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RNA on the brain: emerging layers of post-transcriptional regulation in cerebral cortex development

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Abstract

Embryonic development is a critical period during which neurons of the brain are generated and organized. In the developing cerebral cortex, this requires complex processes of neural progenitor proliferation, neuronal differentiation, and migration. Each step relies upon highly regulated control of gene expression. In particular, RNA splicing, stability, localization, and translation have emerged as key post-transcriptional regulatory nodes of mouse corticogenesis. Trans-regulators of RNA metabolism, including microRNAs (miRs) and RNA-binding proteins (RBPs), orchestrate diverse steps of cortical development. These trans-factors function either individually or cooperatively to influence RNAs, often of similar classes, termed RNA regulons. New technological advances raise the potential for an increasingly sophisticated understanding of post-transcriptional control in the developing neocortex. Many RNA-binding factors are also implicated in neurodevelopmental diseases of the cortex. Therefore, elucidating how RBPs and miRs converge to influence mRNA expression in progenitors and neurons will give valuable insights into mechanisms of cortical development and disease.

INTRODUCTION

The neocortex is an anatomically complex brain structure that controls our higher cognitive functions such as abstract thought and language. This structure develops over the course of embryogenesis in a multi-step process called corticogenesis. In mice, corticogenesis initiates mid-gestation at embryonic day (E) 10, when progenitors of the neuroepithelium expand their population via symmetric self-renewing divisions. Beginning around E11.5, these progenitors are replaced by radial glial progenitor cells (RGCs) which over 7 days, sequentially produce excitatory neurons to form distinct layers of the cortical plate (CP).¹⁻³ This occurs in an inside out fashion in which deeper layers are formed first and superficial layers are formed later. Newly made neurons use the RGC basal process as a scaffold to

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migrate. The basal process extends from the ventricular zone (VZ) where the cell body resides, through the CP to the pia. RGCs undergo neurogenic divisions to generate neurons directly or indirectly via intermediate progenitors (IPs), which themselves produce neurons within the sub-ventricular zone (SVZ).^{4–7} Postnatally in the mouse, RGCs shift their potency, to produce glia including astrocytes and oligodendrocytes.⁸ Defective progenitor proliferation, differentiation, and neuronal migration underlie diverse neurodevelopmental disorders including autism, intellectual disability, and schizophrenia. Understanding how corticogenesis is properly orchestrated at a molecular level is critical for elucidating the underpinnings of neurological pathologies.

Corticogenesis is controlled at many molecular levels, including transcription, translation, signaling, and epigenetic. Post-transcriptional regulation has emerged as a key node controlling cortical development (Figure 1). This includes alternative splicing (AS), stability, localization, and translation, the molecular details of which are summarized in recent reviews.^{9,10} RNA regulation is mediated via *cis*-elements encoded by RNA sequence or secondary structure. These *cis*-elements are bound by *trans*-acting factors including RNA binding proteins (RBPs) and non-coding RNAs, both small (<200 nt) microRNAs (miRs) and long (>200 nt) non-coding RNAs (lncRNAs).

In this review, we comprehensively catalog RBPs and miRs with established *in vivo* functions in prenatal mouse cortical development (Figure 2). Emerging techniques for studying mRNA expression, binding, localization, stability, and translation are also highlighted (Box 1 and Box 2). We do not review lncRNAs, as their roles in cortical development have been highlighted recently.¹¹ We discuss, when relevant, convergent findings in human genetics, as well as the interplay between RBPs and miRs in post-transcriptional regulation.

GENOME WIDE ANALYSIS OF RNA EXPRESSION AND ALTERNATIVE SPLICING

Over the course of mouse neocortical development, RNA undergoes dynamic spatial and temporal transcript control, as illustrated by genomic studies. Vast differences in both RNA steady state levels and AS are evident between embryonic and postnatal stages.¹² Moreover, transcriptome studies of laser-capture microdissected E14.5 regions (VZ, SVZ/IZ, and CP), or isolated populations, reveal transcripts and AS events specific to progenitors and neurons.^{13–15} AS enables production of proteins with distinct functional domains, as exemplified with Ninein (*Nin*) isoforms, which exhibit distinct sub-cellular localization patterns and functions in differentiation.¹⁵ Aberrant AS during cortical development can be deleterious, as evidenced by inclusion of pathogenic *Flna* exons, which cause periventricular nodular heterotopia. This is mediated by splicing factors, RBFOX3 and PTBP1, both of which influence cortical development, as described below. These studies demonstrate the importance of understanding trans-regulation of AS and steady-state RNA levels.

DIVERSE mRNA BINDING PROTEINS IN CORTICOGENESIS

Numerous RBPs are expressed in the developing neo-cortex as evidenced by both *in situ* expression profiling and transcriptome-wide studies.^{23–25} As described below, RBPs orchestrate diverse processes of cortical development including progenitor proliferation, survival, fate specification, and neuron migration and maturation (Table 1).

Trans-Splicing Regulators in Cortical Development

The polyprimidine tract-binding (PTB) splicing regulators, PTBP1 and PTBP2, both influence progenitor proliferation and differentiation. PTBP1 (PTB, hnRNP1) is expressed in RGCs but downregulated in differentiated neurons.²⁶ Conditional *Ptbp1* ablation in RGCs, mediated by *Nestin*-Cre, causes post-natal hydrocephalus. *Ptbp1*-deficient embryonic brains show a gradual loss of adherens junctions, precocious neuronal differentiation and defective interkinetic nuclear migration (INM), suggesting roles in RGC cell cycle. Several candidate PTBP1 targets are proposed to explain these defects including *Flna*¹⁵ and *Pnky*, a progenitor-specific lncRNA, which phenocopies *Ptbp1*-mutant phenotypes.²⁷ However, although genome-wide PTBP1 targets of adult progenitors are known, they have not been described during embryonic development.

In contrast to *Ptbp1*, *Ptbp2* (brPTB, nTPB) is expressed in both RGCs and differentiated neurons. *Ptbp2* germline knockouts share several phenotypes with *Ptbp1* mutants, including defective INM, disrupted RGC maintenance, precocious differentiation, and neuronal heterotopias.²⁸ Notably some of these germline phenotypes are not reported in RGC conditional (*Emx1*-Cre and *Nestin*-Cre) *Ptbp2* models,²⁹ warranting further investigation. Genomic analyses of germline and conditional *Ptbp2* brains has been performed, revealing that this RBP broadly controls AS targets important for neuronal differentiation and function, such as *Psd95*.^{28–30}

During neuronal differentiation, *Ptbp1* and *Ptbp2* compose a regulatory network, which includes a microRNA, *Mir-124*³¹ (Figure 3). Intact microRNA biogenesis is required for proper expression patterns of *Ptbp1* and *Ptbp2*, and for downstream AS. In progenitors PTBP1 controls *Ptbp2* splicing, generating an isoform with a premature stop codon, which is degraded by nonsense-mediated decay (NMD). Thus, PTBP1 is postulated to inhibit neuronal differentiation in part by controlling PTBP2 levels, and to promote differentiation in the presence of high levels of *Mir-124*. Empirical testing of this model *in vivo* is still needed, however, it illustrates how RBPs and miRs can coordinately regulate AS in progenitors and neurons.

Another class of splicing regulators controlling neuronal differentiation and cell survival are serine and arginine-rich (SR) proteins. For example, *Tra2B* is expressed in both RGCs and post-mitotic neurons. Conditional *Tra2B* ablation in RGCs (using either *Emx1*-Cre or *Nestin*-Cre) causes massive apoptosis during early stages of development, ultimately resulting in a thin, disorganized cortex.^{32,33} Microarray analyses of E16.5 *Nestin*-Cre; *Tra2B*^{lox/lox} cortices identified potential TRA2B splicing targets, however, it remains largely unknown which targets are functionally relevant for development.³²

Another SR rich protein expressed in the developing cortex is NSR100 (SRRM4). NSR100 is required for postnatal viability and development of the corpus callosum.³⁴ During embryonic development, *Nsr100*-deficient mice exhibit shifts in neuronal differentiation, evidenced by an expansion of TBR1+ deep layer neurons and concomitant reduction in SATB2+ upper layer neurons. At E18.5, NSR100 modulates widespread AS, including expression of neuronal microexons, which are short 3- to 27-nucleotide protein-coding exons that can influence protein–protein interactions.^{34,35} As neuronal micro-exons are misregulated in autistic brains, identification of functionally significant microexons in cortical development may be valuable for understanding disease.³⁵

Another splicing regulator which influences neuronal differentiation is SAM68. SAM68 is expressed at peak neurogenesis and required to prevent premature cell cycle exit.^{36,37} In primary cells, SAM68 AS targets include genes involved in neuronal and glial differentiation, as well as the metabolic factor *Aldh1a3*.³⁶ Interestingly, both *Aldh1a3* and *Sam68* influence differentiation by controlling glycolysis. This link between AS, metabolism, and the brain, is strengthened by the discovery of human *de novo* mutations in the splicing factor SON in intellectual disability syndromes.^{38,39} Patients with *SON* loss-of-function mutations exhibit mis-splicing of both metabolic factors and other cortical development regulators, such as *FLNA* and *TUBG1*.³⁹ Thus, both SAM68 and SON exemplify emerging relationships between splicing machinery and metabolic pathways in brain development.³⁶

Beyond proliferation and differentiation, AS is critical during neuronal migration, as evidenced by studies of NOVA2. *Nova2* is expressed in all cortical layers and *Nova2* knockout mice exhibit cortical lamination defects, notably disorganization of late-born upper layer neurons but not early-born neurons.⁴⁰ Importantly, progenitor proliferation and morphology are not disrupted in these mutants, suggesting NOVA2 regulates neuronal migration cell-autonomously, although this has not been formally tested. Use of HITS-CLIP and exon-junction arrays led to discovery of NOVA2-mediated direct and splicing targets, including *Dab1*, which encodes a cytoplasmic-adaptor protein for REELIN (*Reln*) receptors. This pathway was of interest because *Nova2* lamination defects phenocopy *Reln* pathway mutants. The authors overexpressed a *Dab1* isoform in embryonic brains to show that *Dab1* AS is required for neuronal migration. However, this is only one of many NOVA2 AS targets, as recent RNA-seq and HITS-CLIP studies discovered NOVA2 AS targets related to axon guidance.⁴¹ This further supports the notion that NOVA2 is especially important for controlling AS of transcripts encoding regulators of cell migration. Given that *Nova2* is required primarily for migration of late-born neurons, this suggests that other AS-regulatory RBPs may orchestrate migration of early-born neurons.

Another splicing factor implicated in neuronal migration is RBFOX1. *In utero* knockdown of this splicing factor disrupts neuronal organization, likely due to aberrant neuronal migration.⁴² *Rbfox1* is also required post-natally for synapse formation. Given that *RBFOX1* mutations are associated with autism spectrum disorder, it will be of interest to elucidate if either or both of these developmental defects contribute to disease.

From the Nucleus to the Cytoplasm

Some RBPs essential for corticogenesis influence mRNA metabolism in the nucleus and cytoplasm. One such class of proteins are core components of the exon junction complex (EJC), which are implicated in mRNA splicing, NMD, translation, and localization.⁴³ The core EJC is composed of MAGOH, RBM8A, and EIF4A3. The first EJC component implicated in neurogenesis was *Magoh*, deriving from analysis of an N-ethyl-N-nitrosourea (ENU) mouse mutant.⁴⁴ Either germline or conditional *Magoh* haploinsufficiency causes microcephaly and overall neuronal depletion, due to loss of IPs and increased apoptosis.^{44,45} These phenotypes may be explained by prolonged progenitor mitosis, which directly correlates with reduced progenitor generation and increased production of apoptotic progeny in mutants.⁴⁶ *Emx1*-Cre mediated haploinsufficiency for *Eif4a3* and *Rbm8a* causes similar phenotypes to *Magoh*, including fewer RGCs, increased neurons, massive apoptosis, and microcephaly.^{47–49} In contrast, a fourth EJC component, *Casc3*, appears to be largely dispensable for cortical development.⁵⁰ Consistent with overlapping cellular phenotypes of the core EJC components, transcriptome analyses of E10.5 mutant embryonic brains demonstrated common alterations of ribosome, proteasome, and p53 signaling components.⁴⁸ Genetic ablation of *p53* significantly rescued microcephaly of all three EJC mutants, implicating p53 activation as a major node of neurodevelopmental pathogenesis following EJC impairment. Significant AS changes were also observed, with disproportionate AS of RNA-regulatory proteins. This suggests that the EJC could act as a central node of post-transcriptional regulation by influencing other RBPs.

Several fascinating questions regarding the EJC remain to be understood. While genome-wide targets of the EJC are identified in immortalized cells, they have not been deduced in RGCs or neurons, and thus it is unclear if transcripts affected in EJC mutants are subject to direct regulation. Another fascinating question is how the EJC influences mitotic progression and the mechanism by which *p53* loss rescues EJC-mediated microcephaly. Moreover, it is interesting to consider how EJC components cooperate with other RBPs. For example, in proteomics studies in immortalized cells, TRA2B can form a macromolecular complex with the EJC,⁵¹ begging the question of whether these RBPs bind and regulate identical targets. Given that point mutations and copy number variations of EJC components are associated with aberrant brain size, intellectual disability, and brain malformations, continued studies of the EJC are clinically relevant.^{43,52}

Emerging Roles for RNA Localization

RBPs controlling subcellular RNA localization within RGCs indicate that this post-transcriptional regulatory mechanism is relevant for neuronal differentiation. This is evidenced by STAU2, which controls RNA localization in both neurons and RGCs.^{59,60} *Stau2* is expressed in RGCs and asymmetrically segregated to IP daughter cells following mitosis. Depletion of *Stau2*, or other members of its mRNP complex, *Ddx1* and *Pum2*, causes premature differentiation and depletes RGCs. RIP-CHIP and RIP-PCR of E13.5 cortices reveal STAU2 binds transcripts encoding cilia components and fate determinants including *Trim32*, *Prox1*, and *Actb*.^{59,60} Amongst these, asymmetric segregation of *Prox1* mRNA in RGCs is lost following *Stau2* depletion.⁶⁰ These studies raise several intriguing questions, including what additional STAU2 targets contribute to maintenance of RGCs

during corticogenesis? As STAU2 is also expressed in post-mitotic cells, it is interesting to consider how the repertoire of locally regulated mRNAs changes across developmental stages and cell types.

Another RBP implicated in RNA localization is adenomatus polyposis coli (APC). *Apc* is essential for RGC polarity, proliferation, and laminar organization.^{61,62} Although best known as a microtubule plus-end binding protein, it can bind RNA.^{62–64} HITS-CLIP in neocortices uncovered APC-associated transcripts related to microtubule organization, cell motility, and neurological disease.⁶² Blocking APC binding to the 3'UTR of *Tubb2b* leads to defective radial neuron migration *in vivo*, further highlighting APC's function as an RBP. Given the association of *APC* mutations with autism and schizophrenia, it will be fascinating to dissect how and when its roles as a microtubule-binding protein and RBP are important during corticogenesis. Towards this, identification of APC targets specifically in progenitors and neurons will be valuable.

Another RBP strongly implicated in RNA localization in other systems is IMP1 (IGF2BP1/ZBP1). *Imp1* deficiency causes precocious production of IPs at the expense of RGCs, premature differentiation, and microcephaly.⁶⁵ *Imp1* expression is regulated by wingless-type MMTV integration site family member (WNT) signaling and by the miR *Let-7*, illustrating another example of interplay between RBPs and miRs. Because *Imp1* deficiency is also associated with premature glial differentiation, it is proposed to regulate the timing of neuron and glia differentiation. Although IMP1 is best known for roles in RNA localization, its exact post-transcriptional functions and targets in the developing cortex are largely unknown.

An RBP with diverse roles in mRNA metabolism, including RNA localization, is Fragile X Mental Retardation Protein (FMRP) encoded by the gene *Fmr1*. Although best known for its roles in synaptogenesis, FMRP is highly expressed in RGCs and immature neurons. Use of *Fmr1*^{-/-} mice demonstrated roles for FMRP in the multipolar to bipolar transition of migrating neurons.⁶⁶ One key effector molecule of FMRP is *Cdh2*, which encodes N-cadherin. In the absence of *Fmr1*, *Cdh2* mRNA is less stable, and *Cdh2* overexpression rescues loss-of-function migration phenotypes. ShRNA studies also show FMRP represses the transition of RGCs to IPs during early corticogenesis (E11.5–E14.5).⁶⁷ Saffary et al. showed that FMRP-dependent alterations in the actin polymerization factor PFN1 contribute to these defects. It will be of interest to determine if these are due to canonical roles of FMRP in translation.^{68,69} Although progenitor defects were not reported in *Fmr1*^{-/-} germline mice, this may be due to experimental differences or compensatory mechanisms such that acute *Fmr1* depletion is more detrimental than constitutive knockout.

FMRP is also critical for RNA localization of specific transcripts within basal compartments of RGCs.^{70,71} FMRP-bound transcripts enriched in basal RGC endfeet include those associated with the cytoskeleton, signaling, and implicated in autism. In *Fmr1*^{-/-} endfeet, several target transcripts fail to localize, although their steady state RNA levels are unaffected. For example, FMRP controls active transport and localization of a kinesin transcript, *Kif26a*. Understanding the role of FMRP-mediated localization in cortical development is of clinical interest, given that *FMR1* mutations cause fragile-X syndrome.⁷²

Taken together these studies highlight diverse roles for FMRP in stability, localization, and translation.

RNA-Binding Proteins Influencing RNA Stability and Translation

Beyond FMRP, RNA stability, and translation in the developing cortex are controlled by additional RBPs. Hu Antigen R (*Hur*), also known as *Embryonic lethal, abnormal vision-like 1* (*Elavl1*), is highly expressed in both RGCs and upper- and deep-layer neurons. In neuroepithelial progenitors, HUR regulates Notch-Delta signaling, which is predicted to modulate lateral-inhibition, limiting neuronal differentiation.⁷³ In later stages of development, conditional *Hur* depletion causes a thinner cortex and defective neuronal lamination. Using ribosome fractionation followed by mass spectrometry and RNA-sequencing, Kraushar et al. characterized protein composition and mRNA translation in *Hur* mutants.⁷⁴ Notably, the composition of ribosomal proteins within polysomes shifted upon *Hur* loss. Moreover, they noted coordinated HUR-dependent translation of mRNAs, including upregulation of translation-related genes and downregulation of transcription-related genes. In embryonic cortices, HUR binds autism-associated transcripts, including *Foxp1* and *Foxp2*.⁷⁵ It is noteworthy that HUR targets are shared with FMRP, however, how these RBPs coordinate during development is unknown. Together, these findings indicate *Hur* controls translation of a related cohort of transcripts relevant for neuronal fate specification, and lay the groundwork for further investigating *Hur* function in corticogenesis.

Interestingly corticogenesis functions may be specific to select ELAV proteins. For example, the ELAV family member *Hud* primarily controls postnatal development and behavior, including specification and dendritic arborization of a subset of deep-layer neurons.^{76–78} In contrast, another ELAV family member, *Musashi1* (*Msi*), is highly expressed in RGCs⁷⁹ and *Msi1* null brains are hydrocephalic indicating that it may impact cortical development. Indeed, *Msi1*^{−/−} neurospheres indicate *Msi1* promotes progenitor proliferation redundantly with the closely related homolog *Musashi2*.⁸⁰ *In vitro* studies suggest that MSI influences translation of key neurogenic targets, *p21* and *M-Numb*.⁸¹ *MSI* is mutated in human microcephaly, further supporting a role for it in cortical development.⁸² However, it remains to be understood how MSI acts a cellular and molecular level during cortical development.

Canonical translation regulators, including eukaryotic initiation factors (eIFs), are also important for corticogenesis.⁸³ EIF4E1 promotes translation by interacting with EIF4G and represses translation via association with EIF4E-T and P-bodies. *Eif4e1* is expressed in presumed RGCs and its depletion causes premature neuronal differentiation, also evident upon *Eif4e-t* knockdown. In contrast, depletion of the translational activator, *Eif4g*, causes opposite phenotypes. RIP-CHIP of embryonic cortices uncovered translationally repressed EIF4E1 targets, including neurogenic bHLH family transcription factors, such as neurogenins. These targets were translationally de-repressed upon depletion of the *Eif4e1/4e-t* complex. This suggests the fascinating notion that translational repression within progenitors could influence their propensity to differentiate. This theme is likewise supported by studies of the translational repressor, SMAUG2, which controls the transition of RGCs to neurons.⁸⁴ In the developing brain, *Smaug2* loss promotes higher levels of

NANOS1, via translational regulation, and *Nanos1* knockdown causes a reciprocal neurogenesis phenotype to *Smaug2*. Since NANOS1 is also an RBP, this is another example of post-transcriptional feedback mechanisms in place to control fate decisions.

Two homologous RBPs, LIN28A and LIN28B, are expressed in RGCs and implicated in both RNA stability and translation. *Lin28a* knockout mice have reduced RGCs and increased cell cycle exit at E17.5 but not E15.5, suggesting a role in late stage progenitor maintenance.⁸⁵ *Lin28a* overexpression causes reciprocal phenotypes, resulting in a larger brain. Interestingly, LIN28A impacts RGCs independent of its canonical target, *let-7d*. Instead, it modulates cortical development in part by regulating *Igfr2* and *Hmga2*, insulin-mTOR signaling pathway components. LIN28A physically binds IMP1, and although their described mutant phenotypes and functions are somewhat different, this raises the question of whether these RBPs work together to control corticogenesis.

Several RBPs associated with stability and translation are implicated in neuronal migration and morphology. One interesting example is Piwi-like RNA-mediated gene silencing 1 (PIWIL-1). PIWIL-1 is abundant in post-mitotic migratory neurons, where it influences multipolar to bipolar transition, but shows no progenitor defect.⁸⁶ *Piwill*-deficient neurons have altered expression of cytoskeletal genes, including the microtubule-associated protein *Map1b*, whose stability depends upon PIWIL-1. PIWI family members are best known for roles in piRNA processing and transposon activity, and are mutated in autism, making this an important area of future research.

Another RBP implicated in migration is UNKEMPT. *Unkempt* is enriched in post-mitotic neurons, and its depletion disrupts neuronal morphology and migration.⁸⁷ Ectopic *Unkempt* expression in heterologous cells promotes bipolar morphology, a phenotype requiring its RNA-binding domain. In embryonic brains, UNKEMPT binds transcripts encoding ubiquitous pathways including protein metabolism and trafficking. This suggests UNKEMPT may promote a generic cellular morphology program through post-transcriptional regulation. Future studies will be valuable to understand mechanistically how UNKEMPT influences RNA.

Beyond these studies in mice, human genetics studies implicate other factors important for translation and RNA decay in cortical development. For example, mutations in the ribosomal subunit *RPL10* cause syndromic microcephaly and intellectual disability disorder.^{88,89} Additionally, mutations in the genes encoding decapping enzyme, scavenger (DCPS) and enhancer of mRNA decapping 3 (EDC3) have been identified in patients with intellectual disability.^{90,91} Future studies identifying the molecular targets of decapping enzymes and how they impact mRNA homeostasis will yield crucial insights into requirements of RNA decay in corticogenesis.

ROLES FOR MICRORNAS IN CORTICOGENESIS

To date, approximately 2500 miRs have been annotated in mice, with a subset exhibiting distinct temporal and spatial expression patterns in the developing neocortex.^{92–97} miRs were first identified as hetero-chronic mutants with roles in developmental timing in

Caenorhabditis elegans.⁹⁸ miR genes can be clustered within the genome, where multiple miRs are transcribed and processed from a single poly-cistronic transcript, or they can be generated from independent loci. Mature 20–25 nt long miRs are produced by initial cleavage events by the RNase III enzyme, DROSHA, in the nucleus, and then by cleavage via DICER in the cytoplasm.⁹⁸ Following this processing event, these are loaded onto Argonaute protein complex, and then base-pair with specific target RNA sequences primarily within the 3′ untranslated region (UTR). Individual miRs can bind many transcripts, inhibiting their translation or promoting their degradation. miR functions can be evaluated using gain- and loss-of-function approaches, as well as dominant-negative sponges to sequester mature miRs from their mRNA targets.

The first evidence of a role for miRs in cortical development came from analysis of knockout mice for *Dicer*, the essential miR biogenesis factor. Although *Dicer* null mice are early embryonic lethal, use of conditional alleles demonstrated requirements throughout corticogenesis.^{99–102} *Emx1-Cre;Dicer^{lox/lox}* brains, in which *Dicer* is depleted beginning at E10.5^{103,104} are microcephalic, with thinner neuronal layers, particularly upper layers.⁹⁹ These phenotypes were largely corroborated in *Foxg1-Cre* and *Nestin-Cre;Dicer^{lox/lox}* mice,^{97,100} and were attributed to defective proliferation of RGCs and IPs and aberrant neuronal differentiation and apoptosis. Cre electroporation of *Dicer^{lox/lox}* brains at E13.5 led to abnormal postnatal production of neurons, highlighting additional roles for DICER in limiting the duration of neurogenesis.¹⁰⁵ *Dicer* depletion from post-mitotic neurons using *Cam-KII-Cre* drivers also uncovered roles in neuronal polarization and potentially migration.^{101,106} These findings demonstrate that *Dicer*-mediated maturation of miRs is critical for virtually all processes of corticogenesis and laid the foundation for study of specific miRs and their targets. Below we highlight the major miRs characterized to date with roles in cortical development (Table 2).

miRs Implicated Throughout Cortical Development

Amongst miRs critical throughout corticogenesis is *Mir-9*. It is produced from three distinct precursors, *Mir-9-1*, *Mir-9-2*, and *Mir-9-3*, and is first detected at E9.0 in the cortical hem, where it negatively regulates *FoxG1* expression to prevent precocious production of Cajal-Retzius neurons.^{107,108} *Mir-9-2/3* double knockout brains also exhibit reduced progenitor proliferation. This may be attributed to *Mir-9*-mediated inhibition of the transcription factor *Meis2*, which decreases levels of the pro-RGC transcription factor, *Pax6*. *Mir-9* may also control cell fate cooperatively with two RBPs, HUB and MSI, to repress expression of a pro-neurogenic transcription factor *Tlx*. It is noteworthy that *Tlx* is itself regulated by *Let-7d* and also controls *Mir-137*.^{109,110} This suggests that *Mir-9* may be part of a larger miR regulatory network during neuronal differentiation. Beyond these stages, *Mir-9* is expressed in post-mitotic neurons, when it is required for migration, outgrowth, and branching, in part by regulating *Map1b* expression.¹¹¹ Neuronal migration phenotypes in *Mir-9* mutants may also be due to ectopic expression of *Foxp2*, which is normally repressed by *Mir-9* in cooperation with *Mir-132*.¹¹² Taken together, *Mir-9* illustrates complex microRNA regulatory loops, which control proliferation, differentiation, and migration.

Another miR with roles across corticogenesis is *Mir-128*, which is expressed in both progenitors and post-migratory neurons.¹¹³ *Mir-128* overexpression depletes RGCs, and increases IPs and neurons. In contrast, *Mir-128* inhibition *in vivo* generates reciprocal phenotypes.^{114,115} At a molecular level, *Mir-128* suppresses expression of *Pcm1*, a centrosome component, whereas *Pcm1* overexpression rescues *Mir-128* phenotypes. This is notable as centrosome dysfunction is thought to underlie cortical development abnormalities such as microcephaly. Beyond progenitors, premature *Mir-128* expression also disrupts neuronal migration, whereas *Mir-128* suppression promotes migration to upper layer positions. These migration outcomes are due in part to regulation of *Phf6*, a transcriptional repressor involved in neuronal migration and the intellectual-disability disorder Börjeson–Forssmann–Lehmann syndrome.¹¹⁶ Altogether, *Mir-128* exemplifies how miR-mediated control of key targets throughout development could contribute to disease.

The *Mir-17-92* cluster is required for the transition of RGCs to IPs.¹¹⁷ A single polycistronic primary transcript encodes four mature miRNAs, *Mir-17*, *-18*, *-19*, and *-92*. *Emx1*-Cre mediated-ablation of the *Mir17-92* cluster results in fewer RGCs and concomitantly more IPs, ultimately generating fewer neurons and microcephaly. This may be due to translational repression of IP-promoting factors *Pten* and *Tbr2* by *Mir-19* and *Mir-92* respectively.¹¹⁸ *Mir-92* also represses expression of the pro-differentiation factor, *Tis21*.¹¹⁹ Consistent with a functional relationship, deletion of the *Tis21* 3'UTR, which contains binding sites for several miRs including *Mir-92*, reduces IP number and causes microcephaly. *Mir-17* alone can also influence the RGC to IP transition by controlling expression of the cell cycle regulator, *p21*.¹²⁰ Going forward, identification of transcriptome-wide targets of the *Mir-17-92* cluster will be valuable for understanding generation of IPs, a key step of neurogenesis.

In addition to this *Mir-17-92* cluster, additional miRs are implicated in progenitor cell cycle regulation. *Mir-7*, which is produced from three distinct genomic loci, is highly expressed in progenitors.¹²¹ Pollock et al. employed a dominant negative sponge approach using a transgenic mouse to express complementary *Mir-7* seed sequence and sequester *Mir-7* from its targets. These mice showed reduced IPs, increased apoptosis, and microcephaly. *Mir-7* inhibited brains showed upregulation of p53-related genes, including *p21* and *Ccng1*, which were subsequently shown to be direct *Mir-7* targets. Cell cycle control is also mediated by other miRs. For example, *Mir-210* promotes progenitor cell cycle exit and controls cell cycle transcripts such as *Cdk7*.¹²² A cluster of six miRs, *Mir-34/449*, is required for cell cycle progression. Knockout mice have reduced neurons and IPs, and increased RGCs, ultimately leading to microcephaly.^{123,124} *Mir-34/449* represses expression of *junction adhesion molecule-A* (*JAM-A*), of interest given mitotic spindle orientation defects are seen in knockout mice. Altogether, these examples highlight how miRs have been co-opted to control cell cycle progression.

Several additional miRs regulate progenitors and neurons by controlling expression of key signaling molecules. For example, *Mir-29b* influences progenitor regulation by modulating Wnt signaling.¹²⁵ Likewise, *Mir-134*, which is expressed in the developing cortex from early to late stages, promotes progenitor proliferation by controlling expression of its target, *Chrdl-1*, a BMP-signaling antagonist enriched in RGCs.¹²⁶ Beyond BMP signaling, *Mir-134*

also regulates neuron migration through suppression of *Doublecortin (Dcx)*. Thus, similar to *Mir-9* and *Mir-128*, this exemplifies how miR function in different stages of development may be mediated by influencing unique molecular targets.

Several miRs control cortical development by influencing epigenetic regulators. Gain- and loss-of-function manipulations reveal that *Mir-15b* promotes neuronal differentiation and inhibits progenitor proliferation.¹²⁷ Lv et al. used target prediction software and follow up analyses to discover that *Mir-15b* suppresses translation of *Tet3*, an epigenetic chromatin modifier. This was further supported by *in vivo* evidence that *Tet3* overexpression rescues *Mir-15b* progenitor proliferation defects. Another miR implicated in epigenetic regulation is *Mir-137*, described above as a target of *Mir-9/Tlx*. *Mir-137* overexpression promotes premature differentiation and migration, in part due to misexpression of its target, *LSD1*, a lysine-specific histone demethylase.¹¹⁰ Likewise, *Mir-124*, together with *Mir-22*, controls expression of components of the REST/Co-REST transcriptional silencing complex.¹⁰⁶ It has been proposed that *Mir-124* promotes neuronal migration by influencing expression of this complex and the downstream target, *Dcx*. Epigenetic regulation by *Mir-9* and *Mir-124* is likely conserved, as these both control expression of subunits of the chromatin remodeling complex in the developing spinal cord.¹²⁸ As described above, *Mir-124* also promotes differentiation by regulating neuron-specific AS of *Ptbp1*³¹ (Figure 3). Clearly, these studies are at the early stages as we discover miRs controlling cortical development, identify their genome wide targets in progenitors and neurons, and their relationship with RBPs.

CONCLUSION

This review highlights recent progress in understanding the functions of RBPs and miRs in mammalian embryonic cortical development. Several themes characterize this developing field. First, RNA regulation impacts virtually every step of corticogenesis. Additionally, we highlight several examples where RBPs and miRs act cooperatively to directly bind and control target transcripts or to regulate each other through regulatory loops. Another emerging theme is that RBPs and miRs often influence transcripts encoding similar cellular functions, a level of control called an RNA regulon.

In many developmental and homeostatic systems, RNA regulons ensure coordinated activation of gene expression.¹²⁹ RNA regulons may be particularly advantageous for coordinating rapid and fine-tuned events of corticogenesis including cell cycle exit, neuron specification, cell migration, and INM. Going forward it will be exciting to identify potential RBPs and miRs, akin to PAX6 and TBR2 transcription factors, that function in regulons or act as master regulators to control a hierarchy of corticogenesis. This role is exemplified by *Mir-9*, *Mir-124*, and PTBP1, in conversion of fibroblasts to neurons, revealing their instructive roles in the nervous system.^{128,130} Likewise, understanding how RBPs and miRs converge to influence target mRNA expression in the developing cortex will be an exciting future area of research.

As the list of RBPs and miRs implicated in cortical development expands, the field faces several future challenges and directions of interest. While hundreds of RBPs are expressed in the developing brain, to date only a small fraction of these have been functionally

assessed *in vivo*. Likewise, for miRs implicated in corticogenesis, although transcriptome-wide targets have been bioinformatically identified, only selected candidates have been validated. Going forward, it will be critical to identify miR targets *in vivo* with both cell and temporal-specificity. It is notable that many studies of RBPs and miRs have focused on single effector targets, yet it is likely that multiple targets contribute to biological functions. Moreover, recent reports indicate that trophic factors can act on core members of the translation machinery.¹³¹ Another future area of interest is thus understanding how extracellular signals influence post-transcriptional controls.

This review described roles for miRs in RNA stability and translation, and for RBPs in splicing, stability, localization, and translation. Going forward it will be important to consider how additional levels of post-transcriptional regulation, such as RNA editing, N6-methyladenosine RNA modifications, and NMD function in cortical development. Several of these processes, particularly NMD, are already implicated in brain development but to date have been primarily investigated *in vitro*.^{115,132} There are dramatic temporal and spatial transcript changes in the cortex of unknown origin. This is often assumed to be due to transcriptional regulation, however, many of these changes may be controlled at the level of mRNA stability.

Why is post-transcriptional regulation so important for corticogenesis? In other stem cell populations, such as embryonic stem cells and the germline, RBPs and miRs also play critical functional roles, where they help fine-tune and coordinate gene expression.¹³³ Recent studies also suggest that RGCs may be primed for differentiation by the presence of pro-neurogenic transcripts, perhaps maintained in a translationally repressed state.¹³⁴ This idea is ripe for study, and will be further challenged as we discover new RBPs and miRs driving corticogenesis. With development of new sophisticated techniques (Box 1 and Box 2), the converging fields of RNA regulation and corticogenesis are poised to continue to rapidly grow.

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BOX 1 TECHNIQUES IN RNA EXPRESSION AND TARGET IDENTIFICATION

Molecular techniques in RNA biology, previously applied to studies of cultured cells, are increasingly used in the developing neocortex to deduce function of *trans*-regulators. Exon-junction microarrays or RNA sequencing technologies are valuable to understand global alterations in transcript levels. When combined with appropriate bioinformatics analyses, such as mixture of isoforms (MISO) analysis,¹⁶ such datasets can be evaluated for AS, including exon and intron retention and skipping events. Such analyses have traditionally been applied to cell populations, but recent technological advances such as Drop-seq now enable investigators to sequence individual cells to gain single-cell transcriptomic resolution.^{17,18} Identification of direct RNA targets is a critical tool for studies of RBPs and miRs. RNA immunoprecipitation (RIP) is a widely used approach to discover transcripts associated with trans-regulators.¹⁹ This can be used in conjunction with microarray (RIP-CHIP), RNA sequencing (RIP-seq), or polymerase chain reaction (RIP-PCR). Under native conditions targets may be indirect, however, recent protocol modifications employ cross linking and other modifications to identify the RNA sequences bound by a given trans-regulator (HITS-CLIP, iCLIP, and DO-RIP-CLIP).^{20,21} These techniques generate complementary information in terms of affinity and binding sites. For a comprehensive discussion of RNA biology techniques, please see recent reviews.^{20,22}

Box 2 TECHNIQUES IN mRNA LOCALIZATION, STABILITY, AND TRANSLATION

RBPs and miRs control their targets at multiple steps of the mRNA lifecycle including splicing, localization, stability, and translation. Beyond measuring steady-state levels of mRNA expression, new techniques enable investigators to examine individual steps of mRNA metabolism. Subcellular changes in mRNA localization can be measured using fluorescent *in situ* hybridization of individual targets. Additionally, multiplexed *in situ* using barcodes can be applied for widespread investigation of mRNA localization.⁵³ RNA stability and decay have been typically measured in immortalized cells with pulse-chase of radioactive nucleotide analogs. For applications *in vivo*, it may be possible to use uridine-analog nucleotides that can be detected by sequencing.⁵⁴ A number of outstanding methods are available for monitoring translation. These include mini-gene assays in which luciferase reporters are used to monitor translation of candidate sequences. Another traditional translational approach is polysome profiling, in which RNA-containing fractions of monosomes and polysomes are collected and quantified by PCR or genomic approaches. Recent development of ribosome footprinting is advantageous for elucidating exact ribosome binding sites on transcripts of interest, and for differentiating between translation initiation and elongation.⁵⁵ TRICK, or Translating RNA Imaging by Coat-protein Knockoff, is another imaging-based technique for monitoring translation initiation, and may enable study of localization and frequency of translation initiation events *in situ*.⁵⁶ Non-canonical amino acid labeling can also be employed to visualize sites of protein synthesis⁵⁷ and to monitor overall translational output *in vivo*.⁵⁸ Photo-convertible proteins are also valuable for visualizing translation.^{70,71} Future applications of these techniques will yield new insights into post-transcriptional control during cortical development.

FIGURE 1.

Cartoon representation of RNA regulation in cortical development. (a) Cartoon representing embryonic brains from about E11.5 and E15.5, with a coronal section shown on the right. (b) Left, cartoon represented region indicated in (a), highlighting a radial glial cell (RGC, blue). Right, cartoon-depicting roles for RBPs (hexagons) in alternative splicing in the nucleus, and translation, localization, and stability in the cytoplasm. microRNAs (red/black lines) work in the cytoplasm to degrade and translationally repress mRNAs.

FIGURE 2.

Post-transcriptional regulation during corticogenesis. (a) Three stages of corticogenesis discussed in this review. Early in corticogenesis, radial glial cells (RGCs, green) undergo symmetric, self-renewing divisions to expand the progenitor pool. Later, RGCs divide asymmetrically to generate a more differentiated cell. RGCs generate excitatory neurons (blue) either directly or indirectly through intermediate progenitors. New born neurons polarize and migrate along the RGC scaffold to reach their final layer in the cortical plate. (b) Trans-regulators implicated in each process. (c) Classes of target transcripts.

FIGURE 3.

An example of a regulatory network in which microRNAs (miRs) and RNA-binding proteins (RBPs) participate in neuronal differentiation and progenitor proliferation. miR-124 is expressed at low levels in radial glial cells (RGCs), allowing for translation of polyprimidine tract-binding variant (PTBP1). PTBP1 activity leads to production of a *Ptbp2* splicing isoform fated for decay. Low levels of PTBP2 result in exclusion of neuron-specific exons in targets, and ultimately repression of neuronal differentiation in progenitors. In the presence of miR-124 in post-mitotic neurons, PTBP1 is translationally repressed, allowing for splicing of functional PTBP2 and inclusion of neuron-specific exons and differentiation. This illustrates how RBPs and miRNAs work co ordinally to post-transcriptionally regulate mRNAs.

TABLE 1

RBPs Involved in Cortical Development

RBP	Best Known Function	Role in Cortical Development	Key Target Genes and Pathways	In Vivo Genomic Analyses	References
APC	Localization	Neuron migration and RGC polarity	<i>β2B-tubulin</i> , microtubule assembly pathway	HITS-CLIP	61,62
EIF4A3	Splicing, translation, stability, localization	RGC proliferation, differentiation, and survival	Ribosome pathway, p53 pathway	RNA-seq	48
EIF4E1, EIF4ET	Translational repressor	Prevent differentiation	Transcriptional factors, <i>neurogenins</i>	RIP-CHIP	83
EIF4G	Translational activator	Promote differentiation	—	—	83
FMRP	Localization, translational repressor	IP transition, neuron migration and polarity	<i>Cdh2</i> , microtubule and signaling associated factors	—	66,67,71
HUR	RNA stability, translation	Progenitor proliferation, neuron lamination	<i>Foxp1</i> , <i>Foxp2</i> , transcript and translational pathways	RIP-CHIP, polysome profiling, RNA-seq	73–75
IMP1	Localization	Progenitor maintenance and differentiation	<i>CyclinD1</i> , <i>Hnga2</i>	—	65
LIN28	Noncoding RNA	Progenitor proliferation	<i>HMG2</i> , <i>IGF1R</i> , insulin-mTOR signaling pathway	—	85
MAGOH	Splicing, translation, stability, localization	RGC proliferation, mitosis, and survival	Ribosome pathway, p53 pathway	RNA-seq	44,46,48,50
MUSASHI	Translation, stability	Differentiation	<i>p21</i> , <i>M-Numb</i>	—	79–82
NOVA2	Splicing	Neuron migration, axon pathfinding	<i>Dab1</i> , axon growth pathway	HITS-CLIP, RNA-seq	40,41
NSR100	Splicing	RGC maintenance, differentiation, neuron maturation	<i>Unc13b</i> , vesicle transport pathway, neurotransmitter secretion pathway, cell projection pathway	RNA-seq	34
PIWIL1	Noncoding RNA	Neuron migration and polarity	<i>Map1b</i>	RNA-seq	86
PTBP1	Splicing	RGC polarity, maintenance, neuron differentiation	<i>Pbp2</i> , <i>Filamin</i> , cell adhesion pathway, synaptogenesis pathway, neurogenesis pathway	RNA-seq	15,26,27
PTBP2	Splicing	RGC polarity, maintenance, neuron differentiation	proliferation and cell fate, actin cytoskeleton , neuron differentiation pathways	HITS-CLIP, exon-exon microarray	28–31
RBFOX1	Splicing	Neuronal migration	—	—	42
RBM8A	Splicing, translation, stability, localization	RGC proliferation and survival	Ribosome pathway, p53 pathway	RNA-seq	47–49
SAM68	Splicing	RGC proliferation, neuron differentiation	<i>Aldh1a3</i> , neuronal differentiation pathway, oligodendrocyte differentiation pathway	Exon-exon microarray	36,37

RBP	Best Known Function	Role in Cortical Development	Key Target Genes and Pathways	In Vivo Genomic Analyses	References
SMAUG2	Translation	Progenitor maintenance, differentiation	<i>Nanos1</i>	—	84
STAU2	Translation	RGC maintenance, differentiation	<i>Trim32</i> , <i>Prox1</i>	RIP-CHIP	59,60
TRA2B	Splicing	RGC survival	<i>Tubd1</i> , <i>Sgol2</i>	Microarray	32,33
UNKEMPT	Unknown	Neuronal morphology	Protein trafficking and metabolism pathway	iCLIP	87

IP, intermediate progenitors; RBP, RNA-binding protein; RGC, radial glial progenitor cells.

Bold denotes direct target.

TABLE 2

miRNAs Involved in Cortical Development

miRNAs	Role in Cortical Development	Key Target Genes and Pathways	References
miR-9	Specification of Cajal-Retzius neurons, progenitor proliferation, neuron migration	<i>Foxg1, Tlx, Pax6, Foxp2, Map1b, Meis2</i>	107,108,111,112
let-7	Progenitor proliferation	<i>Tlx</i> , IMP1	109
miR-17-92	Progenitor proliferation and survival, transition from RGC to IP, progenitor proliferation and differentiation	<i>p21, Tbr2, Tis21, Pten</i>	117–120
miR-7	Progenitor proliferation and survival	<i>p21, Ccng1</i> , p53 pathway	121
miR-210	Progenitor proliferation and differentiation	<i>Cdk7</i>	122
miR-134	Progenitor proliferation, neuron migration	<i>Chrdl-1, dcx</i>	126
miR-137	Progenitor proliferation	<i>Lsd1</i>	110
miR-34/449	Progenitor mitotic spindle orientation, proliferation	<i>JAM-A</i>	123,124
miR-15b	Neuronal differentiation	<i>Tet3</i>	127
miR-124	Neuronal differentiation, neuron migration	<i>Ptbp1, Rcor1 (coREST), Dcx</i>	31,106
miR-22	Neuron migration	<i>Rcor1 (coREST)</i>	106
miR-128	Neuron migration, neuronal differentiation	<i>Phf6, Pcm1</i>	113–115
miR-29b	Progenitor proliferation	Wnt signaling pathway	125

IP, intermediate progenitor; miRNAs, microRNAs; RGC, radial glial progenitor cells.

Bold denotes direct target.