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Regulation of microRNA-124 biogenesis during human neuronal development

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Regulation of microRNA-124 biogenesis during human neuronal development

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ABSTRACT

Regulation of microRNA-124 biogenesis during human neuronal development

By Izabela Suster

Precise renewal and differentiation of multipotent neural progenitor cells (NPCs), the cells of the central nervous system that differentiate into neurons and certain glial subtypes, must be tightly controlled for normal brain development and function. Abnormalities in NPC renewal and neuron-glia lineage establishment are increasingly being recognized as contributors to the pathogenesis of neuropsychiatric diseases, such as schizophrenia and major depression. A number of neuronal-lineage specific microRNAs (miRNAs/miRs), represented by brain-enriched miR-124, have been shown to promote differentiation of NPCs. Despite the well-studied multifaceted regulation of miR-124 biogenesis in rodent neurons, molecular mechanisms that control miR-124 biogenesis during human neuronal development remain largely undefined. In this dissertation, the transcriptional and posttranscriptional mechanisms regulating the biogenesis of pro-neurogenic miRNA-124 in human NPCs (hNPCs) and neurons are explored.

Human and mouse miR-124 are encoded by three distinct loci that give rise to three primary miR-124 transcripts, pri-miR-124-1, -2 and -3, all of which can be processed into mature miR-124. We examined the expression profile of the human pri-miR-124 paralogs in early neurodevelopment. We show that in contrast to mouse embryonic stem cells, which predominantly express pri-miR-124-1, hNPCs predominantly express pri-miR-124-2. We identified a human-specific *cis* regulatory element proximal to the miR-124-2 host gene promoter, which undergoes a developmental change in chromatin

accessibility and scaffolds transcriptional activators and repressors to regulate transcription of the miR-124-2 host gene during neuronal differentiation.

We next explored posttranscriptional regulation of pri-miR-124-2 at the Microprocessor cleavage step. We discovered that pri-miR-124-2 harbors a binding site for the RNA-binding protein, Quaking (QKI), which is selectively expressed in NPCs and glia. Our data demonstrate that elimination of the nuclear QKI isoform in a hNPC cell line model increases levels of mature miR-124. Furthermore, we detect rapid downregulation of QKI upon hNPC differentiation into neurons, which should lead to increased miR-124 production. Together, these studies define novel mechanisms that underlie miR-124 biogenesis to advance early human neuronal development.

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Table of Contents

Abbreviations

AD	Alzheimer's Disease
AGO	Argonaute
ASD	Autism spectrum disorders
ATAC	assay for transposase-accessible chromatin
BAF	Brg/Brm-associated factor
BRE	TFIIB recognition element
CAGE	cap analysis of gene expression
ChIP	chromatin immunoprecipitation
circRNA	circular RNA
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CTD	carboxy-terminal domain
DGCR8	DiGeorge syndrome critical region 8
DPE	downstream promoter element
EB	embryonic body
EMX1	empty spiracles homeobox 1
ENCODE	Encyclopedia of DNA Elements
ESC	embryonic stem cell
EZH2	Enhancer of zeste homolog 2
FANTOM5	Functional Annotation of the Mammalian genome, 5th edition
FOXG1	Forkhead box G1
gRNA	guide RNA

HD	Huntington's disease
hNPC	human neural progenitor cell
lncRNA	long non-coding RNA
lnc-pri-miR	long-noncoding RNA containing miRNA
Inr	initiator
iPSC	induced pluripotent stem cell
M17	BE(2)-M17 cell line
m^6A	N^6-methyladenosine
MAFK	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K
MDD	major depressive disorder
METTL3	methyltransferase-like 3
miR-124	microRNA-124, miR-124-3p
miR-124*	microRNA-124*, miR-124-5p
MIR124-2HG	MIR124-2 Host Gene
mESC	mouse embryonic stem cell
mNPC	mouse neural progenitor cell
NEAA	non-essential amino acids
NPC	neural progenitor cell
OTX2	Orthodenticle homeobox 2
PAX3	Paired box 3
PAX6	Paired box 6
PNS	peripheral nervous system
PPE	proximal promoter element

Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNA
PTBP1	polypyrimidine tract binding protein
QKI	Quaking I
QRE	Quaking response element
RA	*trans*-retinoic acid
RBP	RNA binding protein
RE1	repressor element 1
REST	repressor element 1 silencing transcription factor
RhoG	Ras homolog family member G
RIP-seq	RNA-interacting protein immunoprecipitation sequencing
RISC	RNA-induced silencing complex
RNAPII	RNA Polymerase II
Rncr3	Retinal non-coding RNA 3, murine miR-124-1 host transcript
SCP1	small C-terminal domain phosphatase 1
SCZ	schizophrenia
siRNA	short interfering RNA
SP1	specificity protein 1
TF	transcription factor
TSS	transcription start site
USP14	ubiquitin-specific protease 14

Chapter 1: Introduction to Dissertation

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Introduction

MicroRNAs (miRNAs) are a class of small, non-coding RNAs (20-24 nt) that suppress mRNA targets through mRNA degradation and translational repression (Jonas and Izaurralde, 2015). Mature miRNAs are highly conserved across species and broadly expressed but display variable tissue-specific abundance, with a subset of miRNAs enriched in or specific to the brain (Lagos-Quintana et al., 2002; Sempere et al., 2004). As such, the expression profiles and function of brain miRNAs in normal neuronal development have been the focus of numerous investigators in the past decades. From the resultant studies, neural-specific/brain-enriched miRNAs have been demonstrated to govern all stages of neuronal lineage development, from proliferation of neural progenitor cells (NPCs), neuronal fate commitment, morphological differentiation and circuitry assembly to synaptic plasticity (Fiorenza and Barco, 2016; Loya et al., 2010; Ma et al., 2019; Pham and Gallicano, 2012). Despite the large volume of discoveries that document the essential roles of miRNAs in brain development and function, molecular mechanisms that precisely regulate miRNA biogenesis in neuronal development and thus impact normal and diseased brains have just begun to unfold. Additionally, how distinct miRNAs converge on downstream molecular networks to advance neuronal development is a prevailing research question under active investigation. Here we provide an up-to-date and comprehensive review of the recent advancements and outstanding questions regarding mechanisms that control miRNA biogenesis and functional abundance in neuronal development, as well as how diverse neural miRNAs converge on common downstream molecular networks.

1.1 Canonical and non-canonical miRNA processing pathways

1.1.1 The canonical miRNA biogenesis pathway

Decades of investigation have established a canonical miRNA biogenesis pathway beginning with the transcription of a miRNA host gene by RNA polymerase II (RNAPII) to produce a primary miRNA (pri-miRNA) transcript (Lee et al., 2004). Pri-miRNAs contain a local stem-loop structure that encodes miRNA duplexes in the arm of the stem. Cleavage of the stem-loop by the RNase III enzyme Drosha and its RNA-binding protein cofactor DiGeorge Syndrome Critical Region 8 (DGCR8), together termed the Microprocessor complex, produces a 60-80nt stem-loop/hairpin intermediate known as the precursor miRNA (pre-miRNA) (Denli et al., 2004; Lee et al., 2003). Nuclear export of the pre-miRNA is facilitated by Exportin 5 and Ran-GTP (Lund et al., 2004). In the cytoplasm, a second RNase III enzyme, Dicer, cleaves the pre-miRNA terminal loop to produce a miRNA duplex (Bernstein et al., 2001; Hutvagner et al., 2001). Each miRNA duplex produces two mature miRNAs: one from the 5' stand and one from the 3' strand. Using miR-124 as an example, these miRNAs are termed miR-124-5p and miR-124-3p, respectively. Generally, one of the arms/strands of the duplex, namely the guide strand, is preferentially loaded onto Argonaute (AGO) to form the RNA-induced silencing complex (RISC), which acts as an endogenous gene silencer directed by miRNA base-pair complementarity with the 3'UTR of target mRNAs (Kobayashi and Tomari, 2016; Mourelatos et al., 2002). The other strand of the miRNA duplex, the passenger strand denoted as miRNA*, is often found in much lower abundance (Ha and Kim, 2014). Nonetheless, mutually exclusive expression of guide and passenger strands is rarely observed (Biasiolo et al., 2011). For certain miRNA species, the passenger strand is not

subjected to rapid degradation but rather, may acutely target mRNAs and play functionally important roles. A notable example is miR-9-3p (miR-9*), which targets anti-neural transcriptional machinery (Packer et al., 2008; Yoo et al., 2009).

Mounting evidence has demonstrated the crucial roles of the canonical miRNA biogenesis enzymes in proper brain development and function. Conditional knockout of *Drosha* in mouse NPCs resulted in a loss of multipotency status and induced precocious neuronal differentiation, which was phenocopied by depletion of DGCR8 (Knuckles et al., 2012). Dicer depletion profoundly impairs the morphological and proliferative characteristics of NPCs (Nigro et al., 2012), mature neurons (Chmielarz et al., 2017; Davis et al., 2008; O'Toole et al., 2017), oligodendroglia (Shin et al., 2009), microglia (Varol et al., 2017) and astrocytic glia (Sun et al., 2019; Tao et al., 2011). Developmental regulation of canonical miRNA biogenesis enzymes in human neurons has not been precisely characterized. In mature neurons, Drosha is restricted in the soma while Dicer and RISC components are found in distal dendrites and axons (Kim et al., 2015; Lugli et al., 2005), potentially allowing for spatially-restricted maturation of miRNAs. Indeed, a recent study demonstrated biogenesis of mature miRNAs from fluorescently labeled pre-miRNAs in neuronal dendrites upon synaptic stimulation, which reduced local protein synthesis of predicted mRNA targets (Sambandan et al., 2017). Thus, despite the ubiquitous expression of the canonical miRNA biogenesis machinery, neurons harbor more sophisticated spatial regulation of canonical miRNA biogenesis that govern the development and function of the normal brain.

1.1.2 Emerging roles of non-canonical miRNA biogenesis pathways in the central nervous system

Alternative noncanonical pathways for miRNA biogenesis exist, which can bypass steps of the aforementioned canonical pathway. Mechanisms for both Drosha-independent and Dicer-independent miRNA biogenesis have been reported, reviewed by Yang and Lai (Yang and Lai, 2011). Mirtrons, derived from splicing of short introns that carry pre-miRNA-like structures, are recognized and processed by Dicer, constituting one group of Drosha-independent noncanonical miRNAs. Of note, a number of mirtron-derived miRNAs are highly expressed in the mouse hippocampus and cerebral cortex, including miR-877 and miR-1981 (Babiarz et al., 2011). On the other hand, Drosha/DGCR8-dependent but Dicer-independent miRNA biogenesis has also been observed, represented by miR-451 (Cheloufi et al., 2010; Cifuentes et al., 2010; Yoda et al., 2013). Mechanistically, the endonucleolytic slicer activity of Ago2 cleaves the pre-miR-451 hairpin into a ~30 nt intermediate RNA in the cytoplasm, which is trimmed into a conventional ~22 nt miRNA by 3′-exonucleolytic activity of the poly(A)-specific ribonuclease (PARN) (Yoda et al., 2013). Notably, miR-451 was reported to drive glioma tumorigenesis (Nan et al., 2010; Zhao et al., 2017). Moreover, miR-451 exerts neuroprotective effects against cerebral ischemia/reperfusion injury in stroke patients (Fu et al., 2019). These emerging studies suggest potential roles of non-canonical miRNA-biogenesis pathways in normal and diseased brains. Lastly, another class of Dicer-independent miRNA-like molecules are derived from Argonaute-associated short introns of 80-100 nucleotides, termed agotrons, which are stabilized by AGO proteins and

capable of repressing mRNAs via sequence seed-matching in the 3'-UTR of target genes (Hansen et al., 2016).

1.2 MiRNAs play key roles in governing neuronal development

1.2.1 The most abundant miRNA in the brain: miR-124 and its anti-neurogenic targets

The mature miR-124 (miR-124-3p) is one of the most well-characterized pro-neurogenic miRNAs, which governs various aspects of neuronal development and function, including neurogenesis, neuronal network assembly and synaptic plasticity (Cheng et al., 2009; Hou et al., 2015; Kutsche et al., 2018; Makeyev et al., 2007). The sequence of miR-124 is highly conserved from worms to humans (Guo et al., 2009). Brain-specific expression of miR-124 was identified in the first large-scale vertebrate miRNA expression profiling panel (Lagos-Quintana et al., 2002). Brain-specific expression of miR-124 in humans has also been observed (Sempere et al., 2004). During neuronal maturation, miR-124 is minimally expressed in NPCs and immature neurons but is drastically up-regulated to become the most abundantly expressed miRNA in the adult murine brain, accounting for an estimated 25% to 48% of all brain miRNAs (Deo et al., 2006; Lagos-Quintana et al., 2002; Smirnova et al., 2005). Expression of miR-124 has been detected as early as E11.5 in the CNS of mouse embryos (Deo et al., 2006). In contrast to the functional miR-9* mentioned above, miR-124-5p (miR-124*) is not appreciably expressed in the embryonic CNS, suggesting that miR-124* does not play a significant role in pre-natal neurodevelopment (Deo et al., 2006). Analysis of a transgenic miR-124 activity sensor mouse model at E13.5 and in adult brain supported CNS neuron-

specific miR-124 expression, with no activity detected in astrocytes, microglia and the peripheral nervous system (PNS) (Akerblom et al., 2012).

The abundant pool of miR-124 generated in the CNS targets numerous well-defined repressors of neuronal differentiation, including but not limited to RhoG, PAX3 and BAF53a (Franke et al., 2012; Wei et al., 2018; Yoo et al., 2009). Of note, miR-124 has also been reported to target anti-neural epigenetic regulator, histone methyltransferase EZH2, to be discussed in more detail in a subsequent section. Importantly, miR-124 targets master repressors of neuronal-specific gene expression at both the transcriptional and posttranscriptional levels. More specifically, miR-124 targets small C-terminal domain phosphatase 1 (SCP1), associated with the anti-neural repressor element 1 (RE-1)-silencing (REST) transcription factor and the well-characterized splicing factor polypyrimidine tract binding protein (PTBP1/PTB/ hnRNP I) (Makeyev et al., 2007; Visvanathan et al., 2007). Interestingly, REST and PTBP1 regulate expression of miR-124 host genes and processing of miR-124 precursors, respectively. In mouse primary cortical progenitors, REST targets all three miR-124 loci for transcriptional silencing, forming a double-negative miR-124-REST/SCP1 feedback loop (Conaco et al., 2006). In mouse neuroblastoma cell lines, miR-124-mediated repression of PTBP1 initiates a transition to alternative splicing and biogenesis of neuronal specific mRNAs (Makeyev et al., 2007). In 2018 Yeom *et al.* elucidated a negative feedback loop between these two molecules, whereby PTBP1 binding and blockade of DROSHA/DGCR8 dependent cleavage of pri-miR-124-1 represses miR-124 biogenesis in mouse embryonic stem cells (mESCs) (Yeom et al., 2018). An additional layer of complexity is the reported competition between PTBP1 and miR-124 for miRNA target

sites in the 3'UTR of the *SCP1* gene in HeLa cells (Xue et al., 2013). Thus, a secondary source of miR-124 or miRNA targeting REST and/or PTBP1, or both, must trigger downregulation of these molecules and break the aforementioned negative feedback loops to allow for neuronal lineage commitment (Figure 1-1).

An early study found that *in vitro* overexpression of miR-124 duplexes and inhibition by anti-miR-124 2'-O-methyl oligonucleotides in murine neural precursors had no significant effects on the neuron/astrocyte ratio ($Tuj1^+/GFAP^+$) during lineage differentiation (Krichevsky et al., 2006). However, recent miR-124 deletion studies performed in human and mouse models have reported modest neural lineage commitment impairment. Deletion of all miR-124 encoding alleles in human induced pluripotent stem cells (hiPSCs) and *in vivo* inhibition of miR-124 in neonatal mouse brains reduced neuronal lineage commitment and neurogenesis, respectively (Akerblom et al., 2012; Kutsche et al., 2018). In the mouse model, the authors observed increased gliogenesis in the adult olfactory bulb (Akerblom et al., 2012). One mechanism by which miR-124 is reported to orchestrate the fate between neuronal and glial differentiation is through regulation of EZH2 expression, either directly or indirectly through USP14 (Lee et al., 2018; Neo et al., 2014). The discrepancies regarding whether miR-124 is essential in neuron-glia lineage establishment, between these studies, may be due to the experimental models utilized.

1.2.2 Convergence of distinct pro-neurogenic miRNAs on key inhibitors of neuronal differentiation

The modest effects of miR-124 deletion on neuronal lineage development (Akerblom et al., 2012; Kutsche et al., 2018) supports the theory that multiple neuronal

miRNAs may orchestrate neuronal differentiation by acting in a cooperative fashion to target master regulators of transcriptional and post-transcriptional programs. Theoretically, convergence of miRNAs on key mRNA targets increases the robustness of developmental programs while simultaneously preventing aberrant differentiation. Additionally, coordinated miRNA targeting may help ramify the cellular pool of individual miRNAs amongst hundreds of target mRNAs (Lim et al., 2005). Evidence supporting a cooperative neuronal miRNA network has begun to emerge as miR-128, -124 and -137 have been proposed to serve as a triad of pro-neurogenic miRNAs with extensive overlap in key predicted anti-differentiation transcription factor (TF) targets (Santos et al., 2016). Analysis of overlap in differentially expressed targets under antagomir treatment of each of the above miRNAs identified specificity protein 1 (SP1), a well-studied transcriptional activator, as a central node at which all three miRNA target networks converge. Supporting this finding, miR-124 has been shown to target *SP1* for downregulation during neurogenesis (Mondanizadeh et al., 2015). Additionally, in non-neuronal systems, miR-128 and miR-137 have been shown to directly target SP1 (Dai et al., 2016; Dong et al., 2014; Zeng et al., 2016).

Despite the fact that miR-124 deletion alone failed to significantly impact neuron-glia fate in cultured neural precursors (Krichevsky et al., 2006), miR-124 can promote neuronal lineage establishment in tandem with miR-9/9*. Simultaneous overexpression of miR-9/9* and miR-124 duplexes in neural precursors caused a significant reduction in astrocytic $GFAP^+$ cells, thereby increasing the neuron/glia ratio ($Tuj1^+/GFAP^+$) upon differentiation *in vitro*, compared to control. These effects on cell differentiation were not observed when either duplex was delivered separately (Krichevsky et al., 2006),

highlighting the combinatorial potency of neural miRNAs to advance differentiation. Later, Yoo *et al.* elucidated the molecular mechanism underlying the pro-neurogenic role of miR-9/9* and -124. Ectopic expression of miR-9/9* and miR-124 directly reprograms primary human dermal adult fibroblasts into functional neurons by targeting a subunit of the Brg/Brm-associated factor (BAF) chromatin remodeling complex, BAF53a, of the neural-progenitor-specific BAF (npBAF53) complex (Yoo et al., 2011), allowing for de-repression of neuron-specific homolog BAF53b.

Analysis of multiple independent miRNA studies establishes the repressor element 1 (RE-1)-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) complex as another anti-neural transcriptional hub (Ballas et al., 2005) at which brain-enriched miRNAs seemingly converge. This complex is comprised of the namesake RE1 silencing transcription factor (REST) which binds to the 23bp repressor element 1 (RE1) element and co-factors mSin3A and CoREST (Andres et al., 1999; Grimes et al., 2000). The REST complex recruits histone deacetylases (HDACs) to actively repress transcription of neuronal genes (Ballas et al., 2005). Neural miR-9 and -9* have been shown to target REST and CoREST, respectively (Packer et al., 2008). Additionally, as discussed earlier, miR-124 targets SCP1 (Visvanathan et al., 2007), which is recruited to genes harboring RE1 elements to silence neuronal genes (Yeo et al., 2005). In addition to directly targeting components of the REST complex, miR-9/9* and -124 target ubiquitin-specific protease 14 (USP14), which leads to the destabilization and repression of EZH2 and subsequently, destabilization of REST (Lee et al., 2018). Additional upstream targeting of the REST complex by miR-9/9* and -124 may serve as an additional mechanism to enforce acquisition of neural fate. In addition to driving neural-specific

transcription networks, neural miRNAs may additionally converge on a broad spectrum of targets to drive pro-neural posttranscriptional networks, but this paradigm remains unexplored.

1.3 Neural miRNA multigene families

Numerous miRNAs that control neuronal development are derived from multigene families, represented by miR-9, -128, -125, and -124 (Rodriguez et al., 2004). In contrast, miR-137 is encoded by a single gene, which harbors numerous genetic alterations that are causatively associated with a number of neuropsychiatric diseases (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Willemsen et al., 2011). Therefore, miRNA multigene families may represent an evolutionary fail-safe to protect against perturbations/mutations in an essential miRNA gene leading to disease. Additionally, parallel transcription and processing of the same miRNA from different genes allows for robust upregulation and abundant expression of the miRNA, by overcoming the limiting rate of RNAPII transcription of a single gene. Another potential advantage of miRNA multigene families is to allow for different spatiotemporal expression of individual miRNA paralogs in different neuronal cell types or even within the same neuron, possibly through different rates of transcription and/or processing efficiencies. Excitingly, a recent study by Bofill-De Ros *et al*. reported that pri-miR-9-1 undergoes alternative cleavage by Drosha to generate an isomiR with a unique 5’ end and seed sequence (Bofill-De Ros et al., 2019). This finding indicates that miRNA paralogs within the same multigene family can produce two distinct miRNAs from the same pri-miRNA, which may be functionally advantageous in different cellular contexts.

The exemplary miR-124 multigene family undergoes cell type- and developmental stage-specific regulation of miRNA paralogs to drive neuronal differentiation. Both human and mouse miR-124 are encoded by three paralogous genes, giving rise to pri-miR-124-1, -2 and -3 (Deo et al., 2006; Rodriguez et al., 2004). In the mouse genome, the host transcripts of *miR-124-1* (*Retinal non-coding RNA 3*, *Rncr3*) and *miR-124-2 (Mir124-2host gene, Mir124-2hg)* have been annotated, while *miR-124-3* is intergenic. In the human genome, only *miR-124-2* (8q12.3) is annotated as an intronic miR of a long-noncoding pri-miRNA (lnc-pri-miR) (Dhir et al., 2015), while *miR-124-1* (8p23.1) and *miR-124-3* (20q13.33) are intergenic. As a multigene family with paralogous miRNAs located in distant regions and/or different chromosomes, the miR-124 paralogous genes likely arose from a non-local gene duplication event during vertebrate evolution (Figure 1-2) (Berezikov, 2011). Despite differences in chromosomal location and genomic position, processing of all three miR-124 paralogs is Drosha, DGCR8 and Dicer dependent (Babiarz et al., 2008; Kim et al., 2017), indicating that each miR-124 paralog undergoes canonical miRNA processing.

In mouse embryonic stem cells (mESCs), pri-miR-124-1 is the predominantly expressed pri-miR-124 paralog (Yeom et al., 2018). The expression of pri-miR-124-1 continues to increase as mESCs are differentiated into cortical neurons (Yeom et al., 2018). Similarly, pre-miR-124-1 is abundantly expressed in the midbrain, frontal- cortex, cerebellum and hippocampus of human adults (Landgraf et al., 2007). Homozygous deletion of the miR-124-1 host transcript (*Rncr3*$^{-/-}$) in mice results in increased neuronal apoptosis, axonal mis-sprouting and an overall smaller brain size (Sanuki et al., 2011), demonstrating that miR-124-1 is necessary for proper murine brain development. Indeed,

pri-miR-124-1 is the predominantly expressed paralog in P6 mouse hippocampus, cortex and retina (Sanuki et al., 2011). Similar to pri-miR-124-1, pri-miR-124-2 increases from mESCs to cortical neurons (Yeom et al., 2018), which, in tandem with pri-miR-124-1 upregulation, may underlie the robust increase in mature miR-124 observed in differentiating neurons. The role of pri-miR-124-3 in neural lineage has not been characterized as reports show this transcript is negligibly expressed in mESCs and minimally expressed in cortical neurons (Yeom et al., 2018). Whether similar or distinct regulation of miR-124 paralogs occurs during human neuron development, as compared to that in rodent, has not been reported.

1.4 Regulation of miRNAs in neural development: divergence by biogenesis and convergence on key targets

1.4.1 Transcriptional regulation of miRNA genes

As miRNA host genes are primarily transcribed by RNAPII, pri-miRNAs are generally capped and polyadenylated (Cai et al., 2004) (Figure 1-3). Important exceptions are long non-coding transcripts containing miRNAs (lnc-pri-miRNAs), which were found to be predominantly non-polyadenylated (pA^-) (Dhir et al., 2015). Processing of pri-miRNAs is coupled to RNAPII transcription, with pri-miRNA processing occurring co-transcriptionally, prior to splicing (Morlando et al., 2008; Pawlicki and Steitz, 2008; Yin et al., 2015). Due to the functional integration of these processes, pri-miRNAs generally do not accumulate in the nucleus and are present in low abundance at steady-state. Identification of miRNA gene promoters and transcription start sites (TSSs) is challenging and therefore, incomplete.

Ozsolak and colleagues reported that the promoters of RNAPII-transcribed miRNAs share the same features as protein-coding gene promoters, namely a CpG island and eukaryotic core promoter elements including a TATA box, TFIIB recognition element (BRE), initiator (Inr) and downstream promoter element (DPE) (Ozsolak et al., 2008) (Figure 1-3). Of note, these eukaryotic promoter elements are present only in the minority of miRNA promoters, hence a genome-wide analysis of these elements will preclude the majority of miRNA gene promoters (Ozsolak et al., 2008). Previous studies have systematically characterized miRNA host gene TSSs and promoters by RNAPII ChIP and histone modification ChIP, namely trimethylation of Lys4 of histone H3 (H3K4me3) (Marson et al., 2008; Wang et al., 2010). H3K4me3 modifications deposited around the TSS of miRNA genes are indistinguishable from protein-coding TSSs (Ozsolak et al., 2008). Functionally, this modification regulates transcription of protein-coding and miRNA genes by a similar mechanism as reported by genome-wide analysis of ChIP-seq data in mouse ESC and NPCs cells (Cheng and Gerstein, 2012). These studies reveal an intricate regulatory feedback loop between miRNAs and epigenetic machinery when considered in tandem with neural miRNA targeting of epigenetic regulators discussed in section **1.2.2**.

As the majority of pri-miRNAs harbor a 5' cap, these transcripts are also candidates for cap analysis of gene expression sequencing (CAGE-seq) (Marsico et al., 2013), which identifies TSSs and promoter regions in a high-throughput manner. CAGE-seq has been used to quantify miRNA expression levels by the proxy measure of pri-miRNA expression (de Rie et al., 2017). The 5th edition of the Functional Annotation of the Mammalian genome (FANTOM5) project included CAGE profiling data of pri-miRNA

promoter regions and expression in numerous neurodevelopmentally relevant samples, including human iPSC differentiated neurons, human embryonic and adult brain samples, as well as time-course data of the mouse cerebellar miRNA transcriptome from E11 to P9 (de Rie et al., 2017). For pri-miRNAs encoded by multigene families, these data are particularly useful in identifying cell- and tissue-specific expression among multiple pri-miRNA paralogs, as well as miRNA host transcript promoters for investigation of transcriptional regulation.

The significance of miRNA host transcript analyses remain dubious as the correlation between expression of miRNA host transcripts and embedded intragenic miRNAs remains unclear. Early human and mouse miRNA studies across different organs revealed frequent coordination between intronic miRNAs and host gene mRNAs (Baskerville and Bartel, 2005; Rodriguez et al., 2004). More recently, next-generation sequencing revealed a limited correlation between host gene and mature miRNA expression and rather indicated that processing of pri-miRNAs by Microprocessor is a more accurate determinant of miRNA abundance (Conrad et al., 2014). These inconsistencies may be remedied by accounting for phylogenetic age/evolutionary conservation of miRNAs, as one report found conserved intragenic miRNAs are co-expressed with host genes whereas non-conserved miRNAs are uncoordinated with their host genes (He et al., 2012). Additionally, discrepancies may be, in part, due to incorrect annotation of miRNA host genes (Chiang et al., 2010). Another important consideration is the reported use of transcription sites independent from the host gene by one-third of intronic miRNAs (Ozsolak et al., 2008) (Figure 1-3). Future reciprocal analyses of

transcriptional and post-transcriptional regulation of miRNA expression will better define the functional determinants of miRNA expression.

Regulatory feedback loops between a miRNA and transcription factors modulating expression of the miRNA host gene during neuronal lineage specification have emerged from several independent transcription factor-miRNA focused studies. One example is the double-negative miR-124-REST/SCP1 feedback loop, which controls miR-124 expression in mouse neural progenitor cells (Conaco et al., 2006; Visvanathan et al., 2007). Another potential double-negative feedback loop is formed between miR-9 and REST. Interestingly, all three miR-9 loci are occupied by REST in a murine kidney cell line (Conaco et al., 2006) and miR-9 targets REST in HEK293 cells (Packer et al., 2008). Moreover, miR-9 has been shown to participate in double-negative feedback loops with Hes1 and TLX to regulate neuronal differentiation (Bonev et al., 2012; Zhao et al., 2009).

1.4.2 Posttranscription regulation of miRNA biogenesis

In recent years, a growing number of studies have investigated specific pri-miRNA characteristics for Microprocessor substrate recognition and cleavage (Alarcon et al., 2015; Auyeung et al., 2013; Roden et al., 2017). Within the burgeoning epitranscriptomics field, methyltransferase-like 3 (METTL3) was found to catalyze N^6-methyladenosine (m^6A) modifications of pri-miRNAs to enhance DGCR8 recognition and efficient processing by the Microprocessor complex (Alarcon et al., 2015) (Figure 1-3). Importantly, the deposition of m^6A is not dependent on genomic position as m^6A modifications were found on both inter- and intragenic pri-miRNAs (Alarcon et al., 2015).

Additional *cis* motifs within pri-miRNAs which influence Microprocessor efficiency have been identified, namely UG at the 5' basal junction of the stem-loop and UGUG/GUG

in the apical loop (Auyeung et al., 2013) (Figure 1-3). Another form of posttranscriptional regulation is imparted by *trans* acting factors which may bind to pri-miRNAs to modulate cleavage by Microprocessor. Some *trans* acting co-factors, which enhance processing of specific pri-miRNAs, include SRp20 (SRSF3), QKI, p72 (DDX17), KSRP, hnRNP A1, BRCA1, FUS and SF2/ASF (Auyeung et al., 2013; Guil and Caceres, 2007; Kawai and Amano, 2012; Michlewski et al., 2008; Mori et al., 2014; Morlando et al., 2012; Ruggiero et al., 2009; Trabucchi et al., 2009; Wang et al., 2017; Wu et al., 2010) (Figure 1-4). These RBP co-factors have been reported to promote processing of pri-miRNAs by interaction with and recruitment of Microprocessor to the local stem-loop (Kawai and Amano, 2012; Morlando et al., 2012; Trabucchi et al., 2009; Wang et al., 2017) and inducing a relaxed conformational change in the stem-loop, favorable for Microprocessor cleavage (Michlewski et al., 2008).

Conversely, known *trans* acting co-factors which repress processing of specific pri-miRNAs include PTBP1, NF90-NF45, MSI2/HuR, hnRNP A1 and Lin28B (Choudhury et al., 2013; Michlewski and Caceres, 2010; Piskounova et al., 2011; Sakamoto et al., 2009; Yeom et al., 2018) (Figure 1-4). Mechanisms by which these RBPs negatively affect miRNA processing are similar to those employed by RBPs which promote processing, including binding proximally or directly to the stem-loop, effectively blocking Microprocessor access to the cleavage site (Sakamoto et al., 2009; Yeom et al., 2018). Additionally, these RBPs can induce a rigid conformational change in the stem-loop, which is unfavorable for Microprocessor cleavage (Choudhury et al., 2013) and compete with RBPs which promote processing for binding to shared pri-miRNA targets (Michlewski and Caceres, 2010). Therefore, variations in pri-miRNA sequence composition between

miRNA family paralogs and species, combined with differential RBP abundance in specific cell-types and systems, allow for differential pri-miRNA processing and expression of the derivative miRNAs (Conrad et al., 2014).

During brain development, lineage-specific and/or developmental stage-specific expression has been reported for some RBPs mentioned here. Notably, QKI expression has been detected in neural stem/progenitor cells but is selectively silenced in maturing/differentiating neurons (Takeuchi et al., 2020). On the contrary, QKI is markedly upregulated during oligodendroglia and myelin development (Hardy et al., 1996; Wu et al., 2001). Another example is the decreased expression of DDX17 during *in vitro* differentiation of human SH-SY5Y neuroblastoma cells (Lambert et al., 2018). Additionally, PTBP1 is abundantly expressed in NPCs and translationally repressed by miR-124 during neuronal differentiation (Makeyev et al., 2007). At this moment, how developmental regulation of these RBPs impacts processing of numerous pri-miRNA targets to affect brain maturation remains to be determined.

1.4.3 Posttranscriptional regulation of miRNA activity

Circular RNAs (circRNAs) are single-stranded, closed loop RNA structures, produced by non-canonical ‘back-splicing’ that covalently links a downstream splice donor site to an upstream splice acceptor site (Jeck et al., 2013; Zhang et al., 2014). These RNA molecules are highly expressed in the brain. Notably, the human brain expresses significantly more circRNA species than rodent brains in various brain regions, including the cortex, cerebellum and hippocampus (Rybak-Wolf et al., 2015). Increasing evidence suggests functional roles for circRNAs as miRNA sponges (Hansen et al., 2013; Memczak et al., 2013). Indeed, knockout of neuron-specific circRNA CDR1 antisense

(CDR1as), which harbors multiple conserved miR-7 binding sites, in mice led to a brain-specific significant decrease in miR-7, upregulation of miR-7 target genes, specifically immediate early genes (IEGs) and subsequent dysfunction of excitatory synaptic transmission (Piwecka et al., 2017). In a broader context, altered circRNA expression has been documented in post-mortem brains of schizophrenia and Alzheimer's disease patients (Dube et al., 2019; Mahmoudi et al., 2019). Perturbations in circRNA-mediated sponging of disease-relevant miRNAs have been proposed to underlie disease pathogenesis yet further investigations are needed to reach definitive conclusions (Dube et al., 2019; Mahmoudi et al., 2019).

A second class of non-coding RNAs with the potential to regulate miRNA function/abundance are long non-coding RNAs (lncRNAs). Defined as RNAs more than 200 nucleotides in length without protein-coding capacity, lncRNAs are highly expressed in the brain with known roles in transcriptional and posttranscriptional regulation of coding genes (Mercer and Mattick, 2013; Tripathi et al., 2010; Tsai et al., 2010). Currently, there are four paradigms of lncRNA-miRNA interactions: 1) lncRNAs sequestering/sponging miRNAs, 2) miRNAs affecting lncRNA stability, 3) lncRNA-miRNA competition for mutual target mRNAs and 4) lncRNAs as miRNA precursors (Yoon et al., 2014). Precise mechanisms and functional importance of lncRNA-miRNA interaction paradigms are under active investigation.

1.5 Emerging evidence for miRNA dysregulation in brain disorders: dysregulation, etiology and therapeutic potential

Abnormalities in neuronal development underlie the pathogenesis of numerous neuropsychiatric diseases, including schizophrenia, major depression, autism and fragile

X intellectual disability (Hagerman et al., 2017; Hoffmann et al., 2019; Pan et al., 2019). Given the spatiotemporal function of miRNAs in governing normal neuronal development, genetic alterations and dysregulations that result in abnormalities of numerous miRNAs have been reported in various brain disorders, which are well documented in recent reviews (Gruzdev et al., 2019; Ivanova et al., 2018; Juzwik et al., 2019). Well-characterized examples include miR-137 in major mental illnesses (Schizophrenia Working Group of the Psychiatric Genomics, 2014), miR-125a in fragile X intellectual disability (Muddashetty et al., 2011) and the miR-124 in neuropsychiatric, neurological and neurodegenerative disorders (Angelopoulou et al., 2019; He et al., 2016; Juzwik et al., 2019).

Beyond neurodevelopmental disorders, the role of embryonic and adult neurogenesis in neurodegenerative disorders is increasingly being recognized. In Parkinson's disease (PD), malfunction of PD-related genes affects neural stem cell proliferation and maintenance (Le Grand et al., 2015). Thus, abnormalities in early neuronal development not only underlie neurodevelopmental disorders but may also contribute to age-related neurodegenerative disorders. Besides the well documented miRNA abnormalities identified in postmortem brains of neurodegenerative disorder patients (Aloizou et al., 2020; Wang and Zhang, 2020; Zhao et al., 2020), the rapid advancement in iPSC technology has enabled identification of mechanisms of miRNA dysregulation in iPSC-derived neurons of various neurodegenerative disease patients, such as amyotrophic lateral sclerosis (ALS) and PD (Rizzuti et al., 2018; Tolosa et al., 2018). Moreover, the therapeutic potential of miRNAs in stem cell-based treatments for neurodegenerative diseases has gained significant traction in basic and clinical

neuroscience. In particular, miR-9/9* and miR-124 have been shown to generate disease-relevant neuronal subtypes, including striatal medium spiny neurons, cortical neurons, and spinal cord motor neurons, providing promise for miRNA-based stem-cell therapy (Church et al., 2021).

Perturbations in miR-124 are implicated in several neuropsychiatric and neurodegenerative diseases, including schizophrenia, Alzheimer's disease (AD), PD, hypoxic-ischemic encephalopathy, Huntington's disease, and ischemic stroke (Ghafouri-Fard et al., 2021). The human miR-124-1 locus is located in chromosome 8p23.1, which harbors genes that have been implicated in schizophrenia, microcephaly and epilepsy (Bassett et al., 2010; Baulac et al., 2008; Claeys et al., 1997; Takahashi et al., 2005). Increased expression of mature miR-124 has been found in the hippocampus of AD patients and the hippocampus of Tg2576 mice, a model of AD, as well as in the prefrontal cortex of major depressive disorder (MDD) patients (Roy et al., 2017; Wang et al., 2018). However, other studies have reported decreased expression of mature miR-124 in the frontal cortex of sporadic AD patients (An et al., 2017). Mechanistically, miR-124 is reported to target and repress beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) (An et al., 2017; Du et al., 2017), an enzyme which cleaves amyloid precursor protein (APP) to β-Amyloid (Aβ). Therefore, in AD pathology, downregulation of miR-124 may indirectly lead to accumulation of Aβ and subsequently, amyloid plaque formation. In this model, miR-124 is reported to be neuroprotective (Du et al., 2017). However, it is important to measure differences in miR-124 across all major brain regions in AD patients compared to controls before more definitive conclusions can be made.

Rncr3$^{-/-}$ mice, homozygous for deletion of the miR-124-1 host transcript, exhibit abnormal front and hind limb clasping, commonly observed in mouse models of neurodegenerative disorders (Sanuki et al., 2011). In line with the disease phenotype observed in *Rncr3*$^{-/-}$ mice, decreased expression of mature miR-124 has been reported in a mouse model of and in the frontal cortex of subjects with behavioral variant frontotemporal dementia (bvFTD) (Gascon et al., 2014). Decreased expression of mature miR-124 has also been measured in the cortex of R6/2 mice, a model of HD but importantly, there was no significant dysregulation of miR-124 in the cortex of HD patients as compared to controls (Johnson et al., 2008). In human and mouse models of PD, miR-124 has been proposed to serve as a neuroprotective molecule by targeting numerous different disease-relevant targets. In a mouse model of PD, miR-124 targets p38 and p62 to inhibit activation of the microglial inflammatory response in PD (Yao et al., 2019). In human PD cell models, miR-124 targets signal transducer and activator of transcription 3 (STAT3) and phosphorylated 5' adenosine monophosphate-activated protein kinase (p-AMPK) of the AMPK/mTOR pathway, which play roles in microglial activation and cell apoptosis and autophagy, respectively (Geng et al., 2017; Gong et al., 2016). Moreover, reduced miR-124 has been proposed as a diagnostic biomarker for PD and the therapeutic potential of miR-124 in the treatment of PD has been recognized (Angelopoulou et al., 2019). A comprehensive review of the therapeutic potential of miR-124 in other neurodegenerative disorders can be found elsewhere (Han et al., 2019). Taken together, these human and mouse data indicate that precise expression of miR-124 is crucial for proper brain development and function with disease phenotypes arising under elevated and decreased miR-124 conditions.

In summary, numerous brain-enriched and brain-specific miRNAs that orchestrate neuron-glia fate selection have been identified (Jovicic et al., 2013). However, the molecular mechanisms that regulate miRNA biogenesis of these miRNAs, especially within distinct neural cell-types, remain elusive.

1.6 Questions Addressed in This Work

The major question addressed in this work is how the first pool of mature miR-124 is generated in human NPCs to initiate miR-124-mediated neuronal differentiation. During neural development of the murine central nervous system, miR-124 is expressed at low levels in mNPCs and significantly upregulated in neurons, to become the most abundantly expressed miRNA in the adult murine brain (Deo et al., 2006; Lagos-Quintana et al., 2002; Smirnova et al., 2005).

In both the human and mouse genome, miR-124 is encoded by three paralogous genes, giving rise to pri-miR-124-1, -2 and -3 (Deo et al., 2006; Rodriguez et al., 2004). The miR-124 paralogs undergo cell developmental stage-specific induction during murine neuronal differentiation. Pri-miR-124-1 was identified as the predominantly expressed miR-124 paralog in mouse embryonic stem cells (mESCs) and cortical neurons (Yeom et al., 2018). However, whether any of the human pri-miR-124 paralogs are expressed and/or are subject to transcriptional and posttranscriptional regulation in human NPCs is unknown. Therefore, to understand the mechanism underlying miR-124 biogenesis in early human neuronal lineage development, **my dissertation focuses on the human-specific mechanism underlying miR-124 biogenesis in human NPCs and neurons.** In Chapter 2 of this dissertation, we addressed the question: "**In human NPCs, which of**

the three pri-miR-124 paralogs (s) are expressed and responsible for functional mature miR-124 production?". We identified a human NPC-specific pri-miR-124 paralog and characterized expression of this transcript during neurodevelopment. We next asked: "**What is molecular mechanism underlying robust expression of this paralog in human NPCs and development regulation of this gene during neurodevelopment?**" In Chapter 2 of this dissertation, we use a combination of siRNA-mediated knockdown in cultured cells and luciferase assays to explore transcriptional regulation of the hNPC-specific pri-miR-124 host transcript.

Pri-miRNAs are generally processed co-transcriptionally, prior to splicing (Morlando et al., 2008; Yin et al., 2015). Therefore, the detection of a pri-miR-124 paralog in human NPCs, along with the reported minimal expression of miR-124 in NPCs, indicates that this transcript is not rapidly processed into a mature miRNA but rather is subject to posttranscriptional regulation. Several RNA binding proteins (RBPs) have been reported to enhance and/or repress processing of pri-miRNAs by Microprocessor. Therefore, we next asked: "**Which RBPs are predicted to bind to our pri-miR-124 paralog of interest to repress miRNA processing?**" We first used *in silico* prediction to identify Quaking (QKI) as a candidate *trans* acting co-factor. QKI is a selective RBP, which is selectively expressed in NPCs and is highly expressed in myelinating glia but is not detected in mature neurons in the brain. In Chapter 3 of this dissertation, we address the role of QKI in regulating the processing of the pri-miR-124 paralog of interest through co-immunoprecipitation and CRISPR-Cas9 genome editing. Using these methods, we establish a potential functional connection between nuclear QKI5 and processing of our pri-miR-124 paralog of interest in hNPCs.

Figure 1-1: The miR-124-PTBP1 regulatory loop. During early murine neural development, the RNA splicing factor, PTBP1, inhibits processing of pri-miR-124-1. In young murine neurons, mature miR-124 targets PTBP1, for de-repression of neuron-specific alternative splicing. However, how the first wave of mature miR-124 is produced in human NPCs to initiate this switch remains elusive.

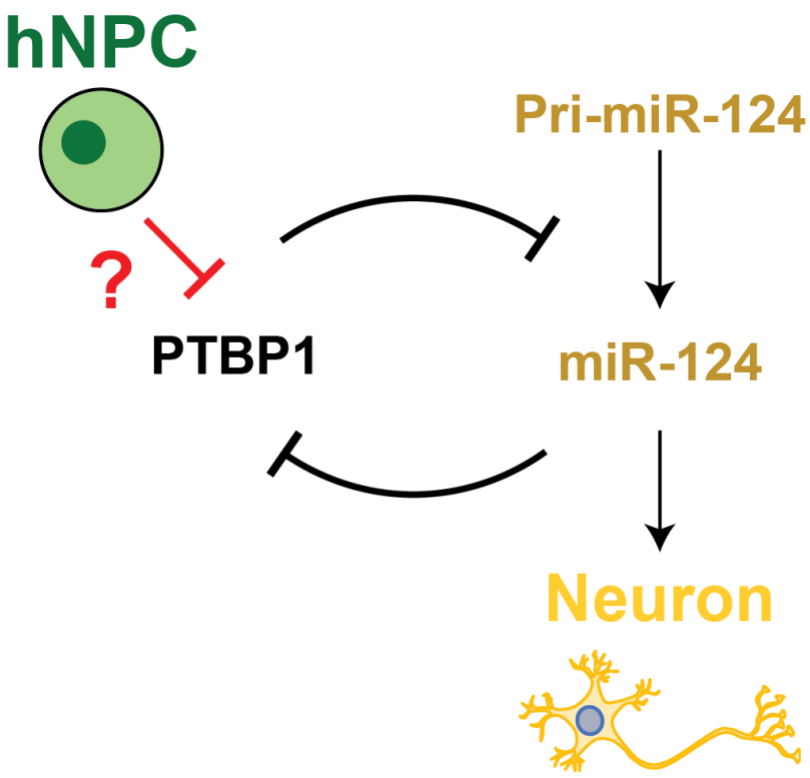
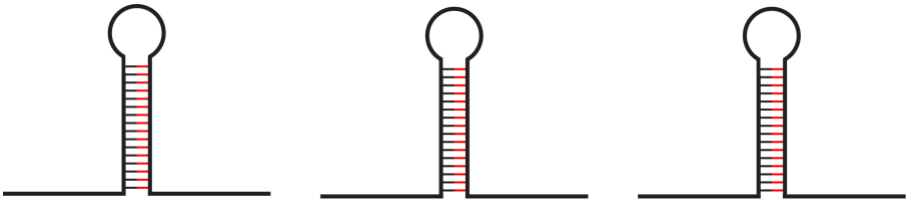

hNPC
Pri-miR-124
?
PTBP1
miR-124
Neuron

Figure 1-2: The human microRNA-124 multigene family. Human mature miR-124 is encoded by three paralogous loci. Coordinates are shown in hg19 assembly. Canonical processing of all three pri-miRNA paralogs contributes to the cellular pool of mature miR-124.



miR-124-1
8p23.1
chr8:9,760,898-
9,760,892

miR-124-2
8q12.3
chr8:65,291,706-
65,291,814

miR-124-3
20q13.33
chr20:61,809,852,
61,809,938

Figure 1-3: Transcriptional regulation of microRNA host genes. RNAPII-mediated transcription of a miRNA host gene from an upstream host gene promoter or independent transcription start site produces two variant pri-miRNAs. Transcription factors bind to transcriptional regulatory elements proximal to the transcription start site (TSS) to activate or repress gene expression. At the TSS, miRNA host gene promoters harbor the same features as eukaryotic protein-coding gene promoters. *Cis* elements, UG at the 5' basal junction and UGUG/GUG in the apical loop, discriminate pri-miRNAs from other secondary structures in the transcriptome and recruit Microprocessor for cleavage. The methyltransferase METTL3 deposits m^6A modifications on pri-miRNAs to enhance recognition by DGCR8. Drosha and DGCR8 bind the pri-miRNA as a heterotrimeric complex to cleave and generate a pre-miRNA for further downstream processing. Regulatory element, Reg. element; TFIIB recognition element, BRE; TATA box, TATA; Initiator, Inr; Downstream promoter element, DPE; RNA Polymerase II, RNAPII; Exon, Ex; Methyltransferase-like 3, METTL3.

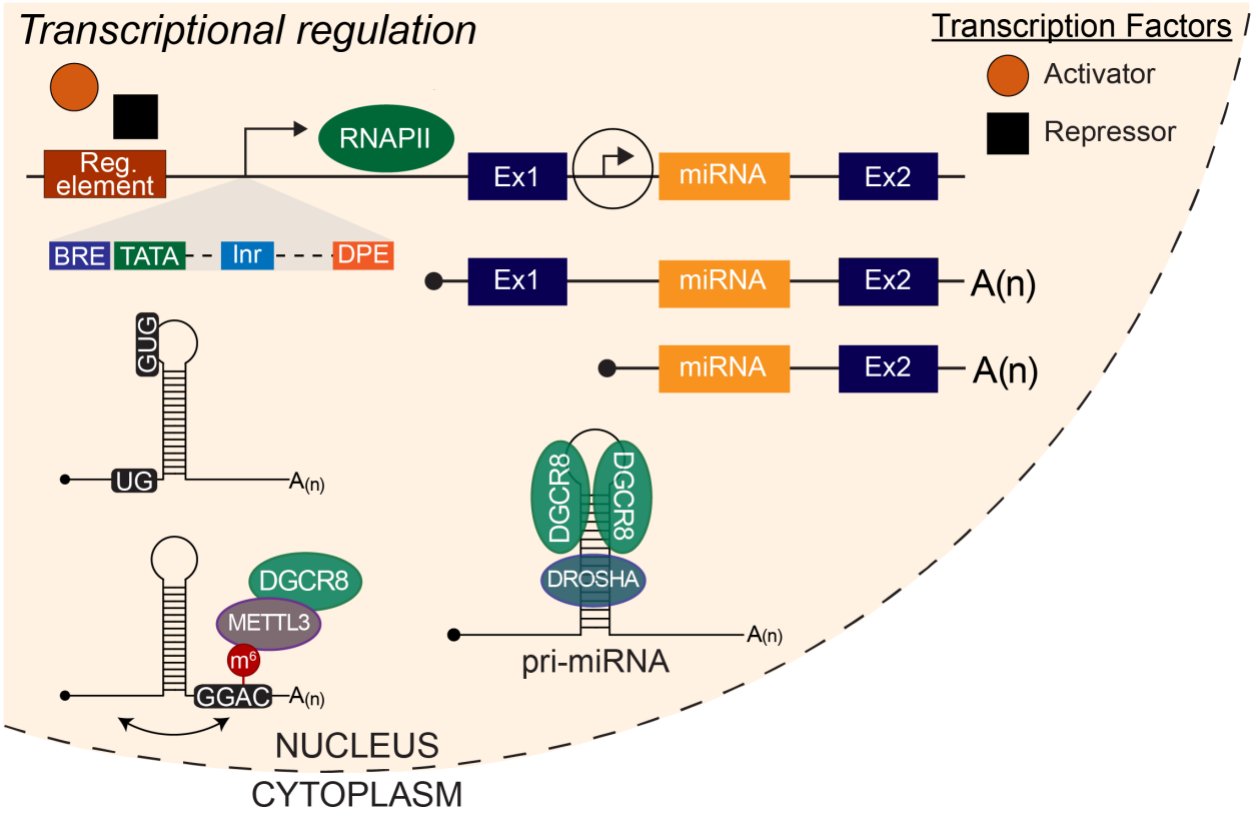
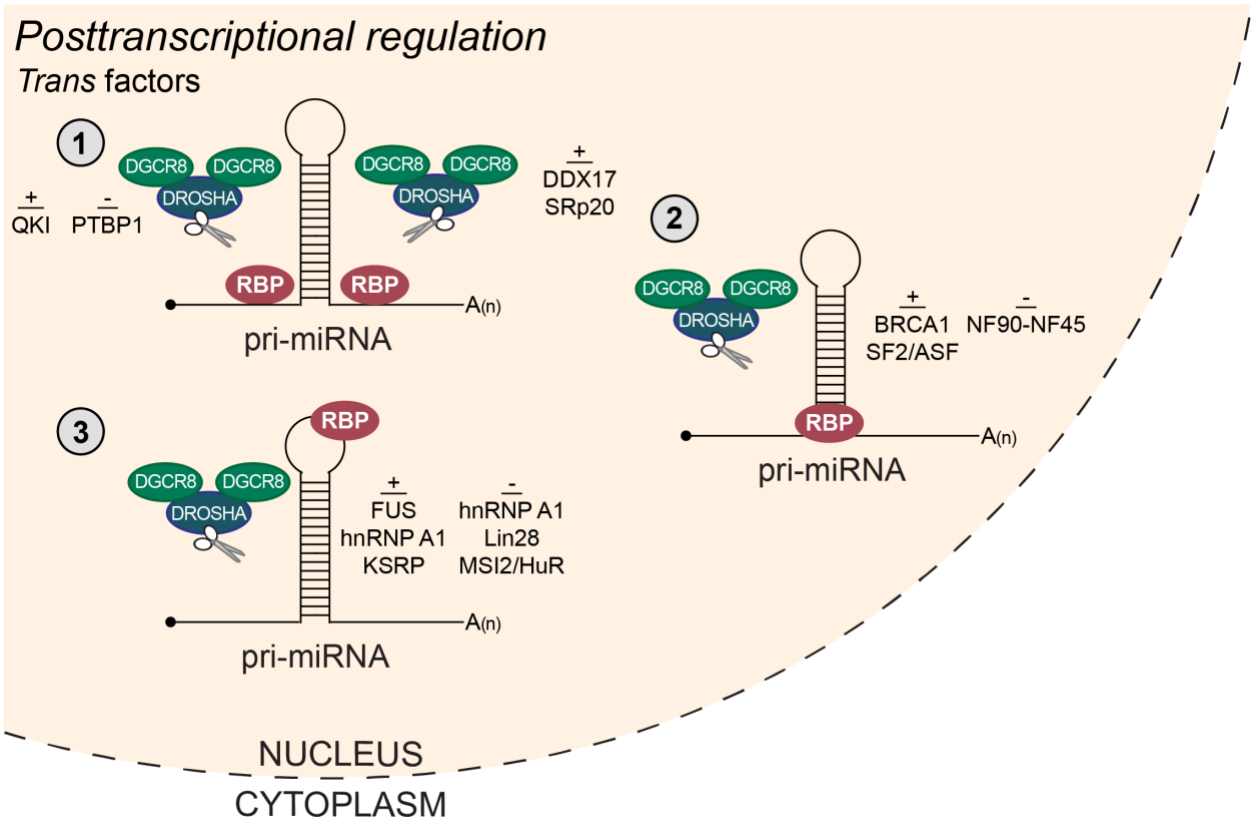

Transcriptional regulation
Transcription Factors
Activator
Repressor
Reg. element
RNAPII
Ex1
miRNA
Ex2
BRE
TATA
Inr
DPE
Ex1
miRNA
Ex2
A(n)
miRNA
Ex2
A(n)
GUG
UG
A(n)
DGCR8
DGCR8
DROSHA
A(n)
pri-miRNA
DGCR8
METTL3
m6
GGAC
A(n)
NUCLEUS
CYTOPLASM

Figure 1-4: Posttranscriptional regulation of pri-miRNAs. *Trans*-acting factors known to bind pri-miRNAs that positively (+) or negatively (-) regulate Microprocessor cleavage. 1) Factors which bind to the single-stranded RNA flanking the stem-loop. Binding toward the 5’ or 3’ end of the pri-miRNA is specified. 2) Factors which bind to the stem-loop. 3) Factors which bind to the apical loop.


Posttranscriptional regulation
Trans factors
1
DGCR8
DGCR8
DROSHA
DGCR8
DGCR8
DROSHA
+
QKI
-
PTBP1
+
DDX17
SRp20
RBP
RBP
A(n)
pri-miRNA
2
DGCR8
DGCR8
DROSHA
+
BRCA1
SF2/ASF
-
NF90-NF45
RBP
A(n)
pri-miRNA
3
RBP
DGCR8
DGCR8
DROSHA
+
FUS
hnRNP A1
KSRP
-
hnRNP A1
Lin28
MSI2/HuR
A(n)
pri-miRNA
NUCLEUS
CYTOPLASM

Chapter 2: Transcriptional regulation of a hNPC-specific pri-miR-124 paralog in early human neuronal lineage development

2.1 Abstract

MicroRNAs (miRs) are a class of small, non-coding RNAs essential for brain development and function, among which miR-124 is a key driver for neuronal lineage establishment by suppressing inhibitors of neuronal differentiation. However, molecular mechanisms that govern the biogenesis of human miR-124 remain largely unknown. Among the three pri-miR-124 paralogs, namely pri-miR-124-1, -2, and -3, which transcript is responsible for the biogenesis of mature miR-124 and initiation of human neuronal lineage development is undetermined. We discovered that pri-miR-124-2 is the primary miR-124 precursor in human iPSC-derived neural progenitor cells (hNPCs), whereas pri-miR-124-1 and pri-miR-124-3 are negligible, suggesting that pri-miR-124-2 is poised to initiate the onset of miR-124 biogenesis and differentiation of hNPCs. We further demonstrated that robust transcription of the *MIR124-2 Host* Gene (*MIR124-2HG*) is the underlying mechanism for predominant pri-miR-124-2 expression in hNPCs, which is achieved by a complex functional interplay between developmentally regulated chromatin accessibility and transcription factors. Moreover, we identified a human-specific proximal promoter element that acts as a scaffold for transcription activator SP1 and repressor MAFK to regulate *MIR124-2HG* transcription during hNPC development. Our results revealed novel mechanisms that underlie the biogenesis of miR-124 in hNPCs to trigger human neuronal lineage development.

2.2 Introduction

MicroRNAs (miRNAs) are a class of small, non-coding RNAs encoded by primary microRNA (pri-miRNA) transcripts, which undergo a series of enzymatic processing steps in the nucleus and cytoplasm. The mature miRNAs derived from these transcripts play pivotal roles in brain development by suppressing key mRNA targets at important developmental transition points. One such important mammalian brain-enriched miRNA is microRNA-124 (miR-124, miR-124-3p) (Lagos-Quintana et al., 2002; Sempere et al., 2004). During murine neuronal maturation, miR-124 is expressed at low levels in neural progenitor cells (NPCs), but is dramatically up-regulated to become the most abundantly expressed miRNA in the adult brain, accounting for an estimated 25% to 48% of all brain miRNAs (Deo et al., 2006; Lagos-Quintana et al., 2002; Smirnova et al., 2005). Within the murine brain, abundantly expressed miR-124 displays neuron-specific activity (Akerblom et al., 2012).

Earlier studies in rodents established several canonical targets of miR-124, including repressors of neuronal differentiation and morphology, such as the GTPase RhoG, the transcription factor Pax3 and the chromatin remodeling complex BAF53a (Franke et al., 2012; Makeyev et al., 2007; Wei et al., 2018; Yoo et al., 2009). Additionally, miR-124 targets a global repressor of neuronal transcription, the small C-terminal domain phosphatase 1 (SCP1) subunit of the anti-neural RE1-silencing transcriptional factor (REST) pathway, as well as a global repressor of neuronal-specific pre-mRNA splicing, polypyrimidine tract binding protein (PTBP1, PTB, hnRNP I) (Makeyev et al., 2007; Visvanathan et al., 2007). A recent study also confirmed PTBP1 as a target of miR-124 in human iPSC-derived neurons (Kutsche et al., 2018).

In the human and mouse genome, miR-124 is derived from three paralogous loci (Deo et al., 2006; Rodriguez et al., 2004). Interestingly, both the transcription factor REST and the splicing factor PTBP1 independently repress expression of miR-124 precursors in murine systems. Mechanistically, REST targets all three *miR-124* loci for transcriptional silencing, forming a double negative miR-124-REST/SCP1 feedback loop (Conaco et al., 2006). Separately, PTBP1 forms a negative feedback loop with miR-124-1 in mouse embryonic stem cells (mESCs), in which pri-miR-124-1 is highly expressed and bound by PTBP1, inhibiting miRNA processing by Drosha/DGCR8 (Yeom et al., 2018). Thus, additional repressive mechanisms are needed to target either SCP1 or PTBP1 to trigger the downregulation of these molecules and break the negative feedback loops, allowing for an increase in miR-124 biogenesis and subsequent commitment to neuronal lineage.

Interestingly, miR-124 paralogs show differential and stage-specific expression in the developing murine central nervous system (CNS). Pri-miR-124-1 is the predominantly expressed pri-miR-124 paralog in mESCs with expression increasing as mESCs differentiate into cortical neurons (Sanuki et al., 2011; Yeom et al., 2018). Similar to pri-miR-124-1, pri-miR-124-2 levels increase as mESCs differentiate to cortical neurons (Yeom et al., 2018), which, in tandem with pri-miR-124-1 upregulation, may underlie the robust increase in mature miR-124 observed in differentiating neurons (Deo et al., 2006; Smirnova et al., 2005).

However, the expression of miR-124 biogenesis intermediates (pri/pre-miR-124) has not been examined during human neuronal lineage development. Therefore, the mechanisms underlying the onset of miR-124 biogenesis to initiate miR-124-mediated neuronal differentiation in human neurons are not yet known. Thus, while early studies

revealed key functions of miR-124 in mice, defining the regulation of pri-miR-124 paralogs within human neurons is important to understanding how miR-124 governs human brain development. Importantly the human miR-124-1 locus, which is located at chromosome 8p23.1, harbors genes implicated in schizophrenia, microcephaly and epilepsy (Bassett et al., 2010; Baulac et al., 2008; Claeys et al., 1997; Takahashi et al., 2005). Furthermore, dysregulation of miR-124 expression has been reported in several neurological diseases (Ghafouri-Fard et al., 2021). Thus, elucidating mechanisms that regulate miR-124 biogenesis in human neurons may provide critical insights into the pathogenesis of neurological diseases caused by miR-124 malfunction.

Here, we report that pri-miR-124-2 is the predominant pri-miR-124 paralog expressed in human NPCs, which is driven by the transcription of the *MIR124-2 Host Gene* (*MIR124-2HG*), to serve as the initial source of mature miR-124 during neuronal differentiation. Mechanistically, transcriptional activator SP1 acts on *MIR124-2HG* to sustain robust transcription in hNPCs. Furthermore, we found that *MIR124-2HG* harbors a human-specific proximal promoter element which undergoes a change in chromatin accessibility and scaffolds transcription factor(s) to repress transcription of *MIR124-2HG* during neuronal differentiation. Our studies elucidate the complex developmental regulation of distinct pri-miR-124 paralogs within the same pro-neurogenic miRNA multigene family during neurodevelopment to orchestrate biogenesis of the mature miRNA.

2.3 Results

2.3.1 Pri-miR-124-2 is the primary source for miR-124 biogenesis in hNPCs

To determine which pri-miR-124 paralogs are expressed in hNPCs, we performed RT-PCR. Abundant pri-miR-124-2 was detected in hNPCs derived from three independent iPSC lines, whereas pri-miR-124-1 was minimally detected (Fig. 2-1A). Moreover, in a human NPC model cell line, BE(2)-M17 (henceforth called M17), pri-miR-124-2 is prominently detected with minimal pri-miR-124-1 and undetectable pri-miR-124-3 expression. To explore whether transcription of the miR-124-2 host transcript underlies the high level of expression of pri-miR-124-2 in hNPCs, we mined the 5th edition of the RIKEN FANTOM project (FANTOM5) datasets for cap analysis of gene expression (CAGE) sequencing (Lizio et al., 2015), which captures the 5' 7-methylguanosine (m^{7}G) cap of miRNA host transcripts/pri-miRNAs as a proxy measure of mature miRNA expression (de Rie et al., 2017). As shown in Fig. 2-1B, only the *MIR124-2 Host Gene* (*MIR124-2HG*) transcript is appreciably expressed in hNPCs, with negligible expression of both the miR-124-1 and -3 host transcripts (Supplemental Table S2.1). Thus, pri-miR-124-2 likely serves as the primary precursor for miR-124 biogenesis in hNPCs.

We next investigated whether miR-124-2 is the predominantly expressed miR-124 paralog throughout neuronal development, underlying the sustained increase of mature miR-124 reported in neurons (Busskamp et al., 2014; Deo et al., 2006; Smirnova et al., 2005). Further analysis of FANTOM5 data revealed that in contrast to hNPCs, which predominantly express *MIR124-2HG*, the miR-124-1 host transcript is the primary miR-124 paralog present in human newborn and adult cerebellum (Fig. 2-1C; Supplemental Table S2.1). In agreement with these data, levels of pri-miR124-2 decreased significantly

when hNPCs undergo four weeks of differentiation into cortical neurons (Fig. 2-1D), further suggesting that pri-miR124-2 is specifically expressed in hNPCs. RNA-seq analysis of M17 cells having undergone differentiation induced by *trans*-retinoic acid (Fig. 2-1E) confirmed the up-regulation of pri-miR-124-1, coinciding with the downregulation of pri-miR-124-2 (Fig. 2-1F). This developmental switch from prominent pri-miR-124-2 to pri-miR-124-1 is also observed during mouse cerebellar development (Supplemental Fig. S2.1B; Supplemental Table S2.2).

2.3.2 Unlike pri-miR-124-1, pri-miR-124-2 processing is not inhibited by PTBP1

A recent discovery has demonstrated a role of PTBP1 in repressing miR-124 biogenesis in mESCs by blocking Drosha/DGCR8-mediated processing of abundantly expressed pri-miR-124-1 (Yeom et al., 2018). However, whether biogenesis of miR-124 from human pri-miR-124-1 is inhibited by PTBP1 has not been studied. In addition, it is not known whether pri-miR-124-2 processing is also regulated by PTBP1. Analysis of the nucleotide composition upstream of the human pri-miR-124-1 and -2 hairpins reveals ~75% and 68% identity with murine pri-miR-124-1 and -2 upstream sequences, respectively (Fig. 2-2A). Importantly, long stretches of nucleotides in the CU-rich tract of pri-miR-124-1 are conserved, which are crucial for PTBP1 binding to mouse pri-miR-124-1 (Yeom et al., 2018). By contrast, neither human nor mouse pri-miR-124-2 contains CU-rich tracts in the sequence upstream of the hairpins, suggesting that unlike pri-miR-124-1, pri-miR-124-2 may not be a target of PTBP1.

To examine whether PTBP1 regulates processing of human pri-miR-124 paralogs, we performed acute knockdown of PTBP1 using siRNA in M17 cells. Depletion of PTBP1 was confirmed by immunoblot (Fig. 2-2B, C). Knockdown of PTBP1 resulted in a

noticeable decrease of pri-miR-124-1 as compared to control, as measured by semi-quantitative RT-PCR (Fig. 2-2D), suggesting enhanced processing of pri-miR-124-1. This result is consistent with the reported function of PTBP1 in blocking Drosha-mediated cleavage of mouse pri-miR-124-1 (Yeom et al., 2018). Thus, PTBP1-mediated inhibition of cleavage and processing of pri-miR-124-1 appears to be conserved between human and mouse. In contrast, pri-miR-124-2 levels were unaffected by knockdown of PTBP1 (Fig. 2-2D). These data suggest that Microprocessor-mediated processing of pri-miR-124-2 is not negatively regulated by PTBP1 in human NPCs.

2.3.3 Identification of a human-specific proximal promoter element that regulates *MIR-124-2HG* transcription during differentiation of hNPCs

We next investigated whether transcriptional regulation underlies the prominent expression of pri-miR-124-2 in hNPCs and the subsequent decline of this transcript in mature neurons. To address this question, we analyzed changes in chromatin accessibility surrounding the *MIR-124-2HG* transcription start site (TSS) during differentiation of hiPSC-derived neurons. Human pri-miR-124-2 FANTOM5 CAGE tags mapped to the 5' end of *MIR124-2HG* (Supplemental Fig. S2.2A), validating the gene TSS. We identified an initiator element at the TSS but otherwise did not note other mammalian core promoter elements (Supplemental Fig. S2.2B). Notably, no TATA sequence was found near the TSS. Assay for transposase-accessible chromatin by sequencing (ATAC-seq) data collected from hiPSC-derived NPCs and cortical neurons subjected to 1- and 2-weeks (wk) of differentiation (Forrest et al., 2017) revealed no significant changes in chromatin accessibility immediately flanking the *MIR124-2HG* TSS (Fig. 2-3A**,** *bottom green bar*). However, we observed a distinct ATAC-seq peak located

−1386 to -625 nt upstream of the *MIR124-2HG* TSS that appeared exclusively in 2-wk old neurons (Fig. 2-3A, *bottom orange bar*), indicating increased chromatin accessibility at this site during neuronal differentiation. Analysis of the genomic DNA at this ATAC-seq peak revealed characteristic signatures of a proximal promoter element (PPE), including a GC box and CAAT box (Blake et al., 1990; Graves et al., 1986; Jones et al., 1985) (Fig. 2-3C).

Using the PhastCons scoring algorithm across 100 vertebrates (Karolchik et al., 2008; Siepel et al., 2005), we found that the genomic sequence of this putative human *MIR124-2HG* PPE is poorly conserved, in contrast to the highly conserved downstream sequence extending to the *MIR124-2HG* TSS, which likely harbors the *MIR124-2HG* promoter (Fig. 2-3B). We further compared the 2 kilobase (kb) genomic sequence upstream of the TSSs of the human and mouse miR-124-2 host genes (Fig. 2-3C), as transcriptional regulatory elements are often conserved between human and mouse (Cheng et al., 2014). Strikingly, no sequence similarity was found between the putative human *MIR124-2HG* PPE and the mouse *MiR-124-2hg*. Conversely, two sequence regions surrounding the *MIR124-2HG* TSS, located at -618 to -172 (Region 1) and -208 to +138 (Region 2), are highly conserved with the corresponding regions in the mouse *MiR-124-2hg*, sharing 84% and 78% identity, respectively (Fig. 2-3C).

To identify the *MIR124-2HG* promoter, we inserted the highly conserved genomic fragment encompassing -618 to +138 relative to the *MIR124-2HG* TSS into a pGL3 luciferase reporter. Luciferase activity was increased ~2.5 fold over the parental vector (Fig. 2-3D), indicating that this genomic fragment harbors promoter activity. When inserted upstream of the *MIR124-2HG* promoter region, the genomic fragment (-1386 to

-625) at the ATAC-seq peak in 2 wk. hiPSC-derived neurons (Fig. 2-3A) caused a significant reduction in luciferase activity (Fig. 2-3D). Such a result suggests that this human-specific PPE can suppress transcription. Hence, the developmentally programed increase in chromatin accessibility at the *MIR124-2HG* PPE is predicted to downregulate *MIR124-2HG* transcription. Consistent with this hypothesis, RNA-sequencing reads for *MIR124-2HG* are significantly reduced in hiPSC-derived cortical neurons as compared to NPCs (Fig. 2-3E). In addition, a decrease of *MIR124-2HG* RNA-seq signal was also observed in M17 cells subjected to *trans*-retinoic acid (RA)-induced differentiation (Fig. 2-3E). This result in M17 cells was validated by RT-qPCR (Fig. 2-3F).

2.3.4 Functional coordination of a transcriptional activator and repressor at the *MIR124-2HG* locus during neuronal maturation

As proximal promoter elements (PPEs) scaffold transcription factor (TF) binding adjacent to promoters and modulate gene expression (Lenhard et al., 2012), we sought to identify TF sites within the *MIR124-2HG* promoter and PPE by performing *in silico* TF binding prediction using the JASPAR database (Fornes et al., 2020; Sandelin et al., 2004) (Supplemental Fig. S2.3A). By RNA-sequencing (RNA-seq), we identified significant differential expression of candidate TFs during retinoic acid-induced differentiation of M17 cells (Fig. 2-4A). We also cross-compared these data with RNA-seq data of our candidate TFs during differentiation of iPSC-derived hNPCs to 6 wk. neurons (Hoffman et al., 2017) (Supplemental Fig. 2.3B). In addition, we mined the Encyclopedia of DNA Elements (ENCODE) Project database for TF chromatin immunoprecipitation sequencing (ChIP-seq) data (Consortium, 2012; Davis et al., 2018). We noted binding of V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K (MAFK), as a homodimer with

MAFF, to the *MIR124-2HG* PPE in non-neuronal K562 cells (Fig. 2-4C). Interestingly, pri-miR-124-2 is negligibly expressed in K562 cells (Wang et al., 2017), consistent with the idea that MAFK may act as a transcriptional repressor on *MIR124-2HG*. Upon differentiation of M17 cells, *MAFK* is significantly upregulated as assessed by both RT-qPCR and RNA-seq (Fig. 2-4D; Supplemental Fig. S2.3C). Indeed, deletion of six key nucleotides in the MAFK binding site (Fig 2-4E, *top*) abrogated the negative regulatory activity of the PPE on luciferase expression of a reporter driven by the *MIR124-2HG* promoter (Fig. 2-4E, *bottom*). These data suggest binding of MAFK, as a transcriptional repressor to the accessible chromatin of the *MIR124-2HG* PPE in developing neurons underlies the transcriptional downregulation of *MIR124-2HG* during neuronal maturation.

In parallel, we sought to identify the transcriptional activator driving *MIR124-2HG* transcription in hNPCs, which may also be developmentally decreased to reinforce downregulation of *MIR124-2HG* in mature neurons. We focused on specificity protein-responsive 1 (SP1), a transcriptional activator that regulates TATA-less promoters (Pugh and Tjian, 1990), which was significantly decreased in differentiated M17 cells (Supplemental Fig. S2.4A, B). In contrast to the single MAFK binding site predicted, there are eleven predicted SP1 binding sites in the *MIR124-2HG* promoter and PPE (Fig. 2-5A), consistent with a previous report that SP1 motifs are enriched in miRNA promoters (Ozsolak et al., 2008). To examine whether SP1 could regulate transcription of *MIR124-2HG*, we performed acute knockdown of SP1 by siRNA (siSP1) in M17 cells (Fig. 2-5B, C). We detect a significant reduction of endogenous *MIR124-2HG* upon knockdown of SP1 compared with control (Fig. 2-5D). Importantly, we also detect a significant reduction of mature miR-124 upon SP1 depletion (Fig. 2-5E), indicating the functional miRNA

derived from *MIR124-2HG* behaves concordantly with host gene expression. These results suggest that SP1 acts as a transcriptional activator, driving robust *MIR124-2HG* expression in hNPCs. Thus, these data support a model for a change in chromatin accessibility of a transcriptional regulatory element at the *MIR124-2HG* locus, along with reciprocal developmental regulation of a transcriptional repressor (MAFK) and activator (SP1), underlying abundant expression of *MIR124-2HG* in hNPCs and decreased expression of this gene during neuronal differentiation.

2.4 Discussion

In the present study, we focused on understanding the molecular mechanisms underlying the onset of miR-124 biogenesis during human neuronal differentiation. Previous studies have characterized the expression of pri-miR-124 paralogs and mature miR-124 during murine neurodevelopment (Deo et al., 2006; Makeyev et al., 2007; Smirnova et al., 2005; Yeom et al., 2018). However, the expression profile of pri-miR-124 paralogs in human NPCs and neurons had not been defined. Here we observed human NPC-specific and predominant expression of pri-miR-124-2, driven by transcription of the miR-124-2 host transcript (*MIR124-2HG*).

We propose a working model to summarize our findings about the transcriptional regulation of *MIR124-2HG* during neuronal differentiation (Fig. 2-6). In this model, chromatin at the *MIR124-2HG* promoter-TSS is accessible to abundantly expressed transcriptional activators, represented by SP1, in hNPCs, underlying active transcription of pri-miR-124-2. Upon neuronal differentiation, an increase in chromatin accessibility at the *MIR124-2HG* proximal promoter element recruits the upregulated MAFK, which acts

as a transcriptional repressor, to decrease levels of *MIR124-2HG* and pri-miR-124-2. Thus, the robust transcription of *MIR124-2HG* specifically in hNPCs serves as the initial factor for miR-124-dependent early human neuronal lineage development.

We also investigated the posttranscriptional regulation of pri-miR-124-2 processing by PTBP1, which serves as a repressor of neuron-specific splicing patterns, by binding pyrimidine-rich sequences in pre-mRNAs to inhibit the splicing of nearby neuron-specific alternative exons (Makeyev et al., 2007; Sharma et al., 2005). In mouse ESCs, PTBP1 inhibits processing of pri-miR-124-1, the predominantly expressed pri-miR-124 paralog in these cells (Yeom et al., 2018). Hence, pri-miR-124-1 cannot be processed and is therefore an unviable source of mature miR-124 during early murine neurodevelopment. We demonstrated that negative regulation of pri-miR-124-1 processing by PTBP1 is conserved between human and mouse. However, importantly, PTBP1 does not affect processing of human NPC-specific pri-miR-124-2. Therefore, hNPCs may advantageously express miR-124-2 as a functional source of mature miR-124 as pri-miR-124-2 is not subject to negative posttranscriptional regulation by PTBP1.

Having established miR-124-2 as the primary source of miR-124 in hNPCs, we investigated the molecular mechanism regulating expression of *MIR124-2HG*, and derivative pri-miR-124-2, during neuronal maturation using ATAC-seq. We identified a human-specific proximal promoter element (PPE) upstream of the *MIR124-2HG* TSS, which is poorly conserved among vertebrates and mice, indicating *MIR124-2HG* harbors a human-specific *cis*-regulatory sequence. Furthermore, the increase in chromatin accessibility at the *MIR124-2HG* PPE we observed in immature neurons opposes the general trend of chromatin condensation reported during neurodevelopment (Le Gros et

al., 2016). Furthermore, *in silico* analysis of transcription factor binding indicated that the *MIR124-2HG* promoter region and PPE contained transcriptional activator and repressor binding sites, suggesting developmental regulation of *MIR124-2HG* may involve sophisticated coordination of *trans*-acting factors. Indeed, we identified coordination of SP1 and MAFK at the *MIR124-2HG* locus during neuronal differentiation, which may allow for fine-tuning gene expression not afforded by either transcription factor alone.

Our data suggest that processing of miR-124-2 initiates miR-124-mediated neuronal differentiation in human NPCs. In contrast, the minimal level of pri-miR-124-1 produced in hNPCs most likely does not produce miR-124 due to repression of Drosha/DGCR8-dependent processing by PTBP1 in both humans and mice. We postulate that in mice, as mESCs differentiate into neurons, a decline in levels of PTBP1 releases pri-miR-124-1 for Drosha/DGCR8-mediated processing into mature miR-124. Therefore, the switch to the predominant expression of miR-124-1 host transcript in mature murine neurons allows for rapid biogenesis of mature miR-124. Indeed, the increase in pri-miR-124-1 correlates with a robust increase in mature miR-124 expression, which is conserved between human and mouse. Currently, the mechanism by which pri-miR-124-1 is upregulated as human NPCs differentiate into neurons remains undefined. Transcriptional activation of miR-124-1 host gene is a possible mechanism. However, analyses of transcriptional dynamics of miR-124-1 are currently hindered by incomplete annotation of the human miR-124-1 host transcript.

Our human-specific findings raise an interesting question of why the human pri-miR-124 paralogs exhibit developmental stage specific expression during neuronal maturation. Human miR-124 and other neural-specific or -enriched miRNAs, represented

by miR-9, -128, and -125 are encoded by multigene families (Rodriguez et al., 2004). Although there is functional redundancy among the mature miRNAs encoded by multigene families, the miRNA host genes and pri-miRNA paralogs may be subject to differential transcriptional output and processing efficiencies, respectively, potentially making induction of different paralogs advantageous during various stages of neurodevelopment. Using miR-124 as a relevant example, the expression of individual pri-miR-124 paralogs in our model at different developmental stages, rather than simultaneous expression of multiple pri-miR-124 paralogs may protect against abnormalities in neuronal developmental and fragility to human diseases caused by miR-124 malfunction as other pri-miR-124 paralogs can theoretically serve as secondary sources of mature miR-124. This theory is confounded by the numerous genetic alterations identified in the single gene encoding for miR-137, which are causatively associated with several neuropsychiatric diseases (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Willemsen et al., 2011).

Together our studies have elucidated novel, human-specific expression of pri-miR-124 paralogs during neuronal differentiation. The selective expression of pri-miR-124-2 in human NPCs is achieved by robust transcription of the miR-124-2 host transcript, *MIR124-2HG*. As neuronal differentiation proceeds, a human-specific *MIR124-2HG* proximal promoter element undergoes a change in chromatin accessibility, which underlies developmentally regulated transcription of this gene. Moreover, our studies provide evidence of a functional interplay between a transcriptional activator, SP1 and repressor, MAFK in orchestrating *MIR124-2HG*, pri-miR-124-2 and mature miR-124 expression in human NPCs and neurons.

Figure 2-1: MIR-124-2 is the primary paralog for miR-124 biogenesis in hNPCs.

(A) Semiquantitative RT-PCR detection of pri-miR-124-1 and -2 expression in three independent lines of hiPSC-derived forebrain hNPCs from healthy donors (C1-C3) (*Left*) and in BE(2)-M17 cells (*Right*). RT-PCR detection of pri-miRNA was performed using primers spanning of the stem–loop (*Supplemental Figure*, Fig. S1A). Negative control reactions lacking reverse transcriptase (-RT) were performed in parallel. (B) FANTOM5 pri-miRNA CAGE data for human miR-124-1, -2 and -3 transcripts in day 18 hNPCs derived from hiPSCs of control donors C11-CRL2429 (Donor 1) and C32-CRL1502 (Donor 2) (t.p.m., tags per million; error bars are SEM). (C) FANTOM5 pri-miRNA CAGE data for human miR-124-1, -2 and -3 transcripts of donors 10223 (Newborn) and 10196 (Adult) cerebellum (t.p.m., tags per million). Detailed information of FANTOM5 samples and raw values are available in Supplemental Table 1. (D) RT-qPCR of pri-miR-124-2 from four independent cultures of iPSC-derived forebrain hNPCs and four-week old (4 wk.) cortical neurons (Student's t=test; $*P \leq 0.05$; error bars are SEM). (E) Morphology of BE(2)-M17 cells treated with 10μM RA for 10 days to induce differentiation. Arrows indicate neurite extensions. (F) Normalized RNA-seq FPKM of human pri-miR-124-1 and -2 (hairpin, +/- 200nt flanking sequence) in BE(2)-M17 cell having undergone 0 and 10 days of differentiation induced by retinoic acid (RA).

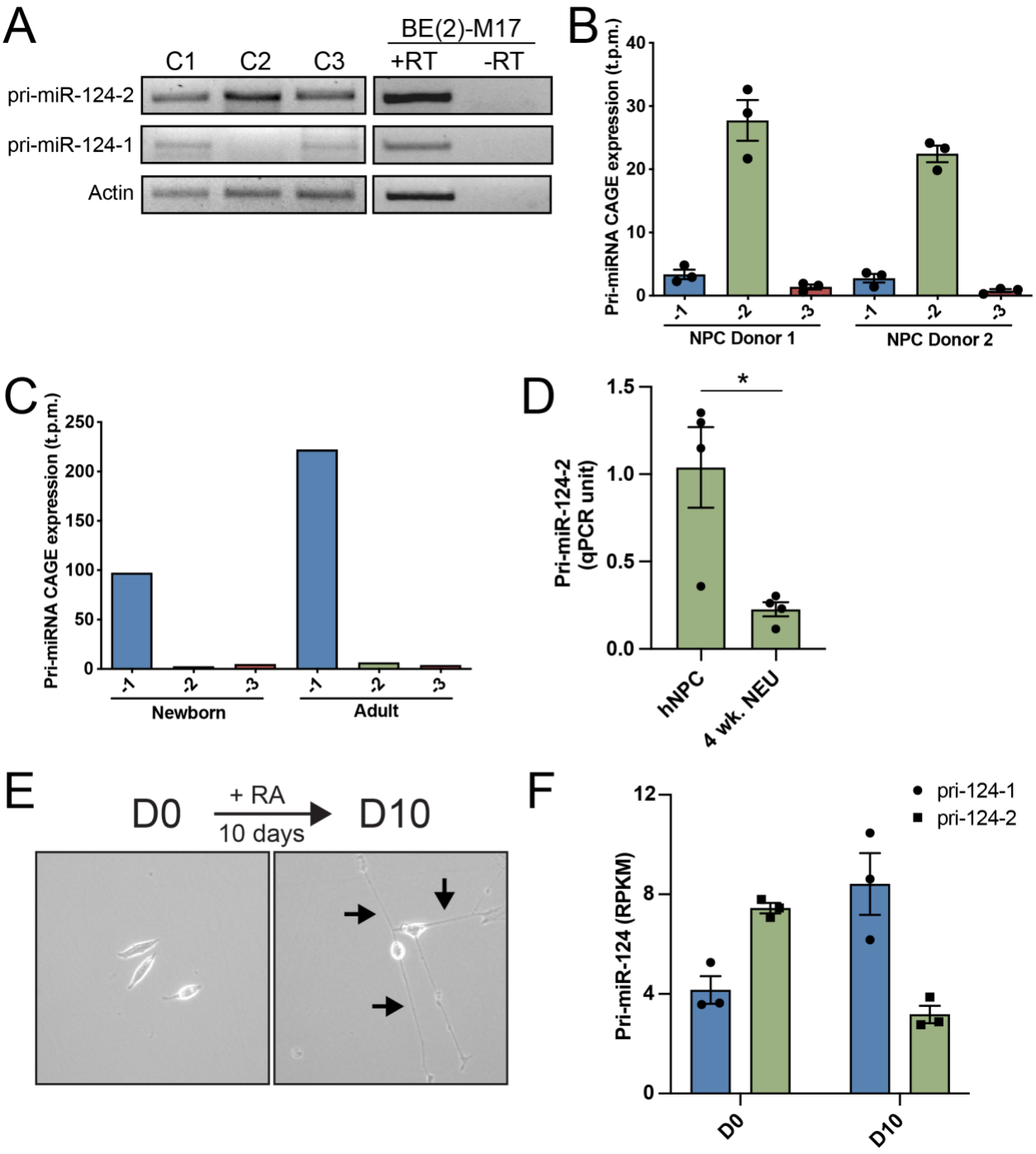

A
BE(2)-M17
C1
C2
C3
+RT
-RT
pri-miR-124-2
pri-miR-124-1
Actin
B
Pri-miRNA CAGE expression (t.p.m.)
40
30
20
10
0
-1
-2
-3
-1
-2
-3
NPC Donor 1
NPC Donor 2
C
Pri-miRNA CAGE expression (t.p.m.)
250
200
150
100
50
0
-1
-2
-3
-1
-2
-3
Newborn
Adult
D
Pri-miR-124-2 (qPCR unit)
1.5
1.0
0.5
0.0
*
hNPC
4 wk. NEU
E
D0
+ RA
10 days
D10
F
pri-124-1
pri-124-2
Pri-miR-124 (RPKM)
12
8
4
0
D0
D10

Figure 2-2: PTBP1 does not inhibit processing of pri-miR-124-2. (A) Sequence alignment of -70bp upstream of human and mouse miR-124-1 and -2 hairpins. (B) Immunoblot detection of siRNA knockdown of PTBP1 (siPTBP1) compared to non-specific control siRNA (siNS) in BE(2)-M17 cells (*Left*). β-actin served as a loading control. (C) Densitometry analysis of PTBP1 signal normalized to β-actin (*Right*). (D) Semiquantitative RT-PCR of pri-miR-124-1 and -2 expression in control (siNS) and siPTBP1 BE(2)-M17 samples (n = 3 samples). Negative control reactions lacking reverse transcriptase (-RT) were performed in parallel.

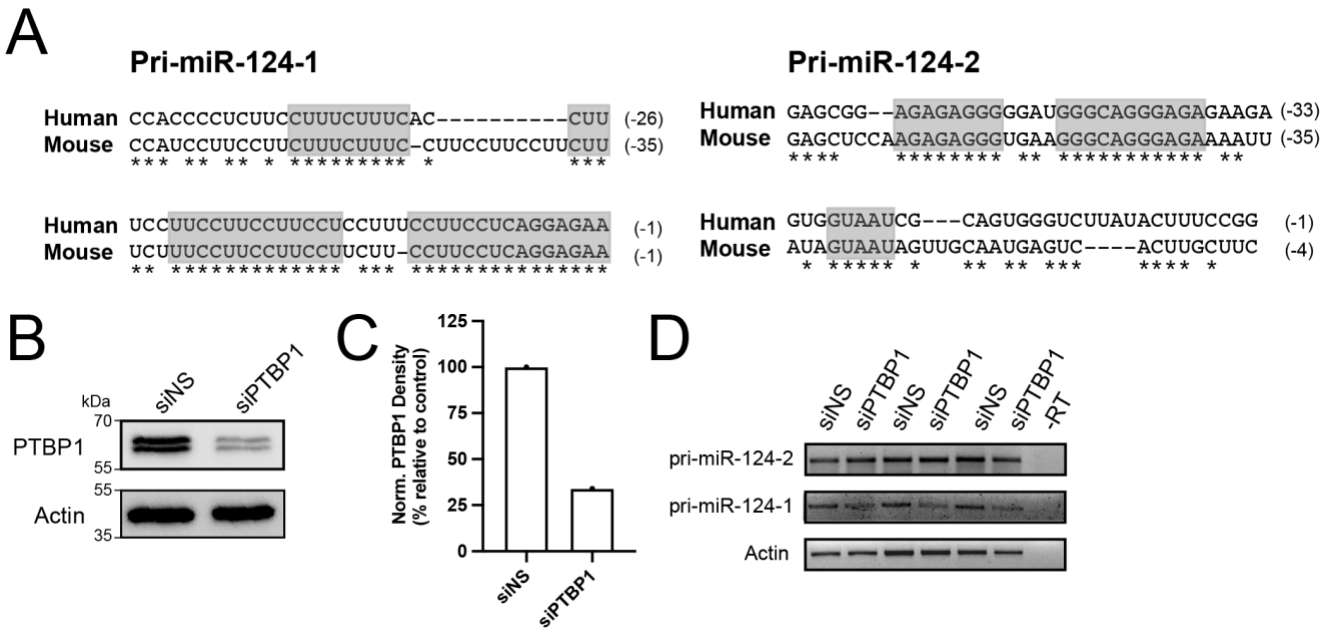

A
Pri-miR-124-1
Human CCACCCCUCUUCCUUUCUUUCAC----------CUU (-26)
Mouse CCAUCCUUCCUUCUUUCUUUC-CUUCCUUCCUUCUU (-35)
Human UCCUUCCUUCCUUCCUCCUUUCCUUCCUCAGGAGAA (-1)
Mouse UCUUUCCUUCCUUCCUUCUU-CCUUCCUCAGGAGAA (-1)
Pri-miR-124-2
Human GAGCGG--AGAGAGGGGGAUGGGCAGGGAGAGAAGA (-33)
Mouse GAGCUCCAAGAGAGGGUGAAGGGCAGGGAGAAAAUU (-35)
Human GUGGUAAUCG---CAGUGGGUCUUAUACUUUCCGG (-1)
Mouse AUAGUAAUAGUUGCAAUGAGUC----ACUUGCUUC (-4)
B
kDa
siNS
siPTBP1
70
PTBP1
55
55
Actin
35
C
Norm. PTBP1 Density (% relative to control)
125
100
75
50
25
0
siNS
siPTBP1
D
siNS
siPTBP1
siNS
siPTBP1
siNS
siPTBP1
-RT
pri-miR-124-2
pri-miR-124-1
Actin

Figure 2-3: Chromatin accessibility of the *MIR124-2HG* proximal promoter element increases during neuronal differentiation. (A) ATAC-seq peaks at the *MIR124-2HG* TSS in iPSC-derived hNPCs and neurons undergone 1 wk and 2 wks of differentiation. An orange bar is drawn under the proximal promoter element peak. A green bar is drawn under the promoter-TSS region. (B) PhastCons DNA sequence conservation of the *MIR124-2HG* proximal promoter element (*Top, orange*) and promoter-TSS region (*Bottom, green*) among 100 vertebrates. (C) Schematic illustration of conserved sequences between the human and mouse miR-124-2 host genes. GC and CAAT boxes identified in the proximal promoter element are depicted. (D) Activity of firefly luciferase reporter constructs driven by the *MIR124-2HG* promoter fragments of varying lengths in transfected BE(2)-M17 cells. Firefly luciferase activity of each construct is normalized to co-expressed Renilla luciferase activity. Results are normalized to the parent pGL3 plasmid. (E) Normalized RNA-seq signal of *MIR124-2HG* in iPSC-derived 1 wk. old neurons, 2 wk. old neurons, and BE(2)-M17 cells having undergone 10 days of retinoic acid (RA)-induced differentiated. (F) RT-qPCR detection of *MIR124-2HG* expression in BE(2)-M17 cells undergone 0 and 10 days of RA-induced differentiation (n = 4 cultures; Student's t=test; $*P \leq 0.05$; error bars are SEM).

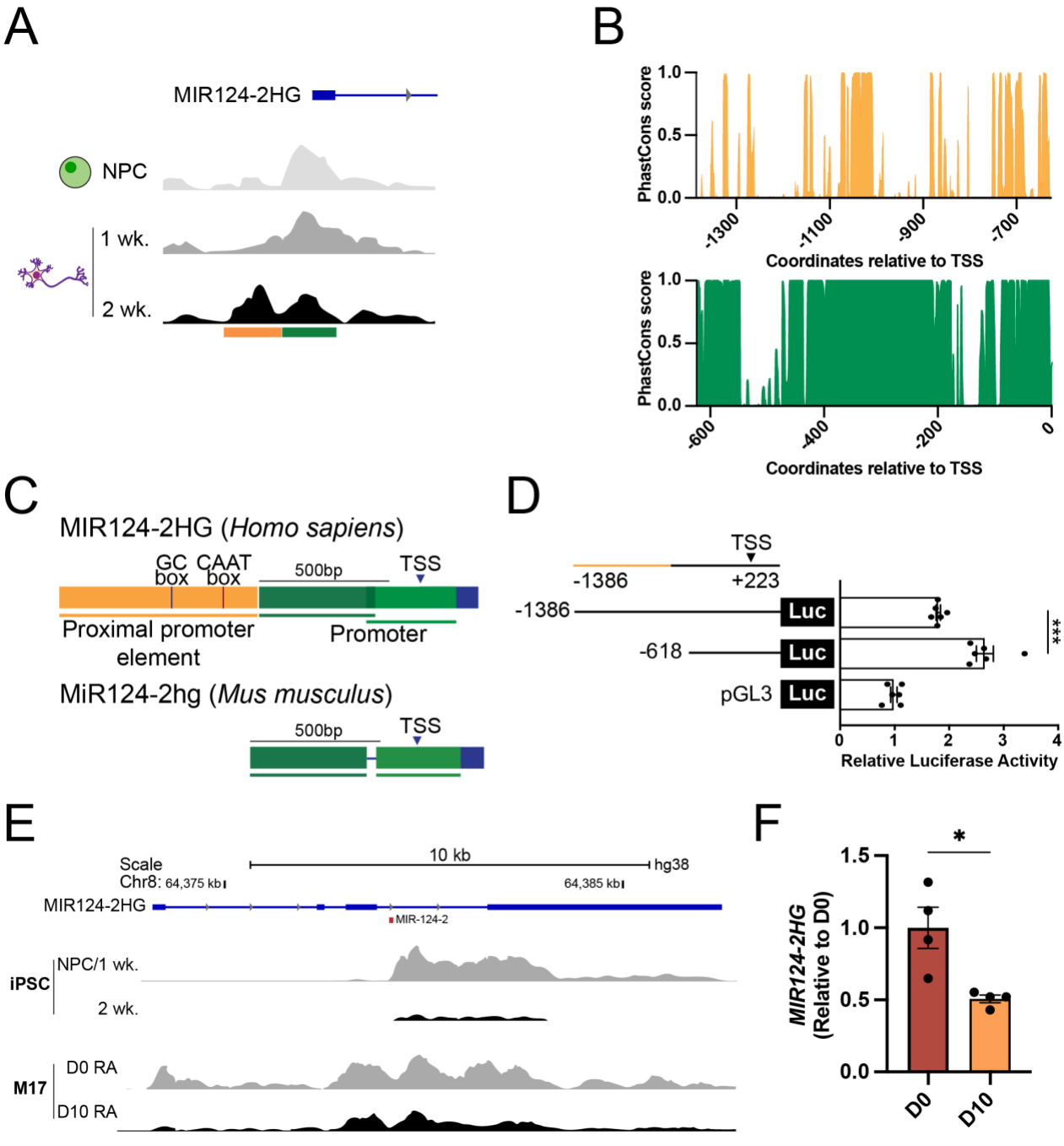

A
MIR124-2HG
NPC
1 wk.
2 wk.
B
PhastCons score
1.0
0.5
0.0
-1300
-1100
-900
-700
Coordinates relative to TSS
PhastCons score
1.0
0.5
0.0
-600
-400
-200
0
Coordinates relative to TSS
C
MIR124-2HG (Homo sapiens)
GC box
CAAT box
500bp
TSS
Proximal promoter element
Promoter
MiR124-2hg (Mus musculus)
500bp
TSS
D
TSS
-1386
+223
-1386
Luc
-618
Luc
pGL3
Luc
***
0
1
2
3
4
Relative Luciferase Activity
E
Scale
Chr8: 64,375 kb
10 kb
hg38
64,385 kb
MIR124-2HG
MIR-124-2
iPSC
NPC/1 wk.
2 wk.
M17
D0 RA
D10 RA
F
*
MIR124-2HG (Relative to D0)
1.5
1.0
0.5
0.0
D0
D10

Figure 2-4: MAFK negatively regulates transcription through the *MIR124-2HG* proximal promoter element. (A) Volcano plot illustrating log(2)-fold steady state mRNA level changes of up-regulated (red) and down-regulated (blue) transcription factors (TFs) following differentiation by retinoic acid over control (RA/CTL) and log10-transformed p-values after Student's t-test in biological triplicate. (B) A schematic representation of MAFK dimer-mediated regulation. MAFK may bind as a homodimer with other small MAF family members, namely MAFF and MAFG, to act as transcriptional repressors. MAFK may also form heterodimers with cap 'n' collar (CNC) and with Bach proteins to act as a transcriptional activator or repressor. (C) ENCODE genome browser view of MAFK and MAFF ChIP-seq signal at the MIR124-2HG locus in K562 cells. Y-axis represents fold change over control. (D) RT-qPCR of *MAFK*, *MAFG* and *MAFF* expression in BE(2)-M17 cells having undergone D0 and D10 of retinoic acid (RA)-induced differentiation (n = 4 cultures; Student's t=test; $*P \leq 0.05$; error bars are SEM). (E) Illustration of partial MAF recognition element (MARE) within the *MIR124-2HG* proximal promoter element (*Top*, bold). Luciferase activity of firefly luciferase reporter constructs driven by the wild type *MIR124-2HG* promoter fragment or promoter and proximal promoter element mutant lacking the partial MARE in transfected BE(2)-M17 cells (*Bottom*). Firefly luciferase activity is normalized to co-expressed Renilla luciferase activity for each construct. Results are normalized to the parent pGL3 plasmid.

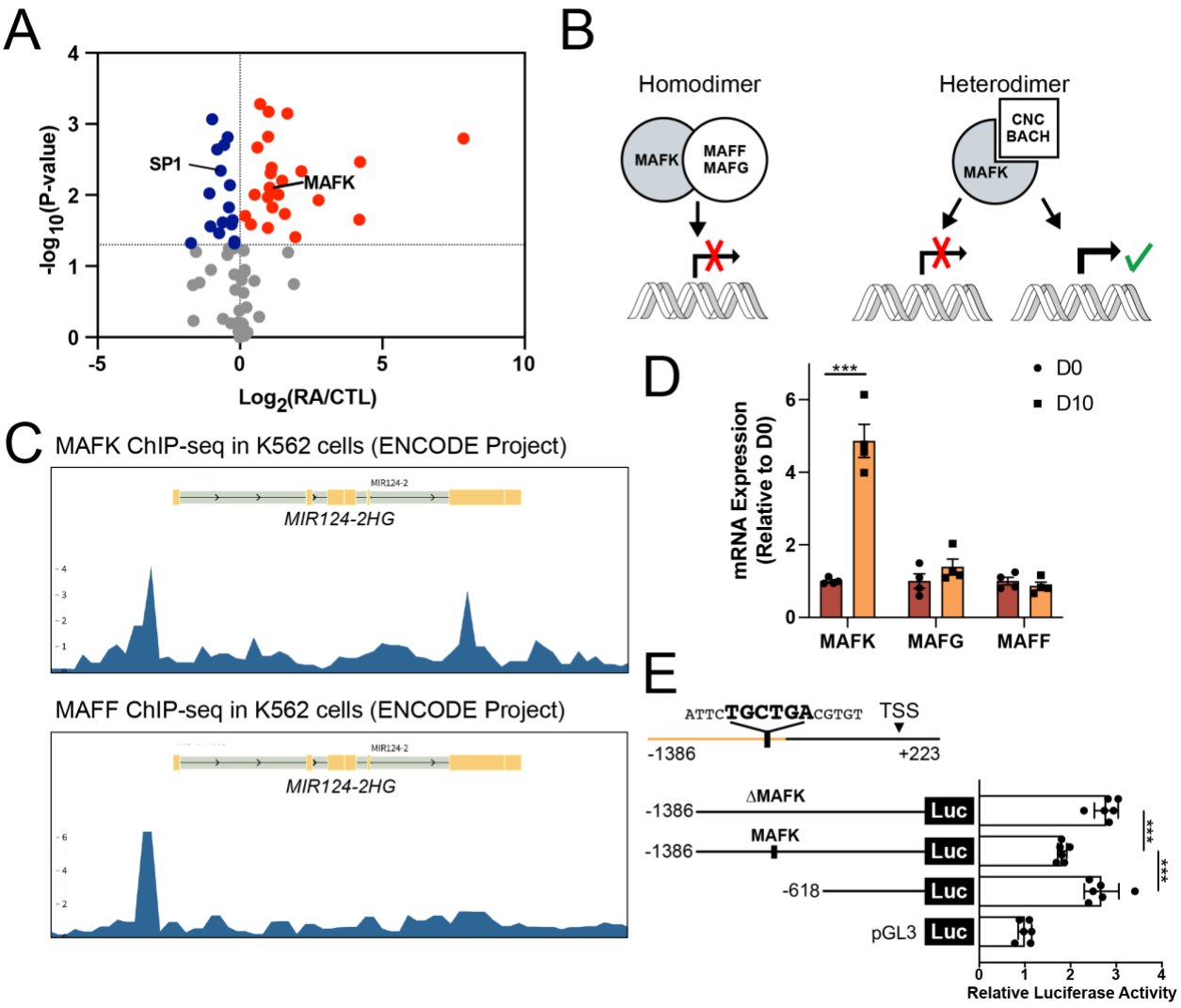

A
SP1
MAFK
-log10(P-value)
Log2(RA/CTL)
B
Homodimer
MAFK
MAFF
MAFG
Heterodimer
CNC
BACH
MAFK
C
MAFK ChIP-seq in K562 cells (ENCODE Project)
MIR124-2
MIR124-2HG
MAFF ChIP-seq in K562 cells (ENCODE Project)
MIR124-2
MIR124-2HG
D
***
D0
D10
mRNA Expression
(Relative to D0)
MAFK
MAFG
MAFF
E
ATTCTGCTGACGTGT
TSS
-1386
+223
ΔMAFK
-1386
Luc
MAFK
-1386
Luc
-618
Luc
pGL3
Luc
***
***
Relative Luciferase Activity

Figure 2-5: SP1 controls miR-124 biogenesis in hNPCs. (A) A schematic representation of the SP1 binding sites (red bars) in the *MIR124-2HG* proximal promoter element and promoter-TSS region identified using the JASPAR database. (B) RT-qPCR of *SP1* mRNA in control siRNA (siNS) and SP1 siRNA (siSP1)-treated BE(2)-M17 cells (n = 4 samples; Student's t=test; error bars are SEM). (C) Immunoblot detection of SP1 knockdown by siSP1 in BE(2)-M17 cells compared to siNS (*Left*). β-actin served as a loading control. Densitometry analysis of SP1 signal normalized to β-actin (*Right*). (D) RT-qPCR of pri-miR-124-2 expression in siNS and siSP1-treated BE(2)-M17 cells (n = 4 samples; Student's t=test; error bars are SEM). (E) RT-qPCR detects mature miR-124 (miR-124-3p) expression in siNS and siSP1-treated BE(2)-M17 cells (n = 3 samples; Student's t=test; error bars are SEM).

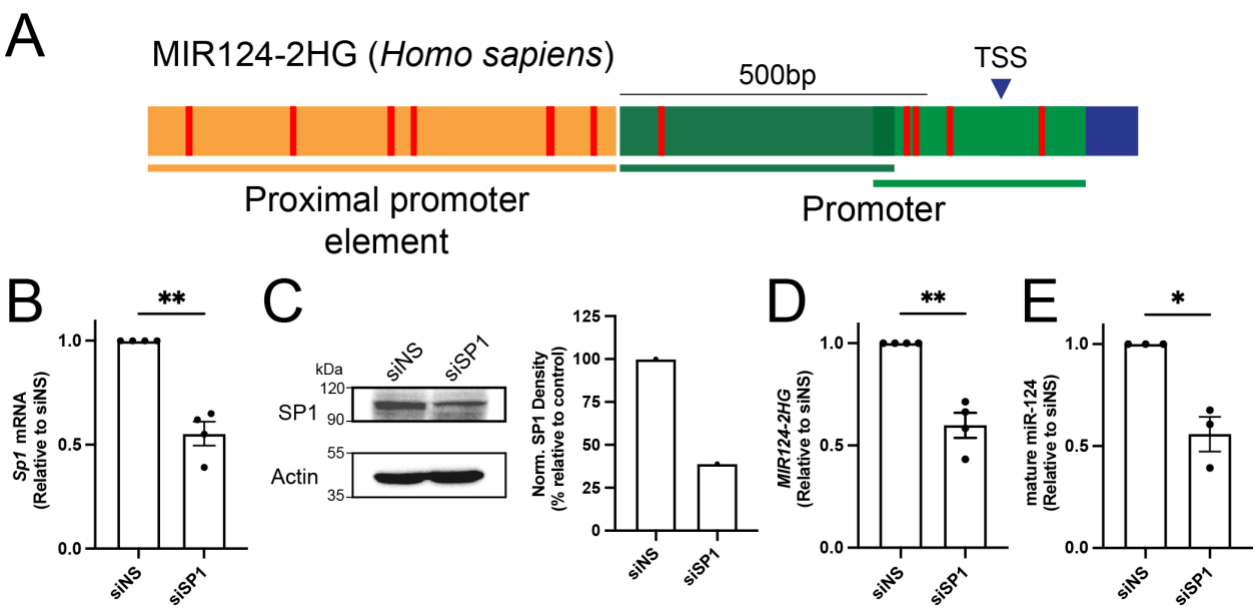
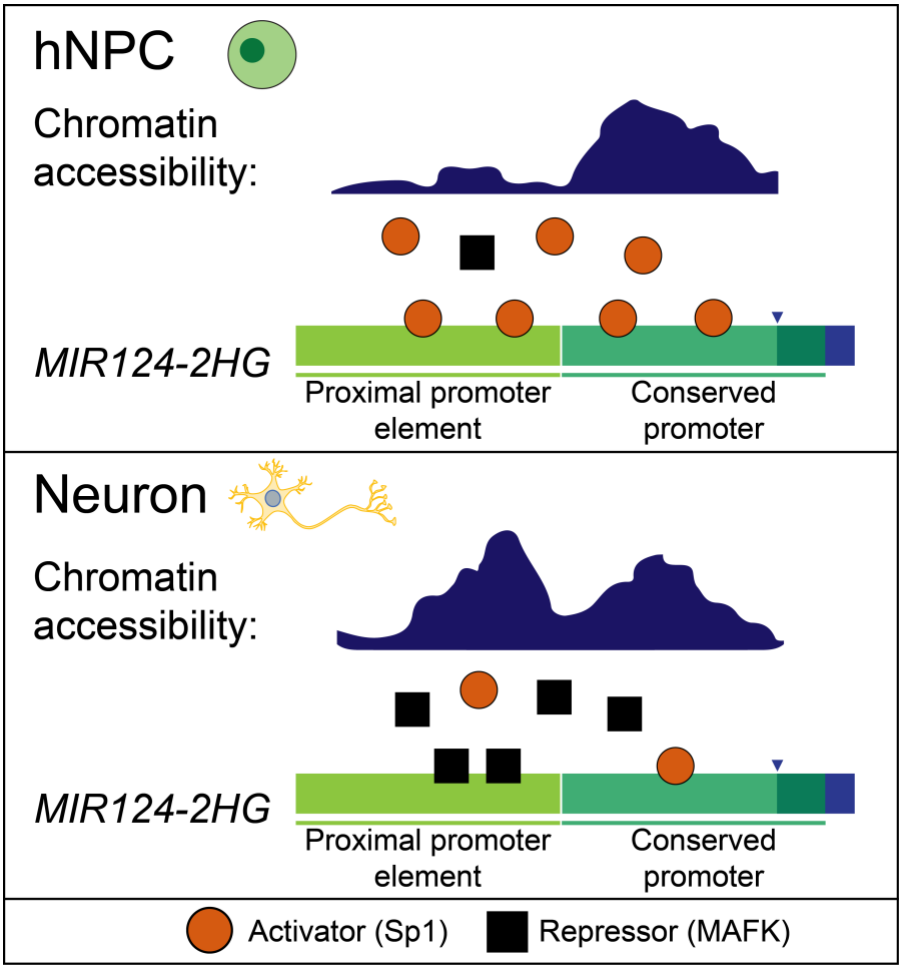
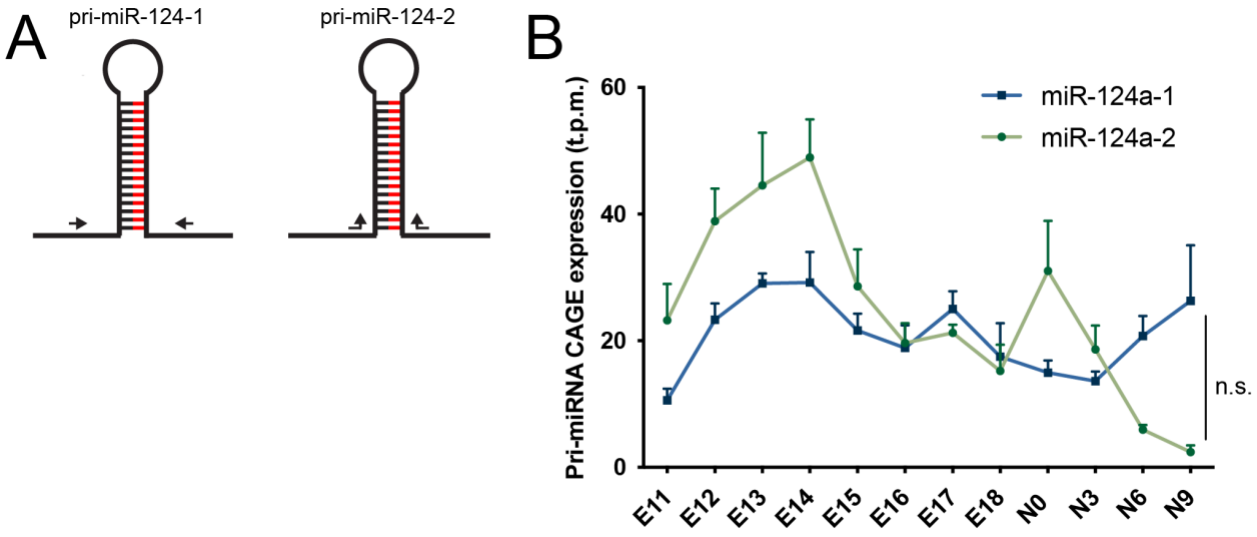

A
MIR124-2HG (Homo sapiens)
500bp
TSS
Proximal promoter element
Promoter
B
**
Sp1 mRNA (Relative to siNS)
1.0
0.5
0.0
siNS
siSP1
C
kDa
siNS
siSP1
SP1
120
90
Actin
55
35
Norm. SP1 Density (% relative to control)
125
100
75
50
25
0
siNS
siSP1
D
**
MIR124-2HG (Relative to siNS)
1.0
0.5
0.0
siNS
siSP1
E
*
mature miR-124 (Relative to siNS)
1.0
0.5
0.0
siNS
siSP1

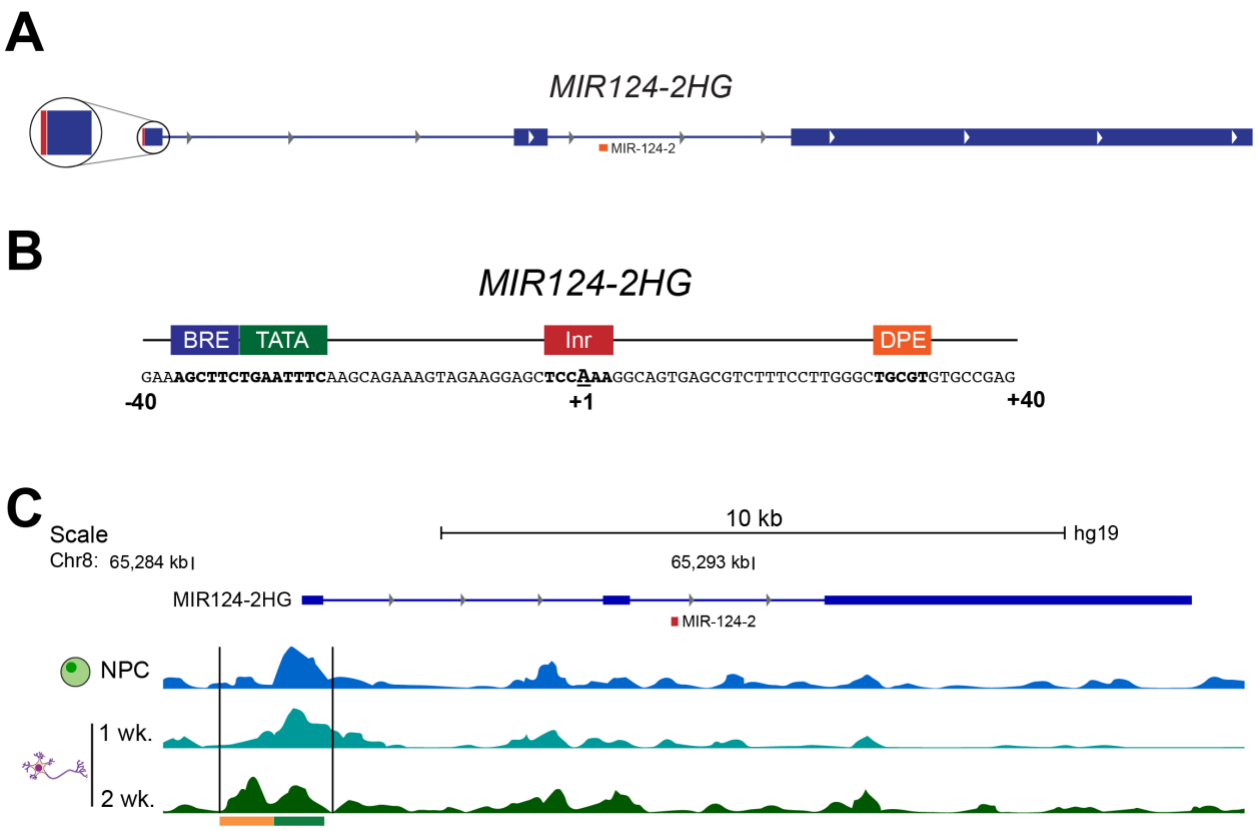
Figure 2-6: Model of developmentally regulated chromatin and transcription factor dynamics at the *MIR124-2HG* locus in human NPCs and neurons.


hNPC
Chromatin accessibility:
MIR124-2HG
Proximal promoter element
Conserved promoter
Neuron
Chromatin accessibility:
MIR124-2HG
Proximal promoter element
Conserved promoter
Activator (Sp1)
Repressor (MAFK)

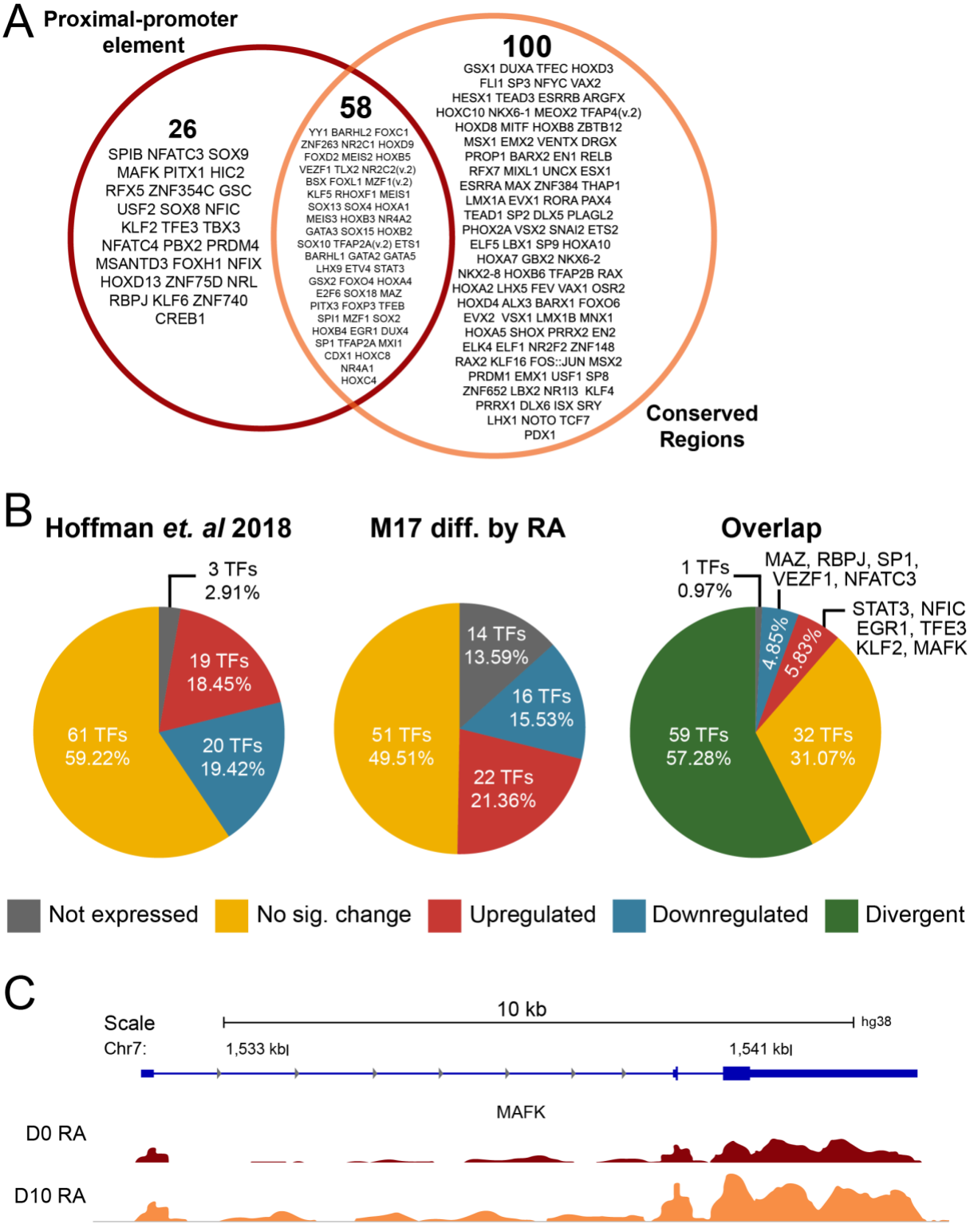
Supplementary Figure S2.1: A switch between pri-miR-124 paralogs in the developing mouse cerebellum, recapitulates human cerebellar development. (A) A schematic representation of the primers used in RT-PCR and RT-qPCR detection of human pri-miR-124-1 and -2 paralogs. (B) FANTOM5 pri-miRNA CAGE data for miR-124-1 and -2 transcripts in embryonic day 11 (E11) to postnatal day 9 (P9) mouse cerebellum (t.p.m., tags per million). Detailed information of FANTOM5 samples and raw values are available in Supplemental Table 2.


A
pri-miR-124-1
pri-miR-124-2
B
Pri-miRNA CAGE expression (t.p.m.)
60
40
20
0
miR-124a-1
miR-124a-2
n.s.
E11
E12
E13
E14
E15
E16
E17
E18
N0
N3
N6
N9

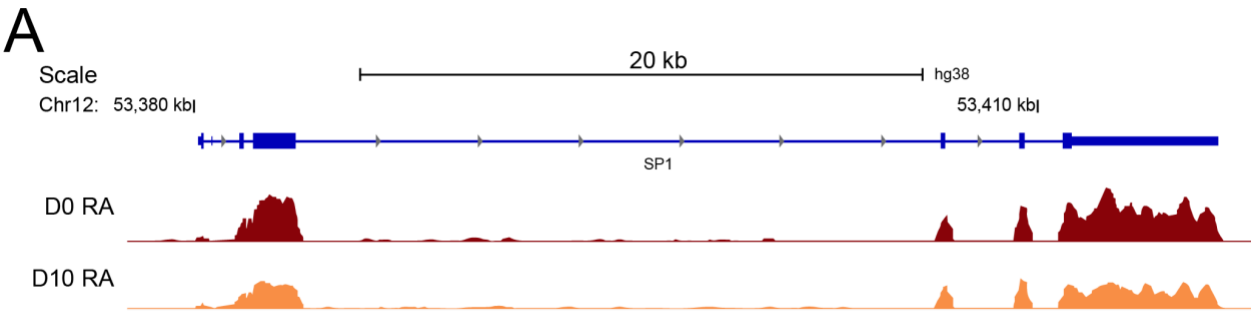
Supplementary Figure S2.2: The human *MIR124-2HG* promoter region and developmentally programed chromatin accessibility. (A) A schematic representation of the FANTOM5 CAGE-seq tag (red) for human miR-124-2 relative to the *MIR124-2HG* (blue). (B) A schematic representation of mammalian core promoter elements at the human *MIR124-2HG* TSS. TFIIB recognition element, BRE; TATA box, TATA; Initiator, Inr; Downstream promoter element, DPE. (C) ATAC-seq signal of *MIR124-2HG* gene body in iPSC-derived NPC, 1 week (1 wk.) and 2 week (2 wk.) old neurons.


A
MIR124-2HG
MIR-124-2
B
MIR124-2HG
BRE
TATA
Inr
DPE
GAAAGCTTCTGAATTTCAAGCAGAAAGTAGAAGGAGCTCCAAAGGCAGTGAGCGTCTTTCCTTGGGCTGCGTGTGCCGAG
-40
+1
+40
C
Scale
Chr8: 65,284 kb
10 kb
hg19
65,293 kb
MIR124-2HG
MIR-124-2
NPC
1 wk.
2 wk.

Supplementary Figure S2.3: Differential expression of candidate TFs during *in vitro* neuronal differentiation. (A) Venn diagram of candidate transcription factors with binding sites identified in the conserved promoter-TSS region, the proximal promoter element or both using the JASPAR database. (B) Pie charts of differential expression, as measured by RNA-seq of JASPAR predicted TFs during differentiation of iPSC-derived hNPCs to 6 wk. neurons (*Left*), differentiation of BE(2)-M17 cells by retinoic acid for 10 days (*Middle*) and the overlap between these two datasets (*Right*). (C) Normalized RNA-seq signal of *MAFK* in BE(2)-M17 cells having undergone 0 (D0) and 10 days (D10) of retinoic acid-induced differentiation.


A
Proximal-promoter element
26
SPIB NFATC3 SOX9 MAFK PITX1 HIC2 RFX5 ZNF354C GSC USF2 SOX8 NFIC KLF2 TFE3 TBX3 NFATC4 PBX2 PRDM4 MSANTD3 FOXH1 NFIX HOXD13 ZNF75D NRL RBPJ KLF6 ZNF740 CREB1
58
YY1 BARHL2 FOXC1 ZNF263 NR2C1 HOXD9 FOXD2 MEIS2 HOXB5 VEZF1 TLX2 NR2C2(v.2) BSX FOXL1 MZF1(v.2) KLF5 RHOXF1 MEIS1 SOX13 SOX4 HOXA1 MEIS3 HOXB3 NR4A2 GATA3 SOX15 HOXB2 SOX10 TFAP2A(v.2) ETS1 BARHL1 GATA2 GATA5 LHX9 ETV4 STAT3 GSX2 FOXO4 HOXA4 E2F6 SOX18 MAZ PITX3 FOXP3 TFEB SPI1 MZF1 SOX2 HOXB4 EGR1 DUX4 SP1 TFAP2A MXI1 CDX1 HOXC8 NR4A1 HOXC4
100
GSX1 DUXA TFEC HOXD3 FLI1 SP3 NFYC VAX2 HESX1 TEAD3 ESRRB ARGFX HOXC10 NKX6-1 MEOX2 TFAP4(v.2) HOXD8 MITF HOXB8 ZBTB12 MSX1 EMX2 VENTX DRGX PROP1 BARX2 EN1 RELB RFX7 MIXL1 UNCX ESX1 ESRRA MAX ZNF384 THAP1 LMX1A EVX1 RORA PAX4 TEAD1 SP2 DLX5 PLAGL2 PHOX2A VSX2 SNAI2 ETS2 ELF5 LBX1 SP9 HOXA10 HOXA7 GBX2 NKX6-2 NKX2-8 HOXB6 TFAP2B RAX HOXA2 LHX5 FEV VAX1 OSR2 HOXD4 ALX3 BARX1 FOXO6 EVX2 VSX1 LMX1B MNX1 HOXA5 SHOX PRRX2 EN2 ELK4 ELF1 NR2F2 ZNF148 RAX2 KLF16 FOS::JUN MSX2 PRDM1 EMX1 USF1 SP8 ZNF652 LBX2 NR1I3 KLF4 PRRX1 DLX6 ISX SRY LHX1 NOTO TCF7 PDX1
Conserved Regions
B
Hoffman et. al 2018
M17 diff. by RA
Overlap
3 TFs 2.91%
19 TFs 18.45%
61 TFs 59.22%
20 TFs 19.42%
14 TFs 13.59%
16 TFs 15.53%
51 TFs 49.51%
22 TFs 21.36%
1 TFs 0.97%
MAZ, RBPJ, SP1, VEZF1, NFATC3
STAT3, NFIC EGR1, TFE3 KLF2, MAFK
4.85%
5.83%
59 TFs 57.28%
32 TFs 31.07%
Not expressed
No sig. change
Upregulated
Downregulated
Divergent
C
Scale
10 kb
hg38
Chr7:
1,533 kbl
1,541 kbl
MAFK
D0 RA
D10 RA

Supplementary Figure S2.4: *SP1* decreases in BE(2)-M17 cells having undergone 0 (D0) and 10 days (D10) of retinoic acid-induced differentiation. (A) Normalized RNA-seq signal of *SP1* in BE(2)-M17 cells differentiated by retinoic acid. (B) Normalized RNA-seq FPKM of *SP1* in BE(2)-M17 cells differentiated by retinoic acid (n = 3 cultures; Student's t=test; $*P \leq 0.05$; error bars are SEM).


A
Scale
20 kb
hg38
Chr12: 53,380 kbl
53,410 kbl
SP1
D0 RA
D10 RA

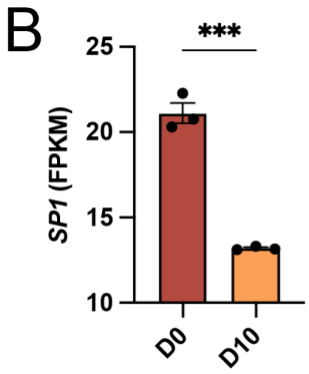

B
***
25
20
15
10
SP1 (FPKM)
D0
D10

Table S2.1 Human FANTOM5 sample descriptions and data values.

Sample	Description	Day	Rep	Pri-miRNA CAGE expression (t.p.m.)
Control donor C11-CRL2429, iPS differentiation to neuron	hsa-miR-124-1	18	1	2.964579
	hsa-miR-124-1	18	2	2.288225
	hsa-miR-124-1	18	3	4.819329
	hsa-miR-124-2	18	1	32.610371
	hsa-miR-124-2	18	2	21.670841
	hsa-miR-124-2	18	3	28.915976
	hsa-miR-124-3	18	1	1.905801
	hsa-miR-124-3	18	2	1.615218
	hsa-miR-124-3	18	3	0.602416
Control donor C32-CRL1502, iPS differentiation to neuron	hsa-miR-124-1	18	1	3.628764
	hsa-miR-124-1	18	2	3.260794
	hsa-miR-124-1	18	3	1.404584
	hsa-miR-124-2	18	1	24.13917
	hsa-miR-124-2	18	2	19.861203
	hsa-miR-124-2	18	3	23.3161
	hsa-miR-124-3	18	1	0.946634
	hsa-miR-124-3	18	2	0.296436
	hsa-miR-124-3	18	3	1.123667
Newborn Donor 10223, Cerebellum	hsa-miR-124-1			97.306187
	hsa-miR-124-2			2.32373
	hsa-miR-124-3			4.64746
Adult Donor 10196, Cerebellum	hsa-miR-124-1			222.232559
	hsa-miR-124-2			6.460249
	hsa-miR-124-3			3.876149

Table S2.2 Mouse FANTOM5 sample descriptions and data values.

Sample	Description	Day	Rep	Value
Mouse E11, Cerebellum	mmu-miR-124-1	E11	1	14.190623
	mmu-miR-124-1	E11	2	8.194173
	mmu-miR-124-1	E11	3	9.412823
	mmu-miR-124-2	E11	1	34.700507
	mmu-miR-124-2	E11	2	17.957444
	mmu-miR-124-2	E11	3	17.044842
Mouse E12, Cerebellum	mmu-miR-124-1	E12	1	24.094162
	mmu-miR-124-1	E12	2	18.543161
	mmu-miR-124-1	E12	3	27.361526
	mmu-miR-124-2	E12	1	49.062207
	mmu-miR-124-2	E12	2	32.646409
	mmu-miR-124-2	E12	3	34.934805
Mouse E13, Cerebellum	mmu-miR-124-1	E13	1	32.14597
	mmu-miR-124-1	E13	2	27.969211
	mmu-miR-124-1	E13	3	27.025139
	mmu-miR-124-2	E13	1	60.832284
	mmu-miR-124-2	E13	2	39.156896
	mmu-miR-124-2	E13	3	33.69368
Mouse E14, Cerebellum	mmu-miR-124-1	E14	1	36.507741
	mmu-miR-124-1	E14	2	30.985418
	mmu-miR-124-1	E14	3	20.06796
	mmu-miR-124-2	E14	1	59.878227
	mmu-miR-124-2	E14	2	47.903836
	mmu-miR-124-2	E14	3	39.132522
Mouse E15, Cerebellum	mmu-miR-124-1	E15	1	26.337329
	mmu-miR-124-1	E15	2	17.088296
	mmu-miR-124-1	E15	3	21.417313
	mmu-miR-124-2	E15	1	40.207283
	mmu-miR-124-2	E15	2	21.504373
	mmu-miR-124-2	E15	3	24.057803
Mouse E16, Cerebellum	mmu-miR-124-1	E16	1	21.580066
	mmu-miR-124-1	E16	2	11.765392
	mmu-miR-124-1	E16	3	23.199341
	mmu-miR-124-2	E16	1	23.062666
	mmu-miR-124-2	E16	2	13.399474
	mmu-miR-124-2	E16	3	22.456962
Mouse E17, Cerebellum	mmu-miR-124-1	E17	1	30.473628
	mmu-miR-124-1	E17	2	23.453425
	mmu-miR-124-1	E17	3	21.113973
	mmu-miR-124-2	E17	1	22.763433
	mmu-miR-124-2	E17	2	18.694759
	mmu-miR-124-2	E17	3	22.265644

Mouse E18, Cerebellum	mmu-miR-124-1	E18	1	24.539283
	mmu-miR-124-1	E18	2	20.808998
	mmu-miR-124-1	E18	3	7.116923
	mmu-miR-124-2	E18	1	21.8315
	mmu-miR-124-2	E18	2	16.184777
	mmu-miR-124-2	E18	3	7.71
Mouse N0, Cerebellum	mmu-miR-124-1	N0	1	18.195073
	mmu-miR-124-1	N0	2	11.532498
	mmu-miR-124-1	N0	3	15.158449
	mmu-miR-124-2	N0	1	46.094185
	mmu-miR-124-2	N0	2	19.320679
	mmu-miR-124-2	N0	3	27.736737
Mouse N3, Cerebellum	mmu-miR-124-1	N3	1	14.872511
	mmu-miR-124-1	N3	2	10.588865
	mmu-miR-124-1	N3	3	15.419672
	mmu-miR-124-2	N3	1	11.154383
	mmu-miR-124-2	N3	2	23.010418
	mmu-miR-124-2	N3	3	21.701761
Mouse N6, Cerebellum	mmu-miR-124-1	N6	1	24.575055
	mmu-miR-124-1	N6	2	23.181091
	mmu-miR-124-1	N6	3	14.451761
	mmu-miR-124-2	N6	1	7.160943
	mmu-miR-124-2	N6	2	6.047241
	mmu-miR-124-2	N6	3	4.624563
Mouse N9, Cerebellum	mmu-miR-124-1	N9	1	10.431958
	mmu-miR-124-1	N9	2	40.773449
	mmu-miR-124-1	N9	3	27.717711
	mmu-miR-124-2	N9	1	2.013185
	mmu-miR-124-2	N9	2	4.414959
	mmu-miR-124-2	N9	3	0.815227

Table S2.3 Oligo sequences used in this study.

Application	Target	Forward Primer (5' → 3')	Reverse Primer (3' → 5')
RT-PCR	pri-miR-124-1 (Human)	CCCTCTTCCTTTCTTTCACCTTTC	TCGGTCGGTCGCTCCTTC
	pri-miR-124-2 (Human)	CAGTGGGTCTTATACTTTCCGGATC	GAGATGCTTTGGTGTCCTTCAAGT
RT-qPCR	pri-miR-124-2 (Human)	CAGTGGGTCTTATACTTTCCGGATC	GAGATGCTTTGGTGTCCTTCAAGT
	MIR124-2HG	GTGAGCGTCTTTCCTTGGG	AGAGAGAGCTGAGAGCTGGT
	MAFK	GACGACTAATCCCAAACCGAATA	CATGGACACCAGCTCATCAT
	SP1	GCCGTTGGCTATAGCAAATGC	CCTCTCCACCTGCTGTGTCA
	MAFF	ATCCCCTATCCAGCAAAGCTC	TTGAGCCGTGTCACCTCCTC
	MAFG	CCCAATAAAGGAAACAAGGCC	GCACCGACATGGTCACCA
	Actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
Cloning	MIR124-2HG Promoter	GCGCGGTACCCGGGTTGATAAAAGGCTCCA	CCCGGGCGTGCGCTCGGGTAATC
	MIR124-2HG Promoter + PPE	GCGCGGTACCGGTCCTAACCTCTCTCGCATC	CCCGGGCGTGCGCTCGGGTAATC
	MIR124-2HG Promoter + PPE △MAFK	GATCAGGCTTGATTCCGTGTGTATTTCAGA	TCTGAAATACACACGGAATCAAGCCTGATC

Underlines indicate restriction enzyme sites.

	Target	Sequence/Identifier	Cat. No
siRNA	siPTBP1	CAGTTTACCTGTTTTTAAA	
	siSP1	Dharmacon ON-TARGETplus SMARTpool siRfNA	Cat #: L-026959-00-0005
	siNS	Ambion™ In Vivo Negative Control #1 siRNA	Cat #: 4457287

Chapter 3: Posttranscriptional mechanisms miR-124 biogenesis during neurodevelopment

3.1 Introduction

MicroRNAs are small, non-coding RNAs that act as post-transcriptional suppressors of gene expression. These small RNA molecules, which play key roles in neuronal development (Olde Loohuis et al., 2012; Xue et al., 2013; Xue et al., 2016), are primarily produced through the canonical miRNA biogenesis pathway, beginning with the transcription of a miRNA host gene by RNA polymerase II (RNAPII) to generate a primary miRNA (pri-miRNA) transcript (Lee et al., 2004). Pri-miRNAs are cleaved by the RNase III enzyme Drosha and its cofactor DGCR8, together termed the Microprocessor complex, to generate a 60-80 nucleotide (nt) stem-loop intermediate known as the precursor miRNA (pre-miRNA) (Denli et al., 2004; Lee et al., 2003). Pre-miRNAs are then exported to the cytoplasm and cleaved by Dicer to produce a miRNA duplex (Bernstein et al., 2001; Hutvagner et al., 2001; Lund et al., 2004). Generally, one strand of the miRNA duplex, the guide strand, is preferentially loaded onto Argonaute (AGO) to form the RNA-induced silencing complex (RISC), which targets and silences gene expression, directed by miRNA partial base-pair complementarity with the 3'UTR of messenger RNAs (mRNAs) (Kobayashi and Tomari, 2016; Mourelatos et al., 2002).

In addition to *cis* elements within pri-miRNAs, which distinguish pri-miRNAs for Microprocessor recognition and cleavage from other hairpins in the transcriptome (Auyeung et al., 2013; Roden et al., 2017), many RNA binding proteins (RBPs), which show neural-lineage specific expression, affect pri-miRNA processing, including polypyrimidine tract binding protein (PTBP1), KH-type splicing regulatory protein (KSRP) and Quaking (QKI) (Giovarelli et al., 2013; Hardy et al., 1996; Makeyev et al., 2007; Trabucchi et al., 2009; Wang et al., 2013; Yeom et al., 2018).

A recent study demonstrated that depletion of PTBP1 converts astrocytes into functional neurons (Qian et al., 2020). In addition, PTBP1 was recently shown to repress processing of a key pro-neurogenic microRNA (miRNA/miR), miR-124, in mouse embryonic stem cells (Yeom et al., 2018). These foundational studies suggest that other RBPs which exhibit neural lineage specific expression may also play important roles in neural fate specification, in part, by regulating processing of key pri-miRNAs. In our study, we focus on one such lineage-specific RBP, QKI, as a potential regulator of miR-124 processing in human neural progenitor cells (hNPCs).

Alternative splicing of the *QKI* gene produces three unique QKI protein isoforms with distinct C-termini, QKI5, 6 and 7, named after their respective mRNA sizes (Kondo et al., 1999). At steady state, QKI5 is primary localized to the nucleus, where Microprocessor-mediated cleavage of pri-miRNAs occurs (Wu et al., 1999). In contrast, QKI6 and 7 are found in the cytoplasm. All three QKI isoforms bind to a consensus sequence known as the Quaking response element (QRE) (Galarneau and Richard, 2005), composed of ACUAAT/C-N_{1-20}-UAAT/C. Binding of QKI to QREs mediates QKI's role in regulating splicing, stability, subcellular localization and translation of mRNAs and in controlling the cellular abundance of certain circular RNAs (Conn et al., 2015; de Bruin et al., 2016; Larocque et al., 2005; Li et al., 2000; Mandler et al., 2014; Saccomanno et al., 1999; Thangaraj et al., 2017; Wu et al., 2002). Therefore, QKI binding to QRE(s) on non-coding RNAs critically regulates the abundance, activity and localization of numerous RNA spcies.

QKI is expressed in rodent neural stem cells (NSCs) and neural progenitor cells (NPCs) but is selectively silenced in neurons of the central nervous system (CNS) (Hardy

et al., 1996; Hayakawa-Yano et al., 2017; Takeuchi et al., 2020). The role of QKI in promoting myelination is well-documented but recent studies suggest QKI may promote glial lineage establishment at the expense of neuronal lineage commitment as neuron-enriched genes are significantly upregulated in *Qk*$^{-/-}$ neural stem cells (NSCs) (Takeuchi et al., 2020). Moreover, knockdown of QKI promotes migration of E13.5 forebrain NPCs out of the ventricular zone into the intermediate zone, thus depletion of QKI also promotes NPC migration (Shu et al., 2017).

Mature miR-124 is a pro-neurogenic miRNA, targeting several repressors of neuronal differentiation, represented by RhoG, PAX3 and BAF53a (Franke et al., 2012; Wei et al., 2018; Yoo et al., 2009). In the human genome, mature miR-124 belongs to a multigene family encoded by three paralogous genes, *miR-124-1*, *miR-124-2* and *miR-124-3* (Rodriguez et al., 2004). A previous study reported that QKI promotes processing of predominantly expressed pri-miR-124-1 in human erythrocytes (Wang et al., 2017). In contrast, we recently showed that pri-miR-124-2 is highly expressed in hNPCs. Therefore, although early studies performed in human non-neuronal systems have defined a role for QKI in regulating miR-124 biogenesis from pri-miR-124-1, further investigation is needed to define whether and how QKI regulates miR-124 biogenesis in human NPCs and neurons.

Here we postulate that Microprocessor-mediated processing of pri-miR-124-2 is inefficient in hNPCs, which may be regulated by nuclear QKI, QKI5. We identified a QKI response element (QRE) ~150nt upstream of the miR-124-2 hairpin, which may coordinate QKI-mediated regulation of miR-124 biogenesis in hNPCs. As neuronal differentiation proceeds, the repressive mechanism regulating pri-miR-124-2 processing

is alleviated, as miR-124 increases to drive neuronal maturation. During this developmental program, we also observe a developmental downregulation in QKI isoforms. Lastly, deletion of nuclear QKI5 by CRISPR/Cas9 gene editing increased mature miR-124 expression. Taken together, these results suggest a role for QKI5 in regulating mature miR-124 levels, potentially via regulation of pri-miR-124 paralog processing, in hNPCs and provide new insights into cell-type specific regulation of paralogs within the same miRNA multigene family by different neural lineage-specific RBPs.

3.2 Results

3.2.1 Expression of miRNA machinery during *in vitro* differentiation of BE(2)-M17 cells

We previously detected high levels of pri-miR-124-2 in iPSC-derived hNPCs and a hNPC cell line model, BE(2)-M17 cells, henceforth called M17 (Figure 2-1A). Although mature miR-124 is abundant in hNPCs, the detection of pri-miR-124-2 in hNPCs suggests that pri-miR-124-2 processing by the Microprocessor is incomplete, as pri-miRNAs are generally processed co-transcriptionally and do not accumulate at steady-state (Morlando et al., 2008; Pawlicki and Steitz, 2008; Yin et al., 2015). We postulate that processing of pri-miR-124-2 may be posttranscriptionally regulated in human NPCs. Mechanistically, a *trans*-acting factor positively regulating pri-miR-124-2 processing may be developmentally upregulated or conversely, a negative regulator of pri-miR-124-2 processing may be subject to developmental downregulation.

Another potential explanation for detectable expression of pri-miR-124-2 in hNPCs is low expression of Microprocessor subunits, Drosha and DGCR8, leading to accumulation of pri-miRNA transcripts and overall low global miRNA production in hNPCs. However, the expression profile of Microprocessor machinery during human neuronal differentiation has not been precisely characterized. We detected no significant change in *Drosha* or *DGCR8* expression by RNA-seq or RT-qPCR during differentiation of M17 cells induced by retinoic acid (RA) (Figure 3-1). These data agree with unchanged expression of *Drosha* and *DCGR8* measured during *in vitro* differentiation of murine P19 cells (Thomson et al., 2006). Additionally, efficient processing of other pro-neurogenic miRNAs from upstream pri-/pre-miRNA transcripts, namely miR-9, has been observed in human ESC-derived NPCs (Delaloy et al., 2010), indicating Microprocessor is active in this developmental stage. Therefore, changes in the expression of Drosha and DGCR8, of the Microprocessor complex, do not underlie the proposed accumulation of pri-miR-124-2 in hNPCs, which is alleviated during neuronal differentiation.

3.2.2 *In silico* prediction program identifies QKI as a candidate regulator of pri-miR-124-2 processing

Having shown that changes in the expression of the Microprocessor subunits do not underlie changes in pri-miR-124-2 expression measured during *in vitro* differentiation of M17 cells, we speculated that an RNA binding protein (RBP) or other *trans*-acting factor may regulate processing of pri-miR-124-2. We used a computational program named circRNAs interact with proteins (CRIP) (Zhang et al., 2019b) to predict RBP binding sequence motifs in the human miR-124-2 hairpin and 200 nucleotides (nt) flanking the hairpin. We focused on a 200 nt window up- and downstream of the miR-124-2 hairpin

as pri-miRNA single-stranded flanking segments are not only necessary for efficient pri-miRNA processing by Microprocessor (Han et al., 2004; Zeng and Cullen, 2005) but are also bound by RBPs to regulate miRNA biogenesis (Auyeung et al., 2013; Kim et al., 2018; Mori et al., 2014; Yeom et al., 2018).

The CRIP program yielded 137 RBPs with one of more putative binding sites on pri-miR-124-2, when applying a moderate cutoff of 0.8 to the data (Supplemental Table S3-1). To screen for functional RBPs that are expressed in our system of interest and undergo significant differential expression during differentiation of M17 cells induced by retinoic acid (RA) we analyzed expression of our candidate RBPs by RNA-seq (Fig 3-2A). We then selected the RBPs that had been previously reported to regulate miRNA biogenesis. We chose QKI as the most relevant candidate as the *QKI* transcript was significantly changed during RA-induced *in vitro* differentiation of M17 cells (Figure 3-2A) and several previous studies have reported QKI as a regulator of miRNA biogenesis (Treiber et al., 2017; Wang et al., 2017; Wang et al., 2013).

In the transcript of interest, the putative pri-miR-124-2 QRE, identified by the CRIP program, is proximal to the stem-loop, ~150 nt upstream (Figure 3-2 B, C). We became interested in the relationship between the position of the QRE, relative to the miRNA stem-loop, and the reported regulatory role of QKI in modulating Microprocessor cleavage. In human erythrocytes, nuclear QKI5 binds to a distal QRE ~350 nt upstream of the pri-miR-124-1 stem loop and recruits the Microprocessor complex the pri-miRNA transcript (Wang et al., 2017). Simultaneously, a local RNA-RNA interaction brings the QKI5-Microprocessor complex into closer proximity to the stem-loop to promote processing of pri-miR-124-1. In contrast, in glial cells , binding of QKI5 near the stem-loop of pri-miR-7

prevents pri-miRNA processing (Wang et al., 2013). Based on the previous studies mentioned, we hypothesized that QKI-mediated binding to the putative pri-miR-124-2 QRE negatively regulates pri-miR-124-2 processing.

3.2.3 QKI is expressed in hNPCs, declining before PTBP1 during neuronal differentiation

Having measured a significant decrease in *QKI* expression during *in vitro* differentiation of M17 by RNA-seq, we next investigated the developmental regulation of individual QKI protein isoforms during neuronal differentiation. By immunoblot, we observed a visible decrease in both QKI5 and -6 isoforms during neuronal differentiation of iPSC-derived NPCs into 8-week-old neurons (Figure 3-3A). QKI7 was not expressed at appreciable levels in these samples (data not shown). These data support our hypothesis that QKI-mediated negative regulation of pri-miR-124-2 processing would be alleviated during early human neuronal differentiation as QKI is downregulated.

Moreover, our lab previously demonstrated that pri-miR-124-2 may serve as a source of mature miR-124 in hNPCs since processing of pri-miR-124-2 is not regulated by PTBP1 (Figure 2-2). However, if, as we propose, processing of pri-miR-124-2 is negatively regulated by QKI5, the question remains of which pri-miR-124 paralog is the initial pool of mature miR-124 processed from to initiate miR-124 mediated neuronal differentiation, as processing of both pri-miR-124-2 and -1 paralogs is attenuated at the Microprocessor step in hNPCs. To address this question, we next assessed the expression profile of the QKI isoforms and PTBP1 during a differentiation time-course of iPSC-derived NPCs to 2- and 8-week-old neurons. Using a pan-QKI-antibody, we observed a decrease in expression of all three QKI isoforms, from hNPCs to 6 wk old

neurons (Figure 3-3B). During this differentiation time course, PTBP1 did not significantly decrease. Thus, the early developmental downregulation of QKI supports our hypothesis that pri-miR-124-2 is processed into the first pool of mature miR-124 as QKI decrease precedes PTBP1 decline.

3.2.4 QKI5 does not associate with DGCR8 of the Microprocessor complex in neural cells

Cleavage of pri-miRNAs by the Microprocessor complex is the initial enzymatic reaction in the miRNA processing pathway, thereby this event defines the abundance of pri-miRNAs and the derivative miRNAs. Previous studies in non-neuronal cells have shown that QKI5 directly associates with the DGCR8 subunit of the Microprocessor complex (Wang et al., 2017). To test this interaction in a neural system, M17 cells were transfected with FLAG-DGCR8. We then performed a co-immunoprecipitation (coIP) experiment to ask whether endogenous QKI5 interacts with DGCR8. We hypothesized that QKI5 may associate with DGCR8 on the pri-miR-124-2 QRE to sequester DGCR8 away from Drosha, inhibiting formation of the Microprocessor complex and thereby locally repress pri-miR-124-2 processing. However, we did not detect an interaction between DGCR8 and native QKI5 (Figure 3-4A). Furthermore, we co-transfected M17 cells with FLAG-DGCR8 and HA-QKI5 to increase the cellular abundance of QKI5 in these cells. As we observed for endogenous QKI5, HA-tagged QKI5 did not interact with DGCR8 (Figure 3-4B). Our results suggest that QKI5 does not regulate processing of pri-miR-124-2 through interaction with the DGCR8 subunit of the Microprocessor complex. We therefore propose that QKI5 may bind to pri-miR-124-2 and sterically hinder Microprocessor from cleaving the miR-124-2 hairpin.

3.2.5 Deletion of QKI5 increases mature miR-124 levels

To further investigate potential regulation of pri-miR-124-2 processing by QKI5, we carried out CRISPR/Cas9 gene editing to delete nuclear QKI5 in BE(2)-M17 cells. We designed two CRISPR guide RNA (gRNAs) flanking the QKI5 specific exon, exon 7c, (Ebersole et al., 1996) to increase target specificity and minimize off-target effects (Figure 3-5A). We transfected our gRNAs into a M17 cell line stably expressing Cas9, as well as a non-specific gRNA as a negative control, in parallel. We performed PCR using QKI5 specific primers to validate on-target editing compared to control (Figure 3-5B). Additionally, we performed RNA-sequencing to validate QKI5 isoform-specific deletion (Figure 3-5C) and to confirm no cross-reactivity of the gRNAs with the other QKI isoforms, namely QKI6. We observed an increase in mature miR-124 in ΔQKI5 M17 cells, compared with control, by stem-loop RT-qPCR (Figure 3-6D). These data support the hypothesis that nuclear QKI5 inhibits processing of pri-miR-124-2, as deletion of QKI5 increases the steady-state pool of mature miR-124.

3.3 Discussion

In this study, we focus on the posttranscriptional regulation of a hNPC-specific and predominantly expressed pri-miR-124 paralog, pri-miR-124-2, which inhibits efficient biogenesis of mature miR-124, in hNPCs. We characterized the expression profile of pri-miR-124-1, -2 and mature miR-124 during an *in vitro* differentiation time-course of M17 cells. We noted a strong correlation between pri-miR-124-2 and mature miR-124 expression. In addition, we characterized the expression profile of the Microprocessor subunits, Drosha and DGCR8, during *in vitro* differentiation of M17 cells and observed no

significant change, indicating that low Microprocessor activity does not underlie accumulation of pri-miR-124-2 in hNPCs.

We next performed an *in silico* search for RNA binding protein (RBP) sites in pri-miR-124-2 and identified QKI as a top candidate, as it is developmentally regulated during neuronal differentiation and has previously been shown to regulate miRNA biogenesis. We propose that posttranscriptional regulation of pri-miR-124-2 may be modulated by binding of QKI to a QRE upstream of the miR-124-2 hairpin to repress processing of the transcript by Microprocessor in hNPCs. For functional validation, we performed CRISPR/Cas9-mediated knockout of nuclear-QKI5 to analyze QKI5-dependent changes in mature miR-124 levels. We further investigated how QKI5 may locally modulate pri-miR-124-2 processing and provide initial evidence that nuclear QKI5 does not interact with DGCR8, as previously observed in erythroid cells (Wang et al., 2017), indicating that QKI5 does not regulate miR-124 biogenesis via sequestration of DGCR8. We propose a working model to summarize our findings about the posttranscriptional regulation of pri-miR-124-2 in hNPCs (Fig. 3-6).

While our study provides preliminary data to support regulation of mature miR-124 biogenesis by QKI5 in hNPCs, more targeted experiments, namely UV-CLIP-RT-qPCR, are needed to confirm an interaction between nuclear QKI5 and pri-miR-124-2 and subsequently, the detailed mechanism underlying the effect of QKI5 binding on pri-miR-124-2 processing.

Figure 3-1: Expression of Microprocessor subunits is unchanged during *in vitro* differentiation of BE(2)-M17 cells. (A) Normalized RNA-seq signal of DROSHA in and BE(2)-M17 cells (D0) differentiated by retinoic acid (D10) (Left). RT-qPCR of *DROSHA* in D0 and D10 BE(2)-M17 retinoic acid (RA) differentiation samples (Right) (n = 4 cultures; Student's t=test; *P $\leq$ 0.05; error bars are SEM). (B) Normalized RNA-seq signal of DGCR8 in and BE(2)-M17 cells (D0) differentiated by retinoic acid (D10) (Left). RT-qPCR of in D0 and D10 BE(2)-M17 retinoic acid (RA) differentiation samples (Right) (n = 4 cultures; Student's t=test; *P $\leq$ 0.05; error bars are SEM).

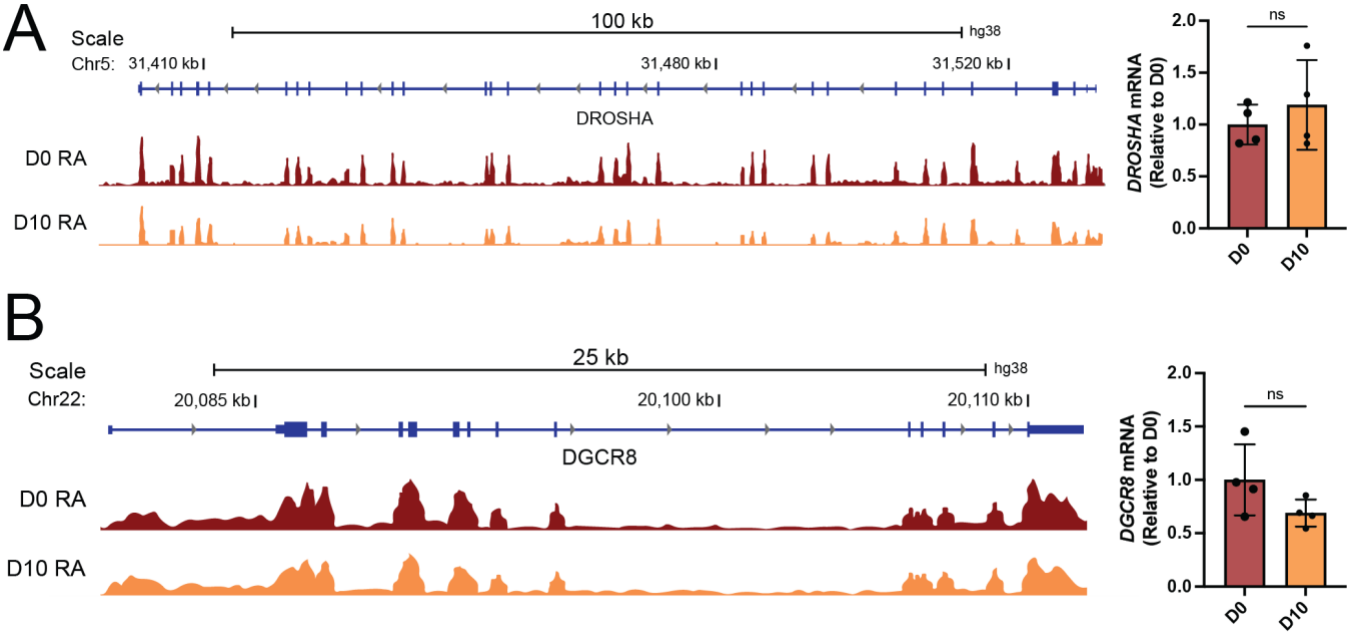
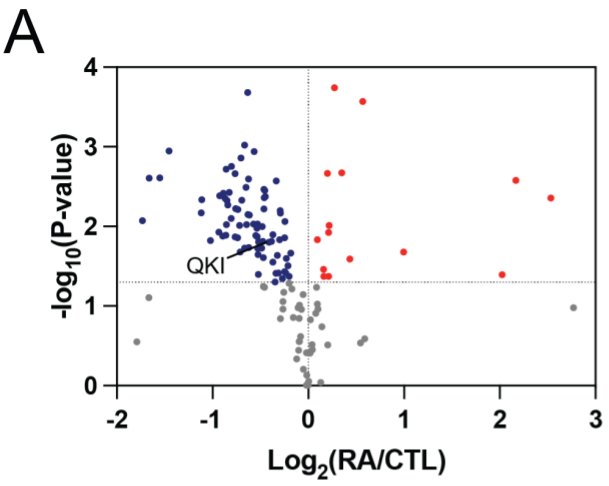
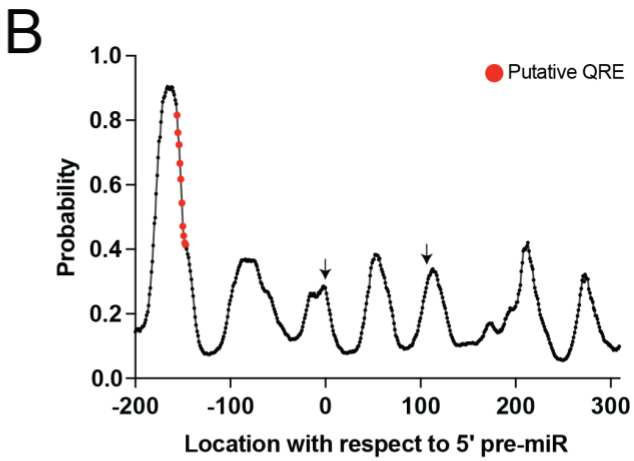

A
Scale
100 kb
hg38
Chr5:
31,410 kb
31,480 kb
31,520 kb
DROSHA
D0 RA
D10 RA
DROSHA mRNA (Relative to D0)
ns
2.0
1.5
1.0
0.5
0.0
D0
D10
B
Scale
25 kb
hg38
Chr22:
20,085 kb
20,100 kb
20,110 kb
DGCR8
D0 RA
D10 RA
DGCR8 mRNA (Relative to D0)
ns
2.0
1.5
1.0
0.5
0.0
D0
D10

Figure 3-2: Human pri-miR-124-2 harbors a QRE. (A) Volcano plot showing log2 abundance fold changes (RA/CTL) and log10-transformed p-values of downregulated (blue) and upregulated (red) RNA binding proteins after Student's t-test across three RNA-sequencing datasets. The data point corresponding to QKI is labeled. (B) Line plot of average probability of QKI binding (window = 101bp) from -200bp to +200up relative to the 5' end of pre-miR-124-2. The putative QRE (red data points) is annotated. Arrows denote the 5' and 3' end of the miR-124-2 hairpin. (C) The nucleotide positions of the QRE (green) and stem loop are indicated relative to the miR-124-2 stem loop.


A
-log10(P-value)
Log2(RA/CTL)
QKI


B
Putative QRE
Probability
Location with respect to 5' pre-miR

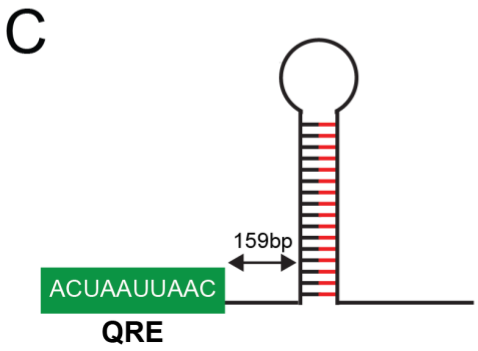

C
159bp
ACUAAUUAAC
QRE

Figure 3-3: QKI is expressed in hNPCs and declines before PTBP1 during neuronal differentiation. (A) Immunoblot of QKI5 and 6 expression in iPSC-derived NPCs and 8 week neurons. β-actin served as a loading control. (B) Immunoblot of pan-QKI and PTBP1 in NPC, 2-, 4-, 6- and 8-week iPSC-derived neurons from two controls donors (C3-1, C2-1). eIF5A served as a loading control.

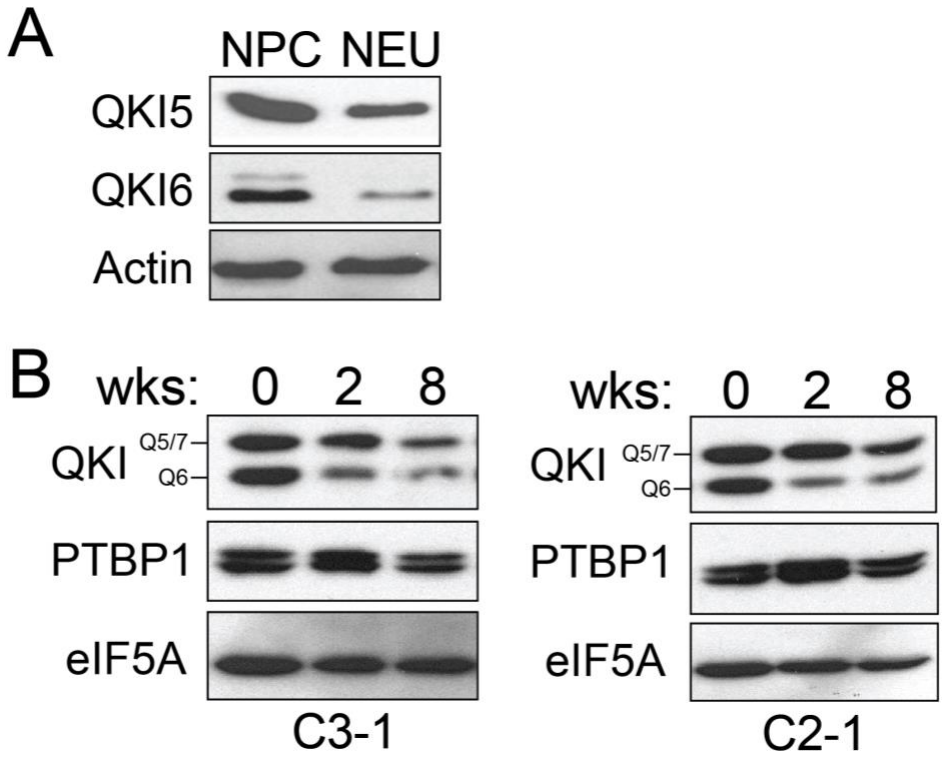

A
NPC NEU
QKI5
QKI6
Actin
B
wks: 0 2 8
QKI
Q5/7
Q6
PTBP1
eIF5A
C3-1
wks: 0 2 8
QKI
Q5/7
Q6
PTBP1
eIF5A
C2-1

Figure 3-4: QKI does not interact with DGCR8 in BE(2)-M17 neuroblastoma cells. (A) Immunoblot of an immunoprecipitation (IP) of recombinant DGCR8-FLAG expressed in SH-SY5Y cells. Co-IP complexes were blotted for FLAG and QKI5. (B) Immunoblot of an immunoprecipitation (IP) of recombinant DGCR8-FLAG and HA-QKI5 expressed in SH-SY5Y cells. Co-IP complexes were blotted for FLAG and QKI5.

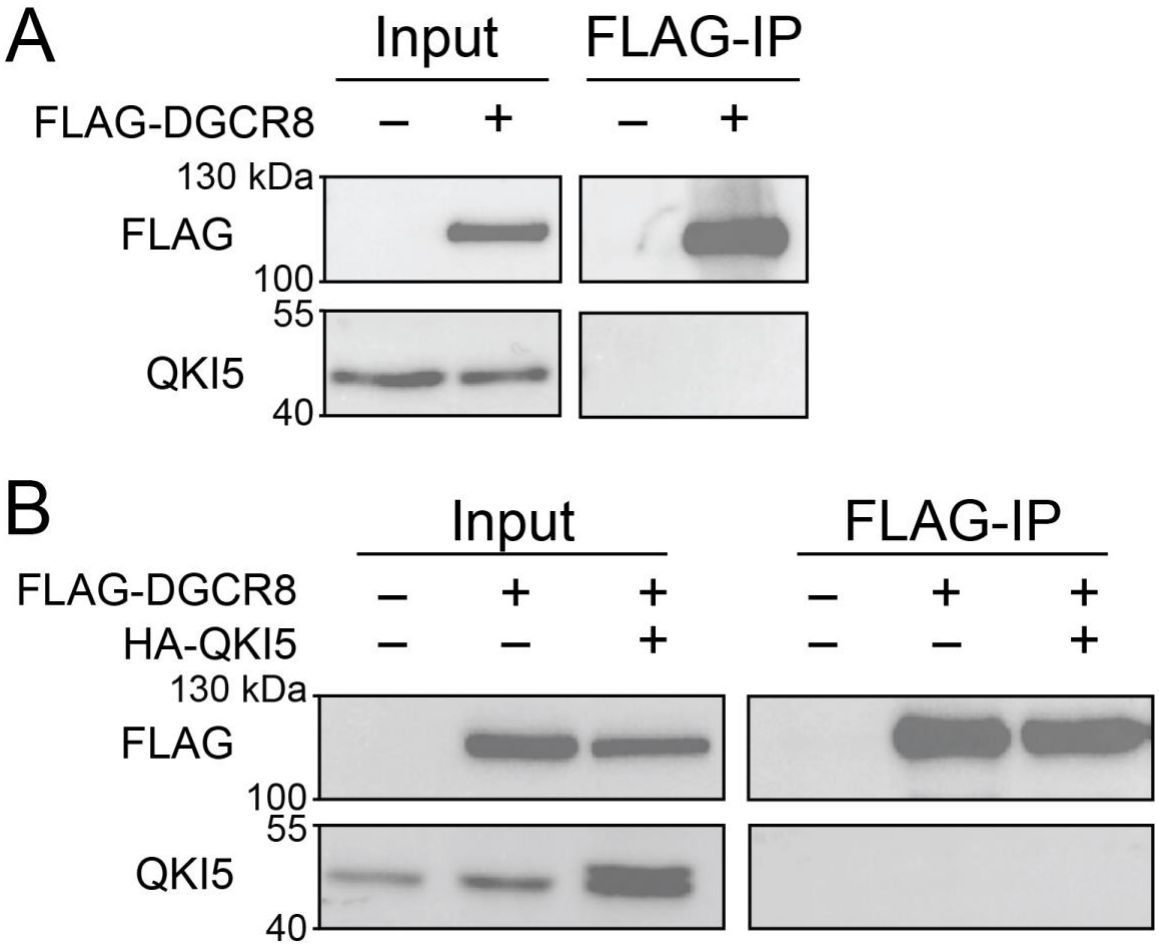

A
Input
FLAG-IP
FLAG-DGCR8
–
+
–
+
130 kDa
FLAG
100
55
QKI5
40
B
Input
FLAG-IP
FLAG-DGCR8
–
+
+
–
+
+
HA-QKI5
–
–
+
–
–
+
130 kDa
FLAG
100
55
QKI5
40

Figure 3-5: Deletion of QKI increases expression of mature miR-124. (A) A schematic representation of QKI gene structure and CRISPR-Cas9 QKI5 deletion strategy. The QKI5 specific exon, exon 7c, was targeted by 5' and 3' flanking gRNAs as indicated by the green lines. Circles indicate stop codons for each isoform. (B) The CRISPR targeted QKI5 exon was PCR amplified from clonal cells using QKI5 specific primers. The expected amplicon size for wild-type (WT) samples is denoted by a thin arrow. The block arrow indicates the band corresponding to the ΔQKI5 PCR-amplicon. (C) RNA-seq data of the *QKI* gene in wild-type (WT) and CRISPR Cas9-edited QKI deletion (ΔQKI5) U-373MG glioblastoma samples. Red arrows mark the QKI6 specific exon (exon 7b) and green arrows mark the QKI5 specific exon (exon 7c). (D) RT-qPCR of mature miR-124 (miR-124-3p) in control cells and a ΔQKI5 BE(2)-M17 clone (n = 1 sample).

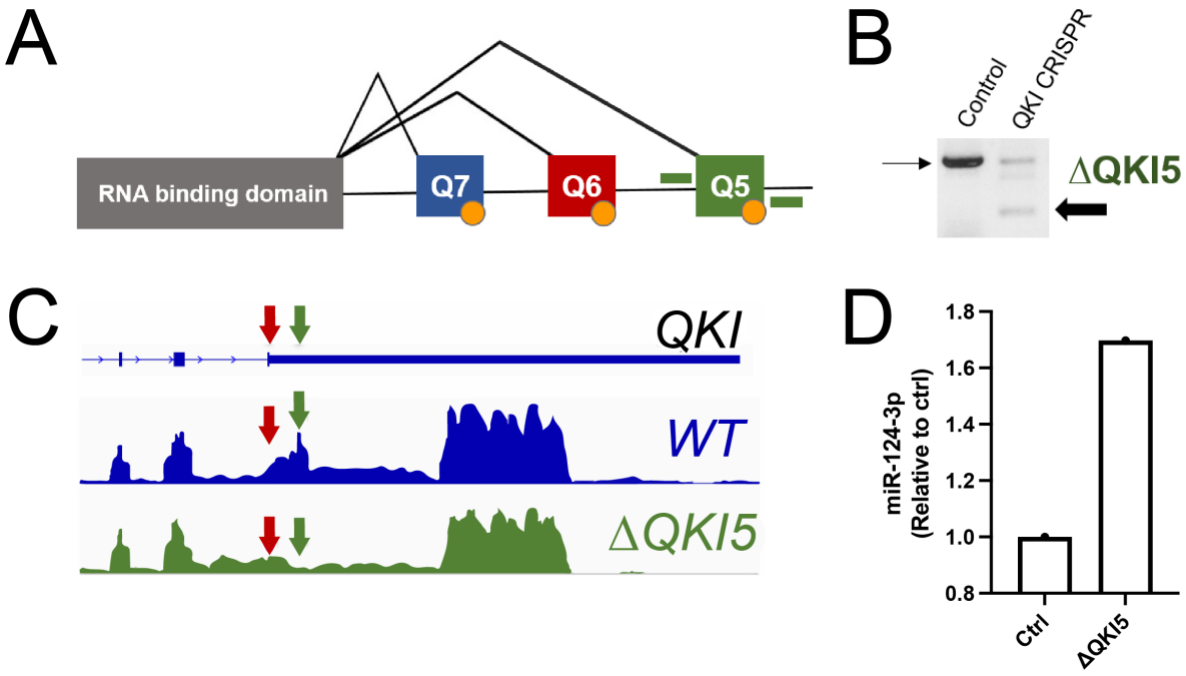
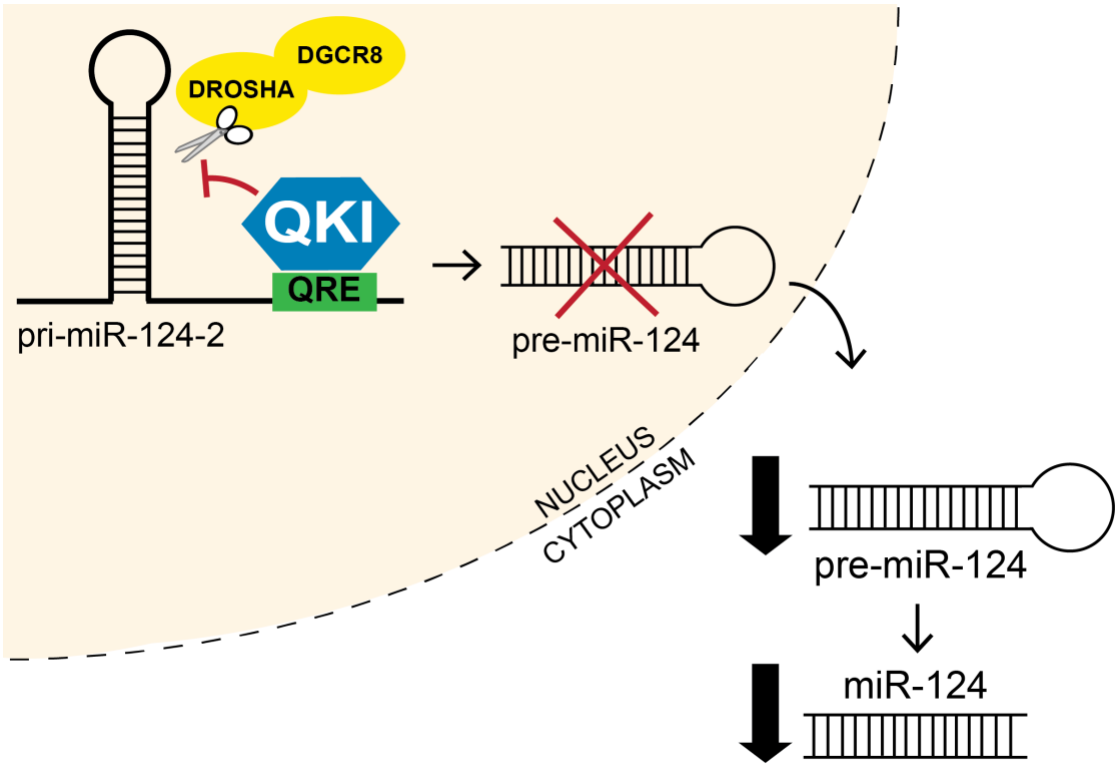

A
RNA binding domain
Q7
Q6
Q5
B
Control
QKI CRISPR
ΔQKI5
C
QKI
WT
ΔQKI5
D
miR-124-3p
(Relative to ctrl)
1.8
1.6
1.4
1.2
1.0
0.8
Ctrl
ΔQKI5

Figure 3-6: A schematic representation of how nuclear QKI5 may regulate processing of pri-miR-124-2 by the Microprocessor complex in hNPCs. Nuclear QKI5 potentially binds a QRE upstream of the miR-124-2 hairpin to prevent cleavage by the Microprocessor complex. QKI5 may then sterically hinder Microprocessor access to the hairpin but does not stably interact with the DGCR8 subunit of the Microprocessor complex as previously reported.


DGCR8
DROSHA
QKI
QRE
pri-miR-124-2
pre-miR-124
NUCLEUS
CYTOPLASM
pre-miR-124
miR-124

Supplementary Table S3.1: List of CRIP predicted RBPs with binding sites in the pri-miR-124-2 region (hairpin, +/- 200nt flanking the hairpin, 509bp total). The average probability that a sequence contains an RBP binding was calculated in a 101 nt sliding window range.

RNA Binding Protein(s)	Possibility of Binding < 0.8
PAPD5, YTHDC2, DDX3X, EIF3G, DHX9, MSI1, NOP56, KHDRBS2, KHSRP, SRSF1, RBM47, TARBP2, DIS3L2, PUM1, TIAL1, TARDBP, KHDRBS3, HNRNPA1, ZFP36, PUM2	409
IGF2BP2, KHDRBS1, HNRNPC	408
WTAP	407
FXR1	404
METTL3	403
QKI, ELAV1	402
MOV10, AGO2, HNRNPD	401
YTHDC1, RC3H1	399
UPF1, BUD13, PTBP1	398
EIF4G1	397
HNRNPUL1	396
ILF3, RTCB	395
SF3A3	394
NOP58	393
LSM11, EWSR1, U2AF2, ELAVL3	392
YTHDF2, HNRNPL	391
CAPRIN1	390
NPM1	389
HNRNPM	388
EIF3D, TRA2A	387
DGCR8, FUS	386
MBNL2, CELF2	385
LIN28B	384
RBM22	382
DKC1	381
LIN28A, SRSF7	379
DICER1	377
LIN28	374
SRSF10	373
SBDS	372
TROVE2, MSI2, RBM10	371
EIF4A1	369
RBM5	368
SLBP, IGF2BP1	365
FTO, ZNF184, SF3B4	364
RBFOX2, SAFB2	363

GTF2F1	362
CBX7, ZC3H7B	361
LARP4B, RBM27, RNF219	358
BCCIP, U2AF1, AIFM1	356
YWHAG, NUMA1	355
DDX54, CSTF2T	354
AGO1	352
IGF2BP3	350
CPSF6, ALYREF	348
SND1, HNRNPU, SRSF9	346
TAF15	345
XRN2	344
FBL	343
RANGAP1, TIA1	342
EIF3B	340
ACIN1	339
FKBP4	337
METTL14	335
NONO	332
HNRNPA2B1, EIF4G2	329
SRSF3	326
DDX42, VIM	325
FAM120A	323
AUH	321
EIF3A	319
FXR2, TNRC6A	318
SMNDC1	312
MBNL1	309
SLTM	308
EIF4A3	306
RBM6	305
HNRNPK	303
GNL3	299
YTHDF1, PRPF8	296
NCBP3	291
ADAR	286
AGO4	280
AGO3	270
ALKBH5, PCBP2	250
CNBP	201
FMR1	183
LARP7	132
RBM39	31

Table S3.2 – Oligo sequences used in this study.

Applicat-ion	Target	Forward Primer (5' → 3')	Reverse Primer (3' → 5')
RT-qPCR	pri-miR-124-2 (Human)	CAGTGGGTCTTATACTTTCCGGATC	GAGATGCTTTGGTGTCCTTCAAGT
	pri-miR-124-1 (Human)	CCCTCTTCCTTTCTTTCACCTTTC	TCGGTCGGTCGCTCCTTC
	DROSHA	CATGCACCAGATTCTCCTGTA	GTCTCCTGCATAACTCAACTG
	DGCR8	TATCAGATCCTCCACGAGTG	TCTTGGAGCTTGCTGAGGAT
PCR	Human QKI exon 7c (QKI5)	CTGCAAGAAAGGCACTTCA	ACCCAACCCTTAAATCTGTC

Chapter 4: Conclusions and Future Directions

In this dissertation, we provide novel evidence that in contrast to mouse embryonic stem cells, in which pri-miR-124-1 is the primary pri-miR-124 paralog expressed (Yeom et al., 2018), pri-miR-124-2 is the predominantly expressed pri-miR-124 paralog in human neural progenitor cells (hNPCs). Transcriptional activation of the *MIR124-2HG* gene by SP1 regulates robust expression of miR-124-2 in hNPCs. During neuronal differentiation we observed markedly decreased expression of *MIR124-2HG* and the derivative pri-miR-124-2. We further establish that *MIR124-2HG* downregulation is accompanied by an increase in chromatin accessibility of the host gene proximal promoter element, which is regulated by transcriptional repressor MAFK. In addition, we present data suggesting that pri-miR-124-2 is subject to post-transcriptional regulation by the hNPC- and glial-specific protein, QKI. In summary, the transcriptional and posttranscriptional mechanisms that mediate miR-124 biogenesis from two distinct pri-miR-124 paralogs in NPCs and neurons require further analysis.

4.1 Potential mechanisms regulating the preferential expression of pri-miR-124-2 in human neural progenitor cells

While the expression profile of mature miR-124 during neuronal differentiation is well-established (Busskamp et al., 2014; Deo et al., 2006; Smirnova et al., 2005), emerging evidence suggests that expression of upstream miRNA biogenesis intermediates (pri-/pre-miRNA) which precede expression of their derivative mature miRNAs are subject to multifaceted transcriptional and posttranscriptional regulation (Choudhury et al., 2013; Michlewski and Caceres, 2010; Yeom et al., 2018). In the second chapter of this thesis, we provide evidence that pri-miR-124-2 is a hNPC-specific miR-

124 paralog which is subject to transcriptional downregulation as NPCs differentiate into neurons, while pri-miR-124-1 concurrently becomes the predominantly expressed pri-miR-124 paralog. These findings raise the question of why pri-miR-124-2 is preferentially and specifically expressed in human NPCs. We have previously proposed that differences in transcriptional and processing efficiencies of miRNA paralogs within the same multigene family may underlie preferential expression of specific paralogs at different developmental stages and in different cellular contexts (Suster and Feng, 2021). Further exploration of the mechanisms regulating expression of the human pri-miR-124 paralogs during distinct neurodevelopmental stages may reveal why pri-miR-124-2 is preferentially expressed in hNPCs.

To address differences in transcriptional dynamics between the miR-124-1 and -2 host transcripts, we could perform RNA Pol II chromatin immunoprecipitation (ChIP) in M17 cells differentiated for 10 days by retinoic acid treatment, followed by PCR with primers tiling the miR-124-1 and -2 promoters. The resultant data may reveal differences in RNA Pol II promoter occupancy in an *in vitro* hNPC and immature neuron cell line model, potentially underlying the hNPC-specific expression of miR-124-2. This experiment is designed under the assumption that miR-124-1 and -2 host transcripts are both transcribed by RNA Pol II, as the majority of mRNA host genes are (Lee et al., 2004). It is important to note that RNA Pol II ChIP does not discriminate between active, inactive and stalled RNA Pol II. Performing ChIP for active RNA Pol II using antibodies against the phosphorylated Ser2 of the repetitive carboxy-terminal domain (CTD) can detect actively elongating RNA Pol II (Komarnitsky et al., 2000), although this experiment is currently hindered by incomplete annotation of the miR-124-1 host transcript.

One major hurdle to the experiments proposed is the comprehensive identification of the miR-124-1 host gene promoter and host transcript. Our initial exploration of miR-124-1 gene transcriptional regulation features did not yield substantive results. One possible explanation for the lack of discernable (above background) miR-124-1 host transcript RNA-seq signal in the neuronal samples we analyzed may be due to degradation of the transcript by the exonuclease XRN2, which is recruited to sites of Drosha-mediated cleavage of pri-miRNAs for intronic cleavage (Morlando et al., 2008). A "torpedo-like model" of XRN2 has been proposed for intragenic miRNA transcripts, wherein XRN2 catches up with RNAPII and rapidly degrades the transcribed miRNA host transcript (Ballarino et al., 2009). For transcripts hosting intronic miR in long-noncoding pri-miRNA (lnc-pri-miR), Drosha-mediated, cleavage and polyadenylation (CPA)-independent transcriptional termination of these transcripts has also been reported (Dhir et al., 2015).

Ectopic expression of dominant negative XRN2 or knockdown of endogenous XRN2 may lead to an accumulation of miR-124-1 transcript, allowing for detection of the host transcript and promoter. Alternatively, expression of dominant negative Drosha in M17 cells may stabilize the miR-124-1 host transcript by inhibiting the co-transcriptional cleavage of pri-miR-124-1 and recruitment of XRN2, thereby potentially allowing for detection of the transcript by RNA-sequencing and/or RT-qPCR.

Previous studies have shown that processing of all three pri-miR-124 paralogs is Drosha-, DGCR8- and Dicer-dependent in human cell lines and mESCs (Babiarz et al., 2008; Kim et al., 2017), indicating that each miR-124 paralog undergoes canonical miRNA processing. An *in vitro* pri-miRNA processing assay (Barr and Guo, 2014; Yang

et al., 2015) of all three human pri-miR-124 paralogs (pri-miR-124-1, -2, -3) would reveal whether these three transcripts undergo differential Microprocessor recognition and cleavage based on structural and sequence features of the pri-miRNA. First, *in vitro* transcription of the pri-miRNA substrates, including 200 nt upstream and downstream of the miRNA stem-loop, followed by incubation of each pri-miRNA with purified Drosha and DGCR8 and Northern blot analysis of the cleavage products, would reveal whether *cis* elements within the transcripts underlie differential processing efficiency between the paralogs. Additionally, pri-miRNA substrates may be incubated with M17 cell nuclear extract to further explore whether pri-miR-124-2 is subject to post-transcriptional regulation by a nuclear RNA binding protein, namely QKI5, or other nuclear *trans* factor(s) in a hNPC model, as we propose in Chapter 3 of this dissertation. Additionally, it would be valuable to generate chimeric RNAs in which the upstream regions of human pri-miR-124-1 and -2 are switched to explore whether these flanking sequences, which potentially scaffold RNA binding proteins, impart the same processing efficiency on the stem-loop as for the native transcript.

The dual transcriptional and posttranscriptional regulation of miR-124-2 in hNPCs, demonstrated in this dissertation suggest that miR-124-2 is a tightly regulated source of mature miR-124 in hNPCs. In theory, these mechanisms prevent aberrant miR-124 production in hNPCs by ensuring each level of regulation, transcriptional and posttranscriptional, acts as a “fail-safe” if either regulatory mechanism is perturbed. In hNPCs, the negligible expression of miR-124, processed from pri-miR-124-2, may partly maintain the multipotency status of these cells and ensure gradual, proper onset and progression of neuronal differentiation. In contrast, pri-miR-124-1 may serve as a robust

and unyielding source of mature miR-124 in neurons, possibly explaining the predominant expression of this paralog in these cells.

4.2 How does neuronal differentiation persist in ∆miR-124 iPSC-derived neurons?

Initial studies of mature miR-124 in neurons suggested that miR-124 induces the onset of neuronal differentiation by targeting several well-characterized inhibitors of neuronal differentiation (Makeyev et al., 2007; Visvanathan et al., 2007; Wei et al., 2018; Yoo et al., 2009). From these early studies, it was presumed that mature miR-124 would be indispensable for neuronal differentiation. However, mice homozygous for deletion of the *miR-124-1* host transcript (*Rncr3*$^{-/-}$) are viable with normal brain morphology but overall smaller brain size (Sanuki et al., 2011). Additionally, a recent study reported that deletion of all *miR-124* alleles in human iPSCs did not affect general neurogenesis (Kutsche et al., 2018). Therefore, the molecular mechanisms that permit neuronal differentiation to proceed, in the absence of mature miR-124, in both human and mouse, remain unclear. We and others have proposed that neural-enriched or neural-specific miRNAs converge on key mRNAs to promote neuronal differentiation (Cherone et al., 2019; Suster and Feng, 2021). Therefore, we postulate that other neural miRNAs may compensate for miR-124 deletion.

A recent study reported that the abundance and activity of eight miRNA species, measured by small RNA-seq and AGO2-RIP-seq, respectively, was significantly increased in ∆miR-124 iPSC-derived neurons (Kutsche et al., 2018). Interestingly, the majority of these eight miRNAs (5/8) are enriched in the glial cell subtypes of postnatal

(P1) rat cortex (Jovicic et al., 2013). Given that the expression profile of these miRNAs is predominantly glial, it is unlikely that the upregulated miRNAs compensated for the absence of miR-124 by overriding the regulatory function of miR-124 in promoting neuronal differentiation. Alternatively, the upregulated miRNAs may employ a different posttranscriptional developmental program to promote neuronal differentiation in ∆miR-124 neurons. *In silico* analyses of the overlap between miR-124 and the predicted targets of the eight human miRNA species may provide an initial idea of whether these miRNAs indeed converge on common target mRNAs shared with miR-124.

Another possibility is that other neuronal-enriched miRNAs are not upregulated in ∆miR-124 neurons. Rather, when the most abundant neuronal miRNA, miR-124, is absent (Busskamp et al., 2014), more AGO2 is theoretically available for loading of other abundant neuronal miRNAs and subsequent mRNA targeting. Indeed, other neuronal-enriched miRNAs, namely miR-9 and miR-9*, share key anti-neuronal targets with miR-124, specifically the repressor element 1 (RE-1)-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) complex (Lee et al., 2018; Packer et al., 2008) and the Brg/Brm-associated factor (BAF) BAF53a subunit of the neural-progenitor (np) BAF chromatin-remodeling complex (Yoo et al., 2009). However, as these hypotheses are unexplored the miRNA-mediated mechanisms that regulate neuronal differentiation in the absence of mature miR-124 are unknown.

4.3 Deciphering the role of individual pri-miR-124 paralogs in advancing neuronal differentiation

As discussed in section 4.2 of this dissertation, deletion of all miR-124 encoding alleles in human iPSC-derived neurons does not significantly affect neurogenesis, as

compared with WT cells (Kutsche et al., 2018). However, by design, this experiment does not allow one to decipher the role of individual pri-miR-124 paralogs in advancing neuronal differentiation. It is important to note that previous studies have reported that pri-miR-124-3 is not expressed in neuronal lineage as the transcript is negligibly expressed in mouse ESCs, cortical neurons and postnatal (P6) murine retina, hippocampus and cortex (Sanuki et al., 2011; Yeom et al., 2018). The data presented in this dissertation strongly support further investigation of the functional role of individual pri-miR-124 paralogs during neuronal differentiation, specifically pri-miR-124-1 and -2, by elucidating the stage-specific expression of these transcripts during human neuronal differentiation.

CRISPR-Cas9 genome editing of the miR-124-1 and -2 hairpins in BE(2)-M17 cells, followed retinoic acid-induced differentiation of these cells, would provide several important insights into the role of the human pri-miR-124 paralogs during neuronal differentiation. First, performing stem-loop RT-qPCR for mature miR-124 in the CRISPR-Cas9-edited and differentiated cells compared to undifferentiated and control samples would provide an initial idea of how much processing of each pri-miR-124 paralog contributes to the cellular pool of mature miR-124 in hNPCs and immature neurons. Secondly, analysis and quantification of neurite outgrowth velocity by time-lapse microscopy, between CRISPR-Cas9 deletion and control cells, would reveal whether deletion of each neural stage-specific pri-miR-124 paralog differentially affects neuronal differentiation as evidenced by differences in neuronal morphology. It would also be important to perform RT-qPCR for the other pri-miR-124 transcripts in the CRISPR-Cas9 deletion samples to assay whether the expression of other pri-miR-124 paralogs are

upregulated to compensate for loss of a miR-124 source, which would also suggest a mechanism of cross-regulation between the pri-miR-124 paralogs.

Our pilot CRISPR-Cas9 ΔmiR-124-1 hairpin experiment in M17 cells arrested cell growth, which was not observed in M17 cells transfected with control gRNAs. Further experiments are needed to investigate the proliferation capacity and apoptotic activity, by Ki-67 and caspase 3/7 activity immunofluorescence, respectively, in these samples before more definitive conclusions can be drawn. Cell cycle arrest and apoptosis in the absence of miR-124-1 expression may lead to widespread neural cell death. Interestingly, increased lethality of retinal neurons was reported in mice homozygous for deletion of the miR-124-1 host transcript (*Rncr3*) (Sanuki et al., 2011). Additionally, cell viability of ΔmiR-124 iPSC-derived neurons was significantly decreased, as caspase 3/7 activity significantly increased, compared to control neurons (Kutsche et al., 2018). Taken together with our initial data, these studies indicate that miR-124-1 may be important for long-term neuronal survival rather than for promoting neuronal differentiation. This hypothesis is also supported by the overall smaller brain observed in mice homozygous for deletion of the miR-124-1 host transcript (*Rncr3*$^{-/-}$) (Sanuki et al., 2011).

Furthermore, it would be interesting to perform CRISPR-Cas9 genome editing of both miR-124-1 and -2 hairpins in iPSC-derived hNPCs differentiated into cortical neurons (Wen et al., 2014). According to our studies, deletion of both miR-124-2 and -1 hairpins would effectively remove the primary source of mature miR-124 in hNPCs and neurons, respectively. Using this experimental model, we would assay for neuronal functions in the CRISPR-Cas9 edited samples – i.e. electrophysiological activity. Additionally, during later stages of neuronal differentiation, dendritic complexity of the CRISPR-Cas9 edited

samples may be quantitatively analyzed by Sholl analysis (Sholl, 1953) and compared to isogenic, control samples. Lastly, the ability to maintain iPSC-derived neurons in culture for several weeks would be especially advantageous in investigating the role of miR-124-1 in long-term neuronal survival as discussed above.

4.4 Potential mechanisms downregulating miR-124-2 during murine neuronal differentiation

We identified a conserved developmental switch from predominant pri-miR-124-2 to -1 expression during human neuronal differentiation and mouse cerebellar development. We revealed that a change in chromatin accessibility of a human-specific *cis*-regulatory element partly underlies the developmental downregulation of *MIR124-2HG* during human neuronal development. However, the question remains of how miR-124-2 is downregulated during murine neuronal differentiation? *In silico* prediction of transcription factor (TF) binding to the *Mir124-2hg* promoter would yield candidate TFs that could be further characterized as transcriptional activators or repressors. Overexpression and knockdown of other candidate transcription factors in conjunction with detection of endogenous *Mir124-2hg* transcript or a luciferase reporter driven by the *Mir124-2hg* promoter would reveal the functional role of these TFs on regulating transcription from the *Mir124-2hg* promoter.

Using the JASPAR database (Fornes et al., 2020), we found that the transcriptional activator Sp1 is predicted to bind the *Mir124-2hg* promoter (data not shown). If Sp1-mediated regulation of miR-124-2 host gene expression in NPCs is conserved between human and mouse, Sp1 downregulation alone may be sufficient to drive downregulation

of *Mir124-2hg* through the gene promoter during neuronal maturation. Indeed, Sp1 is significantly downregulated during both murine and human *in vitro* neuronal differentiation (Milagre et al., 2012; Ravache et al., 2010). To address Sp1 occupancy on the *Mir124-2hg* promoter during mouse cerebellar development, a time-course of Sp1 chromatin immunoprecipitation (ChIP) of embryonic day 11 (E11) to postnatal day 9 (P9) mouse cerebellum, followed by PCR with primers tiling the *Mir124-2hg* promoter would reveal differences in Sp1 occupancy on the gene promoter during cerebellar development in mice, potentially underlying the developmental downregulation of miR-124-2.

Additionally, mature miR-124 targets Sp1, for translational repression, during human *in vitro* neuronal differentiation for translational repression (Mondanizadeh et al., 2015). If this mechanism is conserved between human and mouse, mature miR-124 biogenesis would reinforce downregulation of *MiR124-2hg* as pri-miR-124-1 increases and becomes the predominant source of mature miR-124 in neurons.

Another mechanism potentially underlying *MiR124-2hg* downregulation is a decrease in chromatin accessibility at the *Mir124-2hg* locus during neuronal development, leading to gene silencing. Although the murine *MiR124-2hg* does not harbor a proximal promoter element to scaffold the binding of transcription factors, chromatin compaction at the *MiR124-2hg* promoter would decrease accessibility of the promoter to transcription factors and the transcriptional machinery. To investigate this developmental mechanism, assay for transposase-accessible chromatin by sequencing (ATAC-seq) of embryonic (E11) and postnatal (P9) mouse cerebellum samples may reveal potential differences in chromatin openness at the *MiR124-2hg* locus during cerebellar development. It is important to note that we only searched for a proximal promoter element and not other

transcriptional regulatory elements upstream at the *MiR124-2hg* gene locus. In summary, the mechanisms proposed here that potentially mediate downregulation of *MiR124-2hg* and pri-miR-124-2 during murine neuronal differentiation warrant further investigation.

4.5 Potential mechanisms mediating regulation of human pri-miR-124-2 processing by QKI5

From our study, we identified a putative QRE upstream of the human miR-124-2 hairpin, which may mediate processing of pri-miR-124-2 by nuclear QKI5. We showed that QKI5 does not interact with the DGCR8 subunit of the Microprocessor complex in M17 cells, a hNPC cell model, in contrast to what has been reported in erythroid cells (Wang et al., 2017). Therefore, the precise mechanism by which QKI5 potentially represses processing of pri-miR-124-2 in neural cells remains elusive. Experiments first determining whether QKI5 binds the putative pri-miR-124-2 QRE and previously identified pri-miR-124-1 QRE in neural cells are essential. These experiments involve UV-crosslinking immunoprecipitation (UV-CLIP) of QKI5-RNA complexes, to determine whether these interactions exist in our system of interest. If we observe binding of QKI5 to pri-miR-124-2, our hypothesis would be supported. To further identify the effect of QKI5 binding on pri-miR-124-2 processing, UV-CLIP of DGCR8 under control (siNS) and siRNA-mediated knockdown QKI5 (siQKI5) conditions for DGCR8-pri-miRNA complexes would reveal whether DGCR8 associates with pri-miR-124-2 in the absence of QKI5. Data showing a DGCR8-pri-miR-124-2 interaction under siQKI5 conditions would suggest that binding of QKI5 to the pri-miR-124-2 QRE prevents Microprocessor recognition and cleavage of the miR-124-2 hairpin.

Additionally, a positive result for binding of QKI5 to pri-miR-124-1 would be an interesting result as this transcript is also bound and blocked from Microprocessor cleavage by PTBP1, which binds proximal to the stem-loop (Yeom et al., 2018), whereas the pri-miR-124-1 QRE is distal to the stem-loop (Wang et al., 2017). If future studies reveal that pri-miR-124-1 is indeed subject to posttranscriptional regulation by both PTBP1 and QKI in hNPCs, the functional impact of the QKI5-pri-miR-124-1 interaction may not significantly affect pri-miR-124-1 processing during neurodevelopment. In support of this, PTBP1 protein declines in expression after QKI downregulation as hNPCs differentiate into neurons (Figure 3-3B). In conclusion, while the QKI isoforms are generally accepted as a pro-glial RBPs (Larocque et al., 2009; Larocque et al., 2005; Takeuchi et al., 2020; Zhao et al., 2006a; Zhao et al., 2010; Zhao et al., 2006b), further investigation of the posttranscriptional regulation of pri-miR-124-2 processing proposed in this dissertation may reveal nuclear QKI-5 as an anti-neuronal RBP through QKI-mediated regulation of miRNA biogenesis.

The findings presented in this dissertation provide new insights into the initiation of miR-124 biogenesis from distinct pri-miR-124 paralogs during early human neurodevelopment. In the future, these findings may provide new strategies for stem cell treatment of neurological disorders by allowing researchers to manipulate miR-124 biogenesis for precise control of stem cell differentiation. Furthermore, the expression profile of pri-miR-124 paralogs during neuronal differentiation, which is not conserved between humans and mice, warrants further investigation of the human-specific mechanism underlying preferential expression of a different hNPC-specific pri-miR-124 paralog from mouse. Lastly, identifying the posttranscriptional regulation of pri-miR-124-

2 processing in hNPCs would enhance our understanding of the multifaceted control of an hNPC-specific source of miR-124, which allows for fine-tuning of mature miR-124 expression during neuronal differentiation.

Chapter 5: Materials & Methods

5.1 Chapter 2

Materials and Methods

Cell Culture and Transfection Human BE(2)-M17 neuroblastoma cells (ATCC) were propagated in DMEM/F12 media (Corning) containing 10% (vol/vol) fetal bovine serum (GenClone). The BE(2)-M17 cells were transfected with DNA plasmids and/or siRNAs using Lipofectamine 2000 (Invitrogen). Transfection efficiency was assessed via visualization of EGFP plasmid co-transfected in parallel.

***In vitro* iPSC Differentiation**

Human iPSCs were differentiated into forebrain-specific hNPCs following a previously established protocol (Wen et al., 2014). Briefly, human iPSC colonies were detached from the feeder layer with 1 mg/ml collagenase treatment for 1 hour and suspended in embryonic body (EB) medium, consisting of FGF-2-free iPSC medium supplemented with 2 μM Dorsomorphin and 2 μM A-83 in non-treated polystyrene plates for 4 days with a daily medium change. After 4 days, EB medium was replaced by neural induction medium (NPC medium) consisting of DMEM/F12, N2 supplement, non-essential amino acids (NEAA), 2 μg/ml heparin, and 2 μM cyclopamine. Floating EBs were then transferred to Matrigel-coated 6-well plates at day 7 to form neural tube-like rosettes. Attached rosettes were maintained for 15 days, with NPC medium change every other day. On day 22, rosettes were picked mechanically and transferred to low attachment plates (Corning) to form neurospheres in NPC medium containing B27. Neurospheres were then dissociated with Accutase at 37 ºC for 10 min and placed onto Matrigel-coated 6-well plates at day 24 to form monolayer hNPCs in NPC medium containing B27. These hNPCs expressed

forebrain-specific progenitor markers (Wen et al., 2014), including NESTIN, PAX6, EMX-1, FOXG1, and OTX2.

mRNA Quantification

For cells, total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's protocol. RNA concentration was quantified with the BioDrop μLITE (BioDrop). Total RNA was treated with DNase I, Amplification Grade (Invitrogen), followed by phenol-chloroform extraction. RNA quality was assessed by gel electrophoresis. One microgram of total RNA was subjected to random hexamer-based reverse transcription using SuperScript II RT (Thermo Fisher Scientific). Reactions lacking reverse transcriptase enzyme (-RT) were prepared in parallel as negative controls. Semi-quantitative RT-PCR was performed using Taq polymerase (Thermo Fisher Scientific). Detailed RT-PCR conditions are described in Supplemental Materials and Methods. RT-PCR products were resolved on 3% agarose gels with SafeRed (FroggaBio) staining and visualized using a Gel Doc XR+ Imaging System (Bio-Rad). Real-time PCR was performing using PerfeCTa SYBR Green FastMix (QuantaBio) on a CFX96 Touch Real-Time PCR system (Bio-Rad). Threshold amplification values (C_t) were assigned by the CFX Manager software (Bio-Rad). For detection of mature miRNAs, 1 μg of total RNA was subjected to microRNA-specific reverse transcription and qPCR using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) and TaqMan Universal Master Mix (Thermo Fisher Scientific), respectively.

RNA Interference

The siRNA duplexes were transfected in BE(2)-M17 cells using Lipofectamine 2000 (Invitrogen). ON-TARGETplus SMARTpool siRNA targeting human SP1 was purchased

from Dharmacon. The PTBP1 siRNA duplex (Ambion) sequence is listed in Supplemental Materials and Methods. *In Vivo* Negative Control #1 siRNA (Ambion) was used as a negative control. Each siRNA reagent was prepared as a 200 μM solution. Cells were harvested 48 h post transfection for downstream RNA or protein analysis.

Immunoblot Analysis

Harvested cells were lysed and sonicated in 1X Laemmli buffer as described previously (Mandler et al., 2014). Proteins were than separated on a 10% SDS/PAGE and transferred to a PVDF membrane (GE Healthcare), which was blocked in 10% nonfat, dry milk in phosphate-buffered saline with 0.01% Tween-20. The membrane was then probed with primary antibodies: PTBP1 antibody (1:5000, Invitrogen, 32-4800), , SP1 antibody (1:800, Santa Cruz, sc-17824) and β-actin antibody (1:10,000, Sigma, A5441). Immunoblot images were collected on a ChemiDoc MP Imaging System (Bio-Rad). Densitometry analyses of protein bands were performed using the Image Lab 6.1 software (Bio-Rad). Densitometry values were normalized to β-actin.

RNA-seq data processing

Raw RNA-seq reads were aligned to the human genome build hg38 using TopHat2 (v1.3.3) (Kim et al., 2013). Alignment bam files were used to generate fragments per kilobase million (FPKM) of all RefSeq genes. Differential expression gene analysis was conducted by Cuffdiff with respect to control and *trans* retinoic acid (RA)-treated BE(2)-M17 cells (Trapnell et al., 2012). Mapped reads in pri-miRNA region are counted by bedtools according to the genome coordinates then normalized to total mapped reads to determine its expression (Quinlan and Hall, 2010). The pri-miR-124-2 region analyzed (the hairpin +/- 200nt flanking sequences, 509bp total) spans chr8: 64378949-64379457

(hg38). The pri-miR-124-1 region analyzed (the hairpin +/- 200nt flanking sequences, 485bp total) spans chr8: 9903188 – 9903672 (hg38).

ATAC-seq Data Processing and Analysis

Paired-end ATAC–seq reads (GSE70823) were mapped to the hg19 reference genome using bowtie2 (v2.2.6) (Langmead et al., 2009) with the parameters --no-discordant --no-mixed to prevent discordant alignments and alignments for the individual mates. Flags -F 4 and -q 10 were used in samtools (v 1.9) (Li et al., 2009) to exclude unmapped reads and reads with MAPping Quality (MAPQ) values less than 10 for each replicate in all stages. Genes of interest were visualized using the Integrative Genomics Viewer (IGV v2.3.38) (Robinson et al., 2011).

PhastCons for Conservation

To measure conversation of the *MIR124-2HG* promoter and proximal promoter element, PhastCons scores for the human genome (hg19) regions of interest of 100 vertebrate species alignments were downloaded from the UCSC Genome Browser database (Karolchik et al., 2008; Siepel et al., 2005). The *MIR124-2HG* promoter region analyzed spans chr8: 65285267 - 65286023 (hg19). The *MIR124-2HG* proximal promoter element region analyzed spans chr8: 65284499 – 65285260 (hg19).

Plasmid Construction and Mutagenesis

The *MIR124-2HG* promoter and proximal promoter element (PPE) were PCR-amplified from BE(2)-M17 genomic DNA. Primers were designed with Kpn*I* and Sma*I* sites for cloning into pGL3 Basic plasmid (Promega) lacking an internal promoter element. Site-directed mutagenesis of the MAFK binding site in the *MIR124-2HG* PPE was performed

using the Quick-Change Site-Directed Mutagenesis kit (Stratagene). All primers used for cloning and mutagenesis are listed in Supplemental Materials and Methods.

Luciferase Reporter Assay for Promoter Activity

Luciferase activity of pGL3 constructs of the *MIR124-2HG* promoter alone and with the upstream proximal promoter element (PPE) was measured to assess promoter activity of the promoter and regulatory activity of the PPE, respectively. BE(2)-M17 cells were co-transfected with 900 ng luciferase reporter plasmid and, as a transfection efficiency control, 10 ng Renilla plasmid. Twenty-four hours post-transfection, firefly and Renilla luciferase activities were measured with 20/20^{n} Luminometer (Turner Biosystems) using Dual Luciferase Reporter Assay reagents (Promega). Signals were normalized for transfection efficiency to the Renilla control.

***In vitro* BE(2)-M17 Differentiation**

BE(2)-M17 cells were differentiated as described previously (Andres et al., 2013). Briefly, BE(2)-M17 cells were seeded at 20-30% confluency and treated with 10 μM *trans*-retinoic acid (Sigma) added to the culture medium every day for the first 5 days and every other day thereafter for a total of 10 days. Differentiation was monitored by neurite outgrowth observed using light microscopy. The cells/neurite outgrowth were viewed and photographed at a magnification of 40X with an Olympus IX-51 inverted fluorescent microscope.

***In silico* Transcription Factor Binding Site Prediction**

The eighth release of (2020) JASPAR (http://jaspar.genereg.net/), a database of 810 known *Homo sapiens* transcription factor (TF)-binding sites from the experiment-based literature, was used to predict putative TF binding to the human *MIR124-2HG* promoter

and proximal promoter element (Fornes et al., 2020). Relative profile score threshold was set as 90%. Duplicate profiles of the same transcription factor were combined.

ENCODE ChIP-seq Data Analysis

The “Region Search” function in the ENCODE portal was used to find experiments in which the input region of interest intersects with regions specified in the peaks file of experimental ENCODE projects.

Statistical analysis

All graphs were presented as means ± SEM. All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc.). For two-sample comparisons, independent two-sided *t*-test was applied. For three-sample comparisons, one-way ANOVA analysis and Tukey’s multiple comparison post hoc test were used as indicated in the corresponding figure legends. Statistical significances are indicated by *($P < 0.05$) or **($P < 0.01$).

Supplemental Materials and Methods

RT-PCR conditions

For pri-miR124-1 (amplicon size = 195nt) and pri-miR-124-2 (148 nt), PCR reactions used 45 cycles at an annealing temperature of 62.7°C and 62°C, respectively. Primers designed to amplify pri-miR-124-3 did not detect a PCR amplicon after 45 cycles. The above primer pairs spanned the stem-loop region of each miRNA (spanning primer, Supplemental Fig. S1A). The primer sequences are shown in Supplemental Table S3.

5.2 Chapter 3

Cell Culture and Transfection Human SH-SY5Y and BE(2)-M17 neuroblastoma cells (ATCC) were propagated in DMEM/F12 media (Corning) containing 10% (vol/vol) fetal bovine serum (GenClone). The BE(2)-M17 cells were transfected with DNA plasmids using Lipofectamine 2000 (Invitrogen). Transfection of SH-SY5Y cells was performed using jetMessenger reagents (Polyplus) and following the manufacturer's protocol. The FLAG-DGCR8 construct was a gift from Dr. Zixu Mao (Yang et al., 2015). Transfection efficiency was assessed via visualization of EGFP plasmid co-transfected in parallel.

***In vitro* BE(2)-M17 Differentiation**

BE(2)-M17 cells were differentiated as described previously (Andres et al., 2013). Briefly, BE(2)-M17 cells were seeded at 20-30% confluency and treated with 10 μM *trans*-retinoic acid (Sigma) added to the culture medium every day for the first 5 days and every other day thereafter for a total of 10 days. Differentiation was monitored by neurite outgrowth observed using light microscopy. The cells/neurite outgrowth were viewed and photographed at a magnification of 40X with an Olympus IX-51 inverted fluorescent microscope.

miRNA Quantification

For cells, total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's protocol. RNA concentration was quantified with the BioDrop μLITE (BioDrop). RNA quality was assessed by gel electrophoresis. For detection of mature miRNAs, 1 μg of total RNA was subjected to microRNA-specific reverse transcription and qPCR using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) and TaqMan Universal Master Mix (Thermo Fisher Scientific), respectively. Real-time PCR was

performing using PerfeCTa SYBR Green FastMix (QuantaBio) on a CFX96 Touch Real-Time PCR system (Bio-Rad). Threshold amplification values (C_t) were assigned by the CFX Manager software (Bio-Rad).

RNA-seq data processing

Raw RNA-seq reads were aligned to the human genome build hg38 using TopHat2 (v1.3.3) (Kim et al., 2013). Alignment bam files were used to generate fragments per kilobase million (FPKM) of all RefSeq genes. Differential expression gene analysis was conducted by Cuffdiff with respect to control and *trans* retinoic acid (RA)-treated BE(2)-M17 cells (Trapnell et al., 2012).

***In silico* prediction of RBP sites in pri-miRNA sequences**

The computational package, circRNAs interact with proteins (CRIP), uses a codon-based encoding and hybrid deep neural network, trained by existing CLIP-seq datasets, to predict RBP binding sites to any given RNA sequence (Zhang et al., 2019a). A moderate cutoff of 0.8 probability was applied to the data. The pri-miR-124-2 sequence analyzed (+/- 200nt flanking hairpin and hairpin, 509bp total) spans chr8: 65,291,506-65,292,014 (hg19).

Immunoblot Analysis

Harvested cells were lysed and sonicated in 1X Laemmli buffer as described previously (Mandler et al., 2014). Proteins were than separated on a 10% SDS/PAGE and transferred to a PVDF membrane (GE Healthcare), which was blocked in 10% nonfat, dry milk in phosphate-buffered saline with 0.01% Tween-20. The membrane was then probed with primary antibodies: PTBP1 antibody (1:5000, Invitrogen, 32-4800), FLAG antibody (1:3000, Sigma, F3165), QKI5 antibody (1:5000, Bethyl Laboratories, A300-183A) and

eIF5A (1:10,000, Santa Cruz, sc-282). Pan-QKI and QKI-6 antibodies were custom-made by UC Davis/NIH NeuroMab.

Immunoprecipitation

Immunoprecipitation was performed as described previously (Mandler et al., 2014). Briefly, SH-SY5Y cells were co-transfected with FLAG-DGCR8 and HA-QKI5. Cells were lysed in ice-cold IP Lysis buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.5% Triton X-100, and a cocktail of protease inhibitors including pepstatin, leupeptin, aprotinin and phenylmethanesulfonyl fluoride. Lysates were incubated on ice for 10 min and then centrifuged at 15 700 × g for 10 min at 4°C to sediment cellular debris, and the supernatant was transferred to a fresh tube. Samples were pre-cleared by end-over-end rotation for 1 hour at 4°C with sepharose 4B beads (Sigma) that were pre-balanced in the IP buffer. The samples were then centrifuged for 30 sec at 400 x g to sediment beads and the supernatant was added to fresh tubes. Five percent of pre-cleared supernatant was stored for detecting protein input. Twenty microliters of pre-balanced anti-FLAG-M2 beads (Sigma) were then added to the supernatants and incubated by rotation for 3 hours at 4°C. The samples were washed three times in an elution buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl2, protease inhibitors and 1% Triton X-100 and centrifuged at 400 × g at 4°C. The beads were then resuspended in 50 uL Elution buffer containing 5 mM Tris pH 7.5, 0.32 M sucrose and 2% SDS and 1X Laemmli Buffer and analyzed by SDS-PAGE followed by immunoblot.

CRISPR-Cas9 Editing of BE(2)-M17 cells

CRISPR guide RNA (gRNA) sequences were designed and *in vitro* synthesized by Gene Edit Biolab (Atlanta, GA): gRNA-7c-5-1 TGAATGGATACAATAGGTTA, and gRNA-7c-3-

2 CTTGTCTATAAGAAATGCGT. BE(2)-M17 cells that harbor lentivirus-mediated expression of Cas9 were co-transfected with the above gRNAs and an RFP-construct using Lipofectamine 2000 (Invitrogen). RFP-positive single-cell clones were manually selected. For extraction of genomic DNA from clonal cell lines, cells were resuspended in 500μL proteinase K buffer (containing 2 mg/mL proteinase K) and incubated at 55°C overnight. To deplete RNA, 3μL of 10mg/mL RNase A was added and incubated for one hour at 37°C, followed by addition of 200μL 3M sodium acetate and incubation on ice for 5 minutes. The lysate was centrifuged for 8 min at 15,700 × g at room temperature. To the supernatant, we added 600μL 2-propanol and centrifuged for 1 min at 15,700 g at room temperature to precipitate DNA. The pellet was washed with 70% (v/v) ethanol, dried for 5-10 min at room temperature and resuspended in 50μL water. PCR using the DNA-preparation and primers flanking the deletion site specific to QKI5-specific exon 7c (Table S3.2) was performed to verify on-target editing. RNA-sequencing was performed to confirm the deletion of the QKI5 isoform specifically without affecting other QKI isoforms.

Chapter 6: References

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