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Reciprocal Actions of REST and a microRNA Promote Neuronal Identity

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Ma. Cecilia G. Conaco

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree,

hereby recommend acceptance of this dissertation.

Gail Mandel, Ph.D., Distinguished Professor, Dissertation Advisor
Department of Neurobiology and Behavior

Simon Halegoua, Ph.D., Professor, Chairperson of Defense
Department of Neurobiology and Behavior

Michael J. Hayman, Ph.D., Professor
Department of Molecular Genetics and Microbiology

Maurice Kernan, Ph.D., Associate Professor
Department of Neurobiology and Behavior

Oliver Hobert, Ph.D., Associate Professor
Department of Biochemistry and Molecular Biophysics
Columbia University, New York

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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MicroRNAs regulate diverse cellular processes by mediating the post-transcriptional repression of many mRNAs. Understanding how microRNAs integrate into the complex gene regulatory networks that control cell fate specification and lineage determination requires elucidation of the mechanisms of microRNA gene regulation and the identification of microRNA targets. Here, I show that a family of microRNAs is regulated by REST, a transcription factor best known for its role in controlling the expression of fundamental neuronal traits. One of the REST-regulated microRNAs, miR-124a, exhibits abundant brain-specific expression and has previously been shown to be capable of promoting a neuronal-like mRNA profile in HeLa cells by decreasing the levels of hundreds of non-neuronal transcripts. In this study, we show that miR-124a can also promote neuronal differentiation of cortical progenitors. Extensive experimental validation of putative targets identified a cohort of non-neuronal mRNAs that are directly downregulated by miR-124a. These target transcripts encode various proteins with functions that may be unnecessary, or even antagonistic, to proper neuronal development and activity. Analysis of the characteristics of validated targets revealed the importance of miR-124a seed sites and 3'UTR sequence context on microRNA-mediated repression. The results of this study suggest a model wherein miR-124a and REST, through their reciprocal actions, play a central role in promoting neuronal differentiation of progenitor cells and may be critical for maintaining the stability of the neuronal phenotype.

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GENERAL INTRODUCTION

REST regulation of neuronal differentiation

Multicellular organisms evolved with the emergence of more elaborate gene regulatory networks (Levine and Tjian 2003). Control of gene expression relies on *cis*-regulatory sequences and components of the transcriptional machinery that can recognize these sites. Neuronal development, for example, requires the carefully coordinated action of transcriptional activators and repressors to regulate the spatial and temporal expression of neuronal genes in neurons while maintaining the non-neuronal phenotype of surrounding cells (Munoz-Sanjuan and Brivanlou, 2002; Edlund and Jessell, 1999). A key player in this process is the RE1 silencing transcription factor (REST/NRSF), which regulates the expression of genes that are typically expressed in mature neurons.

REST was initially identified as the repressor protein that restricts the expression of Nav1.2 and SCG10 genes to neuronal cells (Chong et al, 1995; Schoenherr et al, 1995). It is expressed ubiquitously in non-neuronal cells where it serves to silence the expression of its targets by binding to a 23 base pair motif, known as the repressor element 1 (RE1/NRSE), in the regulatory regions of these genes. Many other genes that are also regulated by REST have since been identified. The list includes proteins that are involved in all aspects of neuronal function, such as ion channels and receptors (Schoenherr et al, 1996; Bessis et al, 1997; Mu and Burt, 1999; Brene et al, 2000; Gurrola-Diaz et al, 2003; Mieda et al, 1997; Bai et al, 1998; Andria and Simon, 2001) synaptic vesicle proteins (Lietz et al, 2003; Schoch et al, 1996), neurotransmitter and neuropeptide synthesizing enzymes (Mbikay et al, 2002; Lonnerberg et al, 1996; Quinn et al, 2002; Seth and Majzoub, 2001; De Gois et al, 2000), and structural or signaling proteins (Bieche et al, 2003; Kallunki et al, 1997; Abderrahmani et al, 2001).

The REST cDNA encodes a 116 kDa protein (Chong et al, 1995). It consists of an 8-zinc finger DNA binding domain that when expressed by itself can act as a dominant negative to relieve silencing of target genes (Chong et al, 1995; Schoenherr et al, 1995; Chen et al, 1998). REST repressor activity resides in two domains, one within the 83 amino-terminal residues and another in the carboxy-terminal zinc finger (Tapia-Ramirez et al, 1997). REST represses its target genes by recruiting histone deacetylases through an mSin3 corepressor complex at the amino-terminus, and a CoREST complex at the carboxy-terminus (Andres et al, 1999; Ballas et al, 2001). Additional chromatin modifying factors are recruited by CoREST, including MeCP2 and the histone methylases G9a and SUV39H1 (Lunyak et al, 2002; Roopra et al, 2004). A

negative regulator of RNA polymerase II activity, the C-terminal domain small phosphatase (Ctdsp1/SCP1), is also recruited by REST and is required for neuronal gene repression (Yeo et al, 2005).

Downregulation of REST is important for the acquisition of the neuronal phenotype. As progenitors differentiate into neurons, the REST protein is selectively degraded by the proteasome (Ballas et al, 2005), thus allowing neuronal genes to become expressed. Overexpression of REST in neuronal cell lines blocks neurite growth and prevents the acquisition of membrane excitability (Ballas et al, 2001). In chick neurons, REST overexpression results in axon pathfinding errors (Paquette et al, 2000). On the other hand, inhibition of REST function, using a dominant negative form of REST, results in ectopic derepression of neuronal genes (Chong et al, 1995; Schoenherr et al, 1995; Chen et al, 1998). Similarly, an activating form of REST consisting of the DNA binding domain fused to the VP16 activator (REST-VP16), is sufficient to convert myoblasts into a physiologically active neuronal phenotype (Watanabe et al, 2004). Mice lacking REST exhibit ectopic expression of target genes, retarded growth, and die at an early embryonic stage, indicating that REST is also important during early development (Chen et al, 1998).

An unexpected aspect of REST function is its recently discovered role as a tumor suppressor. Different types of cancer are proposed to arise through various routes of REST dysfunction. For example, medulloblastomas are proposed to arise through the retention of REST in undifferentiated neuroectodermal stem cells (Fuller et al, 2005). In fact, expression of the activating REST-VP16 transgene in medulloblastoma cells was sufficient to inhibit tumor growth (Lawinger et al, 2000; Fuller et al, 2005). In other cancer types, such as colon cancer, small cell lung carcinoma, and prostate cancer, oncogenic transformation was associated with a loss or modification of REST function resulting in aberrant gene expression or activation of oncogenic pathways (Palm et al, 1999; Coulson et al, 2000; Westbrook et al, 2005).

Genome-wide analysis of the REST regulatory network has revealed that REST also controls the expression of genes that encode short regulatory RNAs, known as microRNAs (Conaco et al, 2006; Mortazavi et al, 2006; Wu and Xie, 2006). These RNAs are post-transcriptional regulators of gene expression, and their presence within the REST regulatory network suggests that REST action may have a more widespread impact on the overall genotypic profile of a cell. The identification and validation of these REST-regulated miRNA genes are discussed in Chapter I.

Post-transcriptional regulation of gene expression

Post-transcriptional processes that affect mRNA stability and translation add another layer of complexity to gene regulatory networks. The major determinants of eukaryotic mRNA stability are the 5' 7-methylguanosine cap and 3' poly(A) tail. These structures serve to enhance translation as well as to protect the mRNA from untimely degradation. Loss of the poly(A) tail and subsequent decapping of transcripts paves the way for exonucleolytic degradation (Garneau et al, 2007). mRNAs can also be cleaved internally by endonucleases, yielding fragments that are susceptible to exonuclease digestion. Endonucleases are tightly regulated by the cell and are highly specific for their targets. For example, the Argonaute 2 (Ago2) protein is targeted to specific mRNA sequences by an RNA guide strand (Liu et al, 2004a).

RNA binding proteins can also affect the stability of an RNA transcript. Many of these proteins bind to AU-rich elements (ARE) within the 3' untranslated region (UTR). ARE-binding proteins such as tristetrapolin, KSRP, and AUF destabilize mRNAs by recruiting decay factors (Lai et al, 2003; Gherzi et al, 2004; Chen et al, 2001) while others, such as the ELAV/Hu proteins, may stabilize transcripts by competing for binding sites with destabilizing factors or by inhibiting poly(A) tail deadenylation (Lal et al, 2004; Beckel-Mitchener et al, 2002). In addition to affecting mRNA longevity, neuronal RNA binding proteins, such as Staufen2, Fragile X mental retardation protein (FMRP), and Zipcode binding protein 1 (Zbp1), are important for the proper localization of specific mRNAs to dendrites (Kiebler et al, 1999; Kirkpatrick et al, 2001; Ross et al, 1997).

Components of the mRNA decay pathway, including decapping factors and exonucleases, have been shown to localize to dynamic cytoplasmic foci, known as P-bodies, in an mRNA-dependent manner (Sheth and Parker, 2003; Cougot et al, 2004). P-bodies are distinct from polysomes in that they lack ribosomes and members of the translational machinery (Kedersha et al, 2005; Teixeira et al, 2005). Interestingly, transcripts sequestered in P-bodies can be released back into the translatable pool (Bhattacharyya et al, 2006; Brengues et al, 2005). This suggests that P-bodies may serve as triage centers for mRNAs that are either to be stored and translated at a later time, or that need to be degraded.

MicroRNA biogenesis and mechanisms of translational repression

Short regulatory RNAs, such as microRNAs (miRNAs) and short interfering RNAs (siRNAs), provide another mechanism for post-transcriptional regulation of mRNAs. These ~22nt

regulatory RNAs serve as guide sequences to localize a ribonucleoprotein effector complex to recognition sites on mRNA transcripts. The effector complex can control mRNA expression through direct mRNA degradation or by inhibiting productive translation. siRNAs are double stranded RNAs that can trigger endonucleolytic cleavage of homologous mRNAs in a process referred to as RNA interference (RNAi) that was first described in *C. elegans* (Fire et al, 1998; Timmons and Fire, 1998). RNAi is thought to have evolved as a defense mechanism to protect the cell against exogenous RNAs, such as from viruses (Hannon, 2002). siRNAs may be derived from exogenously added RNA or endogenously transcribed RNAs that form duplexes (Elbashir et al, 2001a; Elbashir et al, 2001b). Binding of the siRNA to a single perfectly complementary sequence, usually within the coding region of the target transcript, triggers mRNA cleavage (Zamore et al, 2000).

MicroRNAs share similar processing enzymes and effector complexes as the siRNAs. However, unlike siRNAs, miRNAs are derived from long stem-loop precursors encoded in the genome. While plant miRNAs and a few animal miRNAs cause mRNA degradation in a manner similar to siRNAs, most mature animal miRNAs bind to imperfectly complementary sites in the 3'UTR of target transcripts and function as translational repressors (Olsen and Ambros, 1999; Doench et al, 2003). miRNAs have diverse expression patterns and have been shown to function in a wide array of developmental and cellular processes (Lim et al, 2003).

The first miRNAs to be discovered, lin-4 and let-7, were identified through a genetic screen for regulators of *C. elegans* developmental timing (Lee et al, 1993; Wightman et al, 1993; Moss et al, 1997; Reinhart et al, 2000). The cloning methods that were used in early miRNA identification studies (Lau et al, 2001; Lagos-Quintana et al, 2001; Lagos-Quintana et al, 2002) have since made way for high-throughput methods involving oligonucleotide microarrays (Thomson et al, 2004; Babak et al, 2004; Liu et al, 2004b) and quantitative PCR (Schmittgen et al, 2004; Chen et al, 2005; Raymond et al, 2005). Notable new developments in global miRNA identification include miRAGE, an assay based on the Serial Analysis of Gene Expression (SAGE) technique (Cummins et al, 2006), and a bead-based flow-cytometric method that is able to distinguish between closely related miRNAs (Lu et al, 2005). The array-based Klenow enzyme assay (RAKE) has the added advantage of not requiring sample manipulation, such as labeling or amplification, prior to array hybridization to avoid introducing biases in the small RNA population (Nelson et al, 2004). More recently, a highly sensitive single molecule detection method was developed that can be used instead of Northern blotting, ribonuclease protection assay (RPA), or quantitative PCR, for precise quantitation of individual miRNA concentrations

(Neely et al, 2006). Using these methods, hundreds of phylogenetically conserved miRNAs have been identified in metazoans and plants.

MicroRNAs are encoded by unique genetic loci that are transcribed by RNA polymerase II to yield capped, polyadenylated transcripts (Cai et al, 2004). miRNA gene expression is controlled by the same set of transcription factors that regulate protein-coding genes (O'Donnell et al, 2005; Sokol and Ambros, 2005; Zhao et al, 2005; Johnston et al, 2005; Vo et al, 2005; Fazi et al, 2005; Conaco et al, 2006). This allows seamless integration of miRNAs into the gene regulatory networks that govern cellular processes.

Primary miRNA transcripts typically contain a terminal loop, a double stranded stem of ~33nt, and flanking single stranded segments. These long primary transcripts (pri-miRNA) are processed in the nucleus into ~70nt precursor miRNAs (pre-miRNA) by the Drosha-DGCR8 complex (Lee et al, 2003; Han et al, 2004). DGCR8 specifically binds to pri-miRNA at the junction of the single stranded segments and the double stranded stem. DGCR8 binding orients Drosha, a nuclear RNAse III enzyme, and allows it to cleave the stem region ~11nt from the junction to release the hairpin pre-miRNA. The hairpin precursors are exported into the cytoplasm in association with exportin-5-GTP (Yi et al, 2003). Once in the cytoplasm, the terminal loop of the pre-miRNAs is cleaved by the cytoplasmic RNAse III enzyme, Dicer, to yield ~22nt RNA duplexes (Bernstein et al, 2001; Hutvagner et al, 2001; Grishok et al, 2001; Ketting et al, 2001). Unwinding of the duplex yields a single stranded mature miRNA that is incorporated into the RNA-induced silencing complex (RISC) where it serves as the guide strand to locate target mRNAs.

The RISC complex mediates target mRNA cleavage or translational repression (Hammond et al, 2000; Hammond et al, 2001). Every RISC contains a member of the Argonaute (Ago) protein family. Ago proteins have two conserved domains, PAZ and Piwi (Carmell et al, 2002). PAZ is an RNA binding domain that recognizes the nucleotide overhangs of the miRNA duplex, possibly to facilitate the loading of the mature miRNA guide strand into RISC (Ma et al, 2004; Lingel et al, 2004). The Piwi domain has structural folding similar to members of the RNAse H family, suggesting that Ago proteins may be involved in cleaving the mRNA-miRNA duplex (Song et al, 2004). In mammals, Ago1-4 is associated with miRNAs, but only Ago2 has been shown to exhibit endonucleolytic activity (Meister et al, 2004; Liu et al, 2004a). Characterization of other RISC components has revealed the presence of various proteins that may be involved in miRNA processing, unwinding and loading of the mature miRNA, mRNA cleavage, and translational repression. Purified *D. melanogaster* RISC contains the putative RNA binding proteins Vasa intronic gene (VIG) and Fragile X-related protein (dFXR), as well as the

putative nuclease, Tudor-SN (Caudy et al, 2002; Caudy et al, 2003; Ishizuka et al, 2002). Affinity purification of human miRNA complexes revealed the association of the human homologue of dFXR (FMRP), along with Dicer, RNA helicases Gemin3-4 and MOV10, and RNA binding proteins TRBP and TNRC6B (Mourelatos et al, 2002; Meister et al, 2005; Chendrimada et al, 2005). The DEAD box helicase RCK/p54 is also associated with Ago1 and Ago2 in human cells and its depletion results in a loss of miRNA-mediated translational repression (Chu and Rana, 2006). Many of the components of the RISC complex also colocalize with P-bodies, further supporting the idea that these dynamic structures play an important role in small RNA-mediated post-transcriptional regulation of messages (Liu et al, 2005).

While there is emerging evidence showing that miRNAs can downregulate target mRNA levels (Bagga et al, 2005; Lim et al, 2005), there are also multiple examples where miRNAs affect protein levels to a much greater extent than can be explained by changes in mRNA concentration (Poy et al, 2004; Chen et al, 2004; Cimmino et al, 2005; Anderson et al, 2006). The exact mechanism by which miRNAs repress translation is still unclear. This effect appears to be independent of the 5' cap and poly(A) tail (Humphreys et al, 2005). Although miRNAs have been observed to accelerate mRNA deadenylation, this does not seem to be the sole mechanism for repression, as an mRNA with a 3' histone stem-loop instead of a poly(A) tail is also effectively repressed (Wu et al, 2006). Translational inhibition is also independent from RNA cleavage activity of the endonuclease Ago2. In fact, translational repression can occur when either Ago2 or Ago4 is tethered to an mRNA (Pillai et al, 2004). In addition, repression is also observed in Ago2 null mice (Liu et al, 2004a).

Recent studies suggest that miRNAs inhibit translational initiation by sequestering mRNAs into the P-bodies, away from the actively translating polysomes (Brengues et al, 2005; Kedersha et al, 2005; Teixeira et al, 2005). It is also predicted that colocalization of mRNAs with mRNA decay factors found in P-bodies may contribute to the often times observed cleavage-independent downregulation of target transcripts (Valencia-Sanchez et al, 2006). A decrease in ribosomal association, indicated by a shift to lighter polysome fractions in density gradient analysis, is evident for endogenous let-7 RISC complexes and Ago2-tethered target mRNAs compared to non-targeted control mRNAs (Pillai et al, 2005). More direct evidence that miRNAs can affect translational initiation is provided by studies where the mode of translation initiation is modified. mRNAs that are tethered to translation factors eIF-4E or eIF-4G become insensitive to miRNA-mediated repression (Pillai et al, 2005). A similar result was observed for *in vitro* transcribed mRNAs containing target sites for the let-7 miRNA, where translation is driven by the internal ribosome entry site (IRES) of either EMCV or HCV. These IRES-driven reporters,

which are able to bypass regular initiation mechanisms, were observed to be insensitive to let-7-mediated translational inhibition (Pillai et al, 2005).

MicroRNAs may also inhibit translation after initiation. The general observation that miRNAs are found associated with the actively translating polyribosome fraction in density gradient sedimentation experiments indicates that translational repression is occurring post-initiation (Kim et al, 2004; Nelson et al, 2004). In addition, it has been observed that lin-14 and lin-28 transcripts, which have binding sites for lin-4 in their 3'UTRs, do not change in polysomal sedimentation profile in the presence of the lin-4 miRNA (Olsen and Ambros, 1999; Seggerson et al, 2002). The lin-41 3'UTR, which is targeted by the let-7a miRNA, is also associated with polyribosomes (Nottrott et al, 2006). A shift in the distribution of repressed mRNAs to lighter polyribosome fractions upon treatment with puromycin, a polypeptide chain terminator (Blobel and Sabatini, 1971), indicates that the ribosomes associated with repressed mRNAs are translationally active (Nottrott et al, 2006; Petersen et al, 2006). It has been postulated that increased ribosome drop-off may be responsible for the lack of full-length protein production (Petersen et al, 2006). Further evidence to support post-initiation inhibition of translation is provided by a study showing that translation of a reporter containing bulged binding sites for the CXCR4 siRNA driven by cap-independent translation through HCV and CrPV IRES is still effectively repressed (Petersen et al, 2006). Interestingly, another study that used the same CXCR4 siRNA (Humphreys et al, 2005) obtained results more similar to those seen by Pillai et al (2005). The cause of these markedly different results is not fully understood. However, it is conceivable that translational repression may occur through different mechanisms depending on cell type, cell condition, the properties of the mRNA target, and the identity of the miRNA (Valencia-Sanchez et al, 2006).

MicroRNA functions

Specific spatial and temporal expression patterns, as well as the observed expansion of miRNA genes in plants and higher animals, suggests that miRNAs play an important role in controlling the transcriptome output during cell fate specification and developmental patterning (Lim et al, 2003). In fact, the classical miRNAs, lin-4 and let-7, were found in a genetic screen for mutations in heterochronic development of *C. elegans* (Lee et al, 1993; Wightman et al, 1993; Moss et al, 1997; Reinhart et al, 2000). Similar mutation screening experiments in *D. melanogaster* led to the discovery of miRNAs bantam and miR-14 that were found to be involved in the suppression of apoptosis (Brennecke et al, 2003; Xu et al, 2003). A more recent study in

flies used antisense 2'O-methyl oligonucleotides to inhibit miRNA activity, revealing a large family of miRNAs involved in the regulation of embryonic apoptosis (Leaman et al, 2005). Other recent studies use a combination of conditional Dicer null mutants, expression profiling, and phenotypic assays to assess the functions of miRNAs.

MicroRNAs play a role in stem cell self-renewal and differentiation. Dicer loss in mouse embryonic stem cells, leading to a defect in miRNA processing, resulted in division and proliferation defects, ultimately causing the death of mouse embryos at 7.5 days (Murchison et al, 2005). Embryoid bodies derived from Dicer null embryonic stem cells fail to differentiate and have defects in centromeric silencing (Kanellopoulou et al, 2005). There is also supporting data from a study done in flies that shows the necessity of the miRNA pathway for germline stem cell (GSC) division. Knocking out Dicer function in GSCs delayed the G1 to S transition, thus drastically reducing the rate of germline cyst production (Hatfield et al, 2005).

MicroRNAs are essential for early vertebrate development. Dicer null zebrafish embryos develop normally through embryogenesis and organogenesis, arresting only after 8 days when the maternally contributed miRNAs are depleted (Wienholds et al, 2003). Surprisingly, zebrafish embryos that lack both maternal and zygotic miRNA contributions still displayed normal axis formation and cell type specification, suggesting that miRNA function may not be critical for initial tissue patterning. However, these embryos did exhibit defects in morphogenesis during gastrulation and at later stages of organ development (Giraldez et al, 2005). Another miRNA with a role in early vertebrate development is miR-196, which may mediate post-transcriptional regulation of Hoxb8 to define its posterior expression boundary (Yekta et al, 2004).

In later development, miRNAs have been shown to be involved in specification of certain cell types in coordination with transcription factor networks. During cardiogenesis, for example, serum response factor induces the expression of muscle-specific miRNA, miR-1, which in turn controls cardiomyocyte proliferation by downregulating the Hand2 transcription factor (Zhao et al, 2005). Interestingly, in flies, miR-1, which is regulated by Twist1 and Mef2, has also been shown to be required for development of larval musculature (Sokol and Ambros, 2005). Another miRNA with a key role in muscle differentiation is miR-206. Overexpression of this miRNA promotes muscle differentiation of C2C12 myoblasts by downregulating DNA polymerase α, which reduces DNA synthesis and cell proliferation (Kim et al, 2006). miRNA function is also important for the regulation of hematopoesis. Overexpression of miR-181 in hematopoetic progenitors leads to an increase in B-lineage cells (Chen et al, 2004). Granulocytic differentiation is enhanced by C/EBPα-induced transcription of miR-223 (Fazi et al, 2005). Adipocyte differentiation is promoted by miR-143, either directly or indirectly through its target, ERK5

(Esau et al, 2004). Other clues to miRNA function in later development are provided by conditional Dicer knockout in mice. Selective loss of Dicer in limb mesoderm revealed that miRNAs are necessary for normal limb growth, but are not required for normal tissue patterning. Loss of Dicer function in the epidermis resulted in evagination and hypoproliferation of hair follicles, as well as a general perturbation of epidermal organization (Andl et al, 2006; Yi et al, 2006).

The first miRNAs shown to have a role in nervous system development were discovered in *C. elegans*. These miRNAs, lsy-6 and miR-273, are part of a gene regulatory cascade that controls the asymmetric expression of guanyl cyclase genes that differentiate the left and right worm chemosensory neurons (Johnston and Hobert, 2003; Chang et al, 2004). In the mammalian nervous system, where cellular diversity is extreme, the importance of miRNA gene regulation is only just emerging (Kosik, 2006). Several studies have used expression profiling to identify miRNAs that may be involved in neuronal differentiation and brain development (Krichevsky et al, 2003; Sempere et al, 2004; Kim et al, 2004). The most abundant brain-specific miRNAs, miR-124a and miR-9, increase the neuron to glia ratio when ectopically expressed in differentiating embryonal stem cells (Krichevsky et al, 2006). However, in an *in vivo* study, inhibition and misexpression of miR-124a did not have any discernible effect on neuronal differentiation, although overexpression did result basal laminae defects (Cao et al, 2007). The variability in these results underlines the need to further elucidate the direct targets of these miRNAs to obtain a better understanding of the points in the differentiation pathway that may be affected by miRNA function. Chapter II explores the role of miR-124a in neuronal differentiation through functional assays and target identification. The results of these studies reveal that the establishment and maintenance of neuronal identity requires both derepression of REST-regulated genes as well as post-transcriptional downregulation of non-neuronal transcripts by miR-124a.

Translational control is another important component of neural function and the maintenance of synaptic plasticity. Certain mRNAs, regulators, and translational machinery are known to localize to the dendrites of neurons where translation is triggered in response to synaptic activity (Jiang and Schuman, 2002). In addition, miRNAs and functional RISC complexes have been observed in neuronal axons (Hengst et al, 2006). The potential for miRNAs to regulate different proteins simultaneously make them likely candidates for the modulation of activity-dependent localized translation. An interesting study in flies revealed a potential role for the miRNA pathway in regulating long-term memory. A component of the RISC complex, Armitage, was shown to colocalize with α-CaMKII in synaptic puncta. In response to neural

activity, Armitage was degraded, which resulted in an upregulation of α-CamKII translation at the synapses (Ashraf et al, 2006). In mammals, several miRNAs that may have activity-dependent regulation have been identified. miR-132 was identified as a target of the cAMP-response element binding protein (CREB) and may regulate neuronal morphology in response to extrinsic signals by downregulating the GTPase-activating protein, p250GAP (Vo et al, 2005). Another miRNA, miR-134, was shown to respond to BDNF signals by releasing its target, Limk1, a kinase involved in dendritic spine development (Schratt et al, 2006).

MicroRNAs have also been implicated in diseases, such as cancer. Many miRNAs are encoded by genetic loci that are associated with genomic alterations frequently observed in tumors. For example, the miR-15a-16 locus is frequently deleted in chronic lymphocytic lymphoma (Calin et al, 2002). The miRNAs encoded by this locus have been shown to target the anti-apoptotic gene Bcl2 (Cimmino et al, 2005). Some miRNAs have been shown to have oncogenic properties. The miRNAs encoded within the miR-17-19 locus cause accelerated lymphomagenesis in a mouse model of Burkitt's lymphoma (He et al, 2005). Another miRNA, let-7, is a tumor suppressor that acts by downregulating expression of the Ras oncogene (Johnson et al, 2005). Gene expression analysis of tissues from many human cancers provides valuable clues to the lineage and differentiation status of these tumors. Interestingly, global miRNA expression in tumors is lower compared to normal cells, suggesting that cancer cells are in a more undifferentiated state (Lu et al, 2005). While the miRNA profile of many tumors is reminiscent of the tissues from which they were derived, tumors express a distinctive miRNA signature, reflecting the gain or loss of different miRNAs (Volinia et al, 2006). Whether the changes in miRNA expression are the cause of oncogenic transformation, or are merely secondary effects, remains to be elucidated.

MicroRNA target prediction

Despite rapid advances in the elucidation of miRNA functions in diverse cellular processes, the exact targets through which they exert their effects is still relatively unknown. Understanding the rules of miRNA target recognition for the development of accurate and sensitive target prediction methods remains one of the main challenges in the field. Identification of miRNA targets is further complicated by emerging evidence that miRNAs can bind multiple targets (Lim et al, 2005) and that multiple miRNAs can regulate a single target mRNA (Doench and Sharp, 2004). Interestingly, this suggests that miRNAs may be capable of integrating signals from different pathways and coordinating simultaneous processes within a cell.

From a comparison of a few validated miRNA-target relationships, it appears that the seed region is an important determinant of miRNA binding (Stark et al, 2003; Rajewsky and Socci, 2004; Lewis et al, 2003). The seed region is a highly conserved stretch of 6-7nt that can pair perfectly to the first 2-8 nucleotides at the 5' end of the microRNA. Perfect pairing at this site is thought to initiate miRNA-mRNA duplex formation to overcome initial thermal diffusion and facilitate annealing of the remainder of the miRNA (Rajewsky and Socci, 2004). It has been reported that the ability of a miRNA to inhibit translation depends on the stability of the binding between the 5' end of the miRNA and the seed region on the mRNA (Doench and Sharp, 2004).

MicroRNA target sites can be classified into three groups (Brennecke et al, 2005). The first group, referred to as canonical sites, have base pairing to the seed region at the 5' end of the miRNA as well as extensive pairing at the 3' end. The second class of sites, referred to as seed only sites, have perfect pairing only at the seed region of the miRNA. The third group, referred to as 3' compensatory sites, have limited 5' end base pairing but compensate with extensive 3' end pairing.

The imprecise base pairing between animal miRNAs and their targets makes it a challenge to computationally predict the genes that are regulated by specific miRNAs. Nevertheless, many target prediction programs have appeared in recent years. The most commonly used mammalian miRNA target prediction programs identify targets based on two major criteria derived from a limited set of experimentally identified miRNA-target interactions. First, they identify potential miRNA binding sites according to certain base pairing rules. TargetScanS (Lewis et al, 2005), the newest version of TargetScan (Lewis et al, 2003), requires only perfect seed pairing to the 5' end of the miRNA. PicTar allows for imperfect seed matches and uses a maximum likelihood approach to incorporate the combinatorial nature of miRNA targeting (Krek et al, 2005). MiRanda and DIANA-microT use a modified dynamic programming approach that does not require perfect seed complementarity. MiRanda scores for either strong single sites or for multiple hits on a gene with any miRNA (John et al, 2004) while DIANA-microT focuses on single strong recognition sites (Kiriakidou et al, 2004). Secondly, the algorithms look at cross-species conservation of 3'UTRs. TargetScanS looks at conservation across five species and requires sequence as well as positional conservation of seeds within the 3'UTR. PicTar compares five vertebrate species but requires only that a seed match occur at overlapping positions in a 3'UTR alignment. MiRanda and DIANA-microT require seed sites to have at least 90% identity and to have corresponding positions in an alignment of human and rodent 3'UTRs. In a comparison of the different target prediction methods to 84 experimentally verified miRNA-mRNA target interactions annotated in TarBase (Sethupathy et al, 2006a), it was

revealed that, individually, the most recent algorithms to be developed (MiRanda, TargetScanS, and PicTar) are able to predict 66-67% of the conserved, experimentally supported miRNA-mRNA target interactions. However, only 40% of the validated targets are predicted in common by all three methods (Sethupathy et al, 2006b). DIANA-microT had even lower sensitivity, predicting only 13% of the validated interactions.

Owing to the small size of miRNA target sites and the different sets of rules that algorithms use to identify targets, a few hundred targets are generally predicted for each miRNA with minimal overlap between methods. One of the biggest problems that affect computational prediction of targets is that each program uses a different 3'UTR annotation to define their search set. Available annotated 3'UTR sequences may not readily reflect the presence of tissue-specific isoforms, splice variants, and alternative adenylation sites (Rajewsky, 2006). Furthermore, it appears that seed sequences by themselves may not be reliable determinants of miRNA-target interaction. This was shown in a study wherein the binding site of the lsy-6 miRNA, which was introduced into different 3'UTR contexts, was not always able to functionally interact with its cognate miRNA (Didiano and Hobert, 2006). This suggests that other factors, such as sequence-dependent 3'UTR accessibility, as determined by RNA secondary structure and RNA binding proteins, may be important to consider in the identification of miRNA targets. Although algorithms have been developed that include RNA accessibility as a criteria (Robins et al, 2005; Zhao et al, 2005), it is not yet clear whether these methods improve specificity and sensitivity. Moreover, it should be noted that RNA folding programs are typically unreliable for long sequences and will not account for structural changes mediated by mRNA binding factors. These challenges all underline the need for more extensive experimental validation of targets to improve our understanding of miRNA target recognition rules. Chapter III presents an analysis of the validated miR-124a-target interactions to show that seed motifs and site accessibility are important determinants of miRNA regulation.

Integrating miRNAs into regulatory networks

Changes in gene expression underlie the differences between cells. Gene expression is controlled at many levels, and although transcriptional regulation is generally considered the primary method of regulating gene expression, post-transcriptional mechanisms also make an important contribution to the overall expression profile of a cell. In fact, regulatory loops of miRNAs and transcription factors appear to be crucial for tissue lineage differentiation, as well as many other biological processes (Hobert, 2004). Elucidation of the workings of these networks is

essential to gain a better understanding of the events that govern cell fate decisions and to provide insights into how defects in these pathways can lead to diseases, such as cancer.

CHAPTER I

REST regulates a family of microRNAs

Introduction

REST was proposed several years ago as a master regulator of the neuronal phenotype due to its ability to repress the expression of select neuronal genes in non-neuronal tissues. This supposition, however, was based on a rather limited number of target genes and on reporter gene analysis. With the advent of technologies for genome-wide analysis of transcription factor binding sites, I have been able to take a more global view of the entire REST regulatory network.

In this chapter, I present findings showing that, in addition to protein coding genes, REST regulates the expression of a family of brain-specific miRNA genes. This regulation is mediated through the binding of REST to RE1 sites on the miRNA promoters. These findings reveal an additional layer of complexity to the pathways governing neurogenesis and neuronal function. The work detailed in this section was done in collaboration with two other graduate students in the laboratory. Stefanie Otto generated the SACO library and Jong-Jin Han performed some of the luciferase assays.

Results

SACO analysis identifies miRNA genes as potential REST targets

Serial analysis of chromatin occupancy (SACO) is an unbiased method for identifying functional transcription factor binding sites genome-wide (Impey et al, 2004). It uses a combination of chromatin immunoprecipitation (ChIP) and serial analysis of gene expression techniques. Essentially, SACO creates a library of small fragments of chromatin (tags) that are immunoprecipitated with an antibody against the transcription factor of interest. Mapping these sequence tags to their locations within the genome reveals regions that are potentially regulated by the transcription factor. Using this method, S. Otto identified REST binding sites in genes using DNA immunoprecipitated from a non-neuronal murine kidney cell line (TCMK1). Upon closer inspection of the SACO library, S. Otto discovered a family of mouse miRNA genes that resided in close proximity to predicted binding sites for the transcriptional repressor, REST

(Table 1, Figure 1A). This family includes miR-9-1, miR-9-2, miR-9-3, miR-34a, miR-124a-1, miR-124a-2, miR-124a-3, miR-132, miR-139, and miR-338, all of which are expressed in the brain (Lagos-Quintana et al, 2002; Krichevsky et al, 2003; Kim et al, 2004; Sempere et al, 2004; Thomson et al, 2004; Smirnova et al, 2005; Wienholds et al, 2005). Each of the miR-9 gene loci gives rise to two mature miRNAs, miR-9 and miR-9*, while the three miR-124a loci give rise to only one mature miRNA, miR-124a. Both miR-9 and miR-9*, as well as miR-124a, are highly enriched throughout the brain and are upregulated during rat brain corticogenesis (Krichevsky et al, 2003) and during neuronal differentiation of P19 embryonal carcinoma cells (Sempere et al, 2004). In addition, miR-9 has been detected by *in situ* hybridization in neuronal progenitors (Deo et al, 2006) and glioblasts (Nelson et al, 2006). In contrast, the products of the miR-34a, miR-132, miR-139, and miR-338 genes are expressed at lower levels and, at least in zebrafish, are restricted to specific cells of the nervous system (Wienholds et al, 2005).

miRNA genes contain functional RE1 sites

Most of the identified miRNA genes contain predicted REST binding sites within 5kb upstream or downstream of the putative miRNA precursor (Table 1). miR-9-2 and miR-124a-1 have more distant RE1 sites, suggesting that these genes may be repressed through propagation of REST silencing. For example, it has previously been shown for the rat 3q22-34 gene locus that REST binding to one RE1 site serves as a point of origin for spreading repression to adjacent chromosomal regions that do not harbor RE1 sites (Lunyak et al, 2002). This repression is mediated by CpG methylation originating from corepressors within the REST complex, and the subsequent recruitment of chromatin remodeling factors, including MeCP2, SuV39H1, and HP1, resulting in condensation of surrounding chromatin.

Chromatin immunoprecipitation (ChIP) analysis was performed in the TCMK1 cells used for SACO to make sure that the predicted binding sites were, in fact, occupied by REST. Using two antibodies directed against different epitopes on the REST protein, we were able to immunoprecipitate the chromosomal region containing the predicted RE1 sites. This verified that the RE1 sites proximal to each of the miRNA genes were indeed associated with REST (Figure 1B).

A luciferase reporter assay was performed to determine if the predicted RE1 site associated with each of the miRNA genes is able to recruit functional REST binding. The RE1 sites, along with up to 500-1000bp of flanking sequences, were cloned upstream of a minimal thymidine kinase (TK) promoter driving the firefly luciferase gene. As a positive control, we also created a reporter vector containing the RE1 of a known REST-regulated gene, glutamic acid

decarboxylase (Gad1). For each miRNA RE1 reporter, a corresponding ΔRE1 construct lacking the 21bp RE1 sequence was created by site-directed mutagenesis. As predicted, when these reporter constructs were transfected into TCMK1 cells that abundantly express REST, constructs bearing the RE1 sites of the miRNAs or Gad1 exhibited lower luciferase activity compared to constructs in which the RE1 site had been deleted (Figure 2A). However, reporters bearing the predicted RE1s of miR-34a and miR-338 displayed the same luciferase activity as their cognate ΔRE1 constructs, indicating that despite their ability to bind REST, these sites by themselves are insufficient to recruit a functional repressor complex. A closer inspection of the RE1 sequences for these two miRNAs (Table 1) revealed that the predicted REST binding sites that were cloned differed more from the canonical RE1 (Figure 1A), lacking either the highly conserved CC at positions 8 and 9, or the GG at positions 12 and 13. While the regulation of miR-34a and miR-338 does not seem to depend on these divergent RE1 sites, it is possible that the promoter regions of these miRNAs may harbor other regulatory sites that may be responsible for their observed expression in specific neuronal subtypes. For example, miR-338, which is found within an intron of the neuronally-expressed Apoptosis-associated tyrosine kinase (Aatk; Baker et al, 2001), may share the same regulatory sequences as its host gene (Table 1). That intronic miRNAs are usually co-expressed with their host genes has been previously demonstrated by microarray profiling (Baskerville and Bartel, 2005).

To confirm that binding of REST to the RE1 sites was responsible for the observed decrease in reporter activity, TCMK1 cells were co-transfected with the reporters and a dominant negative form of REST (dnREST). Dominant negative REST contains the DNA binding domain that allows it to compete for binding sites with endogenous REST. However, because it lacks any repressor domains, it is unable to recruit members of the silencing complex. As expected, co-expression of dnREST resulted in a significant increase, ranging from 2-6 fold, in the luciferase activity for six of the RE1-bearing constructs. A more modest derepression was observed for the miR-124a-2 and miR-139 reporters. Constructs lacking RE1 sites sequences (ΔRE1) were not affected by the expression of dnREST (Figure 2B). The RE1 constructs for miR-34a and miR-338 were not derepressed by the presence of dnREST, which further supports the possibility that these chromosomal regions are not sufficient to recruit REST-mediated repression. Surprisingly, the miR-9-2 RE1 also could not be derepressed by addition of dnREST. The fact that this reporter is clearly repressed by endogenous REST compared to its ΔRE1 counterpart suggests that the inability of dnREST to upregulate its expression may be due to the presence of additional repressor mechanisms, such as the recruitment of chromatin remodeling factors that are responsible for the spreading of repression.

REST directly regulates the expression of miRNAs

To provide evidence that REST regulates the endogenous expression of these miRNAs, we introduced dnREST into mouse embryonic fibroblasts (MEFs) by adenoviral transduction. Quantitative RT-PCR was used to measure the expression level of each miRNA. With the exception of miR-124a, for which it was possible to design a single primer set that could detect the transcripts from all three of its gene loci, primers for the other miRNA genes were designed to detect each unique precursor transcript. Expression of dnREST resulted in an increase in endogenous transcript levels for miR-9-1, miR-9-2, miR-124a, and miR-132 (Figure 3A), an induction comparable to that seen with other REST-regulated genes (Ballas et al, 2005; Lunyak et al, 2002; Bruce et al, 2004; Immaneni et al, 2000). Consistent with results from the luciferase reporter assays, miR-9-2, miR-34a, miR-139, and miR-338 were not significantly derepressed by dnREST. The converse experiment was also performed by ectopically expressing REST in neuronal cells that normally do not express this transcriptional repressor. Ectopic expression of REST in mature cortical neurons resulted in a decrease in the steady RNA levels of miR-9-1, miR-9-2 and miR-124a after 24 hours (Figure 3B), and miR-132 after 48 hours (data not shown).

Discussion

REST regulation has been ascribed largely to neuronal genes that encode proteins expressed in most terminally differentiated neurons. Among these are axon guidance molecules, ion channels, synaptic vesicle proteins, and neurotransmitter receptors (Schoenherr et al, 1996; Bessis et al, 1997; Mu and Burt, 1999; Brene et al, 2000; Gurrola-Diaz et al, 2003; Mieda et al, 1997; Bai et al, 1998; Andria and Simon, 2001; Lietz et al, 2003; Schoch et al, 1996; Mbikay et al, 2002; Lonnerberg et al, 1996; Quinn et al, 2002; Seth and Majzoub, 2001; De Gois et al, 2000; Bieche et al, 2003; Kallunki et al, 1997; Abderrahmani et al, 2001). The unbiased SACO screen in a kidney cell line has now revealed that non-protein-coding miRNAs represent yet another set of target genes for REST. Moreover, our analysis indicates that REST regulation of miRNAs is similar to that of canonical REST-regulated neuronal genes.

Two classes of REST-regulated genes have been described (Ballas et al, 2005). These classes may reflect differences in the stringency of regulation as determined by the affinity of the binding site for REST, local sequence features, and the complement of coregulators that are recruited to the region. Class I genes rely solely on REST repression to control their expression

and are maximally expressed upon loss of REST binding to their promoters. The abundant neuron-specific miRNAs, miR-124a and miR-9, fall under this category. It is conceivable that this class of genes encode gene products required for the initial acquisition or maintenance of the neuronal phenotype.

The other REST-regulated miRNAs exhibit more complex regulation similar to that observed for class II genes. While the RE1 sites on these genes are clearly bound by the REST complex, they also appear to be controlled by additional repressor mechanisms that may be mediated through the binding of the corepressors MeCP2 and CoREST to methylated regions of chromatin (Ballas et al, 2005). Class II genes are minimally expressed after loss of REST binding and are upregulated in response to specific stimuli. For example, BDNF is upregulated in response to cellular depolarization (Martinowich et al, 2003) while calbindin may be regulated through calcium signaling (Arnold and Heintz, 1997). The presence of a binding site for the cAMP-response element binding protein on miR-132 makes expression of this miRNA responsive to neurotrophin signaling (Vo et al, 2005). Whether class II genes are also regulated through the action of cell-specific transcriptional repressors and activators has yet to be determined. Furthermore, it remains to be seen whether the novel REST binding sites identified by Otto et al (manuscript under revision) contribute to the complexity of regulation observed for some REST target miRNA genes.

The results presented in this chapter provide direct evidence that miRNAs, like most protein-coding genes, are regulated by transcription factors, supporting previous findings that miRNAs are transcribed by a mechanism involving RNA polymerase II (Cai et al, 2004). Transcriptional regulation of miRNA expression allows for their integration as key players in the complex signaling pathways that lead to the establishment of cell-type specific gene expression programs. With the large number of miRNAs that are expressed during the progression of cortical development (Lagos-Quintana et al, 2002; Krichevsky et al, 2003; Kim et al, 2004; Miska et al, 2004; Sempere et al, 2004; Thomson et al, 2004; Smirnova et al, 2005), it is possible that their specific spatio-temporal distribution patterns may play a critical role in coordinating the gene expression profiles that characterize the various neuronal subtypes. It remains to be seen how these REST-associated miRNAs function during neuronal development or in the establishment of specific neuronal cell fates.

Materials and methods

Cell culture

The TCMK1 murine kidney cell line was obtained from ATCC. TCMK1 cells were maintained in MEM with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids, and 1.5mg/mL sodium bicarbonate. Primary mouse embryonic fibroblasts (MEFs) were derived from E14.5 embryos and grown in DMEM with 10% FBS, 2mM L-glutamine, and 1% non-essential amino acids. Cortical neurons were isolated from E15.5 mouse embryos as previously described (Ballas et al, 2005) and maintained in Neurobasal media with 2% B27 and 500μM L-glutamine. After three days *in vitro* (DIV), the cortical neurons were treated with 5μM cytosine arabinoside (Sigma) to inhibit the growth of proliferating cells.

Serial analysis of chromatin occupancy (SACO)

A REST SACO library was constructed from TCMK1 cells according to Impey et al (2004). Sequence analysis was performed by the Cold Spring Harbor Laboratory Genome Research Center.

Chromatin immunoprecipitation analyses

Chromatin immunoprecipitation (ChIP) was performed as described previously (Ballas et al, 2001). Crosslinked chromatin was immunoprecipitated using the following polyclonal antibodies: anti-REST-N (Chong et al, 1995) and anti-REST-C (Ballas et al, 2005). Following the reversal of crosslinks, the DNA was purified using the QIAquick PCR purification kit (Qiagen). All DNA samples were subjected to 50 cycles of PCR.

Adenoviral vectors and transduction

Adenoviral vectors expressing full-length REST and a dominant negative REST (dnREST) that lacks both amino- and carboxy-terminal repressor domains have been described previously (Chong et al, 1995). Primary cortical neurons (E15.5) were infected after 5 DIV with adenovirus at a multiplicity of infection (MOI) of 50-100. Calcium phosphate precipitation was used to introduce adenovirus into MEFs (Fasbender et al, 1998). Briefly, virus was resuspended in MEM and precipitates were allowed to form with the addition of 25mM $CaCl_2$. The precipitate was applied to the MEFs for 30 minutes and then replaced with fresh medium.

RNA isolation and quantitative real-time PCR analysis of miRNAs

Total RNA from adenovirus transduced primary cortical neurons and MEFs was extracted using the RNeasy kit (Qiagen) and treated with DNase (DNA-free kit, Ambion). Reverse transcription was performed using Superscript III (Invitrogen). Quantitative real-time PCR (RT-PCR) was performed in an ABI PRISM 7900HT Fast Real-Time PCR System with SYBR green PCR master mix (PE Applied Biosystems). Primers for miRNA detection were designed within the precursor stem-loop for each individual miRNA gene locus, with the exception of miR-124a, for which a single set of primers was able to recognize precursor transcripts from all its gene loci. The relative abundance of each miRNA was determined using a standard curve generated from 10-fold serial dilutions of cortical neuron cDNA and normalized to GAPDH mRNA. To analyze changes in miRNA expression, ratios of the geometric means between control and experimental samples were calculated. Significance was determined using the Student's t-test.

Luciferase assay

Approximately 500-1000bp genomic regions containing the RE1 sites of the miRNA or Gad1 genes were amplified by PCR and ligated into pENTR/D-TOPO (Invitrogen) following the protocol of the directional TOPO cloning kit (Invitrogen). To create the pGL3TK destination vector for firefly luciferase expression, the HSV-TK promoter from the pRLTK *Renilla* luciferase expression vector (Promega) was inserted into the BglII/HindIII sites of pGL3 basic (Promega) and the Gateway cassette was inserted into the SmaI site 5' of the TK promoter. Cloned genomic regions were introduced into the pGL3TK destination vector using LR clonase (Invitrogen) following the manufacturer's guidelines. The RE1 sites on these vectors were deleted using the Quikchange II Site-Directed Mutagenesis Kit (Stratagene) to create the ΔRE1 reporters. Deletion of RE1 sites was verified by sequencing. TCMK1 cells ($1x10^5$ cells per well on a 12-well dish) were transfected with 250ng of each of the reporter vectors along with 20ng of pRLTK and 250ng of an expression vector for dnREST (Chong et al, 1995) or an empty vector (pcDNA, Invitrogen). The ratio of firefly versus *Renilla* luciferase activity was measured after 48 hours using the Dual Luciferase reporter assay system (Promega) following the manufacturer's protocol. Changes in luciferase activity were determined by taking the ratios of the geometric means for reporters co-transfected with dnREST and pcDNA. Significance was determined using the Student's t-test.

Table 1. miRNAs associated with consensus REST binding sites (RE1). Sequences and positions of predicted RE1 sites relative to the miRNA are indicated.

miRNA	miRNA genomic coordinates[a]	Overlapping transcripts	Location of RE1[b]	Predicted RE1 sequence
miR-9-1	**Chr3**: 88301525-88301613 (f)	Intergenic	+5.3kb (f)	TCCAGCACCACGGACAGCTCC
miR-9-2	**Chr13**: 84212016-84212087 (f)	C130071C03Rik	-46.8kb (r)	CGCAGCACTACGGACGGCGCC
miR-9-3	**Chr7**: 79378777-79378866 (f)	Intergenic	-3.2kb (f)	CTCAGCACCATGGCCAGGGCC
miR-34a	**Chr4**: 148912254-148912355 (f)	Intergenic	-212bp (r)	TTCAGCTGGAGGGACAGCGCC
miR-124a-1	**Chr14**: 63544767-63544851 (f)	Intergenic	-22.2kb (r)	TTCAGCACCGAAGACAGCACC
miR-124a-2	**Chr3**: 17987813-17987921 (f)	Intergenic	-3.5kb (f)	ATCAAAACCATGGACAGCGAA
miR-124a-3	**Chr2**: 180823448-180823515 (f)	Intergenic	+427bp (r)	GCCAGCACCGAGGACAGCGCC
miR-132	**Chr11**: 74989877-74989942 (f)	RP23-143A14.3	-160bp (r)	ATCAGCACCGCGGACAGCGGC
miR-139	**Chr7**: 101349197-101349264 (f)	Pde2a	+2.1kb (r)	ATCAGCACCGCAGACAGCGCC
miR-338	**Chr11**: 119830855-119830952 (r)	Aatk	-3.7kb (f)	CTCAGCCCCACCCACAGCTCC

[a] Chromosomal coordinates. Chromosome strand is indicated in parenthesis (f, forward; r, reverse).

[b] Distance and location of RE1 relative to the miRNA precursor (–, upstream; +, downstream). Chromosome strand is indicated in parenthesis.

Figure 1. REST occupies RE1 sites associated with a family of miRNAs. miRNA genes identified as potential REST targets in a REST SACO screen bind REST *in vivo*. (A) Position weight matrix of the canonical REST binding site. (B) Schematic diagram (left) showing chromosomal locations of RE1 sites (vertical lines) with respect to the predicted stem-loop precursors of the miRNA transcripts (grey boxes). Chromatin immunoprecipitation analysis (right) showing REST occupancy at the miRNA loci in TCMK1 kidney cells. REST-N and REST-C refer to antibodies directed against the N- and C-termini of REST. Rabbit IgG served as control.

Figure 1

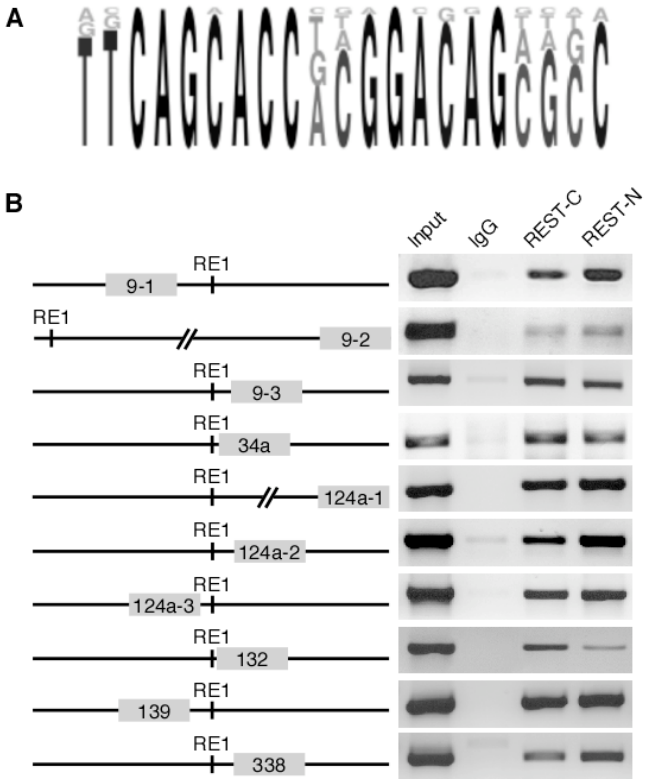

A
B
Input
IgG
REST-C
REST-N
RE1
9-1
9-2
9-3
34a
124a-1
124a-2
124a-3
132
139
338

Figure 2. RE1 sites on miRNA genes can support functional REST binding. (A) Luciferase reporter analysis showing that RE1-containing constructs transfected into TCMK1 kidney cells have lower luciferase activity compared to constructs lacking the RE1 (ΔRE1). Relative luciferase activity was measured after 48 hours. Error bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test). (B) Luciferase reporter assay in TCMK1 kidney cells showing derepression of RE1 reporters upon introduction of dominant negative REST (dnREST). Fold changes in luciferase activity are shown as the ratios of the means of reporter activity with or without dnREST. The RE1 of a known REST-regulated gene, Gad1, served as control.

Figure 2

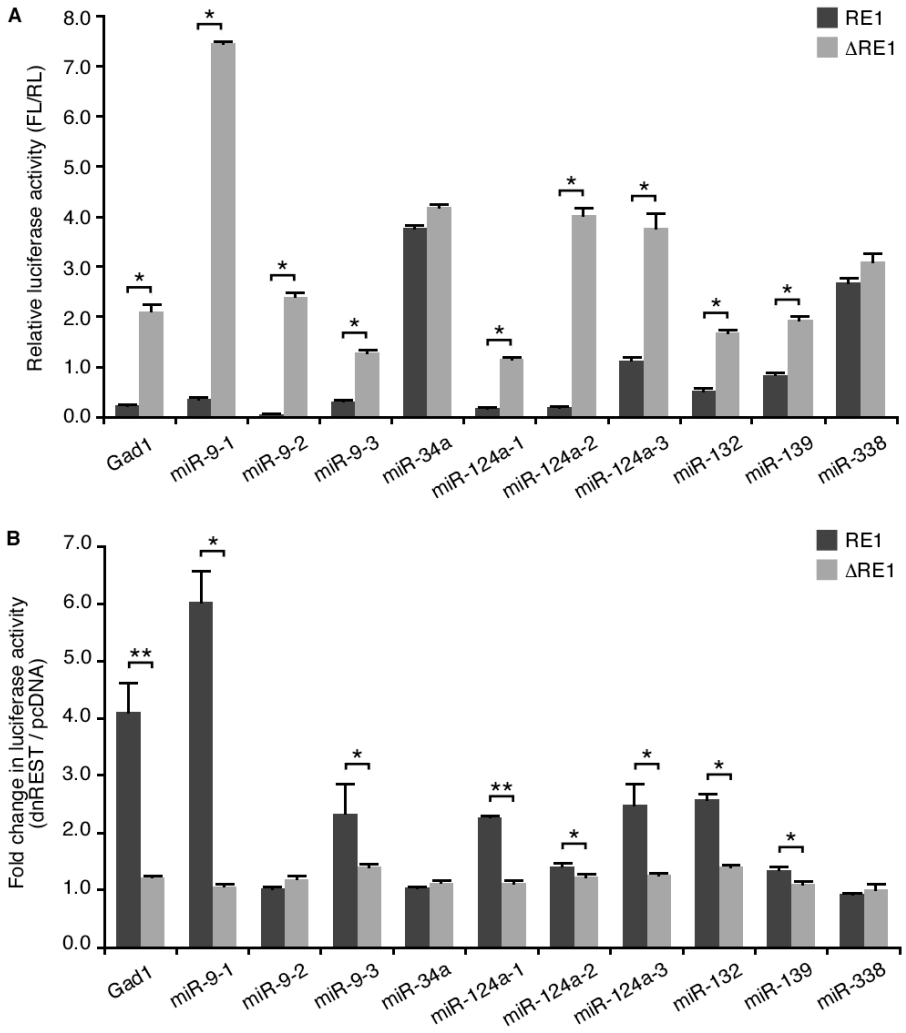
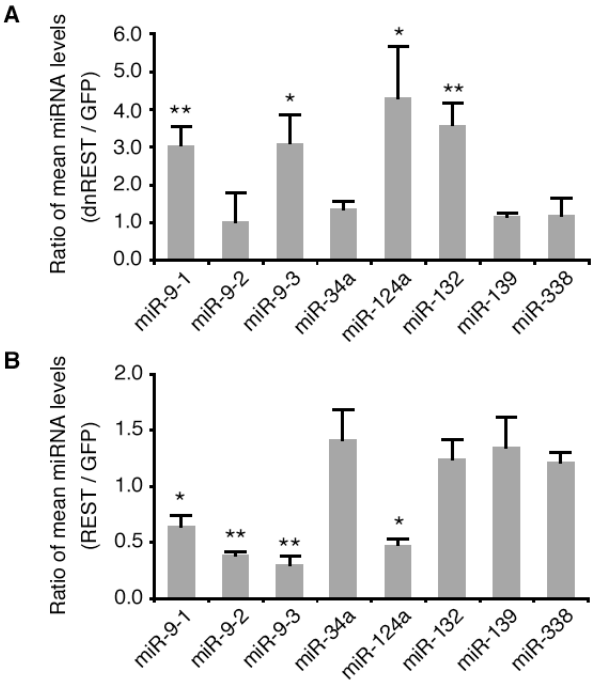

A
RE1
ΔRE1
Relative luciferase activity (FL/RL)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Gad1
miR-9-1
miR-9-2
miR-9-3
miR-34a
miR-124a-1
miR-124a-2
miR-124a-3
miR-132
miR-139
miR-338
B
RE1
ΔRE1
Fold change in luciferase activity (dnREST / pcDNA)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Gad1
miR-9-1
miR-9-2
miR-9-3
miR-34a
miR-124a-1
miR-124a-2
miR-124a-3
miR-132
miR-139
miR-338

Figure 3. Endogenous miRNA genes are regulated by REST. Levels of miRNAs were measured by quantitative RT-PCR 48 hours after transduction of mouse embryonic fibroblasts with dominant negative REST (A) or 24 hours after transduction of E15.5 post-mitotic cortical neurons with REST (B). miRNA levels were normalized to endogenous Gapdh. Fold changes in miRNA expression are shown as the ratio of mean transcript levels in cells expressing dnREST or full-length REST relative to cells transduced with an adenovirus expressing only GFP. Bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test).

Figure 3


A
Ratio of mean miRNA levels (dnREST / GFP)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
*
*
**
miR-9-1
miR-9-2
miR-9-3
miR-34a
miR-124a
miR-132
miR-139
miR-338
B
Ratio of mean miRNA levels (REST / GFP)
2.0
1.5
1.0
0.5
0.0
*
**
**
*
miR-9-1
miR-9-2
miR-9-3
miR-34a
miR-124a
miR-132
miR-139
miR-338

CHAPTER II

miR-124a promotes and maintains neuronal identity

Introduction

MicroRNAs play important roles in cell fate specification and lineage determination. Because they have the potential to regulate the expression of many different mRNAs, miRNA activity can potentially facilitate the shift in gene expression profile that enables a cell to acquire a differentiated phenotype. However, studies aimed at deciphering the involvement of miRNAs in lineage determination have often focused on the consequences of single targets, overlooking the possibility that a miRNA may be exerting its effects through the regulation of an entire network of mRNAs. Identification of miRNA targets is limited, in part, by the paucity of experimentally validated miRNA-target interactions and the inaccuracy of existing target prediction programs.

To overcome these limitations in elucidating miRNA function in neuronal differentiation, we exploited the existing knowledge on the REST-regulated, brain-specific miRNA, miR-124a. Clues to its function have been revealed by expression profiling studies done by other groups. For example, miR-124a has been shown to be upregulated during rat brain corticogenesis (Krichevsky et al, 2003) and during neuronal differentiation of P19 embryonal carcinoma cells (Sempere et al, 2004). In this chapter, I show through extensive target identification analyses and functional assays, that miR-124a can facilitate the shift towards a more neuronal gene expression profile through the downregulation of many non-neuronal mRNAs. The reciprocal actions of miR-124a and REST play an important role in promoting neuronal differentiation of progenitor cells and may be critical for maintaining the stability of the neuronal phenotype. The miRNA target identification assays detailed in this section were done in collaboration with Jong-Jin Han, a graduate student in the laboratory.

Results

REST confers neuronal-specific expression of miR-124a

I chose to study REST regulation of miR-124a in greater depth due to its evolutionary conservation, abundance, and restriction to the nervous system. Vertebrate genomes possess

three highly conserved miR-124a genes while invertebrates only have one. The single miR-124 gene in the invertebrates, *C. elegans* and *D. melanogaster,* gives rise to a mature miRNA of the same sequence as vertebrate miR-124a although their precursor transcripts differ at several nucleotides (Figure 4A). Interestingly, while invertebrates possess a miR-124 gene, they appear to lack REST. However, Dallman et al (2004) have previously shown in flies that REST may be replaced functionally by the transcriptional repressor, Tramtrack 88 (ttk88). Consistent with this finding, a consensus ttk88 binding site is present in the promoter region of the single locus encoding miR-124 in *D. melanogaster*. Moreover, miR-124 is exclusively expressed in the fly central nervous system (Stark et al, 2005). In the mouse, the miR-124a genes (miR-124a-1, miR-124a-2, miR-124a-3) are located on chromosomes 14, 3, and 2, respectively (Figure 4B). Each miR-124a locus is associated with either ESTs or annotated mRNAs. However, these mRNAs do not code for any known proteins, suggesting that they may be part of the primary miRNA transcript. Active transcription at all three miR-124a loci could explain the abundance of this miRNA in neuronal tissues.

I examined the expression of REST protein and mature miR-124a in mouse embryonal carcinoma (P19) cells undergoing neuronal differentiation in response to retinoic acid (RA; Figure 5A). Differentiation was monitored by the appearance of the neuronal marker, neuronal βIII-tubulin (TuJ1). The expression of mature miR-124a at 4 days post RA-treatment coincided with the disappearance of REST protein and terminal differentiation, as evidenced by the expression of TuJ1. In contrast, expression levels of the REST corepressor, CoREST, which is expressed in both neural progenitors and mature neurons (Ballas et al, 2005), were independent of changes in REST levels.

A similar expression pattern was observed in acutely isolated mouse cortices (Figure 5B). miR-124a was barely detectable in dividing cortical progenitors at E12.5 whereas it is abundantly expressed in mature neurons at E16.5. It is interesting to note that the expression of miR-124a persists into adult neurons. The reciprocity of REST and miR-124 gene expression in the developing mouse cortex was accounted for by changes in the occupancy of REST on the chromatin. Chromatin immunoprecipitation analysis (Figure 5C) showed that REST occupied the predicted RE1 sites on miR-124a-1, miR-124a-2 and miR-124a-3, as well as the RE1 of the REST-regulated glutamic acid decarboxylase (Gad1) gene, in primary cortical progenitors, but was dismissed from the chromatin in mature cortical neurons. The dismissal of REST from the RE1 sites coincided with the abundant expression of mature miR-124a in cortical neurons.

miR-124a represses non-neuronal transcripts

miRNAs can regulate their target transcripts either by blocking translation (Poy et al, 2004; Chen et al, 2004; Cimmino et al, 2005; Anderson et al, 2006) or by causing mRNA degradation (Bagga et al, 2005; Lim et al, 2005). In a recent study, Lim et al (2005) demonstrated that overexpression of miR-124a in HeLa cells resulted in selective downregulation of 174 non-neuronal transcripts. Sequence analysis of the 3' untranslated regions (3'UTR) of these transcripts revealed a preponderance of matches to the seed region (nucleotides 2-8) of miR-124a, suggesting that the miRNA was directly targeting these mRNAs for degradation. I observed a similar decrease in mRNA levels for mouse homologues of these transcripts in mouse embryonic fibroblasts overexpressing miR-124a (Figure 6A). However, because these studies are based exclusively on an overexpression paradigm in cells that do not normally express miR-124a, the results have to be interpreted with caution. The high concentrations of miRNA used in these experiments could cause off-target repression of non-targeted transcripts and non-specific downregulation of mRNAs that are not usually co-expressed with the miRNA. To determine if these transcripts are in fact regulated by the miRNA in their native context, I looked at their expression in cortical neurons, which abundantly express endogenous miR-124a. Antisense 2'O-methyl (2'OMe) oligoribonucleotides were transfected into the cortical neurons to deplete miR-124a activity. Due to their resistance to ribonuclease cleavage, the irreversible binding of 2'OMe oligoribonucleotides to cognate miRNAs can efficiently inhibit interaction of the miRNA with its mRNA targets (Meister et al, 2004). Inhibition of miR-124a activity resulted in upregulated expression for some but not all of the transcripts that were downregulated by miR-124a overexpression (Figure 6B). This finding lends support to the idea that miRNA overexpression has some non-specific and off-target effects. To further dissect the regulatory influences on these transcripts, we cloned their 3'UTRs into luciferase reporter vectors and performed sensor assays in cortical neurons. I found that upon inhibition of miR-124a activity in neurons using antisense 2'OMe oligoribonucleotides, the luciferase activity of several transfected 3'UTR constructs were significantly upregulated (Figure 6C). This suggests that these 3'UTRs contain sequences capable of recruiting miR-124a and its associated RISC effector complex.

Through a combination of these gain and loss of function assays, I was able to identify direct targets of miR-124a and distinguish them from transcripts that were being non-specifically downregulated by miRNA overexpression (Figure 6A-C). Endogenous transcripts that are downregulated by miR-124a overexpression, upregulated upon miR-124a depletion in neurons, and upregulated in the sensor assay, were designated direct targets. Transcripts downregulated by miR-124a overexpression that did not exhibit regulation in a cellular context where miR-124a is

normally expressed were considered non-specific targets. Other transcripts that were downregulated by miR-124a overexpression and exhibited upregulation either in neurons depleted of the miRNA or in the sensor assay, but not in both, were classified as potential targets.

Using these assays, I developed a more stringent screen for miRNA target identification and validation. This screen consists of three major steps outlined in Figure 7. The search for targets began with a set of 168 mouse transcripts homologous to the 174 human mRNAs downregulated by miR-124a in the study by Lim et al (2005). The first step of the screen was to ensure that the identified mouse homologues behave in the same manner as their human counterparts. To do this, I overexpressed the miR-124a duplex in mouse embryonic fibroblasts (MEFs) and measured changes in gene expression by quantitative RT-PCR. I found that, similar to what was observed by Lim et al (2005), a majority of the transcripts were downregulated by overexpression of miR-124a (n=146; Figure 7) relative to MEFs that had been treated with an inactive mutated version of miR-124a (miR-124a mut5-6; Lim et al, 2005). Twentytwo transcripts that were not downregulated in this step were omitted from further analysis.

The second step in the screen was to address the possibility that overexpression of miR-124a was causing non-specific downregulation of transcripts. For this step, I used primary cultures of terminally differentiated mouse cortical neurons, which normally express miR-124a in abundance. miR-124a activity was inhibited by transfection of antisense 2'OMe oligoribonucleotides. Quantitative RT-PCR measurement of changes in mRNA expression revealed a significant increase in transcript levels for 85 out of 146 transcripts in neurons treated with an antisense 2'OMe to miR-124a relative to neurons treated with an antisense 2'OMe to a muscle-specific miRNA, miR-1 (Figure 7). The transcript levels for 61 mRNAs were not significantly upregulated, suggesting that some of these mRNAs were non-specifically repressed by miR-124a overexpression.

The third step in the screen was a sensor assay designed to isolate regulatory effects that were being mediated through the binding of miR-124a to recognition sites in the 3'UTRs of target transcripts. To this end, we cloned the 3'UTRs of selected mRNAs next to a luciferase reporter gene driven by a minimal thymidine kinase promoter. We were able to clone the 3'UTRs of 76 mRNAs from the 81 transcripts that were upregulated upon miR-124a depletion in neurons. We also cloned the 3'UTRs of 56 out of 61 mRNAs that were not upregulated under the same conditions. These reporter constructs were co-transfected into cortical neurons with either the antisense 2'OMe to miR-124a or the antisense 2'OMe to miR-1. In this assay, an increase in luciferase activity upon inhibition of miR-124a indicates that the miRNA is binding and actively regulating a specific 3'UTR. Surprisingly, from the set of transcripts that were upregulated by

miR-124a depletion, only 22 had 3'UTRs that exhibited direct regulation by the miRNA. These transcripts were considered direct targets of the miRNA (Figure 7). It must be noted, however, that owing to inherent limitations of the assay, incompletely cloned 3'UTRs, and the possibility of alternatively spliced transcripts, other direct targets may have gone undetected. Thus, the other 54 transcripts that were upregulated by miR-124a depletion in neurons but were unresponsive in the sensor assay were classified as potential targets of miR-124a (Figure 7). Furthermore, eight 3'UTRs derived from mRNAs that were not upregulated in the depletion experiment showed upregulated expression in the sensor assay, indicating that these mRNAs could also be potential targets of miR-124a. Transcripts that were not upregulated by the inhibition of miR-124a activity in cortical neurons and whose 3'UTRs were unresponsive in the sensor assay were all considered non-specific targets of miR-124a (n=48).

Table 2 lists the 22 direct targets of miR-124a that were identified using the three-assay screen. Although many of these genes are not well characterized, closer analysis of the proteins encoded by these transcripts reveals three broad functional classes that appear to be relevant mainly for non-neuronal cells. One group of targets is involved in cell proliferation and in the differentiation of non-neuronal lineages. For example, Nek9 codes for a protein required for mitotic progression (Belham et al, 2003), while the adaptor protein Tom1l1 regulates Src mitogenic signaling in response to growth factors (Seykora et al, 2002; Franco et al, 2006). Mapk14/p38 has been shown to function in the regulation of cell death and survival during the stress response, as well as in cell fate specification (Martin-Blanco, 2000; Nebreda and Porras, 2000; Ono and Han, 2000). Interestingly, Mapk14 has also been shown to control diverse cellular processes in neurons (Takeda and Ichijo, 2002). Other target genes also encode proteins that are needed for cell fate specification. For instance, Cebpa has been shown to be important for granulocytic differentiation (Zhang et al, 1997). Epimorphin (Epim) mediates the epithelial-mesenchymal interactions that regulate differentiation of hepatic stem-like cells (Miura et al, 2003). E2ig4 encodes a secreted protein that mediates breast-tissue remodeling in response to estrogen signaling (Charpentier et al, 2000). Moreover, some of the direct targets appear to have functions that may interfere with normal neuronal development. For example, Ctdsp1 has been identified as a component of the REST repressor complex and is required for silencing of neuronal gene expression in non-neuronal cells (Yeo et al, 2005). Ptbp1 is an mRNA binding protein that inhibits neuron-specific splicing of c-src, γ2 $GABA_A$ receptor, and CGRP/calcitonin (Valcarcel and Gebauer, 1997). Another group of targets is involved in cell adhesion and migration. Vamp3-mediated membrane trafficking and recycling of Itgb1 receptors modulates substrate adhesion and regulates epithelial cell migration (Proux-Gillardeaux et al, 2005). Lamc1

and Itgb1 produced by neural progenitors were shown to be important for maintenance of the basal laminae during neural tube development (Cao et al, 2007). It is possible that changes in the composition of the extracellular matrix and the replacement of vesicle transport proteins, such as Vamp3 and Sypl, with their neuronal isoforms may be important for regulating neuronal migration or axonal extension in the developing nervous system. How the third group of targets, which appear to be involved in different metabolic processes, may be important for creating the distinction between non-neuronal and neuronal cells, remains to be further elucidated.

From the obvious non-neuronal functions described for some direct targets of miR-124a, it was no surprise to find that a majority of these genes exhibit decreased expression levels in neurons (Figure 8) relative to a non-neuronal cell type, such as mouse embryonic fibroblasts. Quantitative RT-PCR measurement of mRNA concentrations revealed that 19 out of 22 direct targets are expressed at significantly lower levels in cortical neurons compared to mouse embryonic fibroblasts. Four targets are found at less than 75% of their expression level in MEFs. Fifteen targets are expressed even lower and are found in neurons at a concentration 50% or less relative to their expression level in MEFs. Fcho2, Mapk14, and Ptpn12, were the only mRNAs expressed in neurons at the same level or higher than in MEFs. However, these proteins may still be repressed at the protein level. In fact, Mapk14 and Vamp3 proteins exhibit an expression pattern that is almost reciprocal to that of miR-124a in differentiating P19 cells and in the developing mouse cortex (Figure 5A and B, Figure 9A and B). Whether Fcho2 and Ptpn12 are also repressed at the protein level remains to be determined. The decrease in expression levels for these mRNAs is most likely mediated by sequences within their 3'UTRs. This is shown by the downregulation of protein expression that is observed for full-length transcripts for Mapk14, Vamp3, and Ctdsp1 co-expressed with an expression vector for miR-124a (Figure 9C). However, when only the coding regions of these genes were co-expressed with the miRNA, the same degree of repression was not observed. The slight decrease in expression of the coding constructs in the presence of miR-124a, which is particularly obvious for Mapk14, may reflect regulation exerted through sites within the coding region, or may be an indirect effect mediated through other targets of miR-124a. It is also possible that this decrease is just an artifact of the experimental conditions. Further studies are necessary to discriminate among these possibilities.

The dramatic repression of miR-124a direct target expression in neurons raises the question of whether this observed downregulation can be attributed solely to the activity of the miRNA. Although miR-124a clearly has a profound effect on the expression of its targets, particularly at the protein level, the contribution of other mechanisms of repression cannot be overlooked. To determine if there is a transcriptional component contributing to repression of

targets, cortical neurons were treated with Trichostatin A (TSA), a general inhibitor of histone deacetylases. Quantitative RT-PCR measurement of mRNA levels revealed that TSA treatment resulted in a significant upregulation of expression for 10 direct targets (Figure 10). With the exception of Hadhb, all of the mRNAs that were upregulated by TSA corresponded to transcripts that were expressed at least 50% less in neurons than in MEFs. This result indicates that in neurons, transcriptional repression contributes to the decreased levels of miR-124a target gene expression. The observed upregulation of these trancripts upon miR-124a depletion in cortical neurons indicates that the target genes are being transcribed at a moderate level in these cells and suggests that the persistence of miR-124a may be important for maintaining these transcripts at low levels in mature neurons.

REST regulation of miR-124a promotes a neuronal phenotype

The coincidental expression of miR-124a and the neuronal marker, TuJ1, during mouse cortical development suggests that the miRNA may be playing an important role in neuronal differentiation of cortical progenitors (Figure 5B). Cortical progenitors isolated from E12.5 mouse embryos and cultured in media supplemented with bFGF maintain their mitotic pluripotent phenotype (Ghosh and Greenberg, 1995) and express very little miR-124a. Precocious overexpression of miR-124a in cortical progenitors by adenoviral transduction resulted in a significant increase in miR-124a levels, as measured by quantitative RT-PCR (Figure 11B). However, even at the maximum multiplicity of infection (MOI) used, the amount of miR-124a produced from the transduced vector was still 6-fold less than the level of miR-124a expression in cortical neurons (Figure 11C). As a measure of the efficiency of miR-124a overexpression, I quantitated the changes in expression of direct targets of the miRNA. In the presence of miR-124a, Mapk14 exhibited a 60-80% decrease at the protein level (Figure 11D and 11E) although at the mRNA level it only showed a 30% drop (Figure 11G). Other miR-124a targets, Ctdsp1, Ptbp1, and Vamp3, all showed significant downregulation, ranging from 60-80%, at the mRNA level (Figure 11G). To determine if miR-124a overexpression had an effect on neuronal differentiation, I examined the expression of several neuronal markers. Neuronal βIII-tubulin displayed increasing expression at both the protein (TuJ1; Figure 11D and 11F) and mRNA levels (Tubb3; Figure 11H) that correlated with increasing expression of miR-124a. Overexpression of miR-124a also resulted in a significant precocious upregulation of the transcript levels of other neuronal markers, including the Type II sodium channel (Nav1.2), Snap25, Celsr3, Nrxn2, and NeuroD1 (Figure 11H). These neuronal genes are known REST targets and their upregulation in response to miR-124a overexpression was not accompanied by a change in REST mRNA levels

(Figure 11B). As expected, the level of induction observed for these genes was relatively low compared to the effects observed in cells treated with dominant negative REST (Ballas et al, 2005; Otto et al, manuscript under revision). It should also be noted that, within the timeframe of the experiment, overexpression of miR-124a was not sufficient to cause an obvious shift to a characteristic neuronal morphology (Figure 11A).

Discussion

In non-neuronal cells, the neuronal phenotype is suppressed by REST repression of neuronal genes. This raises the question of whether there is a reciprocal mechanism in neurons for the suppression of non-neuronal genes. miR-124a is a good candidate to mediate such a mechanism because its overexpression in HeLa cells and MEFs results in the selective downregulation of many non-neuronal transcripts (Lim et al, 2005; Figure 6A and Figure 7). Moreover, as shown in this study, inhibition of miR-124a function in neurons, where it is normally expressed, results in the selective upregulation of non-neuronal transcripts. This provides further support for the role of this miRNA as a constitutive repressor of mRNAs encoding a large diversity of non-neuronal proteins.

An extensive screen for miR-124a targets identified 22 mRNAs that are directly regulated by the miRNA. These target mRNAs are present at higher levels in non-neuronal cells than in neurons and have cellular functions that are either unnecessary in post-mitotic neurons, or that may even be antagonistic to proper neuronal development and activity. Our results indicate that the changes in target expression are mediated not only by the miRNA, but also by the transcriptional regulatory machinery. These results support the findings from other groups showing that most miRNA targets display a pattern of expression reciprocal to that of their cognate miRNAs (Stark et al, 2005). In contrast to the findings by Stark et al (2005), however, the expression of miR-124a and its targets are not mutually exclusive in mouse neurons, at least at the mRNA level. This is consistent with the observed complex expression pattern of miR-124a targets in the cerebral cortex, which perhaps reflects the heterogeneity of neurons in the brain (Farh et al, 2005). Whether any functional protein is indeed produced from these transcripts remains to be determined, as it is conceivable that the abundant expression of miR-124a in neurons is sufficient to suppress the translation of any mRNAs produced by low level transcription of target genes.

Interestingly, not all of the transcripts downregulated by overexpression of miR-124a in non-neuronal cells were affected by depletion of miR-124a in neurons (Figure 6A and 6B). Possible explanations for this are: (1) overexpression causes off-target downregulation of transcripts; (2) downregulation of some transcripts in non-neuronal cells may be an indirect effect mediated through the activity of direct targets of miR-124a, which may include transcriptional activators or RNA binding proteins that can function to stabilize non-neuronal transcripts; (3) in neurons, a single non-neuronal mRNA may be regulated by multiple miRNAs such that blocking miR-124a alone is not sufficient to cause upregulation. Further studies will be required to discriminate among these possibilities.

The expression pattern of miR-124a already provides clues to its functions. The upregulation of miR-124a as cortical progenitors differentiate into neurons suggests that this miRNA plays a role in promoting the neuronal phenotype, while its persistence in adult neurons suggests that it is important for maintaining neuronal identity. Preventing the re-accumulation of non-neuronal gene products through the persistence of miR-124a may be one of the mechanisms through which the terminally differentiated neuron ensures the irreversibility of differentiation. In this study, I have shown that precocious expression of miR-124a in cortical progenitors results in the downregulation of non-neuronal mRNAs, as well as a modest upregulation of neuronal markers independent of changes in REST expression. The observed upregulation in neuronal genes may be attributed, in part, to a downstream effect of miR-124a-mediated repression of Ctdsp1, a small phosphatase inhibitor of RNA polymerase II, which has been shown to be part of the REST complex. Ctdsp1 activity is necessary for the repression of REST-regulated genes and it has been shown that interfering with Ctdsp1 function using either a phosphatase-inactive dominant negative or RNA interference, results in upregulation of neuronal markers and neuronal differentiation of P19 cells (Yeo et al, 2005). Furthermore, expressing a form of Ctdsp1 that is resistant to miR-124a-mediated repression in the developing chick neural tube interferes with cell cycle exit of progenitors and inhibits the expression of neuronal genes (Visvanathan et al, 2007). How the repression of other direct targets contributes to the promotion of neuronal differentiation in cortical progenitors remains to be explored.

Krichevsky et al (2006) reported similar results for miR-124a expression in differentiating embryonic stem (ES) cells. In their study, they found that co-expression of miR-124a and miR-9 in differentiating ES cells caused a slight increase in the neuron to glia ratio as measured by fluorescence activated sorting of cells expressing neuronal βIII-tubulin and glial fibrillary acidic protein (GFAP). That we were able to observe neuronal differentiation without having to co-express miR-9 may reflect the differentiation state of cortical progenitors and ES

cells. Variability in the complement of genes expressed in a cell at a particular window of differentiation may determine the effect of exogenously introduced factors. It is possible that cortical progenitors are at a more advanced differentiation state than the retinoic acid-induced ES cells used by Krichevsky et al (2006). While both these studies indicate that miR-124a can promote neuronal differentiation, the absence of obvious morphological changes suggests that miR-124a upregulation by itself is not sufficient for the establishment of neuronal properties. In fact, neither inhibition nor overexpression of miR-124a in the developing chick neural tube had any noticeable effect on neuronal differentiation, although miR-124a misexpression did result in increased cell death, as well as deterioration of the basal laminae surrounding the neural tube due to downregulation of two direct targets of miR-124a, Lamc1 and Itgb1 (Cao et al, 2007). In this *in vivo* system, neural progenitors were still able to carry out the normal differentiation program initiated by extrinsic signals independent of miR-124a. This is in contrast to the dramatic repression of neuronal gene expression that was observed upon overexpression of REST in chick neural tube (Paquette et al, 2000). Taken together, these findings suggest that while REST regulation of miR-124a may be necessary for the clearance of non-neuronal transcripts during differentiation, the robust upregulation of neuronal genes that are needed to develop a functional neuronal phenotype may rely on downstream transcriptional programs that are activated by the downregulation of REST.

On the basis of our results, and the work of Lim et al (2005), we propose a simple model for how contrast, exemplified by distinct mRNA profiles, is achieved between neuronal and non-neuronal cell phenotypes (Figure 12). In non-neuronal cells, REST binds to consensus RE1 sites in neuronal genes and miR-124a, and blocks their transcription. The repression of miR-124a, in turn, results in the persistence of hundreds of non-neuronal transcripts, thus greatly favoring the non-neuronal phenotype. In neurons, where REST is absent, global derepression of neuronal genes and miR-124a occurs in conjunction with *en masse* downregulation of non-neuronal transcripts mediated by miR-124a. Together, these two events now alter the balance in favor of the neuronal phenotype.

The requirement for persistent downregulation of non-neuronal transcripts might seem uneconomical. In actuality, however, the use of neuronal miRNAs to regulate mRNA concentrations post-transcriptionally allows for basal transcription of these genes in neurons by the same transcription factors and cognate genetic elements used in non-neuronal cells, an economy of genomic function. Furthermore, the synergism between miR-124a and the transcriptional regulatory machinery may help maintain the expression levels of selected non-neuronal mRNAs. This action prevents the accumulation of transcripts that are not needed, or

that may be detrimental to proper neuronal function, and stabilizes the neuronal gene expression profile. It remains to be seen how REST-regulated miRNAs, other than miR-124a, function during mammalian nervous system development or in the fine-tuning of neuronal functions.

Materials and methods

Cell culture

HEK 293 cells were maintained in DMEM with 10% FBS. Primary mouse embryonic fibroblasts (MEFs) were derived from E14.5 embryos and grown in DMEM with 10% FBS, 2mM L-glutamine, and 1% non-essential amino acids. Cortical neurons were isolated from E15.5 mouse embryos as previously described (Ballas et al, 2005) and maintained in Neurobasal media with 2% B27 and 500μM L-glutamine. After three days *in vitro* (DIV), the cortical neurons were treated with 5μM cytosine arabinoside (Sigma) to inhibit the growth of proliferating cells. Cortical progenitors were isolated from E12.5 mouse embryos and grown in Neurobasal medium supplemented with 40ng/ml bFGF (Chemicon), 2% B27 and 500μM L-glutamine (Slack et al, 1998).

Neuronal induction of P19 cells

P19 embryonal carcinoma cells (P19) were obtained from ATCC and cultured in αMEM with 7.5% BCS and 2.5% FBS and maintained subconfluent prior to neuronal induction. Neuronal induction was done essentially as described (Jones-Villeneuve et al, 1983). Briefly, $1x10^6$ P19 cells were allowed to aggregate in polystyrene Petri dishes (Fisherbrand) in the presence of 500nM retinoic acid (Sigma). After four days, the cells were dissociated and plated at $6x10^6$ cells on 10cm tissue culture dishes (Falcon). Cells were collected for protein and RNA extraction every day for 6 days.

Western blotting

Whole cell protein extracts were prepared as previously described (Grimes et al, 2000). Protein extracts were quantitated using the Coomassie Plus Protein Assay Reagent (Pierce) and 50ug of protein was resolved on 7% polyacrylamide gels. The following antibodies were used for Western blotting: anti-REST-N (Chong et al, 1995), anti-CoREST (Andres et al, 1999), and anti-βIII-tubulin (TuJ1, Covance), anti-βactin (ab8227; Abcam), anti-Gapdh (ab9485, Abcam), anti-

myc (clone 9E10; Zymed), anti-Vamp3/cellubrevin (ab5789; Abcam), and anti-Mapk14/p38 (clone 2F11, Upstate).

Ribonuclease protection assay

For ribonuclease protection assay (RPA) of miRNAs, total RNA was extracted using Trizol (Invitrogen). 2μg of RNA was used with the mirVana miRNA detection kit (Ambion) following the manufacturer's protocol. Probes for miRNA detection were synthesized by Integrated DNA Technologies and end-labeled with ^{32}P using the mirVana probe and marker kit (Ambion).

Chromatin immunoprecipitation analyses

Chromatin immunoprecipitation (ChIP) was performed as described previously (Ballas et al, 2001). Crosslinked chromatin was immunoprecipitated using the following polyclonal antibodies: anti-REST-N (Chong et al, 1995) and anti-REST-C (Ballas et al, 2005). Following the reversal of crosslinks, the DNA was purified using the QIAquick PCR purification kit (Qiagen). All DNA samples were subjected to 50 cycles of PCR.

RNA extraction, reverse transcription, and quantitative RT-PCR analysis

RNA for quantitative RT-PCR was extracted using the RNeasy kit (Qiagen) and treated with DNase (DNA-free, Ambion). Reverse transcription with random hexamer primers was performed using Superscript III (Invitrogen). Quantitative RT-PCR was performed in an ABI PRISM 7900HT Fast Real-Time PCR System with SYBR green PCR master mix (PE Applied Biosystems). Relative abundance of selected mRNAs was determined from a standard curve generated from 10-fold serial dilutions of cDNA and normalized to Gapdh. Significance was determined using the Student's t-test.

Overexpression and depletion of miR-124a

For overexpression of miR-124a, MEFs were plated at $1x10^5$ cells per 6cm dish and transfected by calcium phosphate precipitation as described (Sambrook et al, 1989). Briefly, 500μl of 2x HBS was added dropwise to 100nM of miR-124a or miR-124a mut5-6 RNA duplex (Integrated DNA Technologies) in 500μl of 125mM $CaCl_2$ to allow precipitates to form. Precipitates were added to cells and media was renewed after 5 hours. For depletion of miR-124a, E15.5 primary cortical neurons at 4 DIV were transfected with 500nM 2'O-methyl (2'OMe) oligoribonucleotides (Integrated DNA Technologies) using Lipofectamine 2000

(Invitrogen) following the manufacturer's protocol. After the indicated time, relative abundance of selected mRNAs was determined by quantitative RT-PCR as described above using a relative standard curve generated from 10-fold serial dilutions of MEF cDNA.

Luciferase sensor assays

3'UTR sequences were amplified by PCR and ligated into pENTR/D-TOPO (Invitrogen) following the protocol of the directional TOPO cloning kit (Invitrogen). To create the 3'UTR pGL3TK destination vector for firefly luciferase expression, the HSV-TK promoter from the pRLTK *Renilla* luciferase expression vector (Promega) was inserted into the BglII/HindIII sites of pGL3 basic (Promega) and the Gateway cassette was inserted into the XbaI site at the 3' end of luciferase. Cloned 3'UTRs were introduced into the destination vector using LR clonase (Invitrogen) following the manufacturer's guidelines. Cortical neurons ($5x10^5$ cells per well on a 24-well dish) were transfected with 100ng of each of the reporter vectors along with 20ng of pRLTK and 200nM of 2'OMe antisense oligoribonucleotides against miR-124a or miR-1 (Integrated DNA Technologies) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The ratio of firefly versus *Renilla* luciferase activity was measured after 48 hours using the Dual Luciferase reporter assay system (Promega) following the manufacturer's protocol. Changes in luciferase activity were determined by taking the ratios of the geometric means for reporters co-transfected with antisense 2'OMe oligoribonucleotides to miR-124a and miR-1. Significance was determined using the Student's t-test.

Cloning and transfection

Full-length and coding regions of Ctdsp1, Vamp3, and Mapk14 were PCR amplified from MEF cDNA. Vamp3 was inserted into the XhoI/BamHI sites of the pcDNA3.1 vector. Ctdsp1 and Mapk14 were cloned into the BamHI/NotI sites of the pcDNA-myc vector 3' of the myc epitope. 300bp of the miR-124a-3 precursor was PCR amplified from cortical neuron cDNA and cloned into the EcoRI site of the pcDNA3.1 vector. Equal molar ratios of the vectors expressing full-length or coding transcripts for each target gene were co-transfected with either pcDNA-miR-124a-3 or an empty vector into HEK 293 cells using FuGENE 6 (Roche) following the manufacturer's protocol. Protein was extracted after 48 hours as described above.

Drug treatments

Primary cortical neurons were treated with 300nM of Trichostatin A (Sigma) for 15 hours. RNA for quantitative RT-PCR analysis was extracted and processed as described above.

Adenoviral vectors and transduction of cortical progenitors

300bp of sequence surrounding the miR-124a-3 precursor was PCR amplified and cloned into the SalI/XhoI sites of the AdTrack-CMV vector (He et al, 1998). This vector was then linearized with PacI and electroporated into BJ5183-AD-1 *E. coli* (Stratagene) to create the recombinant adenovirus plasmid. The plasmid was purified, linearized, and transfected into 293 cells for packaging and amplification of adenovirus particles. Virus was purified using the AdenoX rapid virus purification kit (Stratagene) and titer was estimated using the AdenoX rapid titer kit (Stratagene). Primary cortical progenitors from E12.5 mouse embryos ($5x10^5$ cells/35mm dish) grown in Neurobasal medium supplemented with 40ng/ml bFGF (Chemicon) were transduced with 40-160 MOI of adenovirus for 48 hours. RNA and protein were extracted as described above.

Figure 4. Conservation of miR-124a. (A) Comparison of miR-124a stem-loop precursor sequences across species. Nucleotides conserved across all species are shown in dark grey while regions conserved in vertebrates but not in invertebrates are in light grey. The box indicates the sequence of mature miR-124a. (B) Predicted stem-loop precursors for each miRNA are depicted as short filled rectangles with a vertical bar representing the mature miRNA. miR-124a-1 and miR-124a-2 are located within GenBank transcripts and miR-124a-3 is located within a mouse EST (arrows indicate direction of transcription). Alignment scores, indicated by black bars in the conservation track, show that the three miR-124a loci are located in conserved regions of the mouse genome (UCSC, Mm5 assembly).

Figure 4

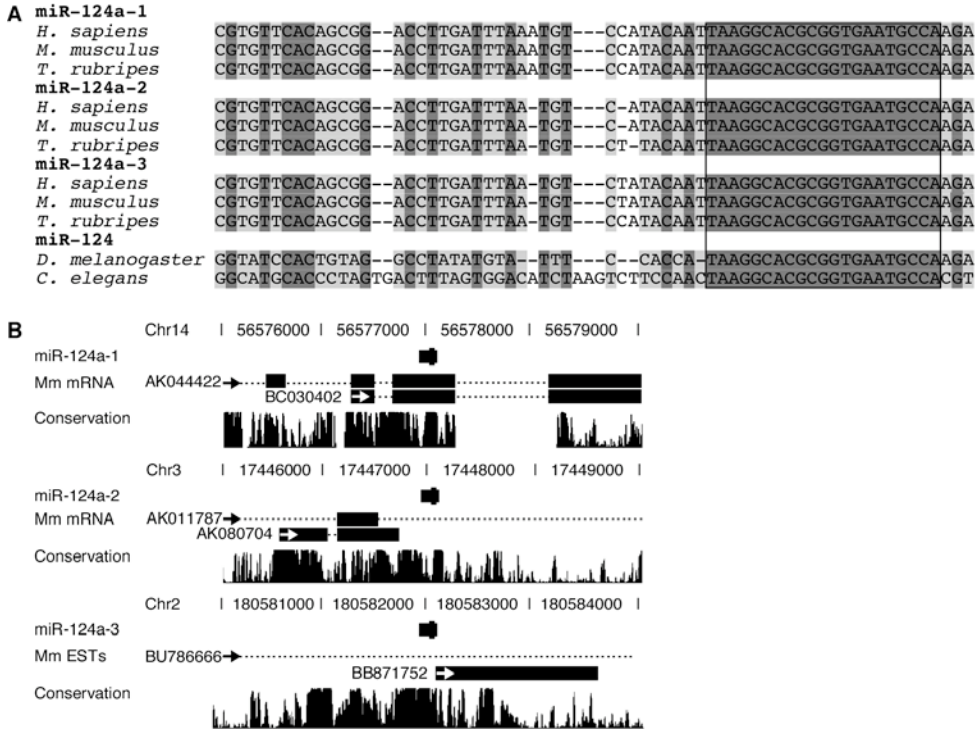

A
miR-124a-1
H. sapiens CGTGTTCACAGCGG--ACCTTGATTTAAATGT---CCATACAATTAAGGCACGCGGTGAATGCCAAGA
M. musculus CGTGTTCACAGCGG--ACCTTGATTTAAATGT---CCATACAATTAAGGCACGCGGTGAATGCCAAGA
T. rubripes CGTGTTCACAGCGG--ACCTTGATTTAAATGT---CCATACAATTAAGGCACGCGGTGAATGCCAAGA
miR-124a-2
H. sapiens CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---C-ATACAATTAAGGCACGCGGTGAATGCCAAGA
M. musculus CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---C-ATACAATTAAGGCACGCGGTGAATGCCAAGA
T. rubripes CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---CT-TACAATTAAGGCACGCGGTGAATGCCAAGA
miR-124a-3
H. sapiens CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---CTATACAATTAAGGCACGCGGTGAATGCCAAGA
M. musculus CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---CTATACAATTAAGGCACGCGGTGAATGCCAAGA
T. rubripes CGTGTTCACAGCGG--ACCTTGATTTAA-TGT---CCATACAATTAAGGCACGCGGTGAATGCCAAGA
miR-124
D. melanogaster GGTATCCACTGTAG--GCCTATATGTA--TTT---C--CACCA-TAAGGCACGCGGTGAATGCCAAGA
C. elegans GGCATGCACCCTAGTGACTTTAGTGGACATCTAAGTCTTCCAACTAAGGCACGCGGTGAATGCCACGT
B
Chr14 | 56576000 | 56577000 | 56578000 | 56579000 |
miR-124a-1
Mm mRNA
AK044422
BC030402
Conservation
Chr3 | 17446000 | 17447000 | 17448000 | 17449000 |
miR-124a-2
Mm mRNA
AK011787
AK080704
Conservation
Chr2 | 180581000 | 180582000 | 180583000 | 180584000 |
miR-124a-3
Mm ESTs
BU786666
BB871752
Conservation

Figure 5. REST confers neuronal specificity of miR-124a. (A) Western blot and ribonuclease protection assay (RPA) showing the disappearance of REST protein and appearance of mature miR-124a during neuronal differentiation of P19 cells with retinoic acid (RA). Neuronal βIII-tubulin (TuJ1) indicates presence of mature neurons. Levels of 5s rRNA and CoREST protein serve as controls. (B) Expression patterns of miR-124a and TuJ1 in the developing mouse cortex from embryonic day 10.5 (E10.5) to post-natal day 1 (P1). 5s rRNA and Gapdh protein serve as controls. (C) Chromatin immunoprecipitation analysis showing REST leaving the chromatin of the three miR-124a loci as cortical progenitors differentiate into cortical neurons. The neuronal-specific Gad1 gene and REST coding sequence serve as positive and negative controls, respectively.

Figure 5

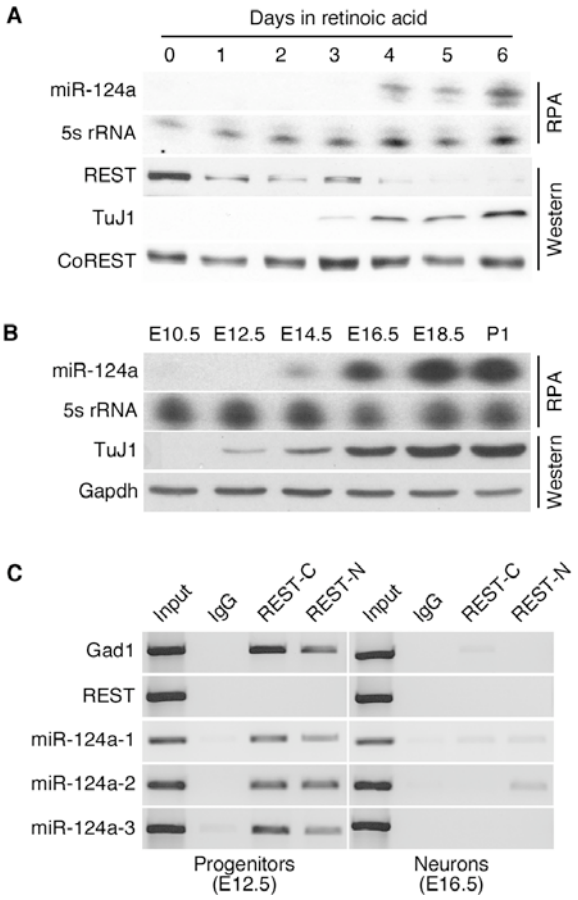

A
Days in retinoic acid
0 1 2 3 4 5 6
miR-124a
5s rRNA
REST
TuJ1
CoREST
RPA
Western
B
E10.5 E12.5 E14.5 E16.5 E18.5 P1
miR-124a
5s rRNA
TuJ1
Gapdh
RPA
Western
C
Input IgG REST-C REST-N Input IgG REST-C REST-N
Gad1
REST
miR-124a-1
miR-124a-2
miR-124a-3
Progenitors (E12.5)
Neurons (E16.5)

Figure 6. Overexpression of miR-124a causes off-target regulation. (A) Quantitative RT-PCR showing that overexpression of miR-124a downregulates many transcripts in mouse embryonic fibroblasts. Expression of target mRNAs was measured after 24 hours and normalized to Gapdh. Fold changes in mRNA expression are shown as the ratio of the geometric means of transcript levels in cells overexpressing miR-124a relative to cells treated with an inactive mutated version of miR-124a (miR-124a mut5-6). Variability is indicated by the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test). (B) Quantitative RT-PCR showing that inhibition of miR-124a in cortical neurons using an antisense 2'OMe results in upregulation of some transcripts. Expression of target mRNAs was measured after 48 hours and normalized to Gapdh. Fold changes in mRNA expression are shown as the ratio of the geometric means of transcript levels in cells transfected with an antisense 2'OMe to miR-124a relative to cells transfected with an antisense 2'OMe to muscle-specific miR-1. (C) 3'UTR sensor assays showing that only a subset of the transcripts that are affected by miR-124a overexpression or depletion are directly regulated through their 3'UTRs. Cortical neurons were co-transfected with 3'UTR sensor constructs and 2'OMe oligoribonucleotides. Relative luciferase activity was measured after 48 hours. Fold changes in luciferase activity are shown in cells co-transfected with an antisense 2'OMe to miR-124a relative to cells co-transfected with an antisense 2'OMe to miR-1. mRNAs that are directly regulated by miR-124a are shown in blue, potential targets are shown in green, and non-specifically downregulated targets are shown in red.

Figure 6

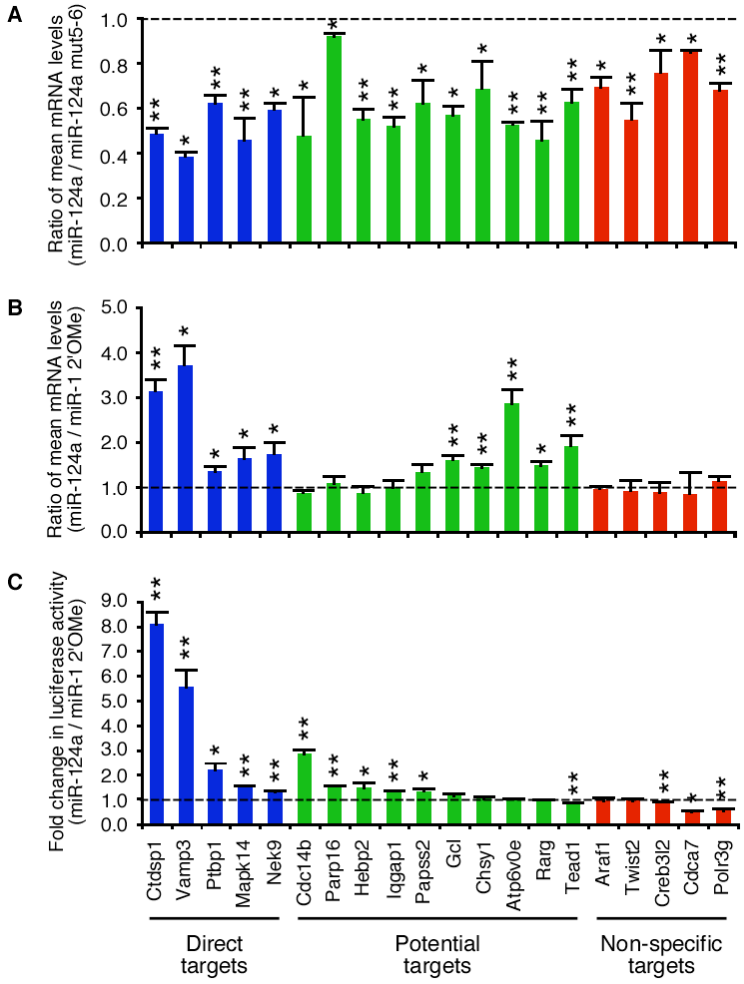

A
Ratio of mean mRNA levels (miR-124a / miR-124a mut5-6)
1.0
0.8
0.6
0.4
0.2
0.0
B
Ratio of mean mRNA levels (miR-124a / miR-1 2'OMe)
5.0
4.0
3.0
2.0
1.0
0.0
C
Fold change in luciferase activity (miR-124a / miR-1 2'OMe)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ctdsp1
Vamp3
Ptbp1
Mapk14
Nek9
Cdc14b
Parp16
Hebp2
Iqgap1
Papss2
Gcl
Chsy1
Atp6v0e
Rarg
Tead1
Araf1
Twist2
Creb3l2
Cdca7
Polr3g
Direct targets
Potential targets
Non-specific targets

Figure 7. Identification of miR-124a direct targets. Using a three-assay strategy, 22 direct targets of miR-124a were identified from a starting set of 168 mouse homologues of transcripts from Lim et al (2005). First, to identify mRNAs downregulated by miR-124a, the miRNA was overexpressed in mouse embryonic fibroblasts (MEFs) and transcript levels were measured by quantitative RT-PCR. Second, to determine if any of the downregulated transcripts were non-specifically targeted by virtue of miR-124a overexpression, cortical neurons that normally express miR-124a were depleted of the miRNA using 2'OMe antisense oligonucleotides. Transcript upregulation was measured by quantitative RT-PCR. Third, to isolate direct targets from the transcripts upregulated in the second assay, selected 3'UTRs were cloned into luciferase reporters and transfected into cortical neurons with or without miR-124a depletion using 2'OMe antisense oligonucleotides.

Figure 7

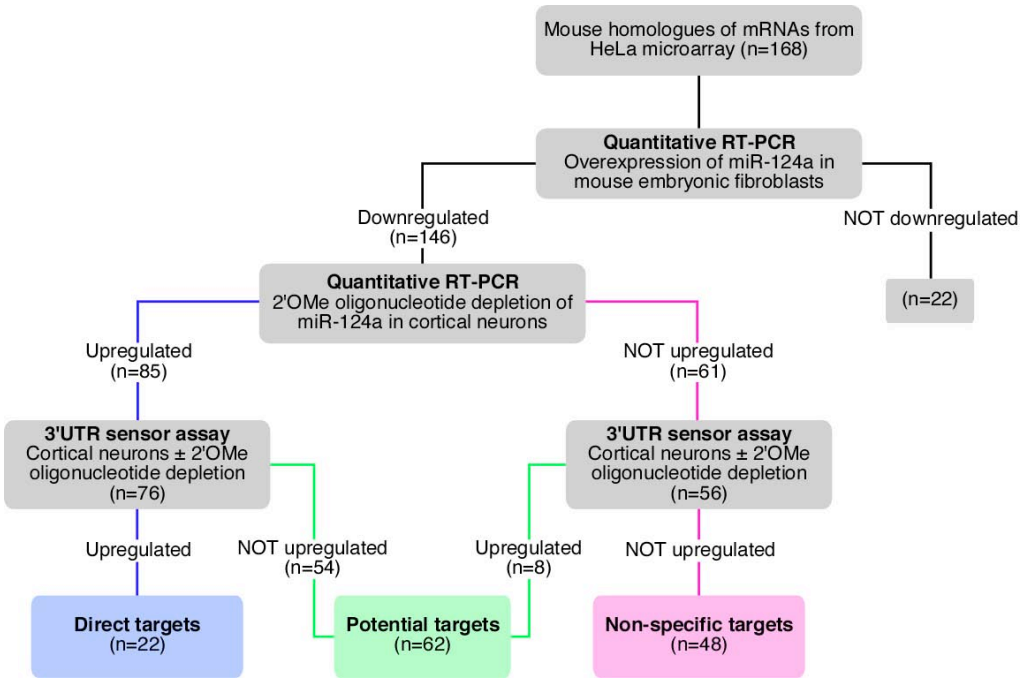

Mouse homologues of mRNAs from HeLa microarray (n=168)
Quantitative RT-PCR
Overexpression of miR-124a in mouse embryonic fibroblasts
Downregulated (n=146)
NOT downregulated
(n=22)
Quantitative RT-PCR
2'OMe oligonucleotide depletion of miR-124a in cortical neurons
Upregulated (n=85)
NOT upregulated (n=61)
3'UTR sensor assay
Cortical neurons ± 2'OMe oligonucleotide depletion (n=76)
3'UTR sensor assay
Cortical neurons ± 2'OMe oligonucleotide depletion (n=56)
Upregulated
NOT upregulated (n=54)
Upregulated (n=8)
NOT upregulated
Direct targets (n=22)
Potential targets (n=62)
Non-specific targets (n=48)

Table 2. miR-124a direct targets. Many of the direct target genes have functions relevant for proliferating and non-neuronal cells.

Symbol	Accession	Gene Name	Function
Proliferation/differentiation			
Nek9	NM_145138	NIMA (never in mitosis gene a)-related kinase 9	Regulator of mitotic progression
Ptpn12	NM_011203	Protein tyrosine phosphatase, non-receptor type 12	Tyrosine phosphatase; metastasis
Mapk14	NM_011951	Mitogen activated protein kinase 14	Kinase; stress response
Fcho2	NM_172591	FCH domain only 2	Cytoskeletal rearrangements
Tom1l1	BC004710	Target of myb1-like 1	Adaptor protein for src signaling
E2ig4	NM_001024619	Leucine rich repeat containing 54	Estrogen response; secreted
Epim	NM_007941	Epimorphin	Epithelial morphogenesis
Ctdsp1	NM_153088	RNA polII C-terminal domain small phosphatase 1	Neuronal gene repression
Ctdsp2	NM_146012	RNA polII C-terminal domain small phosphatase 2	Neuronal gene repression
Ptbp1	NM_008956	Polypyrimidine tract binding protein 1	Splicing factor
Cebpa	NM_007678	CCAAT/enhancer binding protein (C/EBP), alpha	Transcription factor
Adhesion/migration			
Lamc1	NM_010683	Laminin gamma 1	Extracellular matrix
Plod3	NM_011962	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	Collagen modification
B4galt1	NM_022305	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	Protein modification; cell-matrix interactions
Itgb1	NM_010578	Integrin beta 1	Adhesion receptor
Cd164	NM_016898	Cd164 antigen	Adhesion receptor
Vamp3	NM_009498	Vesicle-associated membrane protein 3	Vesicular transport
Sypl	NM_198710	Synaptophysin-like	Vesicular transport
Arfip1	XM_130985	Arfaptin-1	Vesicular transport
Metabolism			
Hadhb	NM_145558	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, beta subunit	Fatty acid beta oxidation
Lass2	NM_029789	Longevity assurance homolog 2	Sphingolipid synthesis
Unknown			
C6orf72	NM_145418	Hypothetical protein LOC215751	Unknown

Figure 8. miR-124a targets are non-neuronal mRNAs. Expression of target mRNAs in cortical neurons or mouse embryonic fibroblasts (MEFs) was measured by quantitative RT-PCR and normalized to Gapdh. Fold changes in mRNA expression are shown as the ratio of the means of transcript levels in neurons relative to MEFs. Bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test).

Figure 8

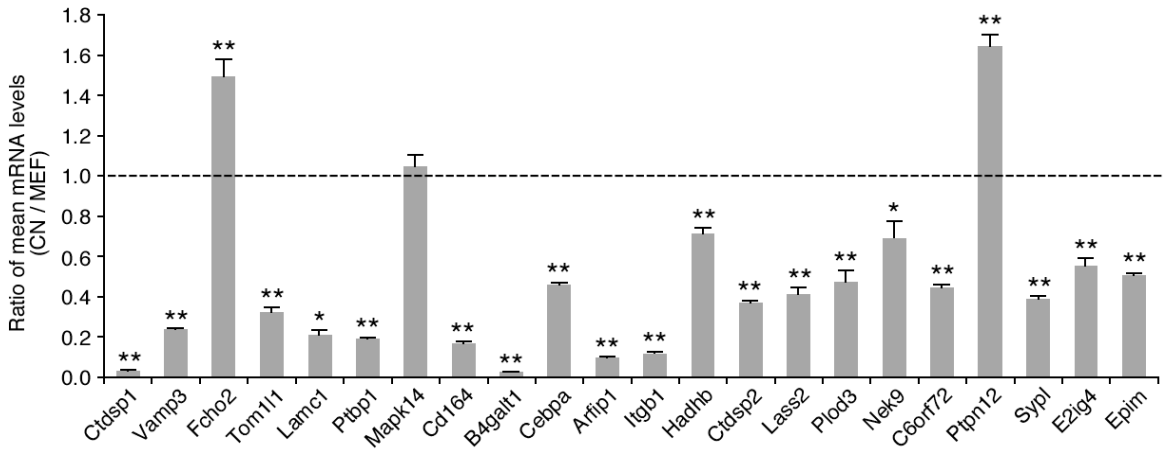

Ratio of mean mRNA levels (CN / MEF)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctdsp1
Vamp3
Fcho2
Tom1l1
Lamc1
Ptpp1
Mapk14
Cd164
B4galt1
Cebpa
Arfip1
Itgb1
Hadhb
Ctdsp2
Lass2
Plod3
Nek9
C6orf72
Ptpn12
Sypl
E2ig4
Epim

Figure 9. Regulation of direct targets during neuronal differentiation. Western blots showing the expression profile of Mapk14 and Vamp3 during retinoic acid-induced neuronal differentiation of P19 cells (A) and in the developing mouse cortex (B). Gapdh serves as loading control. (C) Western blots of full-length or coding regions of selected targets co-expressed with miR-124a. A greater degree of repression is observed for full-length targets, indicating that regulation by the miRNA is mediated mostly through the 3'UTR. Western blot signals were normalized to Gapdh or β-actin loading controls and the relative signal intensities for constructs co-expressed with miR-124a are shown relative to the signal in the absence of miR-124a.

Figure 9

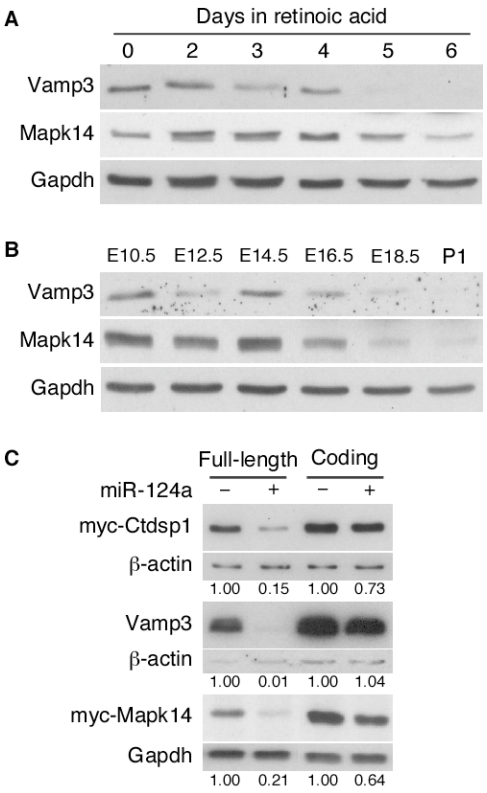

A
Days in retinoic acid
0 2 3 4 5 6
Vamp3
Mapk14
Gapdh
B
E10.5 E12.5 E14.5 E16.5 E18.5 P1
Vamp3
Mapk14
Gapdh
C
Full-length Coding
miR-124a – + – +
myc-Ctdsp1
β-actin
1.00 0.15 1.00 0.73
Vamp3
β-actin
1.00 0.01 1.00 1.04
myc-Mapk14
Gapdh
1.00 0.21 1.00 0.64

Figure 10. Several miR-124a direct targets are transcriptionally repressed in neurons. Cortical neurons were treated with 300nM of the histone deacetylase inhibitor, Trichostatin A (TSA). Expression of target mRNAs was measured after 15 hours and normalized to Gapdh. Fold changes in mRNA expression are shown as the ratio of the means of transcript levels in cells treated with TSA relative to cells treated with vehicle (DMSO). Bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test).

Figure 10

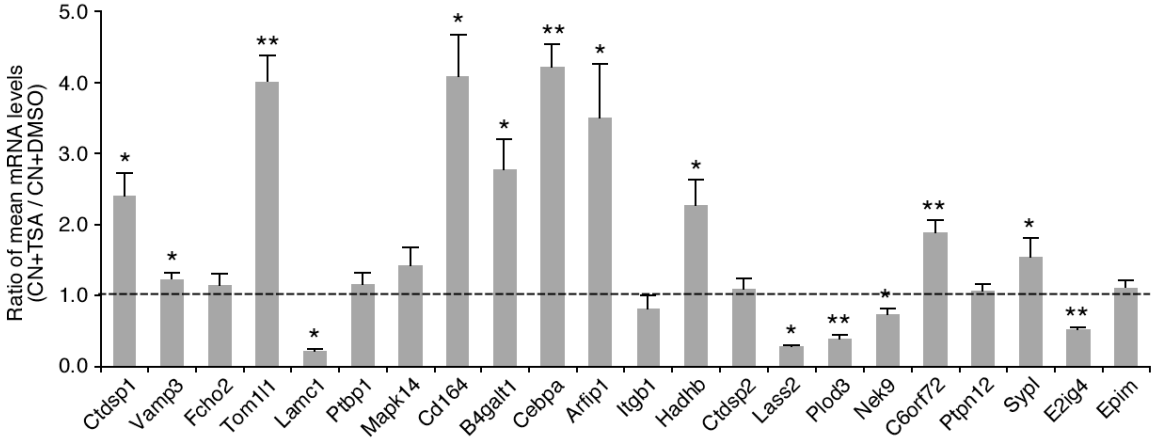

Ratio of mean mRNA levels
(CN+TSA / CN+DMSO)
5.0
4.0
3.0
2.0
1.0
0.0
Ctdsp1
Vamp3
Fcho2
Tom1l1
Lamc1
Ptbp1
Mapk14
Cd164
B4galt1
Cebpa
Arfip1
Itgb1
Hadhb
Ctdsp2
Lass2
Plod3
Nek9
C6orf72
Ptpn12
Sypl
E2ig4
Epim

Figure 11. miR-124a promotes the neuronal phenotype. Cortical progenitors from E12.5 mouse embryos were transduced with different multiplicities of infection (MOI) of an adenovirus expressing either miR-124a or only GFP. (A) Images of transduced progenitors. (B) Levels of miR-124a and REST were measured by quantitative RT-PCR after 48 hours. Transcript levels were normalized to Gapdh. Fold changes in mRNA expression are shown as the ratio of the means of mRNA levels in cells treated with the miR-124a adenovirus relative to cells treated with an adenovirus expressing only GFP. Bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test). (C) Comparison of miR-124a concentrations in adenovirus-transduced progenitors and mature neurons. Values are shown relative to miR-124a expression level in MEFs as measured by quantitative RT-PCR. (D) Western blot showing Mapk14 and neuronal βIII-tubulin (TuJ1) protein expression in progenitors overexpressing miR-124a. Quantitation of Western signals for Mapk14 (E) and TuJ1 (F) are shown. (G, H) Transcript levels for other miR-124a targets (G) and selected neuronal markers (H) measured as in (B).

Figure 11

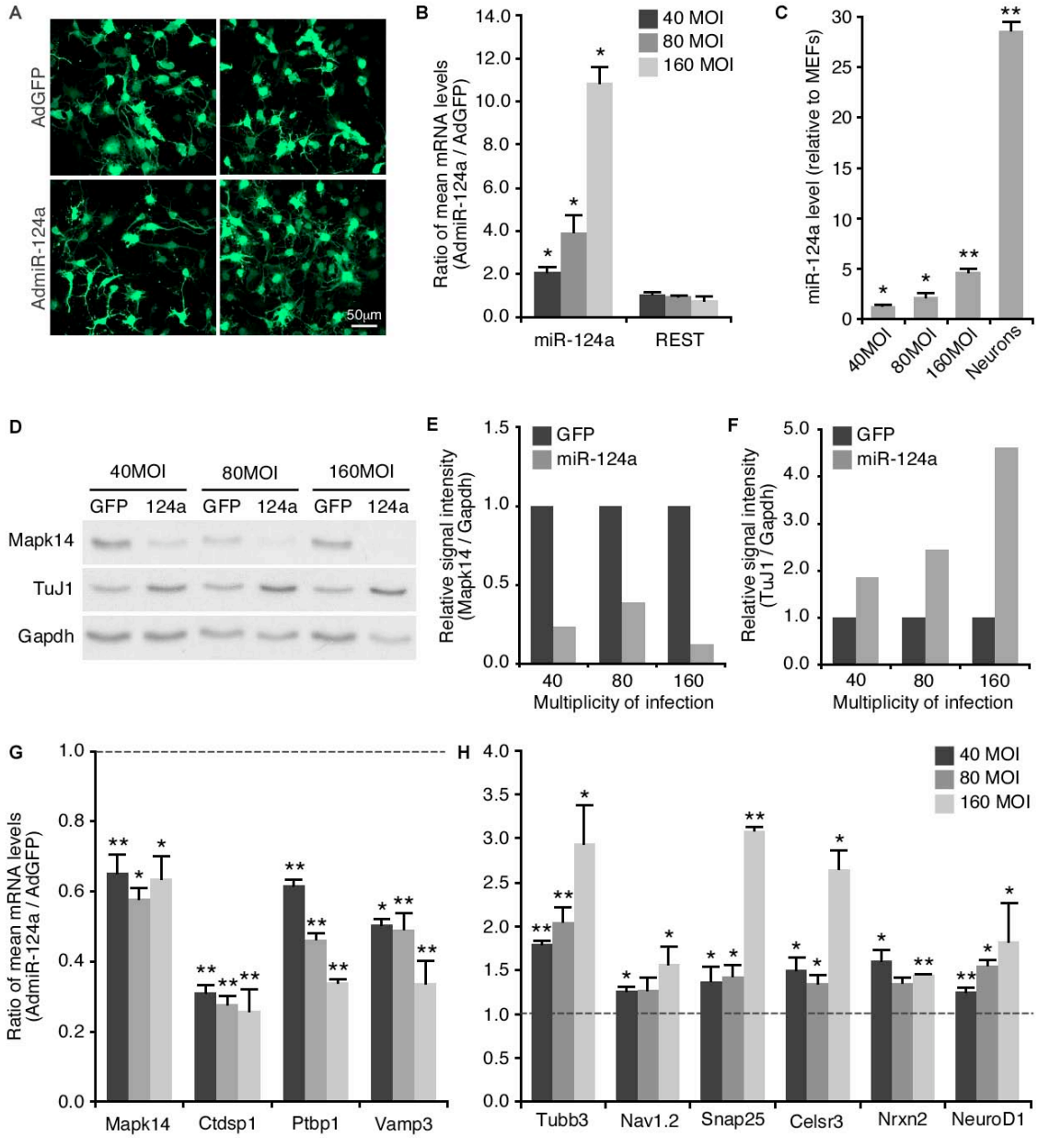
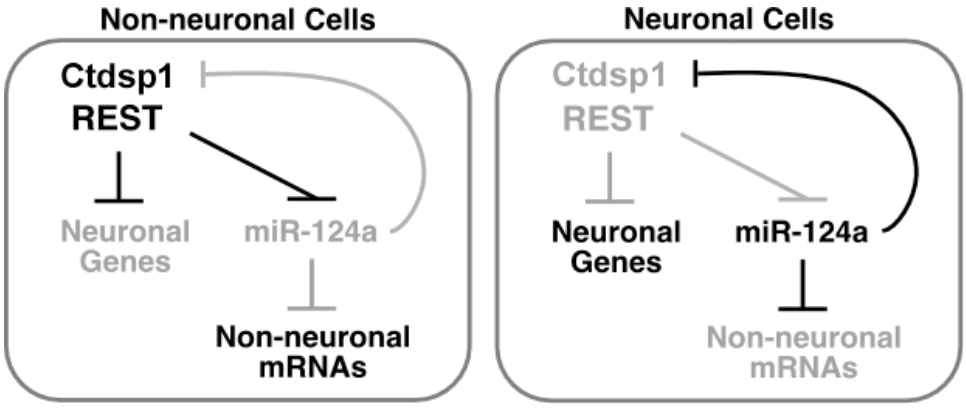

A
AdGFP
AdmiR-124a
50μm
B
40 MOI
80 MOI
160 MOI
Ratio of mean mRNA levels (AdmiR-124a / AdGFP)
miR-124a
REST
C
miR-124a level (relative to MEFs)
40MOI
80MOI
160MOI
Neurons
D
40MOI
80MOI
160MOI
GFP
124a
Mapk14
TuJ1
Gapdh
E
GFP
miR-124a
Relative signal intensity (Mapk14 / Gapdh)
Multiplicity of infection
F
GFP
miR-124a
Relative signal intensity (TuJ1 / Gapdh)
Multiplicity of infection
G
Ratio of mean mRNA levels (AdmiR-124a / AdGFP)
Mapk14
Ctdsp1
Ptbp1
Vamp3
H
40 MOI
80 MOI
160 MOI
Tubb3
Nav1.2
Snap25
Celsr3
Nrxn2
NeuroD1

Figure 12. Model for REST-dependent regulation of neuronal differentiation. A hierarchy of two global negative regulators, REST and miR-124a, promotes the neuronal phenotype. REST transcriptionally represses neuronal genes and miR-124a in non-neuronal cells and neural progenitors (left). The dismissal of REST from chromatin during neurogenesis results in *en masse* expression of neuronal genes and downregulation of competing non-neuronal transcripts through miR-124a function (right). Persistence of miR-124a in adult neurons helps to maintain and stabilize the neuronal gene expression profile.

Figure 12


Non-neuronal Cells
Ctdsp1
REST
Neuronal Genes
miR-124a
Non-neuronal mRNAs
Neuronal Cells
Ctdsp1
REST
Neuronal Genes
miR-124a
Non-neuronal mRNAs

CHAPTER III

Analysis of the interaction of miR-124a with its targets

Introduction

Understanding the role of miRNAs in diverse biological processes requires elucidation of the targets through which they exert their effects. However, because miRNAs typically bind to regions of imperfect complementarity within the 3'UTRs of mRNAs, computational prediction of miRNA targets has been extremely challenging. This is compounded by the lack of sufficient experimental evidence identifying *bona fide* miRNA targets. In fact, existing computational algorithms for predicting miRNA targets are largely based on the rules of miRNA-target interactions inferred from a few experimentally validated miRNA-target pairs. These challenges all underline the requirement for more extensive target identification and validation experiments, as well as the development of alternative methods for target identification, to further our understanding of the factors that affect miRNA-target interactions.

The REST-regulated miRNA, miR-124a, which is abundantly expressed in cortical neurons, provided us with the opportunity to study miRNA-target interactions in their native context. In the previous chapter, I discussed the identification and validation of direct targets of miR-124a. In this chapter, I use the set of validated targets to test known rules of miRNA-target interactions and compare them to target predictions from existing bioinformatic prediction programs. In addition, this set of targets is compared to a novel method for miRNA target identification that is based on the immunoprecipitation of mRNAs associated with Ago2. The Ago2 immunoprecipitation experiments detailed in this section were performed by Fedor Karginov in the laboratory of Gregory Hannon at Cold Spring Harbor Laboratory (CSHL).

Results

In the previous chapter, direct targets of miR-124a were isolated from a set of 168 mouse homologues of transcripts identified through a microarray study conducted by Lim et al (2005). To discriminate between direct and non-specific targets, we employed a three-assay strategy that consisted of the following criteria: (1) downregulation of the transcripts in mouse embryonic

fibroblasts (MEFs) over-expressing miR-124a, (2) upregulation of the transcripts in cortical neurons upon inhibition of miR-124a with 2'OMe antisense oligonucleotides, and (3) upregulation of luciferase activity of 3'UTR sensor constructs upon miR-124a inhibition in neurons. Out of the starting set of transcripts, only 22 mRNAs scored in all three assays, indicating that they are most likely directly regulated by miR-124a (direct targets). A total of 62 transcripts scored in the overexpression assay and in only one of the other assays (potential targets). In addition, 48 transcripts were downregulated by miR-124a overexpression but did not score in either of the inhibition assays, suggesting that these transcripts were non-specifically downregulated (non-specific targets).

Seed sequences are important determinants for miRNA binding

Pairing of the first 2-8 nucleotides in the 5' end of a miRNA to complementary sequences, known as seed regions, in the 3'UTR of a target mRNA (Figure 13A) has been shown to be important for miRNA binding (Doench and Sharp, 2004; Brennecke et al, 2005). In particular, the nucleotides at positions 5 and 6 from the 5' end of the miRNA appear to be critical for miRNA recognition (Doench and Sharp, 2004). Binding to the seed region may initiate miRNA-mRNA duplex formation by overcoming initial thermal diffusion and facilitating miRNA annealing (Rajewsky and Socci, 2004). Analysis of the 7nt and 6nt seed matches to miR-124a in the 3'UTRs of transcripts in each of the three categories obtained using the three-assay screen revealed distinct differences in seed distribution. Although the seed motif frequency was broadly distributed, direct targets had significantly more seed matches than the non-specific targets, while potential targets had a seed distribution that was in between the direct and non-specific target groups (Figure 13B). A majority of the direct targets have 3 or more seed motifs. In contrast, most potential targets have between 1-3 seed sequences and, in general, non-specific target mRNAs have 3 or less. Furthermore, the increase in seed frequency for direct targets correlates with increased 3'UTR length (Figure 13C). There is a significantly greater percentage (30%) of direct targets with 3'UTRs ranging in size from 2-2.5kb compared to the other transcript classes (4-6%). These observations are consistent with findings by Stark et al (2005) indicating that non-targeted mRNAs, such as housekeeping genes, tend to avoid miRNA target sites by limiting 3'UTR length whereas direct targets tend to evolve longer 3'UTRs enriched for miRNA recognition sites.

Seed deletion studies lend further support to the importance of seed motifs in facilitating miRNA-target interactions. Sensor constructs bearing the 3'UTRs of Vamp3 and Ctdsp1, two direct targets of miR-124a with multiple seed matches, were altered by site-directed deletion of

heptameric seed sequences. Transfection of these constructs into mouse kidney cells (TCMK1), together with an expression vector for miR-124a, resulted in a gradual increase in the luciferase activity of reporters as the seed motifs were sequentially deleted, indicating a reduction in the ability of the miRNA to repress targets with fewer seeds (Figure 14A and B). In this heterologous cellular context, seed sequences in both 3'UTRs tested appear to have equivalent contributions to miRNA-mediated repression and are all required for maximal repression.

A similar loss of repression was observed when the seed deletion constructs were transfected into primary cortical neurons expressing endogenous miR-124a (Figure 14C and D). Similar to what was observed in TCMK1 kidney cells, maximal expression of reporters in neurons was exhibited by constructs with the greatest number of deleted seed motifs. Notably, both heptameric and hexameric seed motifs were found to be capable of recruiting miRNA-mediated regulation. The seeds motifs within the Vamp3 3'UTR displayed the same additive contribution to miRNA repression. Surprisingly, however, the Ctdsp1 deletion constructs did not exhibit the same straightforward correlation between sensor activity and seed number. Whereas deletion of the first three heptamers resulted in a three-fold increase in luciferase activity, deletion of all five heptameric seeds resulted in repression. Further upregulation of sensor activity was achieved with the additional deletion of one hexameric seed (Figure 14D). While the exact cause for the variability in the response of these sensor constructs is not known, this finding emphasizes the importance of studying miRNA-mRNA interactions in their native context.

Thus, further characterization of miRNA interaction with other validated targets was performed in primary cortical neurons using endogenous levels of miR-124a to detect only the most robust miRNA-seed interactions. Serial seed deletion constructs were created for the other direct targets of miR-124a and transfected into cortical neurons (Figure 15). Measurement of changes in relative luciferase activity revealed that, in general, most of the direct targets had a single seed motif, typically a heptamer, which appeared to have a dominant role in transcript regulation despite the presence of multiple seeds within the 3'UTR (e.g. B4galt1, Nek9, Epim, Tom1l1, Cebpa, Sypl, Ptpn12, Lass2, Arfip1, Fcho2, Mapk14, E2ig4, and Itgb1). Deletion of this 'dominant' seed sequence from the 3'UTR was required to inhibit miR-124a-mediated repression and to upregulate sensor activity. For Lamc1 and Hadhb, the dominant seed motif was a hexameric sequence. Seed motifs in the Ptbp1 and Cd164 3'UTRs exhibited nearly equivalent contributions to repression, similar to the Vamp3 seed sequences. On the other hand, several deletion constructs had responses similar to that observed for the Ctdsp1 3'UTR. For these 3'UTRs, deletion of specific seed motifs resulted in either a minimal response, or an even greater repression (e.g. B4galt1, Tom1l1, Cebpa, Sypl, Ctdsp2, and Plod3). These observed differences

in seed activity are likely to be influenced by changes in the overall secondary structure of the transcript resulting from the sequence deletions, as well as other factors within the cellular milieu of neurons that can interact with the 3'UTRs of mRNAs.

The ability of a seed sequence to recruit miRNA is context-dependent

The importance of seed sequence accessibility to miRNA regulation is highlighted by seed insertion luciferase assays. Different seed sequences were introduced into the Snap25 3'UTR, which is not targeted by miR-124a, and transfected into cortical neurons (Figure 16). Insertion of a perfect match to miR-124a (miR-124a complement) resulted in a dramatic decrease in reporter expression, indicating that the miRNA was able to bind and repress the reporter. In this case, the miRNA was bound to a perfectly complementary sequence and repression was most likely effected through mRNA cleavage, a mechanism similar to RNA interference. This interaction with the miRNA was lost when positions 5 and 6 within the seed region were mutated (miR-124a mutant complement). Surprisingly, introduction of a single hexameric seed motif (6nt seed) or 1-3 heptameric seed matches (1x7nt, 2x7nt, 3x7nt seeds) into the Snap25 3'UTR did not confer repression. Transplantation of seed motifs with 15bp of upstream or downstream flanking sequences from the 3'UTRs of the *bona fide* direct targets, Vamp3 and Mapk14, was also ineffective. Thus, it seems that the ability of seed matches to recruit miR-124a is dependent on the overall sequence context in which they are found.

miR-124a targets are physically associated with Ago2/RISC

The Argonaute 2 (Ago2) protein specifically interacts with mature miRNAs in the RISC complex (Hammond et al, 2001; Mourelatos et al, 2002; Meister et al, 2004). This specific interaction was exploited by F. Karginov and G. Hannon (CSHL) to biochemically isolate and identify endogenous targets of miR-124a. To this end, 293S cells stably expressing myc-tagged Ago2 (293S(Ago2)) were transfected with miR-124a to load most of the RISC complexes with the miRNA. Transcripts associated with miR-124a-loaded RISCs were then isolated by immunoprecipitation of Ago2 using an antibody against the myc epitope. Purified mRNAs were identified by quantitative RT-PCR or microarray analysis. Because transfection of miR-124a results in a general downregulation of target mRNAs, and enrichment in the immunoprecipitated fraction depends on starting mRNA abundance, the net enrichment for each mRNA was calculated to provide a more accurate measure of miRNA-dependent Ago2 association. Net enrichment was taken as the ratio of the quantity of mRNA in the immunoprecipitated fraction versus total starting mRNA after miR-124a transfection.

Total mRNAs and immunoprecipitated mRNAs from cells transfected with miR-124a, or a control siRNA (GL3.1), were analyzed by quantitative RT-PCR using primers for human homologues of validated target genes, Ctdsp1, Vamp3, and Plod3, as well as primers for housekeeping genes that are unlikely to be miR-124a targets. Levels of mRNA are presented as enrichment or depletion in the miR-124a sample relative to the GL3.1 control (Figure 17). Significant depletion was observed for total mRNAs of Ctdsp1 and Vamp3, but not Plod3, in miR-124a-transfected cells. In the immunoprecipitated fraction, only Ctdsp1 and Plod3 transcripts were significantly enriched. However, upon normalizing to the starting total mRNA levels, an actual enrichment of 2.8 to 9.8 fold was observed for the three target mRNAs tested. In contrast, the net enrichment for housekeeping genes ranged only from 0.8 to 1.1 (Figure 17).

Association of mRNAs with miR-124a-loaded Ago2 is dependent on 3'UTR seed motifs. Vamp3 and Ctdsp1 sensor constructs with serial seed deletions were co-transfected into 293S(Ago2) cells with either the miR-124a duplex or a control siRNA against GFP. Deletion of seed motifs resulted in a gradual loss of miR-124a regulation as indicated by an increase in luciferase sensor activity when co-transfected with miR-124a (Figure 18A). Loss of miR-124a-mediated regulation was accompanied by only a slight improvement in total mRNA stability. More notably, however, seed deletion resulted in the inhibition of mRNA association with miR-124a-loaded Ago2, as indicated by a reduction of transcript enrichment in the immunoprecipitated fraction (Figure 18B).

To identify a transcriptome-wide set of miR-124a targets, total and immunoprecipitated mRNAs from miR-124a- or GL3.1-transfected cells were analyzed on microarrays. By calculating the net enrichment of mRNAs, 172 targets were identified that showed significant miR-124a-dependent association with Ago2 (net enrichment $p<0.005$). Interestingly, several mRNAs identified using this method were not identified in the microarray screen conducted by Lim et al (2005). These transcripts may represent mRNAs that are regulated mainly through translational repression, suggesting that the Ago2 immunoprecipitation method may allow the identification of mRNAs that are not degraded but are nevertheless associated with Ago2 and RISC (Karginov et al, manuscript in preparation). Comparing the biochemically identified targets to validated target sets (Figure 19), we found that the miR-124a direct targets showed stronger net IP enrichment with a mean fold change of 1.92. The potential target set was moderately enriched in the net IP (mean fold enrichment 1.60), and the non-targets showed the least net enrichment (mean fold enrichment 1.41). Therefore, net enrichment of mRNAs in purified RISC preferentially identifies the direct targets of miR-124a.

Comparison of miRNA target identification methods

Present miRNA target prediction programs for vertebrates identify targets based on two major criteria derived from a limited set of experimentally identified miRNA-target interactions. These criteria include the presence of seed motifs and cross-species conservation of 3'UTRs. Because each program starts with a different 3'UTR search set and employs variable methods for filtering targets, these programs tend to predict hundred of targets for each miRNA with little overlap between methods. To see how well the different algorithms can identify miR-124a targets, I compared the targets that were validated in our three-assay screen to mammalian target predictions from TargetScanS (Lewis et al, 2003; Lewis et al, 2005), PicTar (Krek et al, 2005), and MiRanda (John et al, 2004), three algorithms that provide precompiled target lists. I also compared the validated targets to targets identified through a biochemical interaction with Ago2 (Karginov et al, manuscript in preparation).

The TargetScanS algorithm predicted 1007 targets for miR-124a and identified 73% of validated direct targets, 50% of potential targets, and 29% of non-specific targets (Table 3). The PicTar and MiRanda algorithms, which predicted 788 and 1189 targets, respectively, saw similar percentages of transcripts in all three target classes. Each of these two algorithms recognized 40-45% of direct targets, 14-17% of potential targets, and 14-12% of non-specific targets. Combining the common predictions from all three computational programs reduced the number of predicted targets to 215, with only 40% of direct targets identified in common. Compared to computational programs, the Ago2 immunoprecipitation method had improved sensitivity, identifying 31% of the validated direct targets from out of only 172 transcripts that showed significant association with Ago2 (net enrichment $p<0.005$). Performing Ago2 immunoprecipitation in cortical neurons, which express an abundant amount of endogenous miR-124a, may further improve the sensitivity of this method by providing a way to discriminate between real interactions and false positives that may have resulted from miR-124a overexpression in the 293S(Ago2) cells.

Discussion

Characterization of a set of empirically validated targets of miR-124a revealed that seed motifs are important determinants of miRNA regulation. In fact, target 3'UTRs typically contain multiple seed matches to miR-124a. These recognition sites are necessary for mRNA degradation as well as for association with Ago2 in the RISC complex. However, while seed motifs are

required for miRNA target recognition, the mere presence of a seed is not always sufficient to confer regulation. As such, miRNA targets predicted solely through this seed motif should be interpreted with caution. Other transcript characteristics that may need to be considered in predicting miRNA targets include seed site cooperativity, seed spacing, possible RNA-interacting factors, and overall 3'UTR structure.

Cooperativity of miRNA binding has been demonstrated for several miR-124a targets, as well as for the die-1 and lin-41 transcripts in *C. elegans*. Full repression of the latter mRNAs requires two binding sites for miR-273 and let-7, respectively, suggesting a mechanism whereby greater repression of targets is achieved through mutual stabilization of binding by miRNAs acting cooperatively (Chang et al, 2004; Vella et al, 2004). Interaction between miRNA binding sites may explain why single seed sequences introduced into 3'UTRs that are not normally targeted by miRNAs are unable to recruit miRNA-mediated regulation. For example, seed sequences taken from validated miR-124a targets are unable to confer miRNA-mediated repression when transplanted into the Snap25 3'UTR. In a similar study, it was demonstrated that the binding site for lsy-6 could confer regulation when inserted into the lin-28 3'UTR context, but not in the unc-54 3'UTR (Didiano and Hobert, 2006). Because the lin-28 3'UTR bears binding sites for other miRNAs, it is possible that these sites may be contributing to the stabilization of the binding of lsy-6 to the inserted cognate sequence, thus promoting lsy-6-mediated transcript repression.

The importance of seed site spacing for miRNA repression was demonstrated in a recent study that showed that 3'UTRs with seeds separated by 13-35nt exhibited optimal downregulation (Sætrom et al, 2007). Because miRNAs are associated with ribonucleoprotein effector complexes, sites that are too close together may cause steric hindrance that can inhibit miRNA function. On the other hand, when target sites are too distal, complexes may not be capable of physical interaction. Optimally spaced sites, however, can facilitate complex interactions with adjoining sites and may also promote unfolding of the 3'UTR structure, thus improving sequence accessibility (Sætrom et al, 2007). The inability of multiple heptameric seed insertions to promote repression of the Snap25 3'UTR could be attributed, in part, to suboptimal seed spacing.

RNA-binding factors may affect the overall structure of a transcript, accessibility of seed sites, and mRNA stability. The importance of such cofactors on miRNA-target interactions was demonstrated by the *in vivo* regulation of the lin-41 transcript. Repression of the lin-41 mRNA requires two let-7 binding sites, as well as the 27nt spacer sequence in between. Alteration of the spacer sequence resulted in a loss of let-7-mediated repression (Vella et al, 2004). Interaction

with cofactors may also be one of the reasons why insertion of seed sequences into the Snap25 3'UTR did not readily confer miR-124a-mediated repression. Because Snap25 is a neuronally expressed RNA, its 3'UTR may contain sequence features that confer transcript stability in neurons, thus protecting it from miRNA-mediated degradation. Further experiments are required to identify the proteins or RNAs that may be interacting with particular transcripts.

The observation that some targets predicted solely through seed pairing are unresponsive in validation tests suggests that seed sequence accessibility may be important for miRNA regulation. The inclusion of target accessibility criteria in the identification of miRNA targets may greatly reduce the number of false positive predictions. Recently, a method using a two-step model for the hybridization of a miRNA to a structured target was described (Long et al, 2007). In this model, the miRNA hybridizes first to a short stretch of accessible complementary sequence on the mRNA, typically an open block of four or more nucleotides. The hybrid then elongates, disrupting local mRNA secondary structure, to form the final miRNA-mRNA duplex. While this method has consistently been able to account for experimentally validated miRNA-target pairs in *C. elegans* and *D. melanogaster*, it remains to be tested on vertebrate miRNAs and their targets. To uncover other sequence features that may also be contributing to miRNA regulation, including 3' compensatory binding sites and binding sites in other regions of the transcript, it will be necessary to conduct a more in depth computational analysis of validated miRNA targets.

The data presented in this chapter shows that bioinformatic prediction algorithms can identify only a subset of experimentally validated direct targets. Additionally, the large volume of predictions generated by these programs makes it difficult to distinguish genuine targets from background. And while there has been some success in identifying miRNA targets by rationally choosing genes from out of the hundreds of computationally predicted transcripts, this method overlooks the possibility that miRNAs may exert their effects through the regulation of more than one mRNA. This is an issue that has to be considered, especially when studying complex processes, such as tissue lineage determination. On the other hand, combining common predictions from different algorithms does improve the chances of finding real targets, however, the diversity of the methodologies employed by these programs results in the omission of many real targets.

The improvement of the accuracy and sensitivity of target prediction methods lies, in part, on the validation of a more extensive set of miRNA-target interactions on which the rules for target selection are based. The most systematic analysis of miRNA-target relationships, so far, was performed in *D. melanogaster* for 133 miRNA-target pairs that were validated using an

in vivo imaginal wing disc assay or *in vitro* sensor assays (Stark et al, 2005). It should be noted, however, that many strategies for validating targets, including those in Stark et al (2005), make use of exogenously overexpressed miRNA and mRNAs. The artificially high concentrations of miRNAs achieved by overexpression may cause non-physiological interactions with mRNAs that are not normally targeted but may have minimal complementarity to the miRNA. This situation may result in an overestimation of the number of real interactions. Targets should ideally be tested using close to endogenous levels of miRNA and mRNA. Excellent examples are provided by methods developed to test miRNA targets in *C. elegans* (Didiano and Hobert, 2006; Lall et al, 2006). In these studies, native promoters were used to drive the expression of 3'UTR reporters in cells that endogenously express the miRNA of interest. In many cases, however, such elegant analysis of interactions under native conditions may not be feasible. An alternative is to validate targets through a combination of *in vitro* assay methods and phenotypic or functional analysis. Our work on miR-124a is the only study, to date, detailing extensive experimental validation of mammalian targets using endogenous miRNA and expressed reporters. Large-scale experimental analysis of *in vivo* miRNA-mRNA relationships in mammals is yet to be accomplished.

Other developments that will contribute to an improvement of target prediction algorithms includes the creation of a more accurate 3'UTR annotation database that includes splice variants and alternatively polyadenylated transcripts. In addition, the development of advanced RNA folding programs, as well as a better understanding of 3'UTR interacting factors will allow more accurate assessment of seed site accessibility. In a comparative study of genomic regulatory motifs across four mammalian species, Xie et al (2005) found 106 conserved motifs in the 3'UTRs of transcripts. Half of these motifs corresponded to miRNA seed sites. The other half of the motifs were characteristically AT-rich, and included polyadenylation signals and putative consensus sites for RNA-binding proteins (Xie et al, 2005). While the factors that bind to some of the identified motifs are not yet known, this finding does highlight the importance of combinatorial regulation by miRNAs and proteins on transcript expression.

The advent of alternative unbiased methods for miRNA target identification will be crucial for the identification of targets that may not be recognized using the current rules for target prediction. Ago2 immunoprecipitation will identify not only cleaved targets, but targets that associate with RISC and are not degraded (Karginov et al, manuscript in preparation). Identification of all the transcripts that are associated with RISCs will provide a more complete picture of the network of transcripts that are regulated by any miRNA. This method complements recent studies showing that mRNAs are sequestered into P-bodies for storage or degradation and are released in response to specific stimuli (Bhattacharyya et al 2006; Brengues et al, 2005).

Extended applications of the Ago2 immunoprecipitation method may eventually allow us to gain more insight into the changes that occur within non-translating and translating pools of transcripts under different conditions, such as neuronal depolarization. Translationally repressed targets may also be identified through proteomic profiling. This method has been used, with some success, to identify proteins that are deregulated by the loss of miRNAs during maturation of Dicer null *D. melanogaster* oocytes (Nakahara et al, 2005). Comparative genome-wide expression profiling of miRNAs and mRNAs in different cell types and disease stages is another method that is emerging as a powerful tool for deciphering the potential role of miRNAs in specific cellular processes. For example, profiling of miRNAs in tumors revealed miRNA signatures that point to a role for miRNAs as tumor suppressors and oncogenes (Calin and Croce, 2006). Furthermore, profiling of mRNAs in primary cells where a specific miRNA is overexpressed or depleted will provide a generalized view of transcripts that are affected both directly and indirectly (Lim et al, 2005; Krutzfeldt et al, 2005), providing clues to miRNA function. Most importantly, the target lists generated by these unbiased methods will provide us with the necessary data sets wherein we can look for other possible determinants of miRNA binding, including nonconserved sites, 3' compensatory sites, and sites within the open reading frame of transcripts. Genomic profiling in combination with more advanced computational methods will also allow for the examination of possible combinatorial regulation of transcripts by co-expressed miRNAs, and possibly, RNA-binding factors. Ultimately, advances in our understanding of miRNA-mRNA interactions will lead to the incorporation of miRNAs in the complex regulatory networks that govern many cellular processes.

Materials and Methods

Seed deletion and insertion constructs

Cloning of 3'UTRs into the luciferase expression vector, pGL3TK, was described in Chapter II. Deletion and insertion of seed sequences in the corresponding 3'UTR sensor constructs was performed using the Quikchange II Site-Directed Mutagenesis Kit (Stratagene). 3'UTR alterations were verified by sequencing.

Seed deletion and insertion luciferase sensor assays

TCMK1 cells ($1x10^5$ cells per well on a 12-well dish) were transfected with 250ng of each of the reporter vectors along with 20ng of pRLTK and 250ng of an expression vector for

miR-124a or an empty vector (pcDNA, Invitrogen). The ratio of firefly versus *Renilla* luciferase activity (FL/RL) was measured after 48 hours using the Dual Luciferase reporter assay system (Promega) following the manufacturer's protocol. Changes in relative luciferase activity were determined by taking the ratios of the geometric means for reporters co-transfected with miR-124a and pcDNA. Significance was determined using the Student's t-test. Primary cortical neurons ($5x10^5$ cells per well on a 24-well dish) were transfected with 100ng of each of the reporter vectors along with 20ng of pRLTK using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The ratio of firefly versus *Renilla* luciferase activity (FL/RL) was measured as described above. Fold changes in the relative luciferase activity of deletion constructs were determined relative to the wildtype 3'UTR. Significance was determined using the Student's t-test.

Ago2 co-immunoprecipitation and analysis of targets

293S(Ago2) or the parental 293S cells were transfected with the appropriate plasmid constructs (10µg per 10cm dish for experimental plasmids, 1µg for *Renilla* luciferase controls) using TransIT-LT1 (Mirus) and with 100nM miR-124a, let-7 or GL3.1 siRNAs using TransIT-TKO. 293S or 293S(hAgo2) cells were harvested 48 hours after transfection and washed in PBS followed by hypotonic lysis buffer (10mM Tris pH 7.5, 10mM KCl, 2mM $MgCl_2$, 5mM DTT and 1 tablet per 10ml protease inhibitors, EDTA-free (Roche)). Cells were incubated in lysis buffer for 15 minutes and lysed by douncing. Immediately after douncing, the lysates were supplemented with 5X ATP depletion mix (4U/µl RNaseIn (Promega), 100mM glucose, 0.5U/µl hexokinase (Sigma), 1mg/ml yeast tRNA (Invitrogen), 450mM KCl) to a final concentration of 1X. The lysates were cleared by centrifugation at 16,000 g for 30 minutes at 4º C. Radiolabeled targets were incubated with lysates at 30ºC for 1 hour at this stage. Aliquots of total RNA (1/10 for radiolabeled samples, 1/20 for other) were taken and extracted with Trizol. Prior to immunoprecipitation, anti-myc beads (Sigma) were pre-blocked for 30 minutes in wash buffer (0.5% NP40, 150mM NaCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 20mM Tris pH 7.5, 5mM DTT and 1 tablet per 10ml protease inhibitors, EDTA-free (Roche)) supplemented with 1mg/ml yeast tRNA and 1mg/ml BSA, followed by a wash in wash buffer. One volume of wash buffer was added to the lysates, and myc-Ago2 was immunoprecipitated with the pre-blocked beads for 4 hours at 4ºC. The beads were washed three times with wash buffer, transferring the slurry to a fresh tube on the last wash, and bound RNA was extracted with Trizol. The extracted RNA samples were isopropanol-precipitated, washed with 70% ethanol and reconstituted in water. The radiolabeled samples were analyzed on 4% denaturing polyacrylamide gels. For quantitative RT-PCR, the

samples were treated with DNase I (amplification grade, Invitrogen), reverse-transcribed with gene-specific reverse primers (MessageSensor RT, Ambion) and amplified using SYBR Green PCR Master mix (Applied Biosystems). The Vamp3 and Ctdsp1 3'UTR luciferase reporter constructs were quantified with primers against firefly and *Renilla* luciferase sequence. For microarrays, a reference-based two-color microarray design with Universal Human Reference RNA (Stratagene) was used. Six replicate experiments (both total RNA and immunoprecipitated RNA), along with reference RNA, were amplified using the MessageAmp II aRNA kit (Ambion) with direct incorporation of Cy3- and Cy5-labeled UTP. For the first three replicates, the immunoprecipitated RNA were taken through two rounds of amplification. Samples were hybridized to Agilent Whole Human Genome 4x44K chips.

Figure 13. Direct targets have longer 3'UTRs enriched for seed motifs. (A) Schematic diagram of seed motifs in a target 3'UTR. Heptameric and hexameric seed matches to miR-124a are highlighted in the light gray box. Positions 5 and 6 of the seed are highlighted in dark gray. (B) Seed frequency distribution showing that direct targets bear more 7nt and 6nt seed matches to miR-124a in their 3'UTRs. (C) Distribution of target 3'UTR lengths.

Figure 13

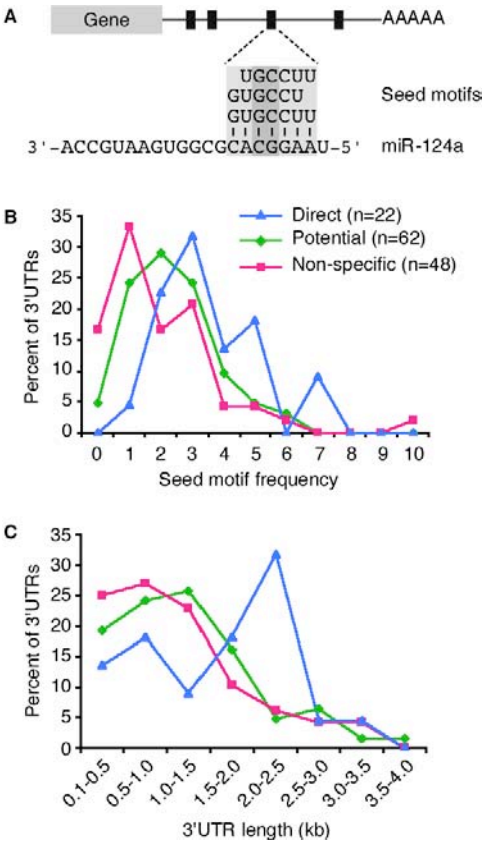

A
Gene
AAAAA
UGCCUU
GUGCCU
GUGCCUU
Seed motifs
3'-ACCGUAAGUGGCGCACGGAAU-5'
miR-124a
B
Direct (n=22)
Potential (n=62)
Non-specific (n=48)
Percent of 3'UTRs
Seed motif frequency
C
Percent of 3'UTRs
0.1-0.5
0.5-1.0
1.0-1.5
1.5-2.0
2.0-2.5
2.5-3.0
3.0-3.5
3.5-4.0
3'UTR length (kb)

Figure 14. Seed sequences in target 3'UTRs confer miR-124a regulation. Sensor assays showing that the seed sequences within the Vamp3 and Ctdsp1 3'UTR are required for miR-124a-mediated repression. (A, B) Firefly luciferase (FL) sensor constructs containing the Vamp3 (A) and Ctdsp1 (B) 3'UTRs with sequential deletions of the seed sequences were transfected into mouse kidney cells together with either an expression vector for miR-124a or pcDNA and a vector expressing *Renilla* luciferase (RL). Relative luciferase activity (FL/RL) was measured after 48 hours. Fold changes in FL/RL are shown as the ratio of sensor activity when co-transfected with miR-124a relative to pcDNA. Error bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test). (C, D) Luciferase activity of sensor constructs bearing the Vamp3 (C) and Ctdsp1 (D) 3'UTRs with sequential seed deletions transfected into primary cortical neurons together with the RL expression vector. Relative luciferase activity (FL/RL) was measured after 48 hours. The relative luciferase activity for each deletion construct is presented relative to the activity of the wildtype 3'UTR.

Figure 14

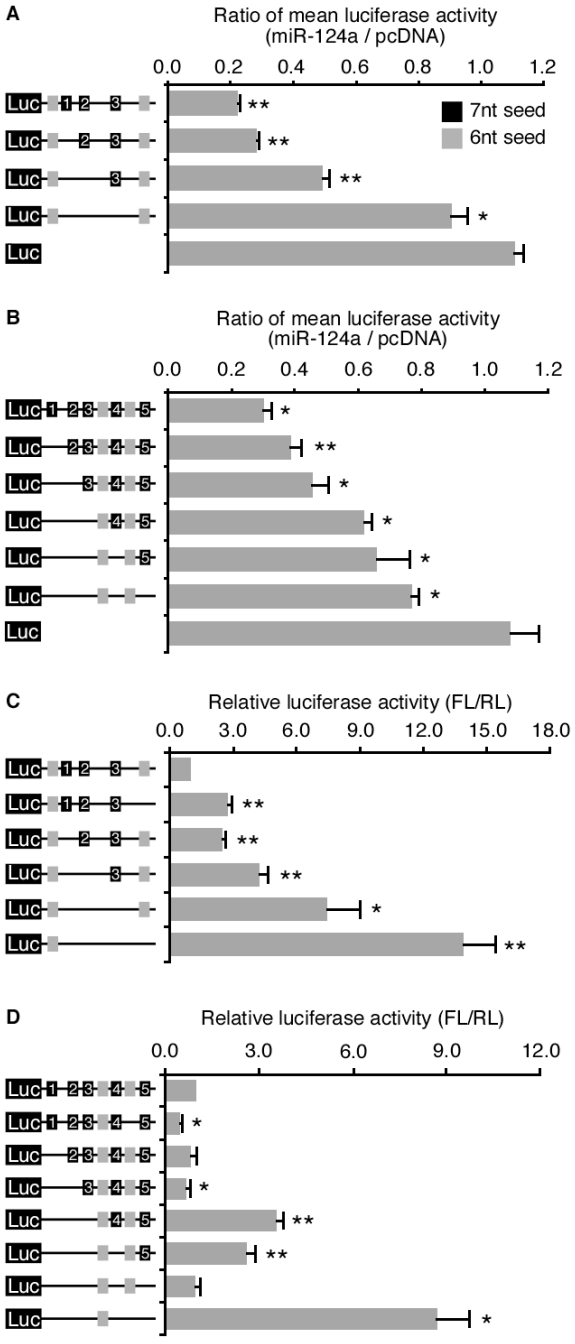

A
Ratio of mean luciferase activity
(miR-124a / pcDNA)
0.0 0.2 0.4 0.6 0.8 1.0 1.2
7nt seed
6nt seed
B
Ratio of mean luciferase activity
(miR-124a / pcDNA)
0.0 0.2 0.4 0.6 0.8 1.0 1.2
C
Relative luciferase activity (FL/RL)
0.0 3.0 6.0 9.0 12.0 15.0 18.0
D
Relative luciferase activity (FL/RL)
0.0 3.0 6.0 9.0 12.0

Figure 15. Many miR-124a direct targets contain a dominant heptameric seed motif. Seed deletion constructs for the 3'UTRs of other validated miR-124a direct targets were transfected into primary cortical neurons together with the *Renilla* luciferase vector. Relative luciferase activity (FL/RL) was measured after 48 hours. Luciferase activities are shown relative to the wildtype 3'UTR of each target. Error bars represent the standard deviation of three independent experiments (* $p \leq 0.05$, ** $p \leq 0.005$, Student's t-test). X-axis labels (x+y) indicate the number of heptameric (*x*) + hexameric (*y*) seed motifs in each sensor construct.

Figure 15

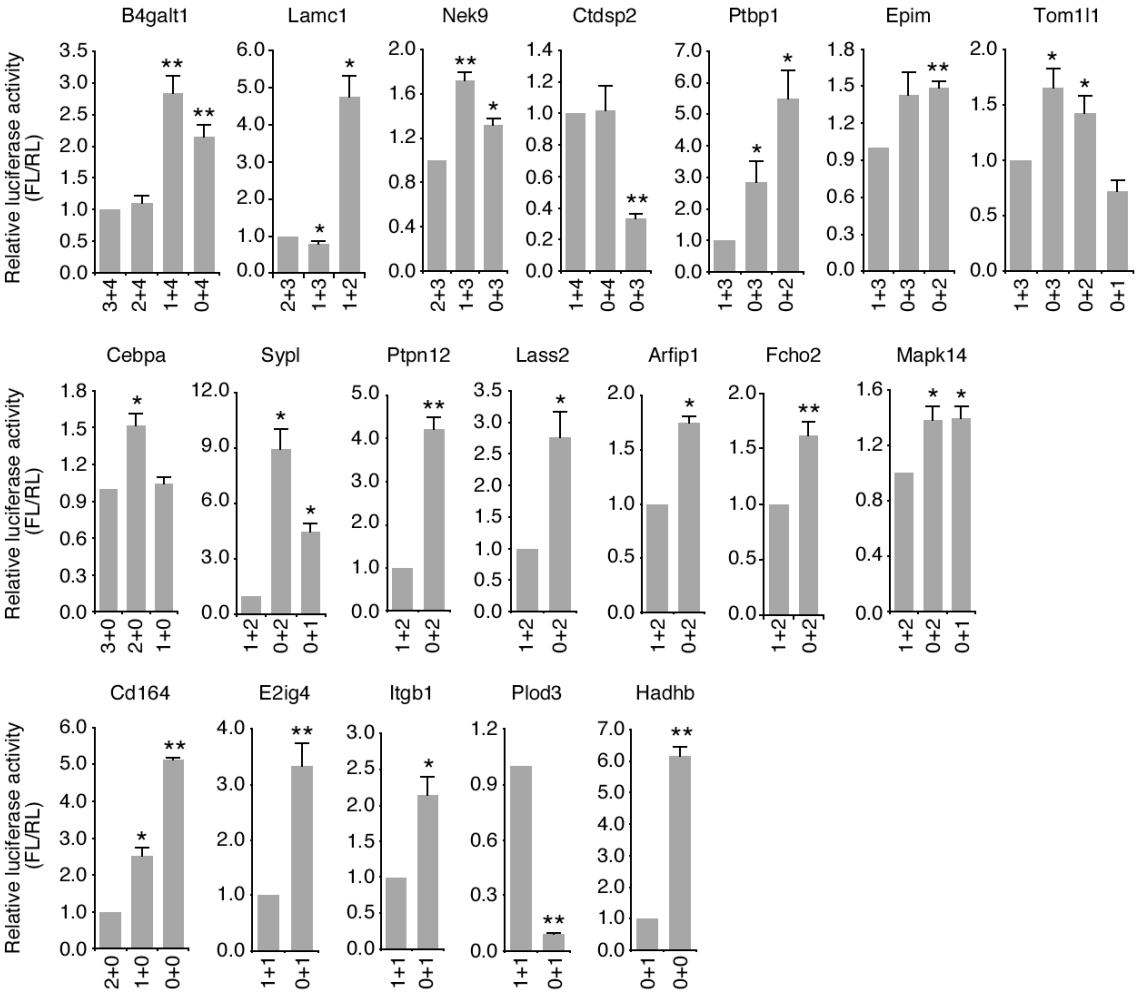

B4galt1
Lamc1
Nek9
Ctdsp2
Ptbp1
Epim
Tom1l1
Cebpa
Sypl
Ptpn12
Lass2
Arfip1
Fcho2
Mapk14
Cd164
E2ig4
Itgb1
Plod3
Hadhb
Relative luciferase activity (FL/RL)

Figure 16. Ability of seed sequences to recruit miR-124a is context-dependent. Sensor assays showing that introduction of different seed sequences into the context of the non-targeted Snap25 3'UTR are not able to recruit miR-124a. Firefly luciferase (FL) sensor constructs containing the Snap25 3'UTR and the indicated seed insertions were transfected into cortical neurons together with a vector expressing *Renilla* luciferase (RL). Relative luciferase activity (FL/RL) was measured after 48 hours. Error bars represent the standard deviation of three independent experiments (** $p \leq 0.001$, Student's t-test).

Figure 16

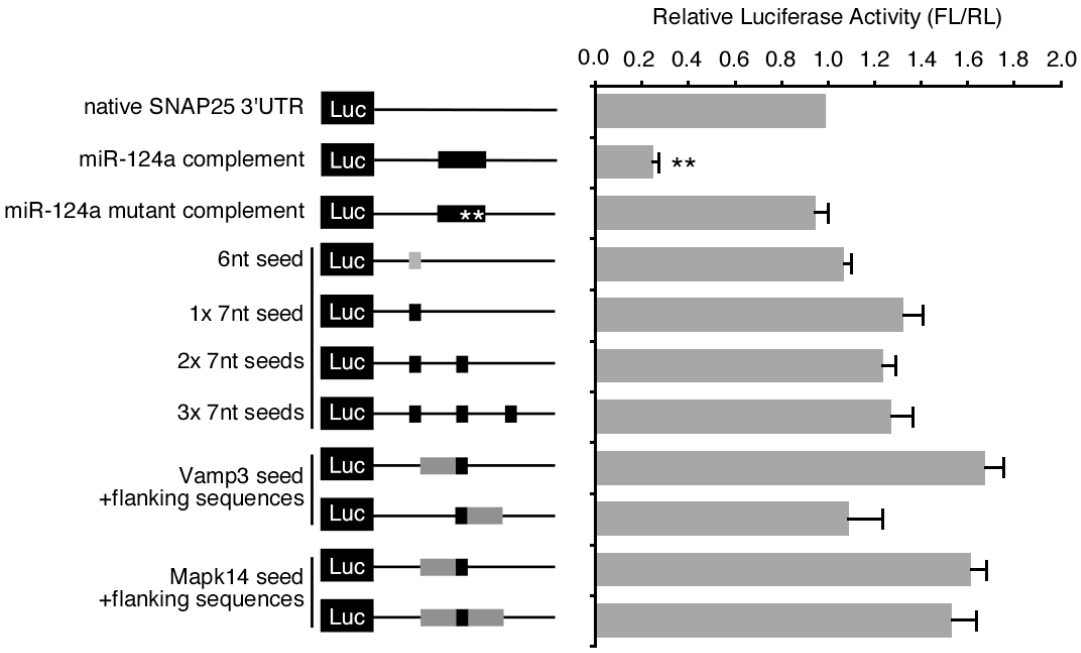

Relative Luciferase Activity (FL/RL)
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
native SNAP25 3'UTR
miR-124a complement
miR-124a mutant complement
6nt seed
1x 7nt seed
2x 7nt seeds
3x 7nt seeds
Vamp3 seed +flanking sequences
Mapk14 seed +flanking sequences
Luc
**

Figure 17. Direct targets of miR-124a interact with miR-124a-loaded Ago2. 293S(Ago2) cells expressing myc-tagged Ago2 were transfected with miR-124a or a control siRNA (GL3.1). Ago2-associated transcripts were isolated by immunoprecipitation (IP) using an antibody against the myc epitope. Fold changes in endogenous mRNA levels of miR-124a direct targets and housekeeping genes were measured by quantitative RT-PCR and are presented as enrichment or depletion in the miR-124a sample relative to the GL3.1 control. The net enrichment of each mRNA in the IP was calculated as the ratio of the quantity of mRNA in the IP fraction relative to total mRNA abundance. Bars represent the standard deviation of three independent experiments. Graph courtesy of F. Karginov (CSHL).

Figure 17

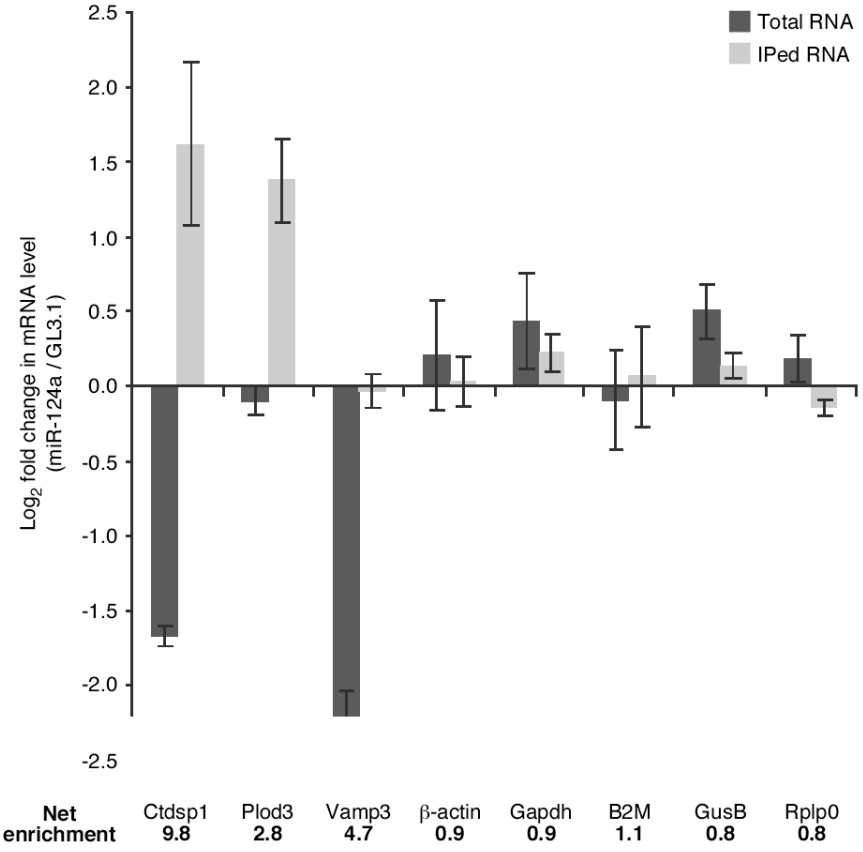

Total RNA
IPed RNA
Log2 fold change in mRNA level (miR-124a / GL3.1)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Net enrichment
Ctdsp1 9.8
Plod3 2.8
Vamp3 4.7
β-actin 0.9
Gapdh 0.9
B2M 1.1
GusB 0.8
Rplp0 0.8

Figure 18. Seed sites are required for interaction with miR-124a-loaded Ago2. Vamp3 and Ctdsp1 3'UTR sensor constructs were transfected into 293S(Ago2) cells together with miR-124a or an siRNA control (GFP). (A) Luciferase activity was measured after 48 hours. Luciferase activity of constructs co-transfected with miR-124a was normalized to the activity of the wildtype 3'UTR co-transfected with GFP siRNA. Error bars represent the standard deviation of three independent experiments. (B) Quantitative RT-PCR measurement of total and immunoprecipitated mRNAs for the Vamp3 and Ctdsp1 3'UTR constructs after 48 hours of transfection. Enrichment or depletion of transcripts is shown relative to the mRNA level of the wildtype 3'UTR co-transfected with GFP siRNA. Error bars represent the standard deviation of three independent experiments. Graphs courtesy of F. Karginov (CSHL).

Figure 18

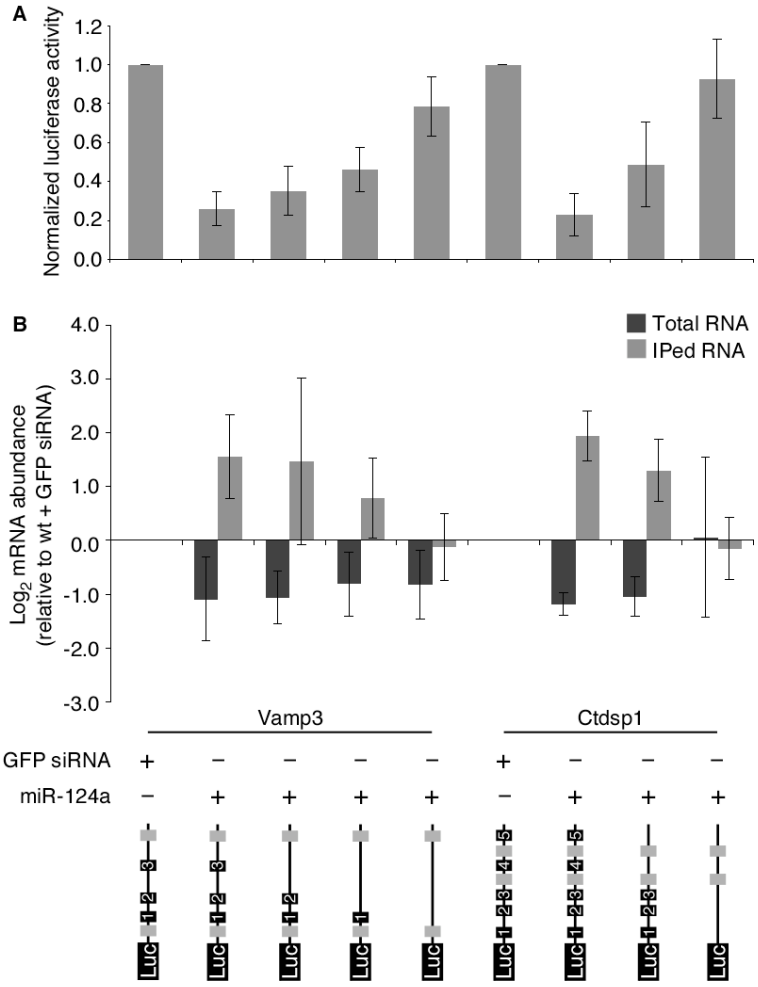

A
Normalized luciferase activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B
Total RNA
IPed RNA
Log2 mRNA abundance
(relative to wt + GFP siRNA)
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
Vamp3
Ctdsp1
GFP siRNA
miR-124a
Luc
1
2
3
4
5

Figure 19. Ago2 IP enrichment of validated miR-124a targets. Histogram of net enrichment of direct, potential, and non-specific targets in the Ago2-immunoprecipitated fraction. Direct targets show a higher degree of co-immunoprecipitation with Ago2. Graph courtesy of F. Karginov (CSHL).

Figure 19

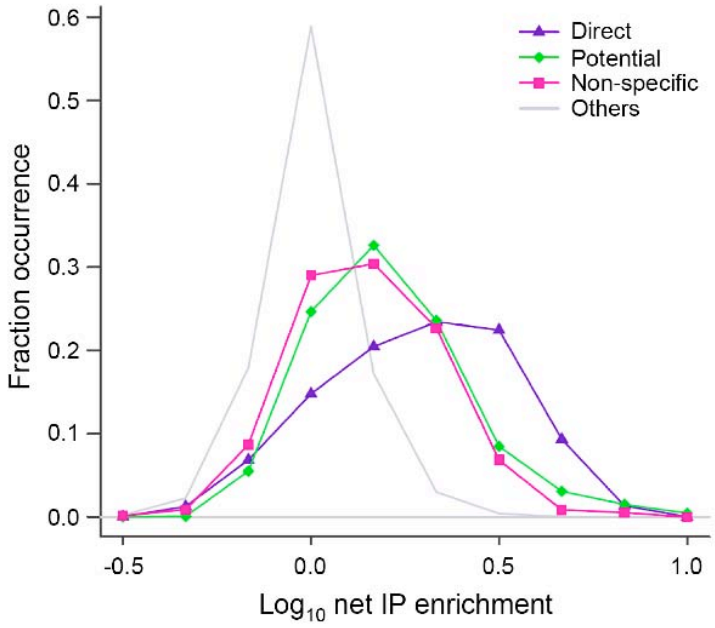

Direct
Potential
Non-specific
Others
Fraction occurrence
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.5
0.0
0.5
1.0
Log10 net IP enrichment

Table 3. Comparison of target identification methods. Predictions from three algorithms and targets identified through Ago2 immunoprecipitation are compared to targets empirically validated using the three-assay screen. The percentage of each target class that is predicted by each method is shown.

Method	Percent of direct targets (n=22)	Percent of potential targets (n=62)	Percent of non-specific targets (n=48)	Total predictions for miR-124a
TargetScanS[a]	72.73	50.00	29.17	1007
PicTar[b]	40.91	17.74	12.50	788
MiRanda[c]	45.45	14.52	14.52	1189
All algorithms	40.91	6.45	8.33	215
Ago2 IP[d]	31.82	3.23	6.25	172

[a]Lewis et al, 2003; Lewis et al, 2005
[b]Krek et al, 2005
[c]John et al, 2004
[d]Targets with significant net enrichment in Ago2 IP ($p<0.005$; Karginov et al, manuscript in preparation)

GENERAL DISCUSSION

Neurogenesis relies on mechanisms that coordinate exit from the cell cycle, downregulation of progenitor phenotype, and the expression of proteins encoding neuronal-specific functions. In this study, I present evidence that miR-124a contributes to neuronal differentiation of cortical progenitors in two ways: expression of miR-124a results in both the downregulation of many non-neuronal mRNAs, as well as a modest upregulation of neuronal markers that is independent of REST. The upregulation of neuronal genes is due, in part, to the downregulation of Ctdsp1, a component of the REST repressor complex. This is supported by a recent finding showing that expression of a Ctdsp1 transcript lacking its 3'UTR, which renders it insensitive to miR-124a repression, antagonizes the ability of miR-124a to induce the expression of neuronal markers in P19 embryonal carcinoma cells (Visvanathan et al, 2007). However, the subtle changes in molecular phenotype resulting from miR-124a expression are not accompanied by obvious morphological transformations suggesting that miR-124a by itself is not sufficient for the establishment of neuronal properties. The robust expression of neuronal genes needed to develop a functional neuronal architecture likely relies on downstream transcriptional programs that are initiated by pro-neural basic helix-loop-helix (bHLH) proteins, and the disappearance of the transcriptional repressor, REST. bHLH transcription factors exert their effects early in development, promoting cell cycle exit, and inducing overt neurogenesis of precursor cells (Farah et al, 2000). REST, on the other hand, acts later in neurogenesis by regulating the expression of neuronal traits. The effects of REST are mediated, in part, through the activity of the pro-neural transcriptional activators, Sox4 and Sox11 (Bergsland et al, 2006). These transcription factors have been shown to be responsible for the expression of REST target genes, including the neuronal markers, TuJ1 and MAP2. These findings all point to a central role for REST as a master regulator for the expression of neuronal genes, as well as for the clearance of non-neuronal transcripts during the terminal stages of differentiation.

The ability of miRNAs to specify cell fate lies in their potential to alter the mRNA profile of a cell. This can be achieved directly through downregulation of large networks of genes, or indirectly, by targeting genes that are central to signaling pathways. In plants, almost two-thirds of known miRNAs are predicted to target the mRNAs of transcription factors regulating crucial steps during plant development (Rhoades et al, 2002). Similarly, the first miRNAs to be identified in *C. elegans*, let-7 and lin-4, functioned as switches for the coordination of developmental timing. Most of the predicted targets of let-7 are transcription factors, including

daf-12, a nuclear hormone receptor in seam cells, pha-4, a forkhead transcription factor in the intestine, and hbl-1, a zinc finger transcription factor in the ventral nerve cord (Grosshans et al, 2005). However, in vertebrates, miRNAs appear to function more as a means to sharpen phenotypic distinctions during the transition between different cell types, rather than as developmental switches. This has been shown to be the case for miR-124a in neurons, and for miRNAs in other tissue types as well. For example, zebrafish embryos that lack both maternal and zygotic miRNA contributions still displayed normal axis formation and cell type specification, exhibiting defects in morphogenesis only during gastrulation and at later stages of organ development (Giraldez et al, 2005). Similarly, loss of miRNAs through conditional Dicer knockout in limb mesoderm did not affect normal tissue patterning, although smaller limbs were formed due to massive cell death and deregulated gene expression (Harfe et al, 2005). Furthermore, mice with conditional loss of Dicer in the epidermis were almost indistinguishable from their wildtype siblings at birth, exhibiting normal cell fate specification and differentiation, with defects in epidermal organization and evagination of hair follicles becoming apparent only after a week (Andl et al, 2006; Yi et al, 2006). Thus, it comes as no surprise that inhibition of miR-124a function in the developing chick neural tube had a very subtle effect on neuronal differentiation (Cao et al, 2007; Visvanathan et al, 2007). Extrapolating from the results of tissue-specific Dicer knockouts, loss of Dicer in the developing nervous system is similarly expected to result in normal patterning and differentiation, eventually resulting in defects in migration, synaptogenesis, and neuronal activity through dysregulation of gene expression. While this study is yet to be performed, the results could provide remarkable insight into the aspects of neurogenesis and neural function that are regulated by miRNAs in general.

The transition between cell states is governed by transcriptional and translational regulatory mechanisms, both of which are subject to random fluctuations, or noise, that results in variability at the gene expression level (Arias and Hayward, 2006). Noise is inherent in the nature of the interactions between proteins and DNA or RNA and needs to be filtered out to stabilize a particular cell state. Development of mechanisms, such as DNA methylation and chromatin remodeling, that can reduce noise due to spurious transcription, are thought to have been critical for the evolution of complex multicellular organisms (Bird, 1995). The abundant, tissue-specific expression profile of some miRNAs, and their potential to downregulate many targets simultaneously, makes them an attractive mechanism for reducing transcriptional noise, as well. In neurons, miR-124a appears to be acting as a noise filter by clearing the cell of mRNAs produced by basal transcription that is occurring even at transcriptionally repressed loci. The activity of the miRNA maintains the stability of the neuronal phenotype by preventing the re-

accumulation of non-neuronal gene products and effectively ensures the irreversibility of neuronal differentiation. In fact, inhibition of miR-124a activity results in increased cell death (Cao et al, 2007), presumably due to misexpression of non-neuronal genes. Interestingly, in neurons undergoing global ischemia, increased cell death is accompanied by an increase in REST mRNA and protein levels (Calderone et al, 2003). This ischemia-induced cell death was postulated to be a result of altered calcium permeability due to REST-dependent repression of glutamate receptor expression. In a related study, it was shown that excitotoxic neuronal death, accompanied by an increase in REST mRNA and activation of the miR-124a target Mapk14/p38, could be antagonized by treatment with a Mapk14/p38 inhibitor (Segura Torres et al, 2006). This suggests that REST upregulation during ischemia may be causing a decrease in miR-124a expression that, in turn, results in the upregulation of non-neuronal targets. It would be of interest to see whether this is indeed the case and whether destabilization of the neuronal phenotype due to miR-124a downregulation contributes to excitotoxic cell death. It may also be worth examining miRNA regulation in the context of neuroregeneration where injured neurons may need to re-express genes that recapitulate the early stages of neurogenesis in order to migrate and reform synapses.

Increased understanding of the mechanisms underlying miRNA function has revealed striking similarities between the principles of miRNA and transcription factor regulation (Hobert, 2004). First, the temporal and spatial expression of miRNAs is regulated by the same transcription factors that control protein-coding genes. Hence, miRNA expression can be readily tied into regulatory networks of transcription factors and signal transducers that govern diverse biological processes. Second, miRNAs and their targets typically form regulatory loops that help establish cell-type specific gene expression profiles. For example, the irreversible differentiation of worm chemosensory neurons is ensured by the miRNAs lsy-6 and miR-273 and their transcription factor targets. Through a double-negative feedback loop, these miRNAs stabilize the asymmetric expression of guanyl cyclase genes that differentiate the left and right worm chemosensory neurons (Johnston and Hobert, 2003; Chang et al, 2004). In granulocytic differentiation, miR-223, which is transcribed by C/EBPα, maintains its stable expression by downregulating a competing transcriptional repressor, NF1-A, in a negative feedback loop (Fazi et al, 2005). miRNAs are also found in signaling cascades. For example, in cardiogenesis, serum response factor induces the expression of muscle-specific miRNA, miR-1, which in turn controls cardiomyocyte proliferation by downregulating the Hand2 transcription factor (Zhao et al, 2005). Interestingly, the finding that many miRNAs are co-expressed within the introns of protein-coding genes hints at the possibility of a regulatory mechanism wherein a single transcript

encodes both a protein that promotes one signaling pathway, as well as a miRNA that represses an antagonistic pathway. Examples of such functional pairs of protein and miRNA derived from single transcripts remain to be discovered. Finally, just like transcription factors, miRNAs also exhibit cooperative and combinatorial regulation of their targets. While a single perfect binding site is sufficient for miRNA-mediated mRNA cleavage (Yekta et al, 2004), translational repression by miRNAs is usually enhanced by the presence of multiple binding sites in the 3'UTR (Doench et al, 2003; Doench and Sharp, 2004). Many mRNA transcripts contain several binding sites for their cognate miRNAs and may require some or all of them for complete regulation. Binding of a miRNA to one site may promote the 3'UTR structure to open up, which then allows more miRNAs to bind to the target, thus enhancing regulation. In addition, mRNAs can also have binding sites for different miRNAs that may act in combination to integrate the signals from multiple pathways.

In contrast to transcriptional regulators, which typically act as on/off switches for gene expression, miRNAs are potentially capable of fine-tuning target concentrations. This type of regulation allows finer control of mRNA levels and translational output in different cells without requiring different sets of promoters and transcription factors for each cell type. Differential regulation of transcripts is dependent on the number of binding sites for a single miRNA, or a combination of miRNAs, within the target 3'UTR (Doench and Sharp, 2004). Thus the expression of the same mRNA is expected to vary from one cell type to another depending on the miRNAs that are present. As such, the variability in miR-124a target expression in the cerebral cortex (Farh et al, 2005) may be attributed, in part, to the heterogeneity of neurons in the brain because each neuronal subtype may express its own unique combination of transcription factors and miRNAs. Local fine-tuning of mRNA levels may also be particularly useful for neurons, where rapid responses to stimuli are often required at sites far removed from the nucleus. One notable example is the release of Limk1 mRNA from miR-134-mediated repression to promote dendritic spine growth in response to BDNF (Schratt et al, 2006). miRNA-mediated sequestration into P-bodies may be an additional mechanism for fine-tuning translational output in neurons. In hepatocarcinoma cells, the cationic amino acid transporter 1 (CAT1) mRNA is relieved of miR-122-mediated repression in response to stress (Bhattacharyya et al, 2006). Derepression of CAT1 is accompanied by a release from P-bodies that is dependent on the association of the RNA-binding protein HuR. It conceivable that a similar mechanism may be present in neurons, given that homologous RNA binding proteins and RISC components known to colocalize with P-bodies are found throughout neuronal axons (Deschenes-Furry et al, 2006; Hengst et al, 2006). Thus, it would be of interest to see how miR-124a and other neuron-specific

miRNAs can work together to fine-tune the expression level of their targets, and whether this regulation is reversible under certain conditions.

A fascinating paradox revealed by the results of genome sequencing projects is that phenotypic diversity is not dependent on the expansion of genome size. Although higher eukaryotes may contain a larger genetic repertoire, many of their genes actually encode variants of the same protein family. Thus, the core proteome size has remained very much the same between worms, flies, and humans (Rubin et al, 2000). This suggests that metazoan complexity emerged from the multitasking of a core proteome of limited size through the development of novel interactions between existing components (Mattick and Gagen, 2001). The additional layers of regulation that may have contributed to the evolution of multicellular organisms includes chromatin modifying factors, alternative splicing factors, and non-protein-coding RNAs, such as miRNAs. miRNAs, which exhibit such striking similarities to the features of transcription factor-mediated gene regulation, are particularly attractive as mediators of network signaling in metazoans for several reasons: (1) miRNAs are sequence-specific regulators that can exert their effects at the level of gene expression; (2) miRNAs can regulate multiple genes simultaneously, allowing tight coordination of the different branches within a regulatory network; and (3) expansion of miRNA genes and their tissue-specific expression correlates well with major developments in metazoan evolution (Sempere et al, 2006; Hertel et al, 2006). In this model of regulation, use of miRNAs provides stability to the core proteome while allowing enough flexibility for the cell to explore phenotypic variations through subtle modifications in network regulation.

REST has recently been shown to function as a tumor suppressor. Although the exact routes through which REST dysfunction leads to cancer is unknown, the discovery that REST also regulates miRNAs opens up a new perspective in which to explore the role of this transcription factor in carcinogenesis. Medulloblastomas, in particular, arise due to the retention of REST activity in neuroectodermal stem cells, which fail to differentiate and continue to proliferate. Expression of an activating form of REST, REST-VP16, inhibits the growth of this type of tumor (Fuller et al, 2005). The connection between REST and tumors arising in non-neuronal tissues is less well established. Several REST-regulated miRNAs, including miR-124a-2, miR-124a-3, miR-9-1, and miR-338, are among the miRNAs that are found in genomic loci associated with copy number gain in several cancer cell types (Zhang et al, 2006). Other REST-regulated miRNAs are potential tumor suppressors, including miR-34a, which induces apoptosis in neuroblastoma cells (Welch et al, 2007), and miR-132, which is found within a region of chromosome that is frequently deleted in hepatocellular carcinoma (Calin et al, 2004).

Identification of the targets of the miR-124a revealed several genes that may have a direct link to tumorigenesis and metastasis. miR-124a regulates genes involved in mitosis, migration, and adhesion, both directly and indirectly. Deregulation of these genes may result in increased cell proliferation and motility, as well as disruption of the basal membrane, all of which are hallmarks of tumorigenic cells (Gupta and Massague, 2006). In a breast cancer precursor model, REST knockdown results in anchorage-independent growth (Westbrook et al, 2005), which may partly be a result of miR-124a-mediated downregulation of the extracellular matrix protein, Lamc1, and its cell surface receptor, Itgb1. Quantitative changes in the concentration and types of integrin expression have been implicated in the processes of malignant transformation (Eble and Haier, 2006). miR-124a also regulates other genes that may be linked to carcinogenesis, including Cebpa, mutations of which are often found in patients with leukemia (Pabst et al, 2001), Tom1l1, a negative regulator of mitogenic signaling by Src family kinases that may have tumor suppressor properties (Franco et al, 2006), and Epim, a morphogen regulating epithelial-mesenchymal transitions that has been implicated in alveolar hyperplasia and mammary adenocarcinoma (Bascom et al, 2005). Further studies are required to determine the role that other miR-124a-regulated genes may have in tumorigenesis. Gene expression analysis of tissues from many human cancers provides invaluable clues on the lineage and differentiation status of these tumors. Interestingly, global miRNA expression in tumors is lower compared to normal cells, suggesting that the cancer cells are in a more undifferentiated state (Lu et al, 2005). While the miRNA profile of many tumors is reminiscent of the tissues from which they were derived, tumors express a distinctive miRNA signature, reflecting the gain or loss of different miRNAs (Volinia et al, 2006). Whether these changes in miRNA expression profile are the cause of oncogenic transformation or merely secondary effects, remains to be determined. Further eludication of the patterns of miRNA expression in tumors, combined with improved methods for miRNA target identification, will contribute to a better understanding of the pathogenesis, markers, and targets for cancer, thus paving the way for novel therapeutic strategies.

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Appendix I. Target validation summary

GENE SYMBOL	MOUSE ACCESSION	GENE NAME	3'UTR LENGTH	ENDOGENOUS mRNA		3'UTR SENSOR	7nt seed	6nt seed		Total seeds	TS[D]	P[E]	M[F]	IP[G]
				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
DIRECT TARGETS														
Ctdsp1	NM_153088	RNA polII C-terminal domain small phosphatase 1	1600	d (0.48)	u (3.36)	u (8.10)	5	1	1	7	x			x
Vamp3	NM_009498	Vesicle-associated membrane protein 3	1674	d (0.39)	u (3.73)	u (5.60)	3	1	1	5	x	x	x	x
Fcho2	NM_172591	FCH domain only 2	2185	d (0.52)	u (1.51)	u (2.87)	1	2	0	3	x			
Ptbp1	NM_008956	Polypyrimidine tract binding protein 1	978	d (0.63)	u (1.36)	u (2.21)	1	2	1	4	x	x	x	
Cd164	NM_016898	Cd164 antigen (sialomucin)	2020	d (0.46)	u (2.37)	u (2.17)	2	0	0	2	x	x	x	
Lamc1	NM_010683	Laminin gamma 1	2426	d (0.41)	u (1.98)	u (2.11)	2	1	2	5	x	x	x	
Cebpa	NM_007678	CCAAT/enhancer binding protein (C/EBP), alpha	1308	d (0.53)	u (1.34)	u (2.10)	3	0	0	3	x			x
Tom1l1	BC004710	Target of myb1-like 1	2807	d (0.53)	u (2.88)	u (2.02)	1	1	2	4	x			
Arfip1	XM_130985	Arfaptin-1 (ADP-ribosylation factor-interacting protein 1)	1590	d (0.52)	u (1.88)	u (2.02)	1	1	1	3				x
Sypl	NM_198710	Synaptophysin-like	2154	d (0.47)	u (1.39)	u (1.90)	1	0	2	3		x		
Hadhb	NM_145558	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	441	d (0.56)	u (1.88)	u (1.84)	0	1	0	1				x
B4galt1	NM_022305	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	2445	d (0.50)	u (1.26)	u (1.77)	3	2	2	7	x	x	x	x
Epim	NM_007941	Epimorphin	1813	d (0.55)	u (1.14)	u (1.75)	1	0	3	4	x			
C6orf72	NM_145418	BC013529 (hypothetical protein LOC215751)	475	d (0.44)	u (1.54)	u (1.74)	2	0	0	2				
Mapk14	NM_011951	Mitogen activated protein kinase 14	2039	d (0.46)	u (1.64)	u (1.54)	1	1	1	3	x	x	x	
Plod3	NM_011962	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (lysyl hydrolase)	262	d (0.56)	u (1.78)	u (1.50)	1	1	0	2	x			
Itgb1	NM_010578	Integrin beta 1 (fibronectin receptor beta)	982	d (0.56)	u (4.09)	u (1.49)	1	0	1	2	x			
Ptpn12	NM_011203	Protein tyrosine phosphatase, non-receptor type 12	759	d (0.67)	u (1.48)	u (1.48)	1	0	2	3	x	x	x	
Lass2	NM_029789	Longevity assurance homolog 2	623	d (0.58)	u (2.07)	u (1.45)	1	0	2	3	x	x	x	
Nek9	NM_145138	NIMA (never in mitosis gene a)-related expressed kinase 9	2256	d (0.59)	u (1.76)	u (1.31)	2	2	1	5				
E2ig4	NM_001024619	Leucine rich repeat containing 54	1247	d (0.60)	u (1.14)	u (1.31)	1	0	1	2				
Ctdsp2	NM_146012	RNA polII C-terminal domain small phosphatase 2	3304	d (0.68)	u (1.46)	u (1.12)	1	2	2	5	x	x	x	
POTENTIAL TARGETS														
Cdc14b	NM_172587	CDC14 cell division cycle 14 homolog B	1352	d (0.48)	ns (0.89)	u (2.90)	2	0	1	3		x		
Mphosph9	XM_893274	M-phase phosphoprotein 9	2901	d (0.61)	ns (1.04)	u (2.09)	2	1	2	5				
Depdc1	NM_029523	DEP domain containing 1a	325	d (0.52)	ns (1.10)	u (1.58)	0	1	0	1				
Parp16	NM_177460	Poly (ADP-ribose) polymerase family, member 16	1063	d (0.92)	ns (1.10)	u (1.53)	1	0	4	5	x		x	
FLJ11259	NM_027878	1200002N14Rik	1854	d (0.57)	ns (1.11)	u (1.49)	2	1	1	4				x
Hebp2	NM_019487	Heme binding protein 2	950	d (0.55)	ns (0.89)	u (1.47)	2	0	0	2				
Iqgap1	NM_016721	IQ motif containing GTPase activating protein 1	991	d (0.52)	ns (1.01)	u (1.36)	1	1	1	3	x		x	

GENE SYMBOL	MOUSE ACCESSION	GENE NAME	3'UTR LENGTH	ENDOGENOUS mRNA		3'UTR SENSOR	7nt seed	6nt seed		Total seeds	TS[D]	P[E]	M[F]	IP[G]
				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
Papss2	NM_011864	3'-phosphoadenosine 5'-phosphosulfate synthase 2	1582	d (0.62)	ns (1.34)	u (1.32)	2	0	1	3				
THG-1	NM_023910	TSC22 domain family 4 (Tsc22d4)	401	d (0.55)	u (1.48)	ns (1.46)	1	0	0	1				
Gcl	NM_011818	Germ cell-less homolog	1011	d (0.57)	u (1.62)	ns (1.16)	2	0	0	2				
Slc30a7	NM_023214	Solute carrier family 30 (zinc transporter), member 7	678	d (0.62)	u (1.31)	ns (1.15)	1	0	0	1	x			
Serp1	NM_030685	Stress-associated endoplasmic reticulum protein 1 (D3Ucla1)	1770	d (0.53)	u (1.85)	ns (1.12)	2	0	1	3	x		x	
MGC17943	NM_001013028	Expressed sequence AI597468	582	d (0.55)	u (1.26)	ns (1.08)	0	0	0	0	x			
Chsy1	XM_194358	Carbohydrate (chondroitin) synthase 1	1350	d (0.69)	u (1.45)	ns (1.05)	1	0	1	2	x	x	x	
Ct120	XM_282971	2310047D13Rik	1130	d (0.49)	u (1.69)	ns (1.04)	1	0	1	2				
Atp6v0e	NM_025272	ATPase, H+ transporting, V0 subunit	372	d (0.53)	u (3.21)	ns (1.02)	0	3	1	4	x			x
Rarg	NM_011244	Retinoic acid receptor, gamma	1025	d (0.46)	u (1.50)	ns (0.97)	2	0	1	3	x			
Hadhsc	NM_008212	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	680	d (0.64)	u (2.23)	ns (0.96)	1	1	0	2	x	x		x
Myh9	NM_022410	Myosin, heavy polypeptide 9, non-muscle	1300	d (0.50)	u (1.29)	ns (0.95)	1	2	3	6	x	x		
Abhd5	NM_026179	Abhydrolase domain containing 5	1750	d (0.66)	u (1.39)	ns (0.94)	1	0	0	1	x			x
Litaf	NM_019980	LPS-induced TN factor	1566	d (0.72)	u (1.61)	ns (0.93)	0	1	2	3	x	x	x	x
Slc16a1	NM_009196	Solute carrier family 16 (monocarboxylic acid transporters), member 1	2671	d (0.62)	u (1.29)	ns (0.93)	1	0	0	1	x	x	x	
Aldh9a1	NM_019993	Aldehyde dehydrogenase 9, subfamily A1	746	d (0.58)	u (1.28)	ns (0.88)	2	0	1	3	x			
Pgrmc2	XM_130859	Progesterone receptor membrane component 2	2116	d (0.70)	u (1.24)	ns (0.87)	1	0	1	2	x		x	
Tead1	NM_009346	TEA domain family member 1	1224	d (0.62)	u (1.93)	ns (0.86)	0	0	1	1	x			
Cav1	NM_007616	Caveolin 1	1750	d (0.73)	u (1.62)	ns (0.84)	1	0	0	1	x			
Plp2	NM_019755	Proteolipid protein 2	445	d (0.45)	u (2.03)	ns (0.83)	1	1	0	2	x			x
Man2a1	NM_008549	Mannosidase 2, alpha 1	2350	d (0.45)	u (1.50)	ns (0.82)	1	0	1	2				
Ryk	NM_013649	Receptor-like tyrosine kinase	500	d (0.73)	u (1.33)	ns (0.77)	0	2	1	3				
Acaa2	NM_177470	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	265	d (0.69)	u (1.25)	ns (0.80)	0	0	2	2		x		
Kiaa1102	BC042189	3732412D22Rik	1751	d (0.87)	u (1.28)	ns (0.76)	1	0	1	2	x			
Ahr	NM_013464	Aryl-hydrocarbon receptor	2564	d (0.61)	u (2.41)	ns (0.75)	1	2	1	4	x	x	x	
Peci	NM_011868	Peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase	133	d (0.86)	u (1.14)	ns (0.74)	1	0	0	1				
C14orf32	NM_178684	C130032J12Rik	3529	d (0.62)	u (1.29)	ns (0.72)	0	0	2	2				x
Dvl2	NM_007888	Dishevelled 2, dsh homolog	2521	d (0.50)	u (1.27)	ns (0.72)	0	5	1	6				
Ak2	NM_016895	Adenylate kinase 2	929	d (0.68)	u (1.56)	ns (0.71)	1	1	1	3	x			
Trip11	XM_127084	Thyroid hormone receptor interactor 11	710	d (0.84)	u (1.53)	ns (0.71)	0	0	0	0				x
Cdk4	NM_009870	Cyclin-dependent kinase 4	182	d (0.82)	u (1.42)	ns (0.70)	0	0	1	1				
Chp	NM_019769	Calcium-binding protein p22 (Calcium-binding protein CHP) (Calcineurin homologous protein) (Sid 470)	1706	d (0.55)	u (1.80)	ns (0.69)	1	1	2	4	x		x	
Rela	NM_009045	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	689	d (0.62)	u (1.56)	ns (0.69)	1	0	0	1				

GENE SYMBOL	MOUSE ACCESSION	GENE NAME	3'UTR LENGTH	ENDOGENOUS mRNA		3'UTR SENSOR	7nt seed	6nt seed		Total seeds	TS[D]	P[E]	M[F]	IP[G]
				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
Nme4	NM_019731	Expressed in non-metastatic cells 4	252	d (0.58)	u (1.40)	ns (0.68)	1	0	1	2	x			x
Podxl	NM_013723	Podocalyxin-like	3439	d (0.59)	u (1.47)	ns (0.67)	0	2	2	4			x	
C9orf88	NM_146119	9130404D14Rik; Niban-like protein	1204	d (0.59)	u (1.46)	ns (0.65)	2	0	0	2	x			
Slc22a5	NM_011396	Solute carrier family 22 (organic cation transporter), member 5	1131	d (0.62)	u (1.34)	ns (0.64)	1	0	0	1	x			
MGC5508	NM_134142	1110006I15Rik (hypothetical protein LOC68539); TMEM109	1002	d (0.62)	u (1.24)	ns (0.64)	1	0	1	2	x			
Kis	NM_010633	U2AF homology motif (UHM) kinase 1 (UHMK1)	1451	d (0.60)	u (1.10)	ns (0.64)	0	0	0	0				
Slc25a30	NM_026232	Solute carrier family 25, member 30	1468	d (0.59)	u (1.65)	ns (0.61)	0	1	0	1				
Spc18	NM_019951	Sec11-like 1	410	d (0.68)	u (1.34)	ns (0.59)	0	1	0	1				
Mad2l2	NM_027985	MAD2 mitotic arrest deficient-like 2	273	d (0.77)	u (1.73)	ns (0.58)	0	0	2	2				x
Slc17a5	NM_172773	Solute carrier family 17 (anion/sugar transporter), member 5	1511	d (0.68)	u (1.59)	ns (0.58)	0	0	1	1	x			x
FLJ46072	NM_134087	Expressed sequence AA409316	733	d (0.65)	u (1.35)	ns (0.58)	1	1	1	3				
FLJ10099	XM_355643	0610007L01Rik (BC033455)	2263	d (0.67)	u (1.32)	ns (0.58)	0	1	1	2				
Tnfrsf21	NM_178589	Tumor necrosis factor receptor superfamily, member 21	1104	d (0.70)	u (1.04)	ns (0.58)	0	0	3	3				
Dnm2	NM_001039520	Dynamin 2	650	d (0.66)	u (1.31)	ns (0.57)	2	1	0	3	x			
Syngr2	NM_009304	Synaptogyrin 2	792	d (0.54)	u (1.42)	ns (0.53)	0	2	0	2				
Btg3	NM_009770	B-cell translocation gene 3	439	d (0.58)	u (1.26)	ns (0.53)	0	1	0	1				
Rnpepl1	NM_181405	Arginyl aminopeptidase (aminopeptidase B)-like 1	924	d (0.67)	u (1.33)	ns (0.52)	2	1	0	3				
Lrrc1	NM_172528	Leucine rich repeat containing 1	964	d (0.60)	u (1.26)	ns (0.52)	2	1	0	3	x	x	x	x
G3bp	NM_013716	Ras-GTPase-activating protein SH3-domain binding protein	1171	d (0.71)	u (1.26)	ns (0.51)	1	0	2	3	x			
Pttg1ip	NM_145925	Pituitary tumor-transforming 1 interacting protein	1620	d (0.44)	u (2.70)	ns (0.50)	1	2	2	5				
Tln1	NM_011602	Talin 1	311	d (0.51)	u (1.62)	ns (0.40)	1	3	0	4	x			
FLJ20847	NM_181403	Vacuolar protein sorting 37C	1167	d (0.56)	u (1.87)	ns (0.37)	2	0	0	2				
NON-SPECIFIC TARGETS														
RAI	NM_001010836	Protein phosphatase 1, regulatory (inhibitor) subunit 13 like	503	d (0.28)	d (0.51)	ns (1.11)	1	1	1	3				
Ahrr	NM_009644	Aryl-hydrocarbon receptor repressor	2472	d (0.92)	ns (1.04)	ns (1.17)	0	1	0	1				
Ankrd15	NM_181404	Ankyrin repeat domain 15	886	d (0.67)	ns (1.16)	ns (1.10)	1	0	0	1	x	x	x	x
Htatip2	NM_016865	HIV-1 tat interactive protein 2, homolog	451	d (0.50)	ns (0.99)	ns (1.06)	2	1	0	3	x			x
Araf1	NM_009703	v-raf murine sarcoma 3611 viral oncogene homolog	394	d (0.69)	ns (0.98)	ns (0.97)	0	0	1	1				
Twist2	NM_007855	Twist homolog 2	666	d (0.55)	ns (0.93)	ns (0.97)	1	0	0	1				
Anxa8	NM_013473	Annexin A8	802	d (0.76)	ns (0.81)	ns (0.91)	0	0	1	1				
Creb3l2	NM_178661	Camp responsive element binding protein 3-like 2	1212	d (0.76)	ns (0.90)	ns (0.88)	0	0	2	2				
D4st1	NM_028117	Dermatan 4 sulfotransferase 1	775	d (0.70)	ns (1.10)	ns (0.85)	1	1	0	2				
Phf19	NM_028716	PHD finger protein 19	1743	d (0.54)	ns (1.06)	ns (0.85)	1	2	3	6	x	x		
Pp1201	NM_027154	Transmembrane BAX inhibitor motif containing 1	1010	d (0.60)	ns (1.02)	ns (0.85)	1	1	0	2				

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				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
		(Tmbim1)												
Arhgef1	NM_008488	Rho guanine nucleotide exchange factor (GEF) 1	245	d (0.33)	ns (0.84)	ns (0.83)	1	0	2	3				
Loc55974	NM_009057	Recombination activating gene 1 activating protein 1 (Rag1ap1)	220	d (0.66)	ns (1.06)	ns (0.82)	1	0	0	1				
F11R	NM_172647	F11 receptor	869	d (0.81)	ns (0.71)	ns (0.82)	0	0	2	2	x			
Arh	NM_145554	Low density lipoprotein receptor adaptor protein 1 (Ldlrap1)	2208	d (0.76)	ns (0.65)	ns (0.82)	1	2	2	5				
Parg1	NM_172525	Rho GTPase activating protein 29 (Arhgap29)	1055	d (0.81)	ns (0.66)	ns (0.79)	0	0	0	0				
Smad5	NM_008541	MAD homolog 5	2953	d (0.68)	ns (0.87)	ns (0.78)	2	1	7	10	x			
C3orf4	NM_171826	Claudin containing domain 1 (Cldndl)	1041	d (0.71)	ns (0.91)	ns (0.74)	1	0	0	1	x			
Gnai3	NM_010306	Guanine nucleotide binding protein, alpha inhibiting 3	1900	d (0.59)	ns (1.14)	ns (0.68)	2	0	1	3	x			
Lmnb1	NM_010721	Lamin B1	742	d (0.78)	ns (1.01)	ns (0.67)	0	0	0	0			x	
MGC20741	NM_198421	USP49	3002	d (0.62)	ns (0.92)	ns (0.65)	1	0	1	2				
Ctgf	NM_010217	Connective tissue growth factor	1040	d (0.61)	ns (0.76)	ns (0.64)	0	1	0	1				
Apex2	NM_029943	Apurinic/apyrimidinic endonuclease 2	253	d (0.73)	ns (0.92)	ns (0.63)	0	1	0	1				x
Gsn	NM_146120	Gelsolin	192	d (0.51)	ns (0.83)	ns (0.62)	1	0	0	1				
Pgm1	NM_028132	Phosphoglucomutase 1	464	d (0.93)	ns (1.03)	ns (0.61)	1	1	1	3	x			
Zbed3	NM_028106	Zinc finger, BED domain containing 3	990	d (0.65)	ns (0.75)	ns (0.61)	1	0	4	5				
Ram2	NM_146040	Cell division cycle associated 7 like	1200	d (0.67)	ns (1.18)	ns (0.60)	0	0	0	0				
Arpc1b	NM_023142	Actin related protein 2/3 complex, subunit 1B	174	d (0.68)	ns (1.18)	ns (0.59)	1	0	0	1				
FLJ20273	NM_178446	9530077J19Rik (BC013481); RNA-binding protein	901	d (0.58)	ns (1.26)	ns (0.65)	0	0	0	0				
Eya4	NM_010167	Eyes absent 4 homolog	1820	d (0.86)	ns (1.18)	ns (0.58)	1	1	1	3	x	x	x	
Elovl1	NM_019422	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	430	d (0.47)	ns (1.05)	ns (0.58)	0	0	1	1		x		
Snai2	NM_011415	Snail homolog 2	952	d (0.61)	ns (1.05)	ns (0.58)	2	1	0	3		x		x
Swap70	NM_009302	SWA-70 protein	1910	d (0.77)	ns (0.86)	ns (0.58)	0	1	2	3				
Cdca7	NM_025866	Cell division cycle associated 7	1060	d (0.68)	ns (1.16)	ns (0.56)	0	2	1	3				
C14orf24	NM_028527	1700047I17Rik	3004	d (0.78)	ns (1.01)	ns (0.55)	1	0	1	2	x	x	x	
NS5ATP13TP2	NM_178644	D9Ucla1	1216	d (0.51)	ns (1.19)	ns (0.54)	3	0	0	3	x			
Cpne3	NM_027769	Copine III	1512	d (0.65)	ns (1.18)	ns (0.51)	0	0	1	1				
TBDN100	NM_053089	NMDA receptor-regulated gene 1 (Narg1)	1100	d (0.76)	ns (1.10)	ns (0.51)	0	0	1	1				
Dnajc1	NM_007869	DnaJ (Hsp40) homolog, subfamily C, member 1	2954	d (0.52)	ns (1.06)	ns (0.51)	0	1	3	4	x	x	x	x
Polr3g	XM_283153	Polymerase (RNA) III (DNA directed) polypeptide G	2112	d (0.85)	ns (0.86)	ns (0.51)	1	0	0	1				
Cd59	NM_181858	Cd59 antigen	112	d (0.58)	ns (1.07)	ns (0.50)	0	0	0	0				
FLJ10420	NM_025383	NECAP endocytosis associated 2	954	d (0.60)	ns (0.91)	ns (0.50)	0	0	1	1				
Dctd	NM_178788	Dcmp deaminase	1015	d (0.79)	ns (1.03)	ns (0.49)	1	0	1	2				
Hspc135	NM_025332	GTP-binding protein 8 (putative)	381	d (0.79)	ns (0.98)	ns (0.48)	0	0	0	0				
Tjp2	NM_011597	Tight junction protein 2	864	d (0.73)	ns (1.06)	ns (0.47)	1	0	1	2	x			

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				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
Dffb	NM_007859	DNA fragmentation factor, beta subunit	830	d (0.74)	ns (0.86)	ns (0.44)	0	0	0	0				
Ifrd2	NM_025903	Interferon-related developmental regulator 2	433	d (0.48)	ns (0.86)	ns (0.42)	0	0	0	0				x
Pldn	NM_019788	Pallidin	1366	d (0.77)	ns (1.07)	ns (0.30)	1	0	3	4	x		x	
NOT DETERMINED														
Ctnnd1	NM_007615	Catenin (cadherin associated protein), delta 1	2010	u (2.26)	ns (1.03)	ns (0.80)	2	0	0	2	x			
Nek6	NM_021606	NIMA (never in mitosis gene a)-related expressed kinase 6	2618	ns (1.08)	u (1.10)	ns (0.86)	1	1	0	2				
Apim2	NM_009678	Adaptor-related protein complex 1, mu 2 subunit	2262	ns (1.25)	u (1.92)	ns (0.85)	1	1	1	3				
FLJ25084	XM_888129	2300003P22Rik	441	ns (1.07)	u (1.17)	ns (0.48)	0	0	2	2				
C18orf37	NM_172625	D030070L09Rik	1738	ns (1.06)	ns (0.94)	ns (0.93)	0	0	0	0				
MGC62100	NM_146185	6330581L23Rik	672	ns (1.01)	ns (1.01)	ns (0.89)	0	0	0	0				
Kiaa0830	NM_028013	2310067E08Rik; Endod1	800	ns (1.41)	ns (0.83)	ns (0.80)	0	0	0	0				
Ssfa2	NM_080558	Sperm specific antigen 2	1166	ns (0.94)	ns (1.00)	ns (0.77)	1	0	0	1				
MGC4083	NM_026473	Tubulin, beta 6	348	ns (0.95)	ns (1.01)	ns (0.76)	0	0	1	1				
Cyp1b1	NM_009994	Cytochrome P450, family 1, subfamily b, polypeptide 1	3031	ns (1.12)	ns (0.99)	ns (0.75)	0	0	1	1				
FLJ20364	NM_027411	2600001J17Rik	242	ns (1.14)	ns (1.01)	ns (0.71)	0	0	0	0				
Fam35a	NM_029389	3110001K24Rik	153	ns (0.92)	ns (0.94)	ns (0.71)	0	0	0	0				x
Cdk6	NM_009873	Cyclin-dependent kinase 6	1412	ns (0.91)	ns (0.93)	ns (0.67)	1	0	4	5	x	x	x	
Usp48	XM_485461	Similar to ubiquitin specific protease 48	2440	ns (1.10)	ns (1.06)	ns (0.66)	0	0	1	1	x		x	
Mtmr6	NM_144843	Myotubularin related protein 6	1724	ns (0.92)	ns (1.06)	ns (0.59)	1	0	0	1			x	
Slc15a4	NM_133895	Solute carrier family 15, member 4	884	ns (0.98)	ns (1.14)	ns (0.54)	1	0	0	1	x	x		
Elf4	NM_019680	E74-like factor 4 (ets domain transcription factor)	1401	ns (0.90)	ns (0.95)	ns (0.50)	0	0	1	1				
Uhrf1	NM_010931	Ubiquitin-like, containing PHD and RING finger domains, 1	904	ns (1.00)	ns (1.02)	ns (0.44)	1	0	0	1	x			
Serpinb6	NM_009254	Serine (or cysteine) peptidase inhibitor, clade B, member 6a	--	d (0.37)	u (2.20)	--	0	0	0	0				x
FN5	NM_133214	FN5 protein (BC017612)	408	d (0.38)	u (1.78)	--	1	0	0	1	x			
Rassf5	NM_018750	Ras association (RalGDS/AF-6) domain family 5	2020	d (0.48)	u (1.78)	--	2	2	1	5	x			
Stom	NM_013515	Stomatin	--	d (0.52)	u (1.64)	--	0	0	0	0				
FLJ21924	XM_130565	4732486I23Rik; Qser1	3615	d (0.57)	u (1.64)	--	2	0	2	4				
Nfic	NM_008688	Nuclear factor I/C	4717	d (0.77)	u (1.60)	--	2	2	2	6				
TTC7A	NM_028639	Tetratricopeptide repeat domain	1627	d (0.65)	u (1.42)	--	2	2	0	4				
Dhcr24	NM_053272	24-dehydrocholesterol reductase	2289	d (0.65)	u (1.32)	--	1	1	2	4	x	x	x	
Ankrd27	NM_145633	Ankyrin repeat domain 27 (VPS9 domain)	--	d (0.60)	u (1.14)	--	0	0	0	0	x			
Katna1	NM_011835	Katanin p60 (ATPase-containing) subunit A1	--	d (0.59)	ns (1.14)	--	0	0	1	1		x		x
Tarbp1	ENSMUST 00000046393	Tar (HIV-1) RNA binding protein 1	--	d (0.58)	ns (1.09)	--				0		x		x
Sp1	NM_013672	Trans-acting transcription factor 1	1336	d (0.72)	ns (1.07)	--	0	0	2	2	x			

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				miR-124a overexpress (MEFs)[A]	miR-124a depletion (neurons)[B]	miR-124a depletion (neurons)[C]	GTGCCTT	GTGCCT	TGCCTT					
Rbms1	NM_020296	RNA binding motif, single stranded interacting protein 1	--	d (0.63)	ns (0.85)	--	0	0	0	0	x		x	
C10orf56	XM_484355	2310047A01Rik	3560	d (0.34)	ns (0.75)	--	1	1	3	5	x		x	
Actr8	NM_027493	ARP8 actin-related protein 8 homolog	--	u (1.46)	ns (0.92)	--	0	0	0	0				
Osbpl8	NM_175489	Oxysterol binding protein-like 8, isoform a	3900	ns (0.26)	u (1.54)	--	2	1	1	4	x			
Gng10	NM_025277	Guanine nucleotide binding protein (G protein), gamma 10	728	ns (0.71)	u (1.51)	--	1	0	1	2				
Trim29	NM_023655	Tripartite motif protein 29	856	ns (0.84)	ns (0.86)	--	0	0	0	0				
C2orf12	NO HOMOLOGUE													
Contig3845_RC	NO HOMOLOGUE													
Hic	NO HOMOLOGUE													
Loc116064	NO HOMOLOGUE													
Loc339924	NO HOMOLOGUE													
Stx10	NO HOMOLOGUE	syntaxin 10												x

[A] Fold change in endogenous mRNA levels in mouse embryonic fibroblasts (MEFs) transfected with miR-124a duplex as measured by quantitative RT-PCR. Significance of changes were determined using the Student's t-test; u, upregulated ($p<0.05$); d, downregulated ($p<0.05$); ns, not significant ($p>0.05$).

[B] Fold change in endogenous mRNA levels in cortical neurons transfected with a miR-124a antisense 2'O-methyl oligonucleotide as measured by quantitative RT-PCR. Notations are the same as in (A).

[C] Fold change in 3'UTR sensor activity in cortical neurons co-transfected with a miR-124a antisense 2'O-methyl oligonucleotide. Notations are the same as in (A).

[D] TargetScanS (Lewis et al, 2003; Lewis et al, 2005)

[E] PicTar (Krek et al, 2005)

[F] MiRanda (John et al, 2004)

[G] Ago2 immunoprecipitation (targets with net enrichment $p<0.005$; Karginov et al, manuscript in preparation)

Appendix II. Target validation primers

Gene	3'UTR Luciferase assay primers		Quantitative PCR primers	
	Forward	Reverse	Forward	Reverse
Abhd5	CACCTGGACTGGACTCTGCAGCTTTC	CAACAATCACTGCACACACC	AGGAGGAGGTGGACTCGGCAGAC	TGTGATGTAGATGTGGGACACCAG
Acaa2	CACCCATCACAAGCACACTGCCCACAC	GGAGTTAAGGCAACATTTATTCAGTTT	TGGTTTCCAGTCCATCGTGAGC	CCTCCACACAAGACGACCTCAGC
Actr8	--	--	CCATCGTACCCGCGTTGGTC	TGACAACAATGAAGTTGCTCTGGA
Ahr	CACCTCCGACTTGAGCAGCATTGG	GGACAGCAAGGAGCTATGTAGACC	CATCACCTATGCCAGCCGCAAG	TCTGTGTCGCTTAGAAGGATTTGA
Ahrr	CACCTTCAGTCCTTGGATAATGGAACA	CAAACCACCACATGAGAAATGC	CGCCGGTAGGAAGAGAAGGAAGC	CCGGTGCCGTTTGGAAGGAT
Ak2	CACCACAGAAGGCCAGGCGAGACC	GGGCCAAACATGGCAGCATT	TCGGACTGTGAGGCAGGC	GCAGCGAGTCTTGGATGCTG
Aldh9a1	CACCGCTGTGGAATGAGTTGACTGCCTTG	TTTATTATTGTGCAGTGTATGATATGG	GTGTGGAGCCGGTGGATGCT	GAACACGCGAAAGTGGCAATCA
Ankrd15	CACCTTATGTAAGCATGGCCCTTGTGTG	TATGGCAAGAGTCTAAGGCACTTCAAA	GGGAATAAAGATTCAAATGGAGCCAAA	CCGAGCTGGATTCGTCACTTG
Ankrd27	--	--	CCACGGAAGGACACTTTCAGACC	GGCACAGAGAGAAGGCAAGCA
Anxa8	CACCGAACAAGGGCAAGAAGTGTGA	TGAAAGTACAAATGTTTATGATTCTGA	CAGGATGGCCTGGTGGAAAGC	TCAGGGTCTGGGTTGAAATGAGAA
Apex2	CACCCAGACTGAGGCATAGGGATGTCTTCG	ACAAAGACGTGACCTTTATTTATGAAC	TTCAGCCGCAGCCGTAGTG	CTTACAGAAGGTAGCCACACCTG
Apim2	CACCGCGAGTGTGTGCAGGGTTAGGG	TGCAATATGTAGCCAAGGATAACACC	TGCACTTCCTGTGGATCAAA	GCATTAGCGTTCTTCAGCGTGGT
Araf1	CACCCTCACTCCCAGCCCACCA	TCTGTGCTTCTTGGTAAAGAGGAGA	TGTCAAAGTGTACCTGCCTAACAA	GGGCCTTGTCCAAAGAGTCA
Arfip1	CACCGGGAGGCCGTGGAGTGACC	AGGACAGGGCAACTGGATTCTGC	AGTAATGGAGAAGTTGACGATGCT	TCCAAGTCCAGATGGTAATGAATGCT
Arh	CACCGGGACGGACCATAAGCCACCA	TCCCAAGAACATAGGCACAGGAGA	CCTTGGTATGACGCTGGTGGA	GCTTCTTCCCGCTGGCCTTG
Arhgef1	CACCAGCTGCCACAGCATCTCACA	AAACATAGCAAAGGCACTTGGG	CAGAGGACAATGGCGAGACTGA	CTCCACCCAGGAGGTTCTTC
Arpc1b	CACCGACTAGACTGCTGCCCTCATCC	GCAATAAAGAGTATTTCTTTAAAAGGCACA	GGAGCACAACGGGCAGGTGA	TCAGCGTCCACACATAGGCATT
Atp6v0e	CACCGATGAAGACGCGCTGCACAG	TCCTCTTGAGCAGGCACTCC	CTGGCACAGCTCAATCCTCTG	GGAGGAAGGTCTGATGTGCAGG
B4galt1	CACCTGGGCTGGTCCCTCTCATTT	TGAATGCCAAGGTCAGAGACA	TGGCTGTATTATTTGCATCCCATCC	CTTAGCTCGATTGAACATGGTGTCT
Btg3	CACCTCCTTTCATTGTGTTGATGTCATGTT	TTGTCATGCCTCAAACTACTTT	GATCCGTGTGAGGTGTGCTG	CAAAGCTGGCAACAATGAACG
C10orf56	CACCAGCTGCCACCTCCCTGTGTTG	TATAGTCTCCAGGAATAAGGCACACAA	GGGCAGGAGTGCATCAAGTGC	CAGGCCGTCAGGCTTCTCCA
C14orf24	CACCAAACCTTCCTTCATTCTTATGCTG	GCCATCTCACCAGCCCTCTGC	AAATACCAGTACGCCATTGATGA	TGTCTTTCTGCTTCTTCTGACATCC
C14orf32	CACCTTGACAGTGGATGAAGAGATGG	TCCAAATACAGCAGCAGGTGACAA	CCGAGTAGCGCAGCACCCTCT	GTCCGGTTGGAGGCATGGAG
C18orf37	CACCCCTCAGAAGTGGAGACGGAGTGG	TTCAAACACATACAGAGTAGACTGGTG	CCAAGCCGTTGCCATTTAAGGA	TCTTCCAGGTTCTGTTCTTCTTGC
C3orf4	CACCTTCCCTTGTCTTCCTCTCAGTCG	TGAATCTGTGAAATGCTACAATAATGG	TATGCGCCACCGGAAAGGAC	GGGTCCACATACTTCTCCATGAACTGC
C6orf72	CACCCCATCTCCAATCATGGACTCTATTGC	TCCTGGTGCATTCAAGGTAAGG	GGGATGGCTGACCACCAACG	CAGGGTAGTTACATTGATTTGGATGC
C9orf88	CACCGGCTTCTGTTGGACTCTGCACCA	AAAGCGTTTATTGATGAGCAGAA	TGTCCACACACCTGGATGACG	TGGTCCTCATAGAAGCGAAGGAA
Cav1	CACCCCCTTACTATTTCCTTGGTGCCAATTC	AAATTTCAGTAACCTTTTGGATCAGCA	CGCACACCAAGGAGATTGACC	GGCCTTCCAGATGCCGTC
Cd164	CACCTCCCACATGGAAGACGCTGT	GCAGCAAGTATGGCAAAGGT	CCGACCACGACCACCAAAGTG	TGGCATTAACACAGGAAACACAGC
Cd59	CACCAGGAGAGCCCACTTGCAGT	TGCCAGGAACTCACTTAACCTGAATCT	GCCGGAAGGCAAGTGTATCAACAG	CCTGCCACGTCTAATCGGCTCAT
Cdc14b	CACCATCTGGACCCTTTGACAGA	TTGTGGCAGCAGATAAATTCTGG	GCACCGCCTGGAGCAGCA	TCGTTTGTTGCACTCTTTGGTCTG
Cdca7	CACCTGAAGATTGTCTGCCCGCCTTC	AATGATTCAAGATCCAACTGAGCACCA	CCCGGAACGGAGAACTCGAC	TTCCTCTGTCTCACCAGCATGT
Cdk4	CACCCTTGGTGGAGAAACCCTCGCTG	GAAGACAGATACACCTGCCCTTT	CCAGATAAAGGGCCACCTCCAGA	ACCAATTTCAGCCACGGGTTCAT
Cdk6	CACCGCTCGTCATCTGAACACATTGG	CAGAAACACATCCTGCGGACAGA	CCGGCGTACCCACAGAAACC	TGTGAGAATGAAGAAAGTCCAGACCTC
Cebpa	CACCGGAGACCCAGAGGATGGTTTCG	GAAGAGAAGGAAGCGGTCCA	GCCGCCTTCAACGACGAGTT	CGCCTTGGCCTTCTCCTGCT
Chp	CACCTGCATGACAACCTGAAGTCTCTCC	AATGGGACAATAAGGCACAACTG	CCACGTTATTGCGGGACGAAGAG	AGGCGCGTGATCTGACTGTGG

Gene	3'UTR Luciferase assay primers		Quantitative PCR primers	
	Forward	Reverse	Forward	Reverse
Chsy1	CACCTCCGGCCCCACTGCGGAGAACGTTT	TCCACTGGTTTATTTGTTCGAGTGTGC	CAGGCGTGCAGTGTGTCTGG	AATGTACCCTTTCTTGTTCTGTTCA
Cpne3	CACCGGACAGTAGCAAGTCCATTTCTGCTCA	GAGGTGCATTGCTTCAGATTAACAGC	CGCTGGCTCTCCACCCTCAC	CAGGAAACATTTAGTTCCACCTTCG
Creb3l2	CACCGCTCCACCCTCCTCTTCTCC	TGTCTCTTTGGATGAGCTGCAA	TCAGAGCCCGGAGAGACTGAGG	CAGGAGCTGACCCAGGACGTT
Ct120	CACCGGGTCAGGCACTCACACTAGC	GGCACCTATTAAAGACTGAAACTCAAA	CCTTGGGCCTGGGAAAGAGT	TGCCCTGAGTGGGTGGGAAG
Ctdsp1	CACCGCATGGAGGACAGTGAGCC	CACTCTGAGGTGCTCAGGTC	CCTGCTGGTGGAGGAAAATGG	GCTTGAAGGAGCTGTGCACC
Ctdsp2	CACCTGCTCCAAGAAACGGCCATCC	GGCATCACGGGAATAGCCAGA	GGCCAAGTATGCTGACCCTGTGA	TGGTGGAACACACAAGCCTCTCG
Ctgf	CACCGCCAGGAAGTAAGGGACACGAA	TTCAACTAGAAAGGTGCAAACAT	GCCAACCGCAAGATCGGAGT	CGACCCACCGAAGACACAGG
Ctnnd1	CACCCGCCACTCTACCTGCTCCATA	CCCTTAGTAGTATTCGGTGGGAGGGTA	ACCAATCAATTACGGGCCTTCTC	CCACAGAGGGAGTAGAAAGGAGGGAGT
Cyp1b1	CACCGCCGGAGGGAGCTGGAATGT	TCTGTGTACGGAGGATCTGTGTGC	GCCAGCCAGGACACCCTTTC	CACGCGGGCCTGAACATCC
D4st1	CACCAGTAGGCCAGCACCTTTTG	GTCAGCCCTTACAAGGATCCT	CATGATCGAGCGAGGCATCC	TTGTGGCTGGGAGGGTGCAG
Dctd	CACCACTTGGAATCTTCTCACCCGTC	AATGCTCCAGAATACGGAGTG	GGACGGTGCAAGCGTGGTCT	TGCAGGAAATGTCACTCATGTTGG
Depdc1	CACCTCAACAAATGAAGCCGTACATG	AAAGAAGCAATCTTAAACAACCAGTTC	CTAAGAAGCGTGGATTACATTTCTCT	TCTTGGTTGTCAGGTGCAATTTCT
Dffb	CACCACAGCCCAGTGTGCAGAGGA	AAATAAACTCAGGTTGGGCGTGGTG	GTAAGGGCTGCGTCCGCTTC	CCGTCACCTCCGTGCCATCT
Dhcr24	CACCGCTGCATGGACCTGCTCTCC	CAGAACCACAGGGACATGAC	CGCCTGGGTGGTGTTCAAGC	TGCCCTGTTCCTTCCATTCC
Dnajc1	CACCGGGCTTTGGATGCTGTTGGTG	AAAGACACCTTCTCCCTCTTCC	CGACTTGGTGGAAGAGGTGCAGTT	CGATATGCTTTTCTGATGTCTGC
Dnm2	CACCTGTAGGACAGCTTTGGTGGTCTGG	GGATATACAGAGGCAGGCACTTAACA	CACAGGCACCAACAAAGGCATCT	TGGGAGGTCGATGAGGGTCAAGT
Dvl2	CACCGGCCACATTGGGTCTGTGC	AAAGCTGGCTCGGATGGCTA	TCGGATGAGGATGACACCATGAG	CTTGAGGAGGCGGGAGGCACT
E2ig4	CACCGACTGCTGCCTGGGCTTGCT	TGGGACCAGCCTCAATGC	CGTGCCGAGGCGACAACTG	CAGGAACAGAGAGCACAGCATGG
Elf4	CACCAAGTATGACCCACCGGGCAAA	TCACATTAACCAAAGTCGCCAGAGG	TGCAAGCAACGGGATGGATG	CACGATCACGGCTGGGAACA
Elovl1	CACCTGGCCTAGACATTGCCCACCTAA	CTGCCTGCCCATTCTGACCTTTG	CGGCCCTGATCCCTTTGAACC	CACAACAGCCTCCATCCTGGCTA
Epim	CACCCGTAGATGGCGCTGGGTGCT	GGCTATGAAGTCAGCAGAGAGATG	CACTGCTGTCGTCATTGTGGAG	CAATCCTGGCTATGCTGCTTC
Eya4	CACCGTGATCCAATGCCGCTGGCTTC	CATTTGTAAACAGGGCCACAGCA	TTCAGTCACAACAAATGGGACAGG	GCGCTCCATCTCCAGTCGTG
F11r	CACCGCTGCGGCTCCTCCGTTG	AAACACACAGAAACACAACCTTGC	TCCAAGGCAGCACAACTGCAC	CACTGGATGAGAAGGTGACTCG
Fam35a	CACCTGCCTGTGTATGTCTGTGTACCATGT	TCATAGGGTTGGATGGCTCA	TCCCGACTCATCCCGAATCC	GGGCCTCCCTCCTGTCCTGA
Fcho2	CACCCCTCGCCTGTTCCTTCCAAACAC	CAACCCAAAGTAGTAAATAACGATCC	GGAGAGGAGCAAGTCAAGTCTCA	TCTGGAGTGCCTGAGTTATATTCTG
FLJ10099	CACCTCATTGCCTGCTGAATCG	GGGAAGGGACGCCATCACAG	CCACCATCCTCGGGCATCAG	ATCGGCGTTCCAGGCAGCA
FLJ10420	CACCGACCCACGGAAGGTGGCAGAGT	TCTGGTCACACAAGGTCAGCACA	GTCAAGCCGGAGGTCCACGTCTA	CCACTCCACGATGGCTGGTC
FLJ11259	CACCGACAGAGGACGGACGGGCTTG	TCTCGGGTACAAAGTCCAAGGAA	AATGGGCATCGTAGCCAACTTCC	CCCGCAGACGAAAGCCAGAA
FLJ20273	CACCGAGCCAAGGCAAGCCACCAA	TGCACTGGAGCTAGGGAAGGAGA	GCAGGAGAAAGGATATGAACTTGTGC	AGCACCGATGGCAGGGATGG
FLJ20364	CACCGCCACGCTAACCATCGCTCCT	TAACCATGTTCAAACTCCACATCCAAC	CATAGGAGCCACCGGCAGGA	GCCTTGCTTCATCCAATTCCA
FLJ20847	CACCTGGTCTGTGTCCACCTTCTTCC	TACCCAGGCAAGGGATGTTTCAA	GGAAATGCAGAATGACCCAGAA	GGCCAGTGCCATCTCTCGTT
FLJ21924	CACCATGTCCACAGAATCCCACGTTT	AAATTGTCTTTCACATTTCCGTACA	GGCACAACTGACTGGTTCACAGC	AAGACGACTGCCTGGATGGTTCC
FLJ25084	CACCCATTTCTTGTTCCCAGCAT	TCAAGGCAAGTGACAGGACAGGA	TGCCTTCGTCCCAGAACCTTTCA	ACTTCAAAGCGGTGCAGCCAAAG
FLJ46072	CACCGGACTCTGCATCACTGCCATACTGAA	TGTCCCGATTCCTTTATTGTACTGC	TACAGCCGCCGCCACCTGAC	CATGTCCATCACCACAGCTACC
Fn5	CACCCACGTCCATTCTGCTTTCCTCTGC	TTTCAAATCCCATCAAGCACAGA	GCAGTGGTTCCTGAGTTTGAAGAAA	CGGCTTGGAGAAAGGCACACA
G3bp	CACCGGCTCTGATCTCCACACAGCACA	TTCCAAGAAACACCCACTGC	GGCGGGAGTTTGTGCGACAG	CCCGTGGGCATAGGAAGAGTT
Gcl	CACCTGCGTTCACGTCCAAGGTGAC	TGGAGGACAATGATCCAGGCTA	TGGAGATTCCTGACCAGAACATTGA	ACAGTGATCCAAATGCGACCTG
Gnai3	CACCACAGGGAGGAGTGTTGAGACCAGATG	TCAACACCATTTTATTAAACCCAAA	TGTGGGCTTTATTGAGAGGATGG	TGCGTGCTGCTGACCCAAGA

Gene	3'UTR Luciferase assay primers		Quantitative PCR primers	
	Forward	Reverse	Forward	Reverse
Gng10	CACCGCTGAGGAAGCCTTGTGTGC	GTCCCCAATTAAATCAAAGGCACATCA	GGTCCTGTGCTCTACTTTGAAGAC	TGCAGTCATCCATCACCATGTGC
Gsn	CACCGCCATCAATGTCACCAATCAGTGC	GGCTTTCATTTCCAAAGTACAGACA	CCCAAAGTCGGGTGTCTGAGG	CCAGGCTCCTTCCCTGCCTTC
Hadhb	CACCAGAAGGAGGCGACCGACTGA	TCTAAGCATTTCAGGTTTAAGAGATGT	GGGTCCGCATTCCATTTCTGC	CCGATGCAACAAACCCGAAAG
Hadhsc	CACCATGGGACAAGGATGAGCAAG	AGTTTATTTATTCCGATTCCAATGTTT	CACCGATGACCAGCCAGAAG	GCACCAAGAGTCGGTTCACG
Hebp2	CACCGGGCAGACCTGCTGCTGAATG	CATTCAAGTTGGAGGGATTTCATGTC	GATGCCGAGCTGGAAAGC	GGTCCGTAGTGCCGGATCTCA
Hspc135	CACCTCAGAGTTCTTCCTGACAACTGGA	TCCTTTCTATTCACATATACACGCTTT	CCGGAACCGCATCGAGTACCT	TCCAACATTGCTTCGGCCTATGA
Htatip2	CACCATGCTGGAGGACATTCGTGA	TTCCTCGGTGAAATACAGAAGC	TTGGTCGGAGGAAGCTCACC	TGAAAGGCAGAAGCATAGACATCCA
Ifrd2	CACCGCCAACGAGAAGGCTACCCATACC	CCAGCACACACATCCATTATAGGC	CTCCGCAAGGGCGGTCAG	GGCCGCTTCATCCTCACTGG
Iqgap1	CACCGCTCGCCGCCCAGAAGAGTTA	TGGAGCTGAACTGTCTTTGTTAGTCA	AGGAGGTTGATGGCCTGGGTGT	CTGCCGTCTCCGCTCATCCAT
Itgb1	CACCACCAAGCTCACAGCAGCAGCATC	GCAAGCATGTAGGAAAGGCATGTG	GTTGGTCAGCAACGCATATCTGG	CACCAAGTTTCCCATCTCCAGC
Katna1	--	--	GGTCCTTGCCTGTACCTGTTGAGC	GCCGGTTACTGTGTGGCTTAGGG
Kiaa0830	CACCAGCAGGTTTCTGTTCCTTGTCAGC	GTTTCAGGAGACAAATCAGACTGT	GGCGAATGCGACCGTTTCTT	CCCGCGAAGCGTTGACAGAT
Kiaa1102	CACCTGGGAGCTTCTGGGCCACTG	GCCGTGGGAACTAGGGAGTGA	ACGCAAGGTCGCAGGAGGAG	TTGGCATCTCCAAGCGTTCG
Kis	CACCAATGCAGGAGCACCCCTTTACC	TGGGAACTTGAACAGCATGGAC	TGGAGCAGTTGCAGGGTCACAG	AGCAGACAGCGTGATGGCACATT
Lamc1	CACCGGCCTCGGTGCCTTGACACA	GCGCTTTATTGCCCTCCCTGA	GCAAAGCCAGACTATGCTGGC	GAGGCGCACGTAAGTGATGTC
Lass2	CACCGCTGCCTCCCACATCAATGC	TTGCCCTTCTACTCCACACCA	TTTCTCGTCATTCGATACTTCTTTGA	ATTGGGAGGTGCCCGCAGTC
Litaf	CACCCCCAGCCAGAAGAGCTTGATGTC	TGCTGCCATTGATGACGACTAAGG	GCAGCAAGATGATCGTGACCC	GGCAGTAGTGGTCCACATCCTG
Lmnb1	CACCCAAGACATGGTCGATCTTCCTCAAGC	TGTCGGATAAGAAATGTAAGCATGT	TGGATTTGGAGAATCGCTGTC	TCTCATGCTTCCTCCTTGTCTCA
Loc55974	CACCCGAGTGCCAACTGGATACC	TAAGGCACATTCTAATACCACCTCAGC	GTGCCTGCGTGCTCTTCACC	ACGCTCCGTGTCCTCTGC
Lrrc1	CACCCCTGCTGTGTCTTCCCTGCTG	GCTTCCTTCGGCAACAGTCATTC	CTGCTGCTGGACGCCAACC	GCTTCCGTAATTTGACTAGCTGGA
Mad2l2	CACCATGCCTGTCACCAGCTACCC	GACAAGGTAGTCAGCCCTTGG	CTCACGCGCCAAGACCTCAA	CCTCGCGCACATAGAGAATCAGG
Man2a1	CACCTCCGGCTTTGTTACCGTCCTTG	CGAGTACAGCAAAGACACGGGATG	CTGCCTCCTTCGGGTGAGGA	AGATCGCGCTGCCAAACACG
Mapk14	CACCGCACCTGGTTTCTGTTCTGTC	CCAGTGGTATTATCTGACATCCTATGG	CCGAACGATACCAGAACCTGTCC	TTCTTAACTGCCACACGATGC
MGC17943	CACCAAGAGAAGTACACTTGCTCTCCACA	AAAGCTCAGTGCATGTTCAGTTGAC	GCAGCTCCCGGCCTCGAC	GGGTTCATCACTAAGGTAAGATGG
MGC20741	CACCAACGGCCTCCAGGTCTCCTCTT	CCCTTAACTCTCATGCAAACCACA	GCAGGGCACAAACGCTGTAGTGA	CTGGCTGGGTTTCCTCTTCTCTG
MGC4083	CACCGGAGCCATAAGATGCTACAGTGAACG	GGATTGTCATAAAGTTCCATAGTGTGC	CCAGTGCGGGAACCAGATCG	TGGTCGATGCCGTGCTCATC
MGC5508	CACCCCAAGAGACACCCTCACCGTACCA	TGCAGCCATGATTCCACATCAAA	GGCTAGGGCCAGAGACCATGC	GGCCACAGAGATGGCTGATGAGA
MGC62100	CACCGCCTATTTCTGGGTCTCAAATCTTA	CATATTTATCTTCATGGGCTTCCTCA	GGGAGGAGTTGGACGGCAGA	GCTGGCTTTCCACAGACCATTT
Mphosph9	CACCTTCTGCCCGTGCGTCATTCA	TCGCGTAGCTGAGGGCTCTTG	CGGCCCTCTCTTCCGAGCA	GGCCTCACGCGACCGTTAAAT
Mtmr6	CACCTCCCTGGATGACTGTAGTGTAAGAAA	TGCCAACGTATGTCCTGTACGTCACT	CACTGTATCTTACGGCCACACACC	GCAAGTTTCTCCACAGAGGCAAT
Myh9	CACCGCAGCCCAGGCGGATGGA	TAGGACATGGTACTCTTTATTGTGACG	TCAAGGAGCGATACTACTCAGGGCTTA	GGGTTGATGACCACACAGAACAGG
Nek6	CACCGCAACACACCTCATCAAAGCCAAG	TGCGTTTGGGCTCCTACTGACC	TTGCCTGCTGGACAGGAAGAC	CCTGGCTTTGGCATCCATC
Nek9	CACCTGAGCCCACATAGCCCTCA	TGCCACACCACTCCATCCTCA	TCAGATTGTTTCAGCAGTGAGCTGT	TCAGATTTGCCTTGGTCAGAAA
Nfic	CACCGGCCCGCTCTGGGAGATGTT	GGGCAGGCATTTACGCTGGA	GGCCCGAGTGCCGTGAAGAC	TCTGGTCCGGGTTGGACAGC
Nme4	CACCTGCCTTCTGCACTTCAGGTCTACCC	AATGTCTCTCCTTTGAGGCAGGTGA	CCACGCTTTCAGTGCCTGCT	CCCATCTGGCTTCACAGCAAC
Ns5atp13tp2	CACCAGAACGGATACTTGGGCCTGCA	TGTCTTCGGTCCTTGCCTCATCA	CGACGGCACTCTCATCTCCTTCA	CGAGGATCAGGGCTCGGAAGAT
Osbpl8	CACCTGGGCTGTGTGTTAAGTGCCAGA	TCTCGTGTATGGCTCATCGCGTA	GATCCCACGGAAGCCTGCTC	GGTTAGCCATCCTATATGTCTGGAA
Papss2	CACCTGGCTCTGGCTTCTTCCTCAA	GTACCAGCGGGACACTGAAACA	GTGTGCAACAGGTGGTGGAA	CGTGGATGCCTTTGATGGTGGT

Gene	3'UTR Luciferase assay primers		Quantitative PCR primers	
	Forward	Reverse	Forward	Reverse
Parg1	CACCTTTGTGAGAGAATGTTTGGACAGG	CGGAAGGCTACTGTGTGTGCTG	TGCAGCCCTCCAAGCCAATA	TCTCCATCTCATTCTTCCTCCCAAG
Parp16	CACCCAGGTGCTTGGAGTGG	TGGCAGGAAGGAGGGATAAACA	TGCGGGTGAAGTACCTGCTG	GGCTGGACAACCAGGAGAGCTG
Peci	CACCAGACCACCACAGCAGCTAGACTCCT	CTGTGAAGAATTCTCATCATAATTGC	CGGGCTCGATGCTGGTGTC	TGCTGACTGGCTCTCATTGTTGG
Pgm1	CACCAGGACTATCCACGCCACCTG	TGTGCAGATGGGAGATGCAAA	GACCGAGGCCATCCAGCTCA	GCCCAATAACCAGGCGACCA
Pgrmc2	CACCTTTGGGTGTGATGCTCCTGCTGT	CCACAAACAGATTTAGGCTCAGTGGA	ACCAAAGGCAGCAAGTTCTACGG	CATCCTTATCCAGGCAGAATGTCG
Phf19	CACCGCTGCCCAGATCCCTGCAAA	CCCTCCTGCCTTCCTGCTCCT	TGCAGGTGGACAGACGGGCTA	GGCAGCTTTGCTTAGGACTG
Pldn	CACCATTGCTGTACACAGAGAGCGAG	CCTATTGACTTGTGCTCTAGGAGTGA	TGCTGCTGCTCCATGAGAAA	GTTGCTGCTCCCTCTCCAGCTCT
Plod3	CACCCACTCAACCAGTCTGCCAAACCT	ATTGAAAGTGAGGCTGAGGAAGCT	CCAGAAGGTCAGGTGGCTCAAG	CAGCAGATGACTGCCACTCTG
Plp2	CACCCTCGCCTTTCCTCCAAGCTCTC	TGTTTATTTAGCCCTTCACAGCTCACC	ACCAGCTTCTCGCGCACCAAA	GGCCGATGTTGTAGATGCACTGA
Podxl	CACCTCTGTCTGCTGGCCACCCCAACTGCACTGC	ATTCCAGTGTACTTTAGACCCCTGTCCACTAC	TCGCCTGCATCTCACTCCCATAA	TGGCACTTTGGTGGCTCTGGAC
Polr3g	CACCGGATTCAGCTTCGAGCGTTTGG	AAATCAGCATGTGGGCAACT	GGGAGAAGTTACCTGATGTTGTGTTGA	GGGCACCGGCTTGTAGTCTG
Pp1201	CACCTGGCCTGGGACTCACCCTTT	ATGTCCTTGGGCTAGTAAGGTTTC	CGTCCATCCAATGCCCATGA	TCCGGTCATCCCATTCTCCA
Ptbp1	CACCGGGCCTGCATCAGCTTCCAC	CTGAGCCTGTAGTCGTTACCAAA	AAGACCGACAGCTCGCCCAAC	ACGCTGCCAAGGCCAGGTTT
Ptpn12	CACCTGCAGGGAGTGAAAGGACACTTC	TGGCAGGTCAAATATCCTGAAACA	TGAGGAAATACCAAGAACATGAAGATG	TCTATGGCACAAATAGCACCTG
Pttg1lp	CACCGGTGGCTGGCACACACTTG	TAAGGCACGGGGTGTGATGGTGTCAGGCTT	CCTGCTTCTCTCTGTAAATTGAGTTCC	CAGGACCGACATGGTGATGA
Rai	CACCGGCATGAAGGATGATTGTAAAGGAGA	AAAGGAAATGTTTCCCATGTCA	AAGGGCCGTCCGTCTTCACC	CGCCCGATCTAGGCTGCTGT
Ram2	CACCACAGTCGAGAGGAGAGGGATCG	TGCATGGAACAATTAAGAGCATCA	GAGAGCTGTGGTAGTTTGGACTCACG	TTTGGAACGGAAGCACACATTC
Rarg	CACCGGCAGCAGACTGACCATTTCC	AAACTCAGCAATATGCGAACAGC	TTAGTGCTATCTGCCTCATCTGTG	CCACCTTCTCGGGCTCTTCCA
Rassf5	CACCAGCCGACTTCCTGTCCTCTC	CAACCATAGAAGGCACTACCAGCA	TTTAAGCGGATACACAAAGATGGA	CCCAGCGAGCAGACGAAGGTAGA
Rbms1	--	--	TGGATAAGGCCACCAACAAGTGC	CCTTCAGGGCGGAGACAGCTT
Rela	CACCGTGCTGACAGCGACCCTGCTCA	TCAGAGCTAGAAAGAGCAAGAGTCCA	TCCTGGCGAGAGAAGCACA	TGTTCCTGGTCCTGTGTAGCCATT
Rnpepl1	CACCCGACCTCCCAGACACCACGATT	TTGAGGAGCTGAGAAGAACAAGGA	CTGAAGGAGCAGAGCGTGGACTG	GGTGGGCCTGTGGCATTGAG
Ryk	CACCTTCTCTCCCAGCTCCGCCACT	TCGTATAAGGCAAACCAGACAACTTCG	CCCAGTGAGACAAACTTCCTGCAC	CATGCCCATAGCCACAAAGT
Serp1	CACCACCATTGAGTAAATCGGCCTGCCTCA	TACAACAGCATCAAATATCCAGGGAAC	CATAACTCAGCGCGGCAACG	CAGGGTCCTACCGACGCCTTT
Serpinb6	--	--	GGGCCTCCTCATCTCTTCTCTGC	CGACTCACTTGGCTGCGTCA
Slc15a4	CACCGGCCTCAGGGCTTGCTAACTACC	CACAGCAACACACACAACAGTTCA	GGCTCGTGCTCGTGGGTCTC	TCCGAGTGGCTTCTGGACCTC
Slc16a1	CACCGCCTGGAGAGAGCAGCGTGTG	GAGGCCATGAATTTGTATGAGAA	CAGGAACTTTACTTGTGCATTGGTG	CAATCATAGTCAGAGCTGGGTTCAAG
Slc17a5	CACCGCCTTTGGTATTTGAATGTGCAAGC	TTTACACAACAGTACATTAACCAATCA	GCGGGAAACGACGATGAGGA	TGTACCGAGCAGAGCAGCACAC
Slc22a5	CACCAGAAATGCTCTCCATGACTGA	TTTGTATTGTAAGTGGGTGAAGCA	ACGTCTTCCTGTCCACCATCG	GGCTTTCCAGTCATCCTTACACACCA
Slc25a30	CACCGCTGCCTCTTACTGCACAGCTCA	TCTGGATTCTAACACTTCAACGGAATG	GGCTGGCCTCCATCACAGCA	TGGCATCATTTGTCTGGCCTTG
Slc30a7	CACCCAAAGGAAGGAAACTGATTGAGGACCA	TGTTGGAAATACACAAAGACAATTATG	CCCAAGTTCAATCTGTTCGGCAAG	GAGGTTCAGGCACAGGAAGAAA
Smad5	CACCTGCATCAACGCTCTGCCTCTTTC	CCGTCACCAGCCCAATGCTT	TGAGCCACAATGAACCGCACA	GGTTGGTGGAAAGAATCGGGAAA
Snai2	CACCTGCATTTCTTCACTCCAATGACAA	CCGTACCTCACTTTCATACAGAGTTC	CAGCGAACTGGACACACACA	GCTCCCGAGGTGAGGATCTCTG
Sp1	CACCGATTAGACACCCAGTGCCAGAGACATATGG	ACTGGCACAAGTTTTGCACTCTACAGGATG	TCAGACTCGAAGCAGCAGCACAG	GCCAGCAGAGCCAAAGGAGATG
Spc18	CACCCTAGGAAGCTGCTGTGCTTGTTG	TGATCGCCTCAATATGTGCCATTC	TGTGCGGCGGATGAACAAGA	CCAGATCATTAGTGCCGAGGAGACA
Ssfa2	CACCACGCCTTCGGGTTGAGATGG	AGAGATCAACCTTTGTGTGATAGTTC	GGACCTCCCAGGCACGAAGC	CGATCTTCTCGTTGGGCACGTT
Stom	--	--	ACGGCAGTCCAGCCATGTCC	TCCATCCACAAGCTCCCAGTTC
Swap70	CACCGTCACCCAGTCAGTCACATCAG	AATTCCTGCAAATAATCTCATCTCTCG	CACCGCGCTCGACCTGGA	CGGGTCATGTGGAACCTTCAGC

Gene	3'UTR Luciferase assay primers		Quantitative PCR primers	
	Forward	Reverse	Forward	Reverse
Syngr2	CACCGGGACAAGAAGGCAGGGAAGG	ACCGTTTATTCCATCTCCAAACT	CTTCCTGTCTCAGCCTCAGG	GGCAGGCATCCTCATTCTGG
Sypl	CACCCACTGTATGAAATGTGTGCTCTAGC	TCCGCTTCTCAGCCTGTGTC	CAACCTCAACCCGCTCAAGGA	TGCCCTTAAACCCACCACAGG
Tarbp1	--	--	GATGGTCCTGCTGGCTGTGGA	CAACACGTTCTGAGTCCTCTGCTT
Tbdn100	CACCACATCATTAAACAAGCAAATGGAAT	AATTACAAAGTCTTGGGCTTGCCTGA	TGAATTGGTTCGCAGAGGTTTGA	CCTTTGAAGAAGGCCATAAACATGC
Tead1	CACCTGACTGTAAACCTCACCGCACAGG	GGCTTATGGAAAGATGCCCTTAGA	TTCTCCGCTTTCCTTGAACA	CAACGGGTCACTGTAAGAATGGTTGG
Thg-1	CACCGCCTTCTTTCCCTCACAATGT	GATACTGTCCATACACCCTCCCTTGG	CCCAGGTCTTCTCTCCAGCTCA	GCTGCTGTCGCCGCTGAAAC
Tjp2	CACCGACTCCTGCGAGGCCACCTG	GGGTGTAGTTCAGTGGTGAAGC	GACCCGCTGATGGCTTGCTT	CGGCAAACGAATGGAGCACA
Tln1	CACCTGCCTGCCACTACCAAAGC	GGGATGAGTTGAGCTGACGTG	GCAGAAGTTGCACACAGACGATGA	CATGCTCTTCCACTCCCTGTTCC
Tnfrsf21	CACCGGGTGATTTCATTTCGTTTCTG	ACTAAGCCCGCCCACGCAAAG	TGGAGAAGATTCGTGGGCTGATG	GGGAGAGCCAGTTTGTCTGTTTCC
Tom1l1	CACCACAAAGGGCCAGCTCTCTAAAGGA	TCTGAAACTAAGGAAGGCTTGTTGC	AACCACAAAGAAATCCAACTTTCC	GCCACAGTTCTGCACGCACAT
Trim29	CACCTTCAGGATGGAGGAAGCTGCAC	CGTCAGCCCAGAGTGGGAGA	GTGACTCGGTGCTGTTCCTG	TGGTAGGTGGGCAGAGGTGGA
Trip11	CACCCCGTACAGAAAGAAAAGCTAACTCACA	TGACAAAGTCTCCTTGGTTCG	CAGCAGAAAGAGGCAAGAGC	TTCATCCTGTAGTGCGAGCTG
Ttc7a	CACCTCTGCAGCTGCAGCTGGCAGCCATATAC	TAGGGCCTCCAAAGAGAGGACGCTTA	AGCCCGAGCGGCAGGTTCAC	TGTCGTGGTTATCCTTCAGACACTGC
Twist2	CACCCGCCGTGCCATCCACCTC	GGCCTCTTTATTGTTCCTGGGTGTG	CGCCGTGCCATCCACCTC	CCGTGGCCGCATCCAGTT
Uhrf1	CACCAGACCCAGGCAGAGGGCTCA	CACAACCCTTAGAACTGTGCTGTCCA	GGATCGCCGCCGTGAGAG	TGTGGGTCTCCTTCCCATCCA
Usp48	CACCTAAATCCCAGACCCGTAAACCCAAG	TGCTTTATTGAAAGATGAGTATAGGTT	TCGAGACCGCCTACCGCATC	GCTCGCCAATGCCAACCAAG
Vamp3	CACCGATGAAACTGAAGCCCGATA	TCCCAGCTAAATGCACAGAGAAGC	AATCGAAGACTCCAGCAGACACAAA	AACACCTTATCCACATTGACTCTCAT
Zbed3	CACCCCGCTCCATGTGTAAACCTAACCA	TTCACGTCGGTCATAGAAACATT	GGCCCTAATGCAGGCGGAAC	TGTAGCTGGTGGCGCTCCTG

Appendix III. Other primers and oligonucleotides

CHAPTER I

RE1 ChIP primers	Forward	Reverse
miR-9-1	GGTCCATCTTTGACCTCAGTCTTGC	GTGCAGGATGTCTGCCTGGAATAG
miR-9-2	GCATATCTCATGATGATGGTAGAGGACATC	CCGAACATAGAATGTAGAACTTCGACCATG
miR-9-3	GAACTTCTGAGCTAGGTGAACTGCTG	ACCCAGCTGACTTTGGCTCTG
miR-34a	CCGCTGTCCTATCTCACTGGC	GTGTAGTGGACCCTACCGAGG
miR-124a-1	GGACTGTGGGTGAGATGCC	CTTCCTGTGTCTCTGAGACGTGAG
miR-124a-2	CAGCTGAATGAAGAAGAAATGGGGC	CGTGGTAGAATGCATACAGTATCCCTTAAATTC
miR-124a-3	GCCTAGGACGTCACGAGAAGG	GGTTGCTGCGGCAACTATCC
miR-132	GCCGCCTTCAGTAACAGTCTCCA	CCCGCCTCCTCTTGCTCTGTATC
miR-139	GAGCAGATGGCAGGTTTGTGAG	GGTCAGTGGTAGTCTGTGGACAG
miR-338	CAGGTAGGATGTCAGGAGCGC	GACTGCTCCAGCTTAGAGCAGAC
GAD1	TCCGTCCAAATTCTTAGGAGG	TCATGACTGGATTAATCTCTG
REST coding	GAAGAGCAGTTTGTGCATCACATCC	GACTTTCCTGGGGAAATGGTTTCTC

Luciferase assay RE1 primers	Forward	Reverse
miR-9-1 RE1	CACCGATACACTCTCCTGTCTGTGCTC	CCTTTGTCTCTAGACTCATGGTG
miR-9-2 RE1	CACCGATGGTAGAGGACATCAAGAAGGA	TGAGGGTGAATCCAGAGGAAGGA
miR-9-3 RE1	CACCGGAGTAACTACCGACAGGGAATAG	GTTATCAGCTCTCTCTCAGGAAGG
miR-34a RE1	CACCCGGAGGCCAGATGAGGGTGT	GGGTGTGGGCGTTGCTGAC
miR-124a-1 RE1 (22.2kb)	CACCGGGCAGAAAGGGCTGGAGAA	CAGGGTGTCAGTCTTGGCAGCA
miR-124a-2 RE1 (3.5kb)	CACCTCCCAGAGGAGTATTGTTCCGTTC	AACCAAACCTGTGCCCTAACCTG
miR-124a-3 RE1	GCCCTCGAGCCGCTCCTTTCTCATGGAAATGG	GCGAGATCTACCTGTGTATCAGGACTCCTTTGC
miR-132 RE1	CACCGACGCAGACACTATGGAGAAAC	TGTAGACTGTTACCTCCGGTTC
miR-139 RE1	CACCAGCAGCAGGGCGAGCAGATG	ATGGGTGGGTGGGTGGACAA
miR-338 RE1	CACCCTCGCTGCTGGGTGCCTCTT	GGTTGGCCGCTCAGTGTGGA
GAD1 RE1	GCTAGCCACAGTGGACGTAGTATGTTTGTCCC	AGATCTCTTCACCTGTGGCACTAGATGGAG

RE1 deletion primers	Forward	Reverse
miR-9-1 ΔRE1 For	ACATGAGGAACATGGTACAGCGGTGAGTGAGCCT	AGGCTCACTCACCGCTGTACCATGTTCCTCATGT
miR-9-2 ΔRE1 For	GCCTGCAGAACAAGATCTCATTTTCACAGCTG	CAGCTGTGAAAATGAGATCTTGTTCTGCAGGC
miR-9-3 ΔRE1 For	ATGCCGGGTGGGCAGTTTGTCAGCAACCATGGGT	ACCCATGGTTGCTGACAAACTGCCCACCCGGCAT
miR-34a ΔRE1 For	CCTGAAGCCCTCCTGGTTCCCGACTAGTGCGCCC	GGGCGCACTAGTCGGGAACCAGGAGGGCTTCAGG
miR-124a-1 ΔRE1(22.2kb)	CGACAGGCAGCCTCCAGGTGGAGCAGCTTGGCGC	GCGCCAAGCTGCTCCACCTGGAGGCTGCCTGTCG
miR-124a-2 ΔRE1(3.5kb)	CTTGTTCCTTCATATGGCGAACTAGTTATTAAGC	GCTTAATAACTAGTTCGCCATATGAAGGAACAAG
miR-124a-3 ΔRE1	CTACCCACGGGCAGTCCTGCGTGGAGTCACCCAGAGAG	CTCTCTGGGTGACTCCACGCAGGACTGCCCGTGGGTAG
miR-132 ΔRE1	CCTCCACGCCTCCCTGCGCAGTGCAAGCGCCTCC	GGAGGCGCTTGCACTGCGCAGGGAGGCGTGGAGG

miR-139 ΔRE1	GACCAGCACACAGGAATGGCAGTCTCAGCCCACA	TGTGGGCTGAGACTGCCATTCCTGTGTGCTGGTC
miR-338 ΔRE1	GGTTCCACAATGGGGTTCTCTGTCCAAGGGGATG	CATCCCCTTGGACAGAGAACCCCATTGTGGAACC
GAD1 ΔRE1	GAAAGCCCCTAACGACAGTCTTCTCTTTGACGCA	TGCGTCAAAGAGAAGACTGTCGTTAGGGGCTTTC

miRNA QPCR primers (mouse)	**Forward**	**Reverse**
miR-9-1	GGGTTGGTTGTTATCTTTGGTTATC	AGACTCCACACCACTCATACAGC
miR-9-2	GCCTTGTGAGGGAAGCGAGTTGTTA	TGAAGACCAATACACTCATACAGCTAGA
miR-9-3	GGAGGCCCGTTTCTCTCTTTGGT	TCTAGCTTTATGACGGCTCTGTGG
miR-34a	TGGCAGTGTCTTAGCTGGTTGTTG	TACTTGCTGATTGCTTCCTTAGC
miR-124a	CTCTGCGTGTTCACAGCGG	CTCTTGGCATTCACCGCGTG
miR-132	CTCCAGGGCAACCGTGGCTTTC	TGGCTGTAGACTGTTACCTCCGGTTC
miR-139	CCAGGACAGGCGCAGGTGTATTC	GTTACTCCAACAGGGCCGCGTCT
miR-338	CAGCTCCAACGCTGCACAGG	TGCACCCACTCAGCACCAGGATA

CHAPTER II

RPA probes	
miR-124a	UGGCAUUCACCGCGUGCCUUAAGGACAGAG
5s rRNA	UAGCUUCCGAGAUCA

Full-length/coding constructs	
miR-124a-3 SalI For	ATTGTCGACAAGAAGCTGGAGCATTCG
miR-124a-3 XbaI Rev	TCTAATCTAGAGCATTGTTCGCCGGATTTGTC
Ctdsp1 BamHI For	GCTCGGATCCTCATGGACAGCTCGGCCGTCAT
Ctdsp1 full-length NotI Rev	CGGTGCGGCCGCGCTCGTGCCATGGTGCATGT
Ctdsp1 coding NotI Rev	CGGTGCGGCCGCCTAGCTCCCGGGCCTAGGCTGTCT
Vamp3 XhoI For	TAGACTCGAGGCCCATCTCCTCAGCCTCGGTT
Vamp3 full-length BamHI Rev	CGGTGGATCCCTGGTCACCATACCGGGAACATT
Vamp3 coding BamHI Rev	CGGTGGATCCTCATTAAGAGACACACCACACGATG
Mapk14 BamHI For	TTCAGGATCCTCATGTCGCAGGAGAGGCCCACGTTC
Mapk14 full-length NotI Rev	TCGAGCGGCCGCCCAGTGGTATTATCTGACATCC
Mapk14 coding NotI Rev	CGTAGCGGCCGCTCAGGACTCCATTTCTTCTTGGTC

miRNA duplexes and 2'O-methyl oligoribonucleotides	**Sense**	**Antisense**
miR-124a duplex	UAAGGCACGCGGUGAAUGCCA	GCAUUCACCGCGUGCCUUAAU
miR-124a mut5-6	UAAGCGACGCGGUGAAUGCCA	GCAUUCACCGCGUCGCUUAAU
miR-124a 2'OMe	UGGCAUUCACCGCGUGCCUUAA	
miR-1 2'OMe	UACAUACUUCUUUACAUUCCA	

Other QPCR primers	Forward	Reverse
REST	GCGGAAGACAAATGCAGGA	TTCGGCTTCGTACTGGCAA
GAPDH	AGAAGGTGGTGAAGCAGGCA	CGAAGGTGGAAGAGTGGGAG
Tubb3	GCGCCTTTGGACACCTATTCA	CCGCGCCCTCCGTATAGTGC
Nav1.2	GGCACAATCAGTGCTGGTACC	CAGCAAGGGATTCCCTGGT
Celsr3	GACTCAGCAGGAAGTTGGACAAC	GCTGTAACACTATGCAGGCCATC
Nrxn2	CACAGATGACCTCCTGGTGGC	GAGGGTCTAAGGAGTCCTCCGTG
NeuroD1	CAAGGTGGTACCTTGCTACTCC	CGCAGGATCTCTGACAGAGC
SNAP25	GAGCTGGAGGAGATGCAGAGG	CTCTTCAACCAGCTGTAGCATGC

CHAPTER III

Seed deletion primers	Forward	Reverse
Ctdsp1 Δ6mer seed1	CAGCTCCTGGGTTTCCTGTTTTTAAAGCCC	GGGCTTTAAAAACAGGAAACCCAGGAGCTG
Ctdsp1 Δ7mer seed1	CTTGGGCCCCTGTGTCACAAACTTCTTCCCCTC	GAGGGGAAGAAGTTTGTGACACAGGGGCCCAAG
Ctdsp1 Δ7mer seed2	GTTTTTGCTGCCCCGGATTCCCCACCCAC	GTGGGTGGGGAATCCGGGGCAGCAAAAAC
Ctdsp1 Δ7mer seed3	GAACAGTGGACACCCAATCATAGAACCCTCTTTAC	GTAAAGAGGGTTCTATGATTGGGTGTCCACTGTTC
Ctdsp1 Δ7mer seed4	CTAAACCCCAGATGTCACCAGAGTATCAACAGG	CCTGTTGATACTCTGGTGACATCTGGGGTTTAG
Ctdsp1 Δ7mer seed5	GATGACCTTCCCAATTAACTCTGTGACCTGAGCAC	GTGCTCAGGTCACAGAGTTAATTGGGAAGGTCATC
Vamp3 Δ6mer seed1	CAGATGTTGAAGATAGCCGCATCAGCCTAGG	CCTAGGCTGATGCGGCTATCTTCAACATCTG
Vamp3 Δ7mer seed1	CAGTTTAGTGCCTAGAAAATTGTTTTTCATATACACTCC	GGAGTGTATATGAAAAACAATTTTCTAGGCACTAAACTG
Vamp3 Δ7mer seed2	CTCCCCACATTTTGCACATACAGATTTCTGGACTG	CAGTCCAGAAATCTGTATGTGCAAAATGTGGGGAG
Vamp3 Δ7mer seed3	GGTGACAATAGCAGGGTATTTTGATAGCTAGGAAAC	GTTTCCTAGCTATCAAAATACCCTGCTATTGTCACC
Arfip1 Δ7mer seed1	CCTTAAATCACAGATACCTCCGATGCAGTAATTC	GAATTACTGCATCGGAGGTATCTGTGATTTAAGG
B4galt1 Δ7mer seed1	CATTCTCCAGCATGATTTTAATTAGAGGTCTAGA	TCTAGACCTCTAATTAAAATCATGCTGGAGAATG
B4galt1 Δ7mer seed2	CAAGATCCTGACTTTTCCAAAAAAAGAAGGGAGACAGACTC	GAGTCTGTCTCCCTTCTTTTTTTGGAAAAGTCAGGATCTTG
B4galt1 Δ7mer seed3	CCCTGGTGTCAACATGCTTCTCTTTCTCTATTTTTG	CAAAAATAGAGAAAGAGAAGCATGTTGACACCAGGG
Cd164 Δ7mer seed1	GTATTAAGAAATCAGAACAAAGACAGACTGAGACGTCGTG	CACGACGTCTCAGTCTGTCTTTGTTCTGATTTCTTAATAC
Cd164 Δ7mer seed2	ACATATATCTAATGTTTTCAAACTTGTTTAAACTCTGACTTC	GAAGTCAGAGTTTAAACAAGTTTGAAAACATTAGATATATGT
Cebpa Δ7mer seed1	CTACCGAGTAGGGGGAGCAAAAATATTTTATTTGGAGGATTCCTG	CAGGAATCCTCCAAATAAAATATTTTTGCTCCCCCTACTCGGTAG
Cebpa Δ7mer seed2	GTCTCTGTGTCCCAGCGGTGCAATGGCAGTGTGC	GCACACTGCCATTGCACCGCTGGGACACAGAGAC
Ctdsp2 Δ6mer seed1	CTCCACCTCCGCATCACAAGTGAGTCCCGCCCAC	GTGGGCGGGACTCACTTGTGATGCGGAGGTGGAG
Ctdsp2 Δ7mer seed1	GGACTCTGCTACACTTGTGGTCAGCCTCAC	GTGAGGCTGACCACAAGTGTAGCAGAGTCC
E2ig4 Δ7mer seed1	GCTCCAGTTGTCCCTAACCCTTTTGTCTGGACTG	CAGTCCAGACAAAAGGGTTAGGGACAACTGGAGC
Epim Δ6mer seed1	GATTTCACAACAGTGGCCTGGTAGCTTTGAAATAGG	CCTATTTCAAAGCTACCAGGCCACTGTTGTGAAATC
Epim Δ7mer seed1	TCAGGGCCCTTCTATACTGGTGACCCTGACTA	TAGTCAGGGTCACCAGTATAGAAGGGCCCTGA
Fcho2 Δ7mer seed1	TATGGACTACATAATGTTTAAAACAATAACAAAAACCAGAAATGT	ACATTTCTGGTTTTTGTTATTGTTTTAAACATTATGTAGTCCATA
Hadhb Δ6mer seed1	CTAAAATACAAACCGATAAGTTAACTTACTATAG	CTATAGTAAGTTAACTTATCGGTTTGTATTTTAG
Itgb1 Δ7mer seed1	GTCAGCTGAGGTCACAGTTTTAACCCTTCCTTCTG	CAGAAGGAAGGGTTAAAACTGTGACCTCAGCTGAC

Lamc1 Δ7mer seed1	CTGTGGCCCTTTTCAATCACTTTAGCTGTTTGCC	GGCAAACAGCTAAAGTGATTGAAAAGGGCCACAG
Lamc1 Δ6mer seed1	CAGTGTTTTGCTCCACCTCGTGTTTGCAGCACCTTCAC	GTGAAGGTGCTGCAAACACGAGGTGGAGCAAAACACTG
Lass2 Δ7mer seed1	GACTTCAAGCCAGGGTTACTGTCCCTTTTCTGGG	CCCAGAAAAGGGACAGTAACCCTGGCTTGAAGTC
Mapk14 Δ6mer seed1	CTTCTCCTGTGGCCCTACCATATCAGTGAC	GTCACTGATATGGTAGGGCCACAGGAGAAG
Mapk14 Δ7mer seed1	CTGTCAGTCTGTTTAAAGGGTGAGAAGG	CCTTCTCACCCTTTAAACAGACTGACAG
Nek9 Δ7mer seed1	GAGGTAACCCTTCCTACAGCCTTGCTATCTGGGAG	CTCCCAGATAGCAAGGCTGTAGGAAGGGTTACCTC
Nek9 Δ7mer seed2	CTGGGAATCTAAGTTCTAGAGACAATTTGCACTTTC	GAAAGTGCAAATTGTCTCTAGAACTTAGATTCCCAG
Plod3 Δ6mer seed1	CAAACCTTCTCTGCCACTGTTGGACAACCTGGGTC	GACCCAGGTTGTCCAACAGTGGCAGAGAAGGTTTG
Ptbp1 Δ6mer seed1	CAGTCAATCCCAGCACCAGAGGGGCTCAGGCGTGAG	CTCACGCCTGAGCCCCTCTGGTGCTGGGATTGACTG
Ptbp1 Δ7mer seed1	CAAACATAGCCGCTTTCTACAAACCAGCGTGCCTC	GAGGCACGCTGGTTTGTAGAAAGCGGCTATGTTTG
Ptpn12 Δ7mer seed1	TCATGGAGATTCAGTAAGGAACAATATTGAATTC	GAATTCAATATTGTTCCTTACTGAATCTCCATGA
Sypl Δ6mer seed1	GGGTTACTAAAAATTAAGGTTTACGTTTGAAATGTTTAAC	GTTAAACATTTCAAACGTAAACCTTAATTTTTAGTAACCC
Sypl Δ7mer seed1	GCTGCTTTTCTACATAAAATTTCTAGAAATTTAAGATGTA	TACATCTTAAATTTCTAGAAATTTTATGTAGAAAAGCAGC
Tom1l1 Δ6mer seed1	CACTGCTGTTCCTGACAAGAGACTTCAGGAAAATC	GATTTTCCTGAAGTCTCTTGTCAGGAACAGCAGTG
Tom1l1 Δ6mer seed2	GTAATGTTTAACATTCTTTATTATAATTCACATTC	GAATGTGAATTATAATAAAGAATGTTAAACATTAC
Tom1l1 Δ7mer seed1	GGGAAAGGAAGAAAATGATATTTAACTGAATCTG	CAGATTCAGTTAAATATCATTTTCTTCCTTTCCC

Seed insertion primers	**Forward**	**Reverse**
SNAP25(3'UTR)	CACCGCTCCTTCATGCTTCTCTCATGG	TGGTGATTAACAAGAGCCAGACG
SNAP25 6nt seed	CATAGTGGTCATGCCTTTCTGGTGGCTC	GAGCCACCAGAAAGGCATGACCACTATG
SNAP25 1x7nt seed (position 262)	CATAGTGGTCAGTGCCTTTCTGGTGGCTC	GAGCCACCAGAAAGGCACTGACCACTATG
SNAP25 2x7nt seed (position 461)	CTTAGGGTGTCAGGCTCTGTGCCTTGAATCTCTCCAAATGTG	CACATTTGGAGAGATTCAAGGCACAGAGCCTGACACCCTAAG
SNAP25 3x7nt seed (position 589)	GATTTATGCATTTATGCGTGCCTTATGAGAACTAAATAGAC	GTCTATTTAGTTCTCATAAGGCACGCATAAATGCATAAATC
SNAP25 miR124a complement	CATAGTGGTCATGGCATTCACCGCGTGCCTTAATCTGGTGGCTC	GAGCCACCAGATTAAGGCACGCGGTGAATGCCATGACCACTATG
SNAP25 mutmiR complement	ATGGCATTCACCGCGTCGCTTAATCTGGTGGCTC	GAGCCACCAGATTAAGCGACGCGGTGAATGCCAT
SNAP25 Vamp3 seed+15nt upstream	CATAGTGGTCACCACATTTTGCACAGTGCCTTTTCTGGTGGCTC	GAGCCACCAGAAAAGGCACTGTGCAAAATGTGGTGACCACTATG
SNAP25 Vamp3 seed+15nt downstream	CATAGTGGTCAGTGCCTTATTTTGATAGCTAGGTCTGGTGGCTC	GAGCCACCAGACCTAGCTATCAAAATAAGGCACTGACCACTATG
SNAP25 Mapk14 seed+15nt upstream	CATAGTGGTCACTGTCAGTCTGTTTGTGCCTTATCTGGTGGCTC	GAGCCACCAGATAAGGCACAAACAGACTGACAGTGACCACTATG
SNAP25 Mapk14 seed 22+15nt upstream and downstream	CAGTCTGTTTGTGCCTTAAAAGGGTGAGAAGGGATCTGGTGGCTC	GAGCCACCAGATCCCTTCTCACCCTTTTAAGGCACAAACAGACTG

Ago2 IP experiments	**Forward**	**Reverse**
GL3.1	CUUACGCUGAGUACUUCGAUU	UCGAAGUACUCAGCGUAAGUU
Renilla luciferase	CCAATGCTATTGTTGAAGGTGCCAAGAAG	GTTCATTTTTGAGAACTCGCTCAACGAACG
Firefly luciferase	CGTCGCCAGTCAAGTAACAACC	CACGGCGATCTTTCCGCC
Ctdsp1 (human)	CCTGCCTCCTATGTCTTCCA	GCCTGAGCACTGAGTACACG
Plod3 (human)	GCGGTGATGAACTTTGTGG	GGGAGGAGATCACACAGTCG
Vamp3 (human)	GCAGCCAAGTTGAAGAGGAA	CAGTTTTGAGTTCCGCTGGT
β-Actin (human)	CGCGAGAAGATGACCCAGAT	ACAGCCTGGATAGCAACGTACAT
Gapdh (human)	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

B2M (human)	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GusB (human)	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA
Rplp0 (human)	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC