of genomic loci was recently introduced (Quinodoz et al., 2018). This method detects proximity between both DNA and RNA molecules and revealed that regions in the active (A) compartment preferentially interact with U1 spliceosomal RNA and Malat1 long noncoding RNA localized at nuclear speckles, whereas those in the inactive (B) compartment interact with rRNA localized at the nucleolus (**Figure 2C**). Consistent with these observations, the contact enrichment

between gene bodies positively correlates with transcriptional level as well as with the numbers of exons and splicing events (Bonev et al., 2017). Given the contribution of nuclear bodies to neural development (Bernard et al., 2010; Hetman and Pietrzak, 2012), these results suggest that the dynamic rearrangements of A/B compartments during neural development may be dependent on or connected to changes in nuclear bodies.

Global Changes in Polycomb Domains

In general, active and inactive histone modifications are associated with A and B compartments, respectively (Lieberman-Aiden et al., 2009; Rao et al., 2014). Interestingly, although H3K27me3, a modification deposited by Polycomb repressor complex 2 (PRC2), is generally considered an inactive histone mark, it is highly associated with the A compartment in ESCs and becomes associated more with the B compartment in neurons (Bonev et al., 2017; **Figure 2D**). This finding can be explained in part by the role of Polycomb group (PcG) proteins in the maintenance of developmental genes in the "poised" state in stem cells for later activation in response to differentiation-inducing cues (Azuara et al., 2006; Bernstein et al., 2006; Zhao et al., 2007). Such poised promoters tend to be "bivalent" in that they possess both active (H3K4me3) and inactive (H3K27me3) marks, and are thus included in the A compartment. Consistent with the notion that PcG proteins, including Ring1B – a major component of Polycomb repressor complex 1 (PRC1) – are associated with many poised developmental genes included in the A compartment in pluripotent stem cells and that such association is attenuated after differentiation, the genomic loci bound by Ring1B manifest strong interactions in ESCs but these interactions become progressively reduced during neural differentiation (Bonev et al., 2017). Furthermore, PcG protein-mediated chromatin interactions can take place beyond TAD boundaries and establish inter-TAD and inter-chromosomal associations in addition to those within TAD boundaries (Denholtz et al., 2013; Schoenfelder et al., 2015; Kundu et al., 2017). The global loss of PRC1-mediated, but H3K27me3-independent, long-range chromatin interactions during neural differentiation may therefore account in part for the global changes in chromatin architecture associated with this process. Conversely, a specific subset of Ring1B-mediated interactions becomes stronger during differentiation so as to allow for persistent repression of certain developmental genes associated with fate restriction (Bonev et al., 2017; Tsuboi et al., 2018). These inactive genes that are persistently silenced by PcG proteins are included in the B compartment. Mechanistically, PcG proteins can mediate high-density chromatin interactions *via* self-aggregation within and between PRC1 and PRC2 (Kim et al., 2002; Francis et al., 2004; Margueron et al., 2008; Eskeland et al., 2010; Grau et al., 2011; Isono et al., 2013). In particular, Phc protein components of PRC1 form nuclear nanoclusters in a manner dependent on polymerization activity of the SAM (sterile alpha motif) domain, with the formation of these clusters facilitating long-range chromatin interactions and persistent silencing (Isono et al., 2013; Wani et al., 2016; Tsuboi et al., 2018).

LOCAL (INTRA- OR SUB-TAD) CHANGES IN 3D GENOME ORGANIZATION DURING NEURAL DIFFERENTIATION

Interactions Between Binding Sites of Neural-Specific Transcription Factors in NPCs and Neurons

Topologically associated domains constitute units of gene regulation (Alexander and Lomvardas, 2014; Dixon et al., 2015; Lupiáñez et al., 2015; Narendra et al., 2016; Symmons et al., 2016; Zhan et al., 2017), with most enhancer–promoter interactions taking place within TADs. High-resolution Hi-C or promoter-capture Hi-C analyses have confirmed that such interactions are highly cell type specific (Rao et al., 2014; Javierre et al., 2016; Bonev et al., 2017; Freire-Pritchett et al., 2017). For example, neuronal enhancers interact with their promoters more strongly in neurons than in ESCs and NPCs (Mifsud et al., 2015; Bonev et al., 2017). Chromatin immunoprecipitation (ChIP)-seq analyses have revealed a link between intra-TAD interactions and cell type-specific transcription factors such as the NPC-specific Pax6 and the immature neuron- and mature neuron-specific NeuroD2 and Tbr1, respectively (**Figure 3A**). The interactions of Pax6-bound sites were thus stronger in NPCs than in neurons or ESCs, whereas those of NeuroD2- or Tbr1-bound sites were stronger in neurons than in NPCs or ESCs (Bonev et al., 2017). Transcription factors may also organize the co-regulation of target genes through homotypic interactions or association with partner molecules such as the BAF chromatin remodeling complex for Pax6 (Ninkovic et al., 2013; Manuel et al., 2015).

PcG Protein-Mediated Enhancer–Promoter Interactions at Neural Gene Loci in ESCs

Polycomb group proteins generally mediate repression of gene expression, as mentioned above. However, recent studies have revealed that these proteins may contribute to gene activation *via* the establishment of enhancer–promoter interactions. PRC1 (Ring1) can mediate the association of a midbrain-specific enhancer and the promoter of the *Meis2* gene during midbrain development, with the subsequent dissociation of PcG proteins resulting in the activation of *Meis2* expression in the midbrain (Kondo et al., 2014; Yakushiji-Kaminatsui et al., 2016). PcG proteins were found to play a similar role in the establishment of "poised" enhancers in ESCs. The poised enhancers were defined by the presence of the histone acetyltransferase p300 and H3K27me3 and the absence of H3K27ac and H3K4me3, and neural genes, especially anterior neural genes, were found to be enriched in poised enhancers in ESCs (Cruz-Molina et al., 2017). Importantly, poised enhancers physically contact their target genes in a PRC2-dependent manner, and the PRC2 components Suz12 and Eed are necessary for the induction of anterior neural genes in NPCs (**Figure 3B**). These findings point to the essential role of PcG proteins in the generation of permissive chromatin topology at such gene loci before their activation, although the molecular basis of their

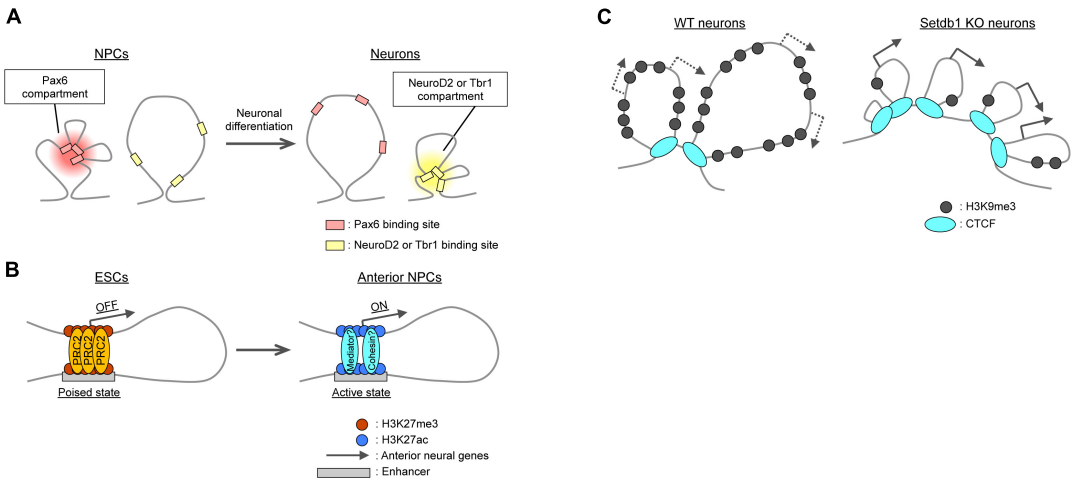

FIGURE 3 | Local changes in 3D genome organization during neural differentiation. **(A)** Intra-TAD interactions between binding regions for cell type-specific transcription factors such as Pax6, NeuroD2, and Tbr1. **(B)** Polycomb (PRC2)-mediated interactions between promoters and poised enhancers lead to the activation of anterior neural genes during differentiation. **(C)** In cortical neurons, H3K9me3 deposition catalyzed by Setdb1 prevents aberrant CTCF binding at Pcdh gene clusters. Knockout (KO) of Setdb1 induces excessive insulation and upregulation of Pcdh gene expression.

differential roles in gene activation and suppression remains to be clarified.

The preferential regulation of anterior neural genes by poised enhancers in ESCs (Cruz-Molina et al., 2017) *per se* is an intriguing finding. Classical developmental models propose that epiblast cells *in vivo* and ESCs *in vitro* are fated toward the neural lineage by "default" (that is, in the "absence" of extrinsic signals) (Levine and Brivanlou, 2007; Gaspard and Vanderhaeghen, 2010). Moreover, induced neural progenitors initially manifest anterior characteristics (that is, those of the forebrain), which must be overridden by extrinsic cues for the induction of more posterior neural fates (such as those of the spinal cord). The readiness of anterior neural genes to be expressed due to their association with poised enhancers in ESCs may explain in part the propensity for default differentiation to an anterior neural lineage.

TAD Boundary Formation in Neural Cells

As mentioned above, most TAD boundaries are conserved between ESCs and neural cells, but a fraction of TAD boundaries appears to emerge and disappear during neural differentiation (Bonev and Cavalli, 2016) – although the interpretation of TAD boundaries depends on the precise definition of TADs (Dixon et al., 2016). Of note, these developmentally regulated TAD boundaries correlate with H3K4me1-positive enhancers (Dixon et al., 2015) and active gene marks (Bonev et al., 2017) as well as with the presence of cohesin, but not that of CTCF (Bonev et al., 2017). Indeed, the emergence of new boundaries in NPCs was found to be associated with Zfp608- and Sox4-dependent transcription, although forced induction of such transcription with the use of the dCas9 system was not sufficient to induce a new TAD boundary (Bonev et al., 2017).

Relevance of TAD Boundaries to Regulation of Pcdh Gene Clusters

Topologically associated domain boundaries can play a role in the regulation of neural genes, most notably in Protocadherin (Pcdh) gene clusters. Pcdh proteins regulate axonal targeting, synapse formation, and dendritic arborization through their homophilic trans-interactions (Yagi, 2012; Chen and Maniatis, 2013). The vast diversity of neurons is generated in part by the stochastic and combinatorial expression of the clustered Pcdh genes, which include Pcdhα, Pcdhβ, and Pcdhγ clusters aligned *in cis*. *In situ* Hi-C experiments with NeuN-positive mouse neocortical neurons revealed that the Pcdh gene clusters are organized as multiple small TADs (∼100 kb in length) nested into a larger TAD that encompasses at least 1.2 Mb. The 5′ end of the Pcdhα cluster is bound to the 3′ end of the Pcdhγ cluster (Jiang et al., 2017). This TAD structure appears to be important for proper regulation of Pcdh genes, given that knockout of CTCF disrupted TADs at this locus and resulted in the aberrant expression of Pcdh genes (Hirayama et al., 2012; Sams et al., 2016). The unique TAD structure of Pcdh gene clusters was also apparent in neurons derived from human induced pluripotent stem cells (iPSCs). Interestingly, a risk haplotype for schizophrenia (according to the Psychiatric Genomic Consortium) has been found to be genetically linked to the 5′ end of the human Pcdhα gene cluster (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Forced dCas9-mediated localization of KRAB or VP64 transcriptional repressor or activator domains, respectively, at the risk gene locus in human iPSC-derived NPCs resulted in dysregulation of Pcdh gene transcription (Jiang et al., 2017). Given the neurodevelopmental functions of Pcdh proteins, an aberrant TAD structure of the Pcdh gene clusters could potentially contribute to the development of schizophrenia.

With regard to the mechanism responsible for the TAD structure of Pcdh gene clusters, deposition of H3K9me3 by the

histone methyltransferase Setdb1 (also known as Kmt1e or ESET) (Schultz et al., 2002) appears to play an essential role. Ablation of Setdb1 in neocortical neurons reduced the level of H3K9me3 and increased the binding of CTCF at the Pcdh gene clusters, resulting in the formation of only small TADs without the large-scale interaction normally apparent between the borders of the clusters (**Figure 3C**; Jiang et al., 2017). Cytosine methylation (5mC) was shown to inhibit the binding of CTCF (Renda et al., 2007; Wang et al., 2012), although this finding is still under debate (see Bonev and Cavalli, 2016). Setdb1 ablation reduced 5mC levels at several residues in the Pcdh gene clusters, which thus may account for the increased CTCF binding and aberrant insulation within these clusters.

Regulation of CTCF binding and TAD structure by Setdb1 is not restricted to Pcdh gene clusters. Loss of Setdb1 in neocortical neurons thus resulted in the emergence of more than 3000 ectopic CTCF-binding sites (Jiang et al., 2017). Setdb1 has also been shown to contribute to the development of several tissues including the mouse neocortex (Tan et al., 2012; Liu et al., 2014; Eymery et al., 2016; Kim et al., 2016; Takikita et al., 2016). Ablation of Setdb1 altered the differentiation potential of neocortical NPCs by reducing neurogenic potential and increasing astrogenic potential. Of interest, transcriptome analysis of Setdb1-deficient NPCs revealed ectopic expression of genes of nonneural lineages as well as of transposons (Tan et al., 2012), implicating Setdb1 in repression of these genes, possibly mediated by inhibition of unwanted CTCF binding and consequent promotion of proper formation of TAD structures in addition to its role in heterochromatin formation through H3K9me3. CTCF binding is also regulated by other factors including YY1, which may control enhancer–promoter interactions and transcription in NPCs (Beagan et al., 2017; Weintraub et al., 2017), although the ubiquitously expressed YY1 alone may not be able to account for cell type-specific CTCF regulation.

CONCLUSION AND FUTURE DIRECTIONS

The regulation of 3D chromatin structure has been studied with regard to its role in determination of gene transcription patterns. New technologies such as high-resolution 3C-based methods have revealed that neural development is accompanied by changes in genome organization at the levels of both interactions between large compartments and local interactions such as those between enhancers and promoters. Such advances in basic knowledge concerning chromatin structure will facilitate our understanding of the mechanisms and relevance of chromatin regulation during neural development and the pathogenesis of related diseases. Given the heterogeneity of NPCs and neurons, analyses at the single-cell level will be especially important for studies of neural development, and the recent implementation of advanced single-cell RNA-seq, ChIP-seq, DamID, ATAC-seq, and Hi-C technologies should prove highly informative in this regard (Nagano et al., 2013; Shalek et al., 2014; Buenrostro et al., 2015; Kind et al., 2015; Macosko et al., 2015; Rotem et al., 2015; Corces et al., 2016; Stevens et al., 2017). The spatial and functional nature of the relation between chromatin domains and nuclear bodies, the nuclear lamina, and other aspects of nuclear architecture also await clarification in future studies (Sutherland and Bickmore, 2009; Quinodoz et al., 2018). Recent developments in advanced microscopic technology, including super-resolution and electron microscopies, as well as in live-cell imaging of specific genomic loci with the use of zinc-finger nuclease, transcription activator-like effector nuclease (TALEN), or CRISPR (clustered regularly interspersed short palindromic repeats)–Cas9 systems may uncover novel principles of 3D organization and genomic localization in the nucleus (Chen et al., 2016; Ricci et al., 2017). We focused in this review on the early developmental process of neural differentiation, but it will also be of interest to determine how chromatin architecture is regulated during neuronal maturation and in association with neural plasticity triggered by changes in neuronal activity (Wittmann et al., 2009; Frank et al., 2015; Thakurela et al., 2015; de la Torre-Ubieta et al., 2018; Gallegos et al., 2018; Preissl et al., 2018).

As suggested in the case of the Pcdh gene clusters, aberrant changes in 3D chromatin structure may give rise to neurodevelopmental disorders (Mitchell et al., 2014). Indeed, mutations in the genes for cohesin components are known to be responsible for Cornelia de Lange syndrome in humans, which is associated with mental retardation (Krantz et al., 2004; Tonkin et al., 2004; Deardorff et al., 2007, 2012; Fujita et al., 2017). Mutations in CTCF and Setdb1 genes also cause severe neural developmental defects in mice (Watson et al., 2014; Sams et al., 2016). Although access to human tissue is limited, the organization of the human genome in both the developing and adult human brain has recently been investigated by Hi-C analyses (Won et al., 2016). Such studies as well as those of neurons derived from iPSCs of patients with neurodevelopmental disorders should provide insight into the pathogenesis of these conditions as well as a basis for the development of new therapeutic strategies.

AUTHOR CONTRIBUTIONS

YK and YG wrote the manuscript.

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