RNA HELICASE MOV10 IS ESSENTIAL FOR GASTRULATION AND NORMAL CNS DEVELOPMENT

BY

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DISSENTATION

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ABSTRACT

Neural circuitry, at its most basic level, is composed of multiple synaptic connections formed between the neurons within a circuit. These synaptic contacts are also the sites of local protein synthesis, which is required for long-term synaptic plasticity underlying learning and memory. The Fragile X Mental Retardation Protein (FMRP) functions as a translational repressor and is critical for fine-tuning local protein synthesis at synapses. Recently, we demonstrated that FMRP associates with the RNA helicase Moloney Leukemia Virus 10 (Mov10) in brain and modulates its translational activity through the microRNA (miRNA) pathway. FMRP is critical for normal cognition and our findings hypothesize a role for Mov10 in brain function. Additionally, Mov10 has a well-documented role of protecting genomic integrity by suppressing actively transposing retroviral elements called LINE-1 (L1) in cell culture systems. This becomes relevant when we consider the studies that propose an increase in endogenous retrotransposition in fetal brain as well as in the hippocampus and other regions of the adult brain during neuronal differentiation. Furthermore, L1 retrotransposition is more common in brain compared to other tissues in the body. Despite the many findings that show a role for Mov10 in suppressing retrotransposition, there are currently no studies describing its role in the developing or adult brain. In order to address these gaps, we generated a knockout mouse model for Mov10 and found that the deletion of Mov10 causes early embryonic lethality. We were able to show that lethality occurs prior to embryonic day 9.5 establishing a crucial role for Mov10 during mouse embryogenesis. We then used the externally developing *Xenopus laevis* embryos to establish the exact stage and cause of the Mov10 embryonic lethality. Our findings show that the blocking
of translation of the Mov10 maternal mRNAs in one-cell stage embryos results in a gastrulation defect at Stage 10 of *Xenopus* embryonic development. RNA sequencing of embryos from Stage 10.5 of the Mov10 knockout shows an increase in mRNAs levels consistent with a miRNA-mediated role of Mov10 possibly at the Maternal to Zygotic Transition (MZT). In addition, we see that knockdown of zygotic Mov10 transcripts results in eye and ventricular neuronal differentiation defects in Stage 36 tadpoles. This suggests an important role for Mov10 in postnatal brain development. In agreement with this, we see that WT mice showed a significant increase in Mov10 protein levels in their brains from Postnatal Day 0 (P0) to P14 suggesting an important function for Mov10 during this critical period of synapse formation and neuronal differentiation. Mov10 expression was seen throughout the P1 brain including cortex, hippocampus and cerebellum and became localized mostly to the hippocampus in the adult brain. Interestingly, the subcellular localization of Mov10 was nucleo-cytoplasmic in P1 brains and predominantly cytoplasmic in the adult brain. The same age-dependent change in cellular localization was also observed in cultured neurons from Day In Vitro 0 (DIV0) and DIV14, suggesting a possible nuclear function for Mov10 at postnatal stages. Because of its critical role during development and postnatal increase in the brain, we further examined the Mov10 heterozygotes in this study. We found an increase in L1 genomic DNA content in the Mov10 heterozygote brains at P2 compared to WT brains from the same stage, corroborating Mov10's role in suppressing L1 retrotransposition. RNA isolation followed by sequencing from Mov10 immunoprecipitates (RNA-IP) at P2 stage in brain shows that Mov10 bound retroelement RNAs belonging to the LINE-1 family. Further verification showed that Mov10 specifically bound a retrotransposition-
competent L1 RNA in mouse brain and that it inhibited the cDNA synthesis of this RNA in an \textit{in vitro} assay. In addition, both the RNA-IP and iCLIP analysis of Mov10 RNA targets at P0-P1 brains followed by functional annotation showed that Mov10 bound significantly more mRNAs involved in cytoskeletal and actin binding. To investigate the role of Mov10 in cytoskeletal dynamics, we created a CRISPR-Cas9 knockout of Mov10 in Neuro2a cells. The deletion of Mov10 in Neuro2a caused abnormally decreased neurite outgrowth on differentiation. We were able to rescue the defect by restoring Mov10 protein levels in the knockout cell line. Furthermore, culturing and staining of hippocampal neurons from Mov10 heterozygotes confirmed these results showing markedly short dendrites as seen in the Mov10 knockout Neuro2a cells. These findings point to an additional stage-specific role for Mov10 in modulating cytoskeletal dynamics that are key to synapse formation and pruning and eventually to the formation of normal brain circuitry. To investigate the behavioral output of reduced Mov10 levels on the brain, we conducted a series of behavioral tests on the Mov10 heterozygotes. We find that the Mov10 heterozygotes show increased activity in a novel environment using the Open-field test as well as increased anxiety in the Elevated- Plus Maze test, suggesting perturbed neuronal circuitry. The Mov10 heterozygotes tested normal in the Trace fear conditioning, Novel Object Recognition and Rotarod tests. These studies along with the neuron culture and knockdown assays suggest that Mov10 is important in developing and maintaining normal brain activity. This is the first study of Mov10 at an organismal level and in the brain.
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“For, we are a sum of the people we meet.”

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CHAPTER 1
INTRODUCTION

RNA binding proteins play principal roles as modulators of synaptic activity in the central nervous system. They perform multiple functions from binding and modifying RNA, to transport and regulation of transcripts at the synapse in response to activity. They also function as part of Ribonucleoprotein complexes (RNPs) and RNA granules to effect various translational decisions and cellular fates [1]. A prominent class among these proteins is the RNA helicases. RNA helicases form a large group of enzymes that are primarily involved in the processing of RNA for regulating gene expression. They are classified into six Superfamilies (SFs) based on their structure and function and can act on a diverse array of substrates as well as perform a wide range of biochemical activities [2, 3]. The helicases of the SF1 and SF2 superfamilies share a catalytic core and act mostly on messenger RNAs (mRNAs) to regulate their function and reorganization [4]. They are present within complexes called messenger RNPs (mRNPs) at the synapse and regulate the synaptic mRNAs within this complex in a dynamic activity-dependent manner. This form of local protein synthesis is important for synaptic plasticity and memory consolidation [5]. Consequently, RNA helicases have been implicated in diseases of the nervous system, like DDX17 in Down syndrome [6, 7]. Thus, RNA helicases play significant roles in ensuring proper synaptic physiology and normal brain function.

The RNA helicase Moloney leukemia virus 10 (Mov10) belongs to this category of synaptic proteins and was found to coordinate the local translation of mRNAs through
the RNA Induced Silencing Complex (RISC) pathway in cultured hippocampal neurons and in brain [8, 9]. Mov10 was first identified from a germline provirus integration study in which the Murine Moloney leukemia virus (Mu-MLV) was used to study the effects of retroviral insertions at random chromosomal locations [10]. The study generated multiple mouse substrains including the Mov-10 strain, named as such because it was the 10th substrain where the retrovirus had integrated. Subsequent studies showed that the provirus had integrated into intron 1 of a transcription unit, which was identified as a protein with a molecular weight of 110kDa and comprising of putative GTP-binding motif [11]. Consequently, the gene was called gb110 and was found to be regulated developmentally and in a cell-cycle specific manner. However, the knockdown of both alleles of gb110 gene in mouse ES cells did not affect the proliferation or differentiation in vitro and no further studies on Mov10 were published for over a decade [12]. Interest in Mov10 was revived in 2005 when it was identified as a novel player in the rising field of miRNA-mediated translational regulation of mRNA targets as an interactor of Argonaute 2 [13]. Importantly, a recent in vivo study from our lab showed that knockdown of Mov10 causes embryonic lethality in both mouse and Xenopus [14] (Chapter 3). In addition, reduced levels of Mov10 in brain resulted in neuronal and behavioral defects [14]. These studies emphasize the crucial role that Mov10 plays at two different stages namely embryonic and postnatal brain development and showcases the complex yet fascinating world of RNA helicases in the cell.
1.1 Characterization of Mov10

Gorbalenya and Koonin first observed in 1988 that proteins with DNA helicase activity contained several highly conserved sequence-motifs that are also shared by viral proteins encoded by RNA viruses [15]. Among these motifs, were the signature NTPase/ATPase motif of the P-loop proteins, which became the hallmark of all helicases. Thus all helicases are P-loop NTPases containing the Walker A (also referred to as Walker I/Motif I) and Walker B (or Walker II/ Motif II) domains for NTP binding and hydrolysis [16].

SF1 and SF2 RNA helicases are characterized by the presence of two conserved domains, which forms the helicase core that is comprised of characteristic sequence motifs. Mov10 is classified as a Superfamily 1(SF1) helicase member based on its conserved helicase core that contains two similar domains that resemble the fold of bacterial RecA protein and are therefore often referred to as RecA-like domains. The arrangement of characteristic motifs within the helicase core results in the RecA like fold of these helicases. Like all helicases, Mov10 has the highly conserved Motif I (GPPGTGKT) and Motif II (DEAGH), which are present in Domain1 of the helicase core (Fig1.1) [17]. Additionally, phylogenetic analysis by sequence similarity clustered Mov10 with the Upf1-like helicases under the SF1 family [2]. Although Mov10 was initially identified as a GTP-binding helicase, recent characterization has shown that Mov10 utilizes ATP and shows a 5’ to 3’ directionality [18]. The domain structure of Mov10 has the conserved helicase core at the C-terminus and a proposed Cysteine-Histidine (CH) domain at the N-terminal end. Cysteine and Histidine rich sequences have been reported in other proteins and are they known to be zinc-chelating residues.
They usually show conservation across species and have roles as Zinc finger domains [19]. Zinc finger domains coordinate a zinc ion to form a secondary structure that binds nucleic acid protein [20]. Here, we have aligned the entire Mov10 protein sequence from Human, Rhesus, Mouse, Xenopus and Zebrafish and find multiple conserved histidine and cysteine residues (Fig. 1.1 -highlighted in yellow). Based on conserved residues across Human and mouse, the proposed CH domain of Mov10 has a consensus of Cys-$X_{15}$-Cys-$X_{10}$-His-$X_{2}$-Cys. Additionally, the Mov10 consensus CH domain does not exactly match with the Upf1 consensus CH domain sequence (Cys-$X_{2}$-Cys-$X_{9}$-His-$X_{3}$-Cys) [21], which is proposed to form a Zinc-finger binding domain based on the NCBI Conserved Domain Database (Fig. 1.2) [22]. It remains to be tested if the proposed Mov10 CH domain can fold in a Zinc-dependent manner or not.

The alignment between multiple species in Fig.1.1 also shows that certain stretches of the N-terminus encompassing the histidines and cysteines that form the proposed CH domain (e.g. amino-acids 115-141,143-159, 163-172,187-194) are conserved between Human, Rhesus and Mouse but differ from other species. These sequences could have important roles in interacting with other proteins as shown for the HIV-1 protein [23]. The C-terminal core however is well conserved, especially the helicase Motif I and Motif II compared to the N-terminus across the different species (Fig. 1.1).

1.2 Tissue expression and distribution of Mov10

In a large-scale study to discover new genes expressed in human adult and fetal brain, Mov10 was identified as one of the new genes (referred to as KIAA1631) found to be differentially expressed in various adult brain regions and moderately expressed in
whole fetal brain [24]. Additionally, according to the Allen Brain Atlas, Mov10 shows low expression in the Olfactory bulb and cortical subplate in adult brain but its expression has not been examined across development in the mouse brain [25]. A recent study from our lab showed a developmentally timed increase in Mov10 protein levels in mouse fetal brain compared to adult. At the subcellular level, Mov10 was found to be nucleocytoplasmic in postnatal brain and became predominantly cytoplasmic in the adult brain and in cultured Day In vitro 14 (DIV) hippocampal neurons [14] [8]. Nuclear localization of Mov10 was described once before in primary human fibroblasts where it was proposed to function in Polycomb-mediated suppression of a tumor suppressor gene [26]. In most studies, where Mov10 was ectopically expressed, a cytoplasmic localization to P-bodies has been reported [27]. The cytoplasmic presence of Mov10 was concluded by the researchers in [26] to be an effect of ectopic overexpression. However, we have examined endogenous Mov10 in HEK293 and in hippocampal neurons and find it to be cytoplasmic, as do others [8]. Thus, it only seems to be nuclear under specific circumstances.

Besides the brain, Mov10 is highly expressed in the testes where it is cytoplasmic [14]. However, a specific function for Mov10 in the testes has not been shown. Interestingly, Mov10 shares very low C-terminal homology with another protein Mov10-Like1 (Mov10L1), which is germline specific and functions in the piRNA pathway [28]. Mov10L1 was also shown to inhibit retrotransposons during spermatogenesis [29, 30], a function shared by Mov10 in brain and cell culture [14] [27].
1.3 Cellular roles of Mov10

1.3.1 Mov10 in retrotransposon biology

Less than 2% of the human genome is protein-coding genes [31]. Approximately 50% of the genome is comprised of repeat elements and subsets of these are transposons. Transposons are defined as mobile genetic elements and can be either DNA transposons or retrotransposons based on their mode of action. While the largely inactive DNA transposons move by excising themselves from one genomic location and inserting at another, retrotransposons move via an RNA intermediate. The RNA intermediate is reverse transcribed and the cDNA is inserted into a new genomic location by a process called Target-Primed Reverse Transcription (TPRT) [32]. Although, the vast majority of retrotransposons are silenced in the genome, a few of them are still active. These retrotransposon-competent elements belong to the autonomous Non-Long Terminal Repeat (Non-LTR) class and are called Long Interspersed Elements-1 or L1. L1s are the only known active elements in eutherians [33]. L1s are also co-opted by other non-autonomous retrotransposons like SINEs (Short Interspersed Elements) and SVAs (SINE-R, VNTR, Alu) that enable them to mobilize in the genome. Needless to say, L1s are tightly regulated in cells to prevent deleterious insertions in the genome. One of the proteins that strongly suppress L1s is Mov10. Many studies have reported this function for Mov10 both in cell culture systems and in the developing brain [27, 34], [14]. The proposed mechanism by which Mov10 suppresses L1 elements is not fully understood. Multiple lines of investigation have shown conclusively that Mov10 binds the L1 mRNA and also associates with the L1 RNP [27, 35], [14]. The proposed mechanism of Mov10 inhibition is linked to its
presence in Processing bodies (P-bodies), where the Mov10 bound L1 RNA is presumably degraded. The presence of Mov10 in the nucleus suggests an additional mechanism where the L1 mRNA is prevented from being reverse-transcribed by the L1 endonuclease and reverse transcriptase ORF2p at the site of insertion. TPRT requires that the nicked DNA strand with the 3’OH overhang be made available for ORF2p to use as a primer for reverse transcribing the L1 mRNA. Additionally, a recent study also showed that G-rich tracts stimulate L1 retrotransposition and we have shown that Mov10 preferentially binds G-rich regions [9, 36]. Based on these findings, we propose a possible model where Mov10 binds the G-quadruplexes found in the 3’UTRs of L1 mRNAs [37]. This binding of L1 mRNAs within the L1 RNP could occur in the nucleus or in the cytoplasm. Additionally, Mov10 also associates with ORF2p through its N-terminal via a protein-protein interaction [14]. Having thus established itself as a part of the L1 RNP, Mov10 then proceeds to unwind the G-quadruplexes within the L1 mRNA in a 5’-3’ direction while ORF2p reverse transcribes the L1 mRNA in the 3’ to 5’ direction at the site of insertion. The opposing movement of Mov10 thus sterically blocks ORF2p from reverse transcribing and inserting the L1 mRNA (Fig.1.3).

1.3.2 Mov10 and viral suppression

Retroviruses are obligate parasites that require host proteins to support their entry and establishment inside cells. As such they interact with host factors that either support or inhibit their life cycle [38]. Of these many factors, Mov10 has been shown to inhibit retroviral activity of HIV-1, SIV, MLV, HCV and HBV retroviruses and more recently Enterovirus 71 and Dengue virus [39-44]. Thus Mov10 is considered to have a broad
anti-retroviral activity. Over the years many more studies have confirmed this finding and added to the molecular understanding of the anti-retroviral properties of Mov10. Overexpression of Mov10 inhibits viral activity both in the virus producing cells and the target cells. It has been shown to decrease the viral particles and infectivity rates in the producer cells possibly by its interaction with the viral Gag protein through its N-terminus [41]. Besides decreasing the infectivity of viral particles, Mov10 is also packaged inside the virions and inhibits the reverse transcription of the viral mRNA in the target cells [39, 40]. Interestingly, this function is similar to its role in inhibiting LINE1 retrotransposons from integrating into the genome [34] [14] and might share molecular features. Although Mov10 prevents the reverse transcription step in L1 retrotransposition and viral suppression, physiological levels of Mov10 were enough to inhibit retrotransposons but did not prevent infection by exogenous viruses [45] suggesting that Mov10 could have been evolutionarily co-opted for suppressing retroelements by being activated through a common cellular signal for example the type I IFN pathway for invading RNA viruses [46]. The activation of Mov10 could be through sequence or structural elements within the single-stranded RNA molecule, be it a virus or a LINE element. This also suggests that Mov10 has an evolutionary past in viral infectivity and a recent study [47] demonstrates this complex role for Mov10, where the authors show that normal endogenous levels of Mov10 enhance nuclear viral mRNA export and subsequently increase the expression of Gag protein during HIV-1 infection. The opposite has been shown for the Influenza A virus, where Mov10 bound the nucleoprotein of the viral complex and prevented its interaction with importin-α.
effectively preventing the nuclear transportation and replication [48]. Thus, the role for Mov10 in suppressing viral infectivity though well established, is far from complete.

1.3.3 Mov10 in miRNA mediated translational regulation

Mov10 is widely described as associating with RISC and was also found to co-immunoprecipitate with the microRNA effector protein Argonaute 2 (Ago2) in an RNA dependent manner [49-51]. Since mRNAs have a high degree of secondary structure and microRNA Recognition Elements (MREs) in their 3’ Untranslated regions (UTRs) cannot be unwound by Ago2, it is possible that Mov10 helps to open up these structures for miRNA mediated regulation [52]. Multiple studies point to a role for Mov10 in miRNA-mediated translational suppression of reporters and neuronal mRNAs at synapses and in the brain [8, 13, 52]. However, the role of Mov10 in miRNA-mediated translation of its targets could be modulatory in nature based on its association with other RNA-binding proteins such as FMRP. Here, the fate of the commonly bound mRNAs depended on the binding site of FMRP and Mov10 and the accessibility of the MRE to AGO2. Thus even though the larger fate of most mRNAs is miRNA-mediated translational suppression, some of the mRNAs are protected from AGO2 and undergo translation. [9, 52]. Additionally, it has been shown in one specific case that endogenous Mov10 is not necessary for the let-7 miRNA-mediated suppression of a reporter construct in HeLa cells [45]. In this particular case, it is possible that the MRE was not buried in a secondary structure or that other helicases are involved in unwinding these specific 3’UTRs. Therefore it is conceivable that Mov10 has more of a modulatory role and regulates a certain pool of targets through the RISC pathway and that this function of
Mov10 depends on the associated protein factors within the RNP complex. In addition to its RISC-mediated function, Mov10 was recently shown to associate with UPF1 and participate in the Nonsense-mediated decay (NMD) pathway [18]. These findings only add to the understanding that RNA helicases rarely have dedicated cellular roles and are often co-opted by different pathways that use RNA metabolites [17].

1.4 Mov10 in embryonic development

Expression of Mov10 in the developing whole embryo was first seen as early as embryonic day 12 in mouse [11]. However an embryonic stem cell knockout of both copies of Mov10 did not affect the differentiation of the embryo into the three germ layers and it was concluded that Mov10 was not required for ES cell proliferation and differentiation [12]. Therefore, it was extremely surprising that a complete knockout of Mov10 in mouse and Xenopus caused early embryonic lethality [14], Chapter 3.

Translation- blocking morpholino based knockouts of maternal Mov10 transcripts in Xenopus shows that the embryos do not complete gastrulation and neural tube formation (Chapter 3). The Mov10 knockout embryos did show formation of the somites (mesoderm) and neural tube (ectoderm) suggesting that germ layer differentiation is proceeding as in the mouse ES cells [12, 53]. However, the Mov10 knockout embryos fail to complete gastrulation at Stage 10 in Xenopus, which is comparable to embryonic day 6.75-7 in mice based on the development of the Spemann’s organizer [54]. These defects could possibly be due to Mov10’s role in miRNA-mediated regulation of the Maternal to Zygotic Transition (MZT) in the developing embryos (Chapter 3). MZT occurs universally in all animals and is the step where maternally deposited transcripts...
are degraded and zygotic transcription is activated [55]. The stage of MZT is species-specific and occurs at the two-cell stage in mouse. MZT in mouse embryos is dependent on the RNAi machinery since a knockdown of Ago2 causes a developmental arrest of embryos at this stage [56]. Ago2 is absolutely critical for miRNA-mediated degradation of maternal transcripts at MZT but Mov10 might have a broader role and it is possible that the gastrulation defects arise due to pleiotropic effects caused by maternal Mov10 knockout. The genes that are largely affected in the Mov10 knockout embryos belong to cytoskeletal and cellular movement categories by Gene Ontology analysis, which explains the observed defect in gastrulation. Gastrulation involves an evolutionarily conserved series of cellular rearrangements and movements that are highly coordinated and dependent on cytoskeletal components [53]. Thus the large scale rearrangements required for proper gastrulation might be perturbed in Mov10 knockout embryos due to a misregulation of mRNAs that are needed at critical time-points and concentrations in the embryo to effect this transition. This could be due to the defective MZT beginning at an earlier stage and leading to an aberrant transcriptional environment at gastrulation.

1.5 Mov10 in the developing brain
Mov10 is present in mature neurons and at synapses. NMDA stimulation leads to its rapid degradation by the proteasome pathway and consequently relieves translation suppression [8, 9, 24]. However, until now, there was no systematic study of this protein in the developing brain [14]. In this recent study published by our group, Mov10 protein levels showed a developmental increase in whole brain and was about 40 fold
higher than the adult brain at postnatal stages P0-P2 [14]. This is an extremely important stage in brain development where a host of important events like neuronal differentiation, synaptogenesis and synaptic pruning occurs to influence brain structure and circuitry [57]. The pattern of Mov10 distribution in whole brain showed a transition from being ubiquitous in P0 brain to becoming restricted to the hippocampus in adult brain. Mov10 also showed a change in cellular localization from nucleocytoplasmic at P0-P2 brain to predominantly cytoplasmic in the adult brain. This developmentally timed increase and nuclear localization of Mov10 at postnatal stages was shown to be important for regulating LINE1 elements that become active during neuronal differentiation [58]. In addition to its nuclear role, cytoplasmic Mov10 also bound cytoskeletal mRNAs in postnatal brain and regulated neurite outgrowth in a knockout cell line, reminiscent of its function in cytoskeletal remodeling during embryogenesis. Interestingly, the Mov10 heterozygote mice show increased genomic LINE1 content, decreased hippocampal dendritic arborization as well as behavioral deficits suggesting an important role for Mov10 in the formation of normal brain circuitry [14]. This is important since several studies have shown that the Mov10 gene is present in Copy Number Variants (CNVs) seen in individuals with intellectual and developmental delay [59-61]. Besides establishing a significant role for Mov10 in brain, this study also raises many interesting questions such as how are the protein levels of Mov10 regulated across development, how does Mov10 shuttle from the nucleus to the cytoplasm and why does its localization and distribution change as the brain develops. In addition, Mov10’s preference for cytoskeletal mRNAs both in embryonic development and in postnatal brain is intriguing and suggests a functional specificity belying this otherwise
multifunctional helicase. The Fragile X mental retardation protein (Fmrp) also preferentially binds RNAs involved in neuron projection [62]. Because Fmrp binds cobound mRNAs before Mov10 [9], this could serve as an initial selection for Mov10 to preferentially bind actin/cytoskeletal/neuron projection RNAs. It also lends precedence to Mov10 as an important candidate in neurological disorders with underlying cryoarchitectural deficits such as Autism and Alzheimer’s disease [63].

1.6 Conclusion

The studies of the RNA helicase Mov10 have rapidly progressed from its earliest categorization as a RISC component to its recent prominence as a critical player in embryonic and normal CNS development. Additionally, as outlined above, Mov10 also participates in many other important cellular events such as retroviral and retrotransposon suppression. These myriad roles of Mov10 is made possible by its association with other RNA binding proteins as well as its assimilation into functional structures such as mRNPs, P-bodies and other cytoplasmic aggregates. The emergence of a preference for Mov10 in cytoskeletal regulation suggests a new direction of research for this fascinating protein.

The work presented here outlines the first study of the RNA helicase Mov10 during embryonic development and postnatal brain development. Chapter 2 deals with the expression and function of Mov10 in the developing mouse brain. Chapter 3 shows the essential role that Mov10 plays during embryonic development using Xenopus laevis model system. Chapter 4 talks about the accessory role that FMRP plays in the miRNA pathway and Chapter 5 is a conclusion of the findings in this thesis.
1.7 Figures

Figure 1.1

<p>| Human   | MPSKFSCKQILGQRLEAGQCFELESFLVRGLDMETDRLRALTNYRDFKISF5FTMPAPG-FS |
| Rhesus  | MPSKFSCKQILGQRLEAGQCFELESFLVRGLDMETDRLRALTNYRDFKISF5FTMPAPG-FS |
| Mouse   | MPSKFSCKQILGQRLEAGQCFELESFLVRGLDMETDRLRALTNYRDFKISF5FTMPAPG-FS |
| Xenopus | MYCSASKDSWNGASRFYSR---RHEKDRIIFPS--QYFGYDKSARAE |
| Zebrafish | MSRYRKRKLSSRDIVAFRDYFLELDRESETDDTRNTLIEQHFRQDRGVDHP-FS |
| Human   | SMLY CKIANLAVYTKVRVRFFLDRW-------------------ADVFRFPEKRM |
| Rhesus  | SMLY CKIANLAVYTKVRVRFFLDRW-------------------ADVFRFPEKRM |
| Mouse   | SMLY CKIANLAVYTKVRVRFFLDRW-------------------ADVFRFPEKRM |
| Xenopus | V5QFQDHLDKEFVSVLQDQIAKK-------------------DRIQQAHAQ |
| Zebrafish | V5FALTRSSRTRVTRCF5VDFDKVRVRQND2SP5RSPYQRNH6AIZRSP5RTTRV |
| Human   | KLGSIDEKHHL---KLSSKFLYDR---AEYHGKHGVDVEQHVHEGDOOLIR |
| Rhesus  | KLGSIDEKHHL---KLSSKFLYDR---AEYHGKHGVDVEQHVHEGDOOLIR |
| Mouse   | KLGSIDEKHHL---KLSSKFLYDR---AEYHGKHGVDVEQHVHEGDOOLIR |
| Xenopus | SADGSĐTGGO---POFTERMOKO---AEIFFDKNIGISSE---COLSDQRIFF |
| Zebrafish | SDP5SVPFENPVRAARRALTMQRNGSDEMSVMFADKYGRYVRS---LQEDGKHMC |
| Human   | LDNLNKEVLTLRLANGGTSSTVLTTHLFLPLRTQPQAF---YNEPDQELPCGPECEYEL |
| Rhesus  | LDNLNKEVLTLRLANGGTSSTVLTTHLFLPLRTQPQAF---YNEPDQELPCGPECEYEL |
| Mouse   | LDNLNKEVLTLRLANGGTSSTVLTTHLFLPLRTQPQAF---YNEPDQELPCGPECEYEL |
| Xenopus | LVPEKTFPIIK5KWSSEESVTFYTHYKLRMFF5FSFKDWEYW5LNLKPKLADODEYEQ |
| Zebrafish | VSDAELHLKLVFNENTGQATLTFC5ALHLYQTYLVLDQ5NVRKONPHRLPNETYEV |
| Human   | VHKTFSYFVGFPATVLWELLGPGSEGEGATFYIARFLAVAHSVPLAAQLKMPKFR- |
| Rhesus  | VHKTFSYFVGFPATVLWELLGPGSEGEGATFYIARFLAVAHSVPLAAQLKMPKFR- |
| Mouse   | VHKTFSYFVGFPATVLWELLGPGSEGEGATFYIARFLAVAHSVPLAAQLKMPKFR- |
| Xenopus | V5SCTVGHYGPTTLLFVEER---SESNSQ---CFOIGRFSSAVNSKLAERLPGSITYP- |
| Zebrafish | LRFKSDQIGVYPAFPAFLFR---ENQFR---PFHIVRFIEAOFRSLTAQGCEOFFFF- |
| Human   | TRITNPVYRTRTREERIERPERDARYKLYDELMASSYPPPLRQQLLP---MLLCQSTIFA |
| Rhesus  | TRITNPVYRTRTREERIERPERDARYKLYDELMASSYPPPLRQQLLP---MLLCQSTIFA |
| Mouse   | TRITNPVYRTRTREERIERPERDARYKLYDELMASSYPPPLRQQLLP---MLLCQSTIFA |
| Xenopus | FQIKFQPKEIEREDGFPPVESLNYELERELQNDPFPNHLRS5RIKGLFVNGTSKF |
| Zebrafish | KRLDPNQPSKEIDEGPSSESSQNFVLKIPVLGNYCSISATLFLVERSQRSS |
| Human   | PKIAEAIKAOETAKLWKNYEVRKLRLHLEEOLEHymiRHYELD5VPMTPDVDVDQNPLR |
| Rhesus  | PKIAEAIKAOETAKLWKNYEVRKLRLHLEEOLEHymiRHYELD5VPMTPDVDVDQNPLR |
| Mouse   | PKIAEAIKAOETAKLWKNYEVRKLRLHLEEOLEHymiRHYELD5VPMTPDVDVDQNPLR |
| Xenopus | SS5GTDHRDRL4SSLQFNYNKKHFHLHLEEOLEHymiRHYELD5VPMTPDVDVDQNPLR |
| Zebrafish | GKQFQNLKQSSLRDOFPDLLLEQDEMLGRMIKRYNKKDOQSNVRDKR |
| Human   | LTLVEPVSEPVSRLGRDHLFALLSATHQEDPTYKGFHVHVELDRKLSF5SMILLSA |
| Rhesus  | LTLVEPVSEPVSRLGRDHLFALLSATHQEDPTYKGFHVHVELDRKLSF5SMILLSA |
| Mouse   | LTLVEPVSEPVSRLGRDHLFALLSATHQEDPTYKGFHVHVELDRKLSF5SMILLSA |
| Xenopus | LTLVEPVSEPVSRLGRDHLFALLSATHQEDPTYKGFHVHVELDRKLSF5SMILLSA |
| Zebrafish | LTLVEPVSEPVSRLGRDHLFALLSATHQEDPTYKGFHVHVELDRKLSF5SMILLSA |
| Human   | FVDGTLTFKVNFTPQRPLQVRHARLELTGRLWLMFLMFVPADVRPDPLLPSDVKLKL5DRS |
| Rhesus  | FVDGTLTFKVNFTPQRPLQVRHARLELTGRLWLMFLMFVPADVRPDPLLPSDVKLKL5DRS |
| Mouse   | FVDGTLTFKVNFTPQRPLQVRHARLELTGRLWLMFLMFVPADVRPDPLLPSDVKLKL5DRS |
| Xenopus | FINGLCFDFVSFTFNRPLKIQHRIVLAKORNL5KIFPEASGIFSITDS---QKLYD |
| Zebrafish | FIDNK5FVEFTINPRLQRVHAR5MVQMLKFVL5FVAP5RPNPV5---SALLRDFQK |</p>
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**Figure 1.1 (cont.)**
Figure 1.1 (cont.) Evolutionary conservation of Mov10- Amino acid alignment of Human (NM_020963.4), Rhesus monkey (NM_001261223.1), Mouse (NM_008619.2), Xenopus (XM_018246602.1) and Zebrafish (NM_001044342.2) are shown. The conserved Cysteine and Histidine residues between human, rhesus and mouse that form a consensus CH domain are highlighted in yellow. The Motif I and Motif II of the helicase core which is required for ATP binding and hydrolysis are in bold. Asterisks marks conservation across all five genera, colon indicates strongly similar group conservation; period indicates weakly similar group conservation and dashes are regions of no conservation.
Human
KLGSISKHHKSLAKIFYDRAEYLIHGKVKHGVDVEVQGPHEARDGQLLRLRDLNLKVLTLRLRNGGTQSVLTLHFLPLCRTPQFAFYNEQELPCPLGPGECEYLHVCKTSFGYFPATVLWELLGPGSSEGAGTFYIARFLAAVASHPLAAQLKPMTPFKRTP

Mouse
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UPF-1 consensus sequence
CXYGCI+XZXXUXCXXXKKWFCNX+XZXXSIXUXHVLXX+XZXXVXLHXXXLXXTVLXCYNCGX+NVFLLGUXAXXVXVVLCCRXP

**Figure 1.2.** Proposed CH domain (amino acid residues 93-305) within the N-terminus of human Mov10 (NM_020963.4, NM_001130079.2, NM_001321324.1) based on [18] along with the same amino-acid residues from mouse Mov10 (NM_008619.2, NM_001163441.1). The Upf1 consensus sequence was adapted from [21] and derived from human UPF1, *S.cerevisiae* UPF1 and *S.pombe* UPF1-like. The conserved residues are shown in red.
Figure 1.3. Proposed model for Mov10 in LINE1 suppression - 1) L1 mRNA is transcribed by RNA PolI and bound by Mov10. 2) Mov10 binds at the G-quadruplex present in the L1 3’UTR, either in the nucleus or the cytoplasm (denoted by question mark). 3) The L1 mRNA is translated and assembled into the L1 RNP (4) and is exported back into the nucleus (5). 6) At the site of insertion, the ORF2p which binds to the polyA tail [64] of the L1 mRNA attempts to reversed transcribe the L1 mRNA into cDNA and encounters steric hindrance from Mov10 which is moving in the 5’ to 3’ direction to unwind the G-quadruplex. 7) This prevents the cDNA synthesis and subsequent TPRT and insertion of the L1 at the genomic locus.
1.8 References


CHAPTER 2

MOV10 SUPPRESSES RETROELEMENTS AND REGULATES NEURONAL DEVELOPMENT AND FUNCTION IN THE DEVELOPING BRAIN\textsuperscript{1}.

2.1 Abstract

**Background:** Mov10 is an RNA helicase that mediates access of the RNA-induced Silencing Complex to mRNAs. Until now, its role as an RNA helicase and as a regulator of retrotransposons has been characterized exclusively in cell lines. We investigated the role of Mov10 in mouse brain by examining its expression over development and attempting to create a Mov10 knockout mouse. Loss of both Mov10 copies led to early embryonic lethality. **Results:** Mov10 was significantly elevated in postnatal murine brain where it bound retroelement RNAs and mRNAs. Mov10 suppressed retroelements in the nucleus by directly inhibiting cDNA synthesis while cytosolic Mov10 regulated cytoskeletal mRNAs to influence neurite outgrowth. We verified this important function by observing reduced dendritic arborization in hippocampal neurons from the Mov10 heterozygote mouse and shortened neurites in the Mov10 knockout Neuro2A cells. Knockdown of Fmrp also resulted in shortened neurites. Mov10, Fmrp and Ago2 bound a common set of mRNAs in brain. Reduced Mov10 in murine brain resulted in anxiety and increased activity in a novel environment, supporting its important role in the development of normal brain circuitry. **Conclusions:** Mov10 is essential for normal neuronal development and brain function. Mov10 preferentially binds RNAs involved in actin binding, neuronal projection and cytoskeleton. This is a completely new and critically important function for Mov10 in neuronal development and establishes
precedent for Mov10 being an important candidate in neurological disorders that have underlying cytoarchitectural causes like Autism and Alzheimer’s disease.¹

2.2 Introduction

Mov10 is a Superfamily 1 (SF1) RNA helicase that binds to G-rich secondary structures and unwinds RNA in a 5‘-3’ direction in an ATP-dependent manner [1, 2]. Mov10 was originally described as associating with RNA-induced silencing complex (RISC) factor Argonaute 2 (Ago2) and was required in microRNA (miRNA)-guided cleavage of a reporter [3]. Mov10 also has roles in nonsense-mediated decay, suppression of viral RNAs and retrotransposition in cultured cells [1, 4, 5]. We found that Mov10 associates with the Fragile X mental retardation protein (Fmrp) in adult brain to regulate translation of a commonly bound set of RNAs by modulating their association with Ago2 [2]. Fmrp is required for normal cognition and our findings suggested a possible role for Mov10 in brain function. Currently, there are no studies describing a role for Mov10 in the developing brain.

In the central nervous system, RNA helicases function by affecting neuronal differentiation, RNA localization, cell morphology and apoptosis [6]. Examples of helicases that are miRNA-related include DHX36, which is required for dendritic localization of pre-miR134 [7] and DDX6, which binds TRIM32 to increase the activity of RISC [8]. Importantly, none of these helicases could functionally compensate for Mov10 since the Mov10 knockout is embryonic lethal in mouse.

¹ This chapter has been accepted for publication in BMC biology and is reprinted under the terms of the Creative Commons Attribution 4.0 International License (https://www.biomedcentral.com/licensing).
Mov10 is also significantly elevated in brain shortly after birth through adolescence. Isolation of Mov10-associated RNAs from P2 brain reveals two critical roles for Mov10 in early brain development: a suppressor of retrotransposition and a regulator of neuronal projections. Two-thirds of the Mov10-associated RNAs encode retroelements including Long Interspersed Nuclear Elements (LINEs) while the rest of the mRNAs encode proteins involved with neurite outgrowth and cytoskeleton.

Mov10 is a strong suppressor of endogenous transposition of L1, an active LINE element in cultured cells [5, 9]. During neuronal differentiation, there is increased L1 retrotransposition in hippocampus and several regions of the adult brain. We hypothesize that Mov10 is elevated in postnatal brain to suppress retrotransposition, which is highly active during this time in brain [10]. As neurons mature and arborize, Mov10 regulates the translation of actin binding proteins and cytoskeleton, which is required for neuronal migration and function. This is the first study to show a role for Mov10 during embryogenesis and in postnatal brain development and function. We propose that Mov10 is vital for viability and for normal CNS development and function.

2.3 Results

Mov10 is elevated in postnatal mouse brain

Since Mov10 functionally associates with Fmrp [2], we examined Mov10 expression in the postnatal murine brain across development. As early as embryonic day 18, there was a higher level of Mov10 in whole brain compared to adult (Fig.2.1A, compare first and last lanes). Mov10 expression continued to rise at birth (P0) and remained elevated over adult levels until P10-P14 when it began to decline (Fig.2.1A).
We observed the same increase in postnatal Mov10 levels in a different mouse strain (FVB, Additional file 2.1A) and it was independent of sex (Additional file 2.1B). We conclude that Mov10 is elevated in postnatal and juvenile mouse brain, suggesting an important role for Mov10 in the developing brain.

To determine the pattern of Mov10 expression, we stained sagittal sections of postnatal and adult brain to examine if Mov10 was elevated in specific brain regions. Mov10 was highly expressed throughout P1 brain, including cortex, hippocampus, cerebellum, midbrain and hindbrain (Fig.2.1C). In contrast, there was very little Mov10 expression in adult brain except in hippocampus (Additional file 2.2A, right). However, the hippocampus and cortex of P0 mice expressed much more Mov10 than did the adult hippocampus and cortex (Additional file 2.2A, 2.2B). In addition, neurons appeared to have both nuclear and cytoplasmic staining in the P0 mice compared to the adult (Additional file 2.2A, see inset).

Since Mov10 has previously been described as cytoplasmic in both cultured cells [3, 5] and in cultured hippocampal neurons [11], we examined Mov10 localization in P2 brain. We observed Mov10 in the nucleus as well as cytoplasm (Fig.2.1D-F, P2). In contrast, Mov10 was primarily cytoplasmic in adult hippocampus (Fig.2.1G-I, Adult). To verify these age-dependent differences in the intracellular localization of Mov10 and using a different Mov10 antibody, we examined hippocampal neurons cultured from P0 mice. We found that Mov10 was distinctly nuclear in Day In Vitro (DIV) 1 neurons (Additional file 2.2C, DIV1) compared to DIV14 neurons, where it was primarily cytoplasmic (Additional file 2.2C, DIV14), as previously reported [11]. We further confirmed the nuclear presence of Mov10 by biochemical fractionation of P2 brain
Mov10 expression was also examined in testes, where it is highly expressed, and found to be cytoplasmic (Additional file 2.2D). We conclude that Mov10 is in the nucleus and the cytoplasm in postnatal brain.

**Mov10 knockout is embryonic lethal**

Mov10, like Fmrp, is expressed throughout the brain. In order to study the function of Mov10 at postnatal stages in brain, we attempted to generate a Mov10 knockout mouse using an ES cell with one copy of Mov10 targeted by a gene trap vector (Additional file 2.3). After screening 156 pups from heterozygote crosses, we found no viable Mov10 knockouts (Table 2.1) and concluded with >95% confidence that the Mov10 knockout has an embryonic lethal phenotype [12]. To determine when Mov10 exerts its crucial effect, we genotyped embryos from E9.5 and E12.5 and failed to detect any Mov10 knockout embryos at these early stages (Table 2.1). Based on this data, we conclude that Mov10 is essential for embryonic development in mouse.

**Mov10 suppresses LINE retrotransposition in the nucleus**

To investigate the role of Mov10 in early brain development, we performed RNA-immunoprecipitation (RIP) from P2 brains and sequenced the RNAs bound to Mov10. The total number of reads was 98,884,367; the number of aligned reads was 71,522,027 (74.59% aligned) and the number of uniquely aligned reads was 57,005,129 (59.45%). We used RIPSeeker to identify RNAs significantly enriched over input RNA [13] and found 2996 RIP peaks: 1313 overlapped with repeat elements from the Long Terminal Repeat Family (LTRs), the autonomous Non-LTR family of LINEs, and the
non-autonomous SINEs in the RepeatMasker database (Fig. 2.2A, Additional File 2.4-see online) and 525 peaks overlapped with RefSeq, indicating that they were mRNAs (Fig. 2.2A, Additional File 2.5-see online). We validated the RIPSeeker result by immunoprecipitating Mov10 from P2 mouse brain and performing RT-PCR on an endogenously active autonomous retrotransposon mL1T_F as well as Prrc2b, a brain mRNA target of Mov10 (Fig. 2.2B) [14]. Although Mov10 has previously been shown to bind the L1 transcript [5] we showed here that it binds L1 transcripts from the retrotransposition-competent T_F subfamily of mouse L1s (Fig. 2.2B). These primers have been used before by others [15]; however, it is possible that the RT-PCR to detect L1 expression is off-targeting to L1 sequence fragments that might be contained in mRNAs. Thus, we cannot rule out the possibility that some of the immunoprecipitated signal could be due to the presence of an mRNA that happens to contain the L1 primer target sequence.

Our hypothesis is that Mov10 binds the RNA of retroelements and inhibits their transposition in developing brain. To test this hypothesis, we quantified the amount of genomic L1 in P2 brains from heterozygous Mov10 knockout mice compared to WT, hypothesizing that the reduction in Mov10 would lead to an increase in L1 retrotransposition events, as observed in the MeCP2 knockout mice [16]. The qPCR was done using genomic DNA treated with Exonuclease1 to remove any unintegrated cDNA and RNase H to remove DNA-RNA hybrids that might artificially contribute to the observed increase in LINE content. Similar to the MeCP2 study, we found a 2-fold increase in L1 genomic content in the Mov10 heterozygotes (Fig. 2.2C, 2.2D, Additional file 2.6-see online), supporting a role for Mov10 in L1 suppression in developing brain.
APOBEC3G is an RNA editing enzyme that plays a key role in regulating retrotransposition by directly binding reverse transcriptases [17, 18] and also by binding RNAs to sterically block reverse transcriptase (RT) activity [19, 20]. To determine whether Mov10 was able to block RT activity, we incubated equal molar amounts of Mov10 and SuperScript III Reverse Transcriptase, an engineered version of M-MLV RT, and then performed a reverse transcription reaction in which total RNA from P2 brains was added. Reverse transcription of both L1 RNA and Prrc2b RNA was blocked by addition of Mov10. In contrast reverse transcription of the Gapdh transcript, which is not bound by Mov10 [2] was only partially inhibited (Fig.2.2E, 2.2F, Additional file 2.6-see online). We also tested purified recombinant human FMRP, another RNA binding protein, in this assay and found that the addition of FMRP did not have an effect on cDNA synthesis (Fig.2.2E). Thus, Mov10 blocked reverse transcription of its bound targets more efficiently than that of non-target RNAs. Others agree that Mov10 is a suppressor of retrotransposition [5] but the mechanism is controversial. The LEAP assay (L1 element amplification protocol) [21], which measures the ability of purified L1 RNP to reverse transcribe the bound L1 RNA was used in [5] to show that Mov10 suppresses reverse transcriptase activity, which agrees with our results using recombinant His-tagged Mov10 purified from Sf9 cells [2]. Our data however contradicts the study in [22] where the LEAP assay was performed from L1 RNPs isolated from HeLa cells and treated with recombinant Mov10 (Origene). Possible explanations for the differing results are that the sources of recombinant Mov10 are different, as are the methods of purification, which could have affected the presence of cofactors. Mov10 purified from mammalian cells may also have post-translational modifications that are
not present in the Sf9 purified Mov10. Li and colleagues concluded that Mov10 blocks retrotransposition by facilitating L1 RNA degradation and its helicase activity is required for this function [9]. We tested the helicase-deficient mutant of Mov10, where a conserved lysine in Motif I has been mutated to alanine [1], in our in vitro assay and found that it does not suppress the cDNA synthesis of either mL1TF or Prrc2b (Fig 2.2F, Additional file 2.6-see online). Thus, the helicase function of Mov10 is required to block RT activity.

To determine if Mov10 directly bound RT, we coupled either SSRTIII or BSA to beads and found that only SSRTIII efficiently captured Mov10. Additionally, we performed the capture using either the C-terminal half or the N-terminal half of Mov10 and found that only the N-terminal half could bind SSRTIII (Fig.2.2H). The unstructured N-terminus of Mov10 has been implicated in inhibiting HIV-infectivity, though the exact mechanism is unclear [23]. Our data suggest that Mov10 binds reverse transcriptase through its N-terminal region and unwinds the L1 RNA using its C-terminal helicase domains. Importantly, Mov10 directly bound ORF2p, which is the RT/endonuclease encoded by L1 (Fig.2.2I). RNAse treatment did not significantly change the amount of immunoprecipitated ORF2p (the difference between the indicated ratios is not significant), suggesting a protein-protein interaction. We conclude that Mov10 is elevated in the nucleus during postnatal brain development when retrotransposition is active to bind retroelement RNAs and block reverse transcription, which is a critical step for retrotransposon insertion.
Mov10 associates with cytoskeletal mRNAs to regulate neurite outgrowth

Approximately one-third of the Mov10-associated RNAs in postnatal brain RIP-seq were mRNAs (Fig. 2.2A). We used the DAVID Bioinformatics database to analyze the Gene Ontology (GO) terms assigned to the 525 RNAs, which revealed axon, neuron projection, growth cone and dendrite among the most significant categories (Fig. 2.3A, p-values 9.1x10^-9, 1.3x10^-8, 7.8x10^-7, 8.6x10^-7, respectively). To independently verify this result, we performed individual nucleotide cross-linking immunoprecipitation (iCLIP) on brains isolated from P0-P1 mice (Fig. 2.3B, right panel) and obtained 92,798,446 reads; after quality trimming and deduplication, there were 5,269,506 reads. Further analysis revealed 61,471 unique tags present in the Mov10 IP compared to 3545 tags in the irrelevant IP. 2988 of the tags aligned to the genome, 2333 uniquely aligned and 729 regions were identified. The gene identities are in Additional File 2.7. GO analysis using a P1 brain transcriptome as background revealed that RNAs encoding proteins involved in neuron projection had the lowest P value (Figure 2.3B, Cellular Compartments category). Under GO category Molecular Function, actin binding and protein binding were the most enriched (Figure 2.3B bottom). In addition, the GO term in the Biological Process category with the lowest P value (9.1x10^-5) was actin cytoskeletal organization. These data suggest a cytoplasmic role for Mov10 in regulating actin and cytoskeletal mRNA expression in the postnatal brain.

To determine if Mov10 functions in neurite outgrowth, we used CRISPR-Cas9 to knockout Mov10 (Additional file 2.4A) in Neuro2a (N2a) cells, a murine neuroblastoma that has long branching processes when grown on a substrate [24]. We induced
differentiation of the WT and Mov10 knockout (KO) N2a cells and found significantly reduced neurite length in the Mov10 KO cell line compared to WT (Fig. 2.4A-D) that could be rescued upon re-introduction of the Mov10 transgene, suggesting that this phenotype was directly attributable to the loss of Mov10 and not an off-target effect.

To determine how Mov10 participates in differentiation, we isolated RNA from undifferentiated and differentiated WT and KO N2a cells and performed high-resolution RNA-seq analysis. Samples were sequenced extremely deep to around 350,000,000 paired-end 100 base pair (bp) to capture lowly expressed genes, with at least 86% reads mapping to the mm10 mouse genome. We identified 16,551 genes and found that 324 genes changed significantly between the differentiated and undifferentiated states of WT N2a with 180 increasing and 144 decreasing (Fig. 2.4E top, Additional File 2.9-see online). GO analysis revealed that RNAs implicated in cell cycle arrest were significantly changed (p-value 4x10^{-4}) under the GO category Biological Process, which is expected since undifferentiated cells proliferate in contrast to differentiated cells (Additional File 2.9-see online). GO terms in the category Cellular Compartments revealed a significant enrichment for RNAs implicated in extracellular region/space and neuron projection (Additional file 2.4B).

To identify Mov10-dependent genes, we compared undifferentiated WT to KO and found 813 significantly changed RNAs (300+513) while a comparison of differentiated WT to KO showed 781 RNAs (513+268) that were significantly changed (Fig.2.4E). 513 genes were shared, suggesting that their expression was regulated by Mov10 and independent of differentiation (Additional File 2.10- see online). GO analysis of these Mov10 target genes revealed strong clustering with terms relating to nervous
system development, axon guidance, and neuron projection with the majority of the genes in the groups being down regulated (Fig.2.4F, orange indicates proportion significantly downregulated and blue indicates proportion significantly upregulated in the KO, see Additional File 2.10 for gene list online). This result suggests a more general function for Mov10 as an RNA remodeler in addition to its role in revealing microRNA recognition elements (MREs). Similar to its function with Fmrp and Ago2, the fate of the mRNA depended on where Mov10 and Fmrp bound in the 3'UTR [2, 25]. The data also show that Mov10 expression is critical for neurite outgrowth in N2a cells and is consistent with the data from mouse brain, where genes representing cytoskeletal components are the predominant functional categories. Additionally, we verified the Mov10 dependence of Microtubule-associated protein 1b (Map1b), a cytoskeletal protein important in neurite outgrowth [26] and found that it was reproducibly reduced in the Mov10 KO and rescued on Mov10 re-expression (Fig.2.4G). Map1b levels went down significantly in the KO cells under both differentiated and undifferentiated conditions, suggesting that it was a direct target of Mov10 irrespective of the differentiation program.

To identify the genes regulated by Mov10 that participate in differentiation, we compared the 324 (144+180) genes that significantly changed during WT differentiation (Fig.2.4E) with Mov10 KO differentiation. There were 64 genes that significantly changed in the opposite direction in the differentiated Mov10 KO compared to WT (Fig.2.4H). This group of 64 Mov10-dependent, differentiation-specific genes include key growth signals such as FGF1 and transcription factors like TEAD2 along with cytoskeletal genes such as actin isoforms and Tnnt1 (Additional File 2.9- see online).
We appreciate that the RNA-seq data include genes indirectly affected by Mov10 loss and are not necessarily directly bound by Mov10, although we do expect there to be some direct mRNA targets of Mov10. The direct binding of Mov10 to cytoskeletal mRNAs from the RIP and iCLIP data suggest that misregulation of those genes in the Mov10 KO leads to reduced neurite outgrowth. In fact some of the same cytoskeletal-related RNAs are found in both the Mov10-dependent genes in N2a (Additional file 2.10- see online) and the mouse brain iCLIP lists (Additional Tables 2.7 and 2.11 as described in the Methods- see online). We conclude that Mov10 plays a key role in neurite development and process formation through its regulation of cytoskeletal and neuroregulatory mRNAs.

Fmrp predominantly binds brain mRNAs that function in neuron projection [27]. Because we have evidence that Fmrp and Mov10 functionally associate in HEK293 cells [2], we examined the effect of Fmrp knockdown on neurite outgrowth in N2a and found that it was significantly reduced (Fig. 2.5A). To ask whether Fmrp and Mov10 functioned in the same pathway, we compared neurite length in the Fmrp/Mov10 double knockdown to the Fmrp knockdown alone and found no difference (Fig. 2.5A). This result suggests that Fmrp and Mov10 function in the same pathway.

In earlier work, we showed that when Fmrp and Mov10 bound the same region in the 3’UTR of cobound mRNAs, binding by Ago2 was blocked [2]. In this subset of mRNAs, Fmrp-Mov10 interaction had a protective effect on the mRNA. To identify commonly bound brain mRNA targets of Fmrp, Mov10 and Ago2, we compared the iCLIP targets of whole brain-derived Mov10, analyzed as described (Methods and [2], Additional File 2.11-see online), which came from postnatal mice (P0, P1). We
compared these genes to previously published lists of iCLIP targets from brain-derived Fmrp [27], which came from mice aged P11-P25 and iCLIP targets from human brain-derived Ago2, which came from adult motor cortex and cingulate gyrus from males aged 44-68 [28] (Fig.2.5B). Despite differences between species and age, we found significant overlaps between the Fmrp and Mov10 targets, between the Mov10 and Ago2 targets, between the Ago2 and Fmrp targets and between all three proteins. All overlaps were highly significant (p=2.15^{-19}, p=5.57^{-26} and, p=4.85^{-159}, p=0.00000 respectively). Using a permutation approach, we also determined that the amounts of overlap in the Venn diagram were significantly more than expected by chance (Fig.2.5B) (see Methods). Thus, Fmrp, Mov10 and Ago2 bind a common set of brain mRNAs (Additional File 2.12- see online). To understand what the functional consequences of such binding might be, we performed GO analysis of the 47 commonly bound Mov10-Fmrp-Ago mRNAs and found an enrichment of dendrite, synapse and neuron projection terms under the GO category Cellular Compartments (Fig. 2.5C). These data suggest a miRNA-mediated function for cytosolic Mov10 in regulating cytoskeletal genes. Map1b was one of the genes present in the Fmrp-Mov10-Ago2 overlap (Fig. 2.5B) and is regulated by Fmrp through the miRNA pathway [29]. Similar to the fate described for the Fmrp/Mov10/Ago2-cobound mRNAs in HEK293, Map1b is reduced in the absence of Mov10 (Fig. 2.4G), suggesting a protective role for Mov10, likely in association with Fmrp.
Role of Mov10 in neuronal maturation and behavior

Because Mov10 is highly expressed in developing brain and is required for normal neurite development, we hypothesized that the Mov10 heterozygote mouse would show a phenotype. This was the case for the microprocessor component DGCR8: loss of both alleles was embryonic lethal but the heterozygotes had a neuronal and behavioral phenotype [30-32]. We verified that the Mov10 heterozygote mouse (HET) expressed half as much Mov10 in brain (Fig.2.6A) and then examined cultured hippocampal neurons from WT and Mov10 heterozygotes (HET). Mov10 heterozygotes had markedly less dendritic branching compared to the WT neurons (Fig.2.6B, C). To quantify the difference between the Mov10 heterozygote and WT neurons, we performed Sholl analysis of all orders of branches (Total Sholl) [33, 34] and observed that a reduction in Mov10 levels significantly decreased dendritic branching at a maximum distance of 120 µM away from the cell body (*p < 0.05) (Fig. 2.6D). Thus, normal levels of Mov10 are required for normal dendritic arborization.

To determine whether reduced Mov10 levels affected neuronal function, we tested the Mov10 heterozygotes in behavioral tests and found that the Mov10 heterozygote showed a significant increase in activity in an open-field compared to WT littermates (Fig.2.7A), suggesting anxiety and/or hyperactive behavior. The Mov10 heterozygotes also spent significantly less time in the open arms in an elevated plus maze test, suggesting an anxiety phenotype (Fig.2.7B, Additional file 2.5B). In contrast we did not see a difference in performance on the rotarod, trace fear conditioning and novel object recognition (Additional file 2.5 A,C,D,E). The increased activity in a novel environment and increased anxiety seen in the Mov10 heterozygotes suggests that an
element of the neuronal circuitry is perturbed in these mice [35]. Thus, WT levels of Mov10 are required for normal neuronal development and function.

2.4 Discussion

We show here two independent and previously undescribed roles for Mov10 in embryonic development and postnatal brain. Like the Ago2 knockout, the Mov10 knockout is also embryonic lethal [36-38] supporting their critical role in miRNA-mediated regulation during development. Since Mov10 is present in both the nucleus and cytoplasm in neurons, we believe that it is co-opted for critical but distinct functions in brain development. We propose that in addition to the cytoplasmic miRNA-mediated function of Mov10 in regulating neurite outgrowth in brain, the developmentally timed increase of Mov10 acts as a defense against nuclear L1 retrotransposition.

Nuclear Mov10 in LINE-1 suppression

There is extensive data in cell culture for Mov10’s role in suppressing LINE-1 retrotransposons [5, 9], although the mechanism is unknown. We present evidence that Mov10 directly binds retrotransposon mRNAs in postnatal brain at stages when neuronal differentiation is high and acts to inhibit their reverse transcription (Fig 2.2A, 2.2B) [39]. Significantly, the consequence of reducing Mov10 in brain increases L1 content in the genome in P2 brains (Fig.2.2C, 2.2D). The mechanism by which Mov10 inhibits reverse transcription could be by a steric block of ORF2p on L1 mRNA. The L1 endonuclease and reverse transcriptase ORF2p binds the poly(A) tract of L1 mRNA to mobilize it to the insertion site where it nicks the DNA to prime reverse transcription in a
3’- 5’ direction [40, 41]. We showed previously that Mov10 binds G-rich regions, including G-quadruplexes [2]. Thus, we suspect that Mov10 binds the G-rich polypyrimidine tracts [2] present in the 3'UTRs of L1 mRNAs [42] and also interacts with ORF2p through it’s N-terminal domain. Subsequently, Mov10 proceeds to unwind in the 5’-3’ direction causing a steric hindrance to the progress of ORF2p. In support of this hypothesis, the helicase-deficient mutant of Mov10 is unable to block reverse transcription of L1 mRNAs (Fig.2.2G). A recent study shows that the G-rich tracts in L1s stimulate retrotransposition [43]. We would hypothesize that Mov10 is elevated in brain postnatally and localizes to the nucleus to suppress this event.

**Mov10 in neurite outgrowth, neuronal development and brain function**

The RNA-IP from postnatal brain also shows a preponderance of actin and cytoskeletal mRNAs, suggesting an important role for Mov10 in regulating cytoskeletal dynamics in the brain (Fig.2.3). The same observation was made in the RNA–seq analysis from Mov10 N2a KO cells further confirming a critical role for Mov10 in neurite outgrowth (Fig.4). We hypothesize that this reflects Mov10’s cytoplasmic role in miRNA–mediated regulation [2]. It likely plays a role with Fmrp in modulating Ago2 association with cobound RNAs (Fig.2.5).

Mov10 has low expression in the adult brain (Fig.2.1) similar to what is reported in the Allen brain atlas [44, 45]. However, there is no report of Mov10 expression in developing brain. We observe an approximately 40-fold increase in Mov10 levels in P0-P3 mouse brain (Fig.2.1) when events like synaptogenesis, synaptic pruning and neuronal differentiation are occurring to shape normal brain circuitry [39]. Mov10 is
important for these events since a 50% reduction in Mov10 levels leads to less dendritic complexity in hippocampal neurons (Fig. 2.6B-D). These data suggest a role for Mov10 in the normal development of brain circuitry. Based on the evidence that Mov10 preferentially binds cytoskeletal mRNAs, we hypothesize that the reduction in Mov10 affects dendritic morphology and synaptic remodeling in the brain. Accordingly the Mov10 heterozygote mice show increased activity in a novel environment and higher anxiety, suggesting that Mov10 is required for normal brain function (Fig. 2.7A, B). It is also possible that the increased retrotransposition activity in the Mov10 heterozygote could be contributing to the neuronal phenotype and behavior. In fact, increased L1 insertions have been implicated in the development or predisposition to psychiatric disorders [46, 47].

The cytoarchitecture of neurons has implications in the neuropathology of autism and neurodegenerative disorders [48, 49]. In fact CNVs containing Mov10 have been found in individuals with developmental delay [50-53]. Our study demonstrates that Mov10 is essential in embryonic development, in normal neuronal development and in brain function. It remains to be determined how Mov10 levels are regulated in brain.

2.5 Conclusions

Mov10 is significantly elevated in postnatal murine brain where it binds retroelement RNAs and mRNAs. Mov10 suppresses retroelements in the nucleus by directly inhibiting cDNA synthesis while cytosolic Mov10 regulates cytoskeletal mRNAs to influence neurite outgrowth. Finally, reduced Mov10 in murine brain results in anxiety
and increased activity in a novel environment. In summary, Mov10 is essential for embryonic viability, normal CNS development and function.

2.6 Methods

Western Blot

Samples from at least three biological replicates were prepared for immunoblotting after quantification by Bradford assay and suspension in 1x sample buffer, resolved by SDS-PAGE and analyzed by western/immuno-blotting. Briefly, membranes were blocked with 5% non-fat dry milk in PBS containing 1% TWEEN-20 for 1 hour at room temperature. Primary antibody was applied for 1hr at room temperature or overnight at 4°C followed by a brief wash in 1% non-fat milk PBS containing 1% TWEEN-20 wash buffer. HRP-conjugated secondary antibody was applied at 1:5000 dilution for 1 hour at room temperature and washed 4x15 minutes using wash buffer. HRP signal was detected using ECL and exposed to film. The antibodies used were anti-Mov10 (Bethyl A301-571A, RRID: AB-1040002) at 1:1000, anti-Cbx7 (Santa Cruz, sc-70232, RRID:AB_2071502) at 1:2000, anti-eIF5 (RRID:AB_631427) (Santa Cruz) at 1:10,000, anti-Gapdh (Abcam ab9484, RRID:AB_307274), anti-LINE-1 (Santa Cruz, sc-67198, RRID:AB_1249550), HRP-conjugated anti-rabbit and anti-mouse antibodies from GE Healthcare (RRID:AB_772191) and Jackson Immunoresearch (RRID:AB_2338512), respectively. The level of significance and tests performed are described in the figure legends for each experiment.

Whole mouse brain fixation, sectioning and staining

Three adult C57BL6 (Envigo, USA {formerly known as ‘Harlan’}) males were euthanized
and whole body fixation was done using 4% Paraformaldehyde in PBS. The brain was
dissected out and fixed with a series of ethanol washes for 30 minutes (25%, 50%,
70%, 83%, 95% and 100%) and left in methyl salicylate for 3 hours to overnight before
embedding in paraffin. For P0 and P1 pups, whole, skinned, heads were fixed in 4%
paraformaldehyde overnight and dehydrated similar to adult brain. Sections were
prepared using a Spencer 820 rotary microtome and dried overnight at room
temperature. The sections were de-paraffinized using xylene and rehydrated through a
series of ethanol washes (100% and 95% followed by 1X PBS) before boiling in 1X
citrate (pH-6.0) for epitope retrieval. The sections were stained using a primary antibody
to Mov10 (Abcam ab60132, RRID: AB_944250) at 1:100 and Alexa fluor 596 at 1:800
(RRID: AB_2340621 Jackson Immunoresearch) before imaging using a Nanozoomer
Slide Scanner (Hamamtsu) and Zeiss LSM700 Confocal microscope. DAB staining was
done using the same antibodies and following the instructions in the DAB staining Kit
(Vector Labs) and counterstained with Hematoxylin before imaging on the Nanozoomer
Slide Scanner. Testes sections were stained using anti-Mov10 (Bethyl A500-009A,
RRID: AB_10950563) and anti-mouse-Cy3 (RRID: AB_2340813 Jackson
Immunoresearch).

Brain IP, RT-PCR and RNA sequencing

For the brain-RIP-seq, brains were harvested from 28 WT P2 pups. For confirming
specific transcripts by brain IP, 3 WT P2 brains were used. All were triturated in HBSS
and then UV-crosslinked thrice for the confirmatory IP. Triturated cells were lysed in
Lysis buffer (50mM Tris-Cl 7.5, 300mM NaCl, 30mM EDTA, 0.5% Triton), cleared by
ultracentrifugation (35000 rpm at 35min at 4°C) and sequentially immunoprecipitated
with an irrelevant rabbit polyclonal antibody (anti-EGFP, Clontech, RRID: AB_10013427) followed by IP with Mov10 antibody (Bethyl A301-571A, RRID: AB_1040002). Both IP’s were washed sequentially for 10min with Lysis buffer and twice with Wash buffer (1X PBS, 0.1% SDS, 0.5% Sodium deoxycholate, 1% NP40). The IP’s were treated with 500 units of RNAse-free DNase I, washed once for 10min with High salt buffer (50mM Tris, 1M NaCl, 1mM EDTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate). To isolate associated RNA, the IP’s were treated with proteinase K followed by TRIZOL (Ambion) extraction for RNA isolation. The ethanol precipitated RNA was quantified and equal amounts were used for cDNA synthesis followed by RNaseH treatment. The RNA was extracted with phenol-chloroform and precipitated in ethanol, converted into cDNA using Oligo dT primer and Superscript III Reverse Transcriptase. qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad) using a StepOnePlus RT PCR machine (Applied Biosystems) with gene specific primers. For the brain IP-RNA sequencing, WT brains were homogenized in the same manner as described above but were not UV crosslinked. Additionally, the Trizol extracted RNA was cleaned using an RNA Clean and Concentrator Kit (Zymo Research) before sequencing.

**RIP-Seq analysis**

Total input RNA, and RNA extracted from the irrelevant IP and the Mov10 IP were used for making libraries and produced over 230 million reads with perfect quality scores. The contribution from the irrelevant IP was negligible and removed from further analysis. Each fastq file was broken into 100 smaller fastq files using a Perl script downloaded from [54]. TopHat2 (version 2.1.1, RRID: SCR_013035) was run on each individual
smaller fastq files using --g 2000 and all default parameters. Setting --g 2000 instructs TopHat2 to allow up to 2000 alignments to the reference (version mm10 that is not masked for repetitive regions, obtained from illumina igenomes) for a given read.

The resulting alignment files in BAM format were merged into a single BAM file for each sample using samtools (version 1.3). BAM files were then sorted based on chromosome coordinates of alignments using novosort (novocraft version 3.02; RRID: SCR_014818). The total number of reads from the Mov10 IP were 98,884,367, the number of aligned reads were 71,522,027 (74.59% aligned), the number of uniquely aligned reads was 57,005,129 (59.45%). From the input sample, the total number of reads were 67,566,885, the number of aligned reads were 59,620,379 (88.24% aligned). The number of uniquely aligned reads was 29,124,706 (43.11%). To identify protein-associated transcripts, a Bioconductor based statistical package RIPSeeker was used [13]. RIPSeeker's (version 3.3; RRID: SCR_006810) function ripSeek was run using the alignments generated by TopHat2 with parameters:uniqueHit=TRUE, assignMultihits=TRUE, rerunWithDisambiguatedMultihits=TRUE, and binSize=NULL. Setting uniqueHit=TRUE requires training Hidden Markov Model (HMM) with only the unique hits, assignMultihits=TRUE enables function disambiguateMultihits to assign each multihit to a unique locus based on the posterior probabilities derived from HMM, rerunWithDisambiguatedMultihits = TRUE tells RIPSeeker to retrain the HMM using the dataset with disambiguated multihits, binSize = NULL enables automatic bin size selection. ripSeek function was run separately for plus strand and minus strand and the output files in GFF3 format were combined into a single GFF3 file that contains genomic coordinates for all the regions identified to be significantly enriched in Mov10 IP
compared to Input. To identify repeat elements and transcripts that overlap with RIP regions identified by RIPSeeker, two tab delimited text files were downloaded from UCSC Genome browser’s table browser interface. One text file contains genomic coordinates of all repeat elements on mouse reference genome mm10 extracted from repeat masker database and another text file contains genomic coordinates of all mouse transcripts on mouse reference genome mm10 extracted from NCBI RefSeq database (RRID: SCR_003496). Bedtools intersect (version 2.25.0) was run to identify repeat elements and mouse transcripts that overlap with RIP regions. 2996 RIP peaks were identified: 1313 overlapped with repeat elements from the repeat masker database and 1683 peaks had no overlaps with repeat masker with 755 peaks overlapping with RefSeq.

**Mouse brain iCLIP analyses**

16 brains from P0 and P1 mice were triturated in HBSS and UV-cross-linked three times (Stratalinker) with mixing between treatments. A published iCLIP protocol was followed [55, 56]. The irrelevant IP was performed with a rabbit affinity purified antibody EGFP (Clontech, RRID: AB_10013427) The Mov10 IP was performed with antibody (Bethyl A301-571A, RRID: AB_1040002). Mov10-CLIP libraries were sequenced by the UIUC sequencing core facility using the Illumina HiSeq2000 platform. The fastq data was trimmed using Trimmomatic (version 0.30, RRID: SCR_011848) to (1st) trim off (crop) the last 14 nucleotides from all the reads, (2nd) trim nucleotides with a quality value lower than 20, from the far (3’ end) of the read, (3rd) trim nucleotides with a quality value lower than 25 from the 5’ end of the read, (4th) remove the adaptor/known contaminant.
Reads with 30 or more nucleotides remaining after the trimming were kept. This data, processed as described below, is presented in Additional File 2.7. Reads with 18 or more nucleotides remaining after the additional trimming were kept and processed as described below. The genes identified are in Additional File 2.11 and were used in the comparisons in Figure 5. The fastq files were converted to fasta files, which were compressed to eliminate duplications, based on the tags. The compressed fasta file of tags was then separated into 2 files—representing the irrelevant and Mov10 immunoprecipitations—each file containing the tags with a specific barcode. This step utilized scripts that did the separation and also removed the barcode from the read, in preparation for the alignment step. The separated samples were aligned to the mouse genome (mm10) using Novoalign (RRID: SCR_014818). The only parameter specified was “-t 60”, this allows for 2 mismatches between the genome and the read. Uniquely mapping reads were extracted from the resulting sam files and the information was converted to bam format using samtools (samtools view; RRID: SCR_002105). The bam format was converted to bed (genome interval) format using bedtools (version 2.17.0; RRID: SCR_006646) (bamToBed). The genome intervals of the reads (bed file) for each sample were merged into larger intervals using bedtools (mergeBed). The new interval/region is a location with a set of overlapping reads. Any regions that had any presence in the control Irrelevant samples (intersectBed) were removed to give the final set of experimental genome intervals that have reads mapping to them in the Mov10 immunoprecipitations, which will be referred to as “Regions” from here on. IntersectBed (bedtools) was used to determine which regions overlap with genes, exons, UTRs, IncRNAs (Ensembl) regions using bed files specific for these features respectively. All
regions that overlap with genes, but not with exons are considered intronic, and all other regions are considered intergenic. DAVID 6.8 analysis [57, 58] (RRID:SCR_001881) was performed on the Mov10 CLIP targets using a P1 C57Bl/6 brain background (GEO numbers GSM417923, GSM417922, GSM417921)

**In-vitro reverse transcriptase assays**

Total RNA from three P2 mice brains was used in the reverse transcription assay with either Superscript III Reverse Transcriptase (Invitrogen ThermoScientific, Carlsbad, CA) alone or with equimolar amounts of recombinant Mov10 or FMRP [2] after pre-incubating the proteins on ice for 5min. cDNA synthesis was carried out at 50°C for 45min, 70°C for 15min. RT-PCR reactions was carried out on the cDNA samples using primers to Prrc2b, mL1T_F or Gapdh (Additional File 14). For q-RT-PCR, iQ SYBR Green Supermix (Bio-Rad) was used and the reactions were set up in a StepOnePlus RT PCR machine (Applied Biosystems) with gene specific primers.

**Purification of the C-term, N-term and Mov10 helicase mutant**

The Myc-tagged Mov10 helicase mutant was generated using site-directed mutagenesis to mutate the conserved lysine in Motif I to Alanine (K531A). The HA-tagged N-terminal half and C-terminal half plasmids was obtained from [23]. Constructs were transfected using PEI (polyethylenimine, # 408727, Sigma) in Freestyle HEK 293F cells (Invitrogen) and cultured according to the manufacturer’s protocol and as described [2]. The cells were harvested after 48 hours and lysed in Lysis buffer (50mM Tris-Cl 7.5, 150mM NaCl, 30mM EDTA, 0.5% Triton) containing Protease inhibitors (Roche, Indianapolis, IN) and spun at 14000 rpm for 5min at 4°C. The supernatant was immunoprecipitated
with anti-HA Magnetic beads (Thermo Fisher Scientific, Carlsbad, CA) for the HA tagged C-term and N-term Mov10 and peptide eluted with HA peptide (2 mg/ml, Protein Sciences, Roy J Carver Biotech Center, UIUC) for 2 hours at 4°C. The Mov10 helicase mutant was immunoprecipitated using agarose beads coupled to myc antibody (RRID: AB_10109522, Sigma-Aldrich, St. Louis, MO) and peptide eluted using the myc peptide (2 mg/ml, Protein Sciences, Roy J Carver Biotech Center, UIUC). Protein concentrations were calculated using Bradford Assay (Bio-rad Laboratories, Hercules, CA) and visualized on silver stain.

**Protein Binding Assay**

For testing direct binding of recombinant WT and helicase deficient Mov10 and SSRTIII, 5ug of BSA or SSRTIII were coupled to M270-epoxy Dynabeads (Life Technologies) overnight at 4°C. The protein-coated beads were washed according to the manufacturers protocol and 10ul of beads (2.5ug protein) was used in a reaction with equimolar amounts of recombinant Mov10 in three independent trials. The reactions were incubated on ice for 30min and washed in 1X PBS. The samples were subsequently processed for Western blotting with the Mov10-specific antibody.

**Genomic DNA isolation and qRT-PCR**

Brains were dissected from three separate WT and Mov10 heterozygote mice and total genomic DNA was isolated using the DNAzol reagent (Invitrogen). The DNA was ethanol precipitated and treated with ExonucleaseI (NEB, Ipswitch, MA) or RNAse H (NEB) as per the manufacturer’s protocol. Equal amounts were used in quantitative RT-PCR using primers for ORF2 and 5S rDNA to estimate total LINE-1 content (see
N2a transfection, neurite analysis and preparation for RNA sequencing

N2a wild type (RRID: CVCL_0470) and Mov10 KO clones were plated in triplicate at a density of 1.5x10^4 cells per well and incubated for 24 hours at 37°C in DMEM (with 10% FCS). One set of the Mov10 ko wells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) with a plasmid bearing full-length mouse Mov10 before differentiating with DMEM (2% FCS) and 20uM Retinoic Acid (Sigma-Aldrich) a day later. Cell were allowed to differentiate for 48 hours and imaged under transmitted light using an EVOS cell-imaging microscope. The images were anonymized and analyzed by an experimenter blinded to the conditions using the Axiovision Image analysis software. 500-800 differentiated neurons were counted from triplicate experiments and a total of 11 images were counted per condition.

For total RNA sequencing, 2x10^5 cells were plated in a 6-well plate and differentiated using 2% FCS and Retinoic Acid after 24hrs. The cells were allowed to differentiate for 48hrs with a media change every 24hrs. Total RNA was isolated using TRIZOL reagent (Ambion) and the RNA quality was checked on a 1% MOPS-Agarose gel. The samples were DNAse treated and cleaned and concentrated using the RNA clean and concentrator Kit (Zymo Research) before sequencing.

RNA-seq analysis of N2A

The Illumina HiSeq4000 sequencer was used in paired-end mode. Library adapters were trimmed and reads were mapped to the Encode mm10 genome using STAR in paired-end mode [59]. Each sample produced ~350,000,000 million paired reads 100bp
in length. These reads mapped to the genome with >85% coverage. CuffDiff was used to identify differential expression between experimental groups [60]. Cuffdiff results were further filtered by P-value (< .005), expression levels (one or both conditions contains the gene at > 1 FPKM), and fold change (log₂ (fold change) > 1). RRID numbers for the software: Cuffdiff: SCR_001647; CummeRbund: SCR_014568; DAVID: SCR_001881; Cytoscape: SCR_003032

**GO analysis**

Gene lists generated from RNA-seq analysis were analyzed for patterns in gene ontology using DAVID 6.8 [57, 58]. An enrichment map of significant ontology terms was generated using the Cytoscape Plugin EnrichmentMaps [61, 62].

**CRISPR-Cas9 knockdown in N2a cells**

Guide RNAs (Additional File 2.14) were designed to the mouse Mov10 locus as described in [63] and cloned into pX459 plasmid (Addgene). Constructs were transfected into WT N2a, serially diluted into 96-well plates and grown under Puromycin (2ug /ml) selection. Puromycin-resistant colonies were selected and screened for Mov10 expression using Western Blot analysis and confirmed by sequencing.

**Nuclei purification and fractionation**

WT brain tissue was extracted from three P2 mice and nuclei preparation was done as described in [64]. 100 mg of the tissue was minced and the nuclei extracted using a nuclei isolation kit (Sigma NUC201), separated by ultracentrifugation at 40,000 rpm for 30min at 4°C (Beckman TL-100). Nuclei were suspended in CSK buffer containing
10mM PIPES, 300mM sucrose, 1mM EGTA, 200mM NaCl, 1mM DTT and Roche protease inhibitor cocktail. The separated nuclei containing CSK buffers were supplemented with 1mM PMSF, left at 4°C for five minutes, centrifuged at 2000xg for five minutes at 4°C to separate the nucleoplasmic and chromatin fractions.

Hippocampal neuron culture

Mov10 heterozygotes were genotyped at P0 using tail samples and DNA was extracted with KAPA Fast Extract Kit (KAPA Biosystems # KK7103). After genotyping, mouse hippocampi were dissected and cultured on embryonic day 20 (E20), or postnatal day 0 (P0), as described [65]. Coverslips were coated overnight with Poly-L-lysine (Sigma, P4707, 10ug/mL) and 10^5 cells/well were plated for immunofluorescence (IF) in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). After 24 hours, medium was switched to Neurobasal (NB) medium (Gibco, 21103049) supplemented with B-27 (Gibco, 17504-044). Half of the media was removed and replaced with fresh NB medium every 3 days.

Immunofluorescence and microscopy of cultured neurons

Neurons grown on coverslips were fixed in 4% paraformaldehyde for 10 minutes at room temperature, either 24 hours after initial culture (DIV0) or 14 days later (DIV14). Samples were blocked in 10% normal donkey serum (Jackson ImmunoResearch, 017-000-121) for 30 minutes at room temperature. Mov10 primary rabbit polyclonal antibody (1:1000, Bethyl, A301-571A RRID: AB_1040002) or MAP2 antibody (1:1000 dilution, Millipore # AB5622, RRID: AB_91939) were incubated overnight at 4°C. Secondary antibody (Alexa 594 goat anti-rabbit {1:4000, Jackson ImmunoResearch, 111-585-144,
RRID:AB_2307325}) was added for 2 hours, room temperature. Coverslips were inverted unto glass slides containing mounting media with 1µg/mL 4', 6-diamidino-2-phenylindole (DAPI). Fluorescence images of DIV0 and DIV14 neurons were obtained with a Zeiss LSM 700 inverted confocal microscope using a 40x and 63x EC Plan-Neufluar 1.30 oil objective respectively. Images were captured with a cooled CCD camera running Zen 2012 software. A total of 10-15 0.2 µM thick section were acquired as z-stacks for each time point.

**Sholl analysis**

Sholl analysis of all orders of branches (Total Sholl) was performed. Confocal z-stacks of either WT or Mov10 heterozygous DIV 14 neurons immunostained for MAP2 were imported into ImageJ. Dendritic complexity, including Sholl analysis, was performed according to the protocol described [33].

**Behavior tests**

The sample size for behavioral testing was estimated using G* power [66] from a pilot study using 5 animals. The analysis recommended a sample size of 4 animals per group and showed an effect size d= 2.522797 and a power of 0.842302. We decided to use at least 10-15 animals per group to account for attrition and outliers. We excluded outliers based on z-test cutoff of +/- 2 Standard Deviations from the mean. 60-65 day old mice were tested at the same hour of the day in the following sequence: open field, novel object recognition, rotarod, elevated plus maze and trace fear conditioning. The experimenter was blinded to the genotypes. Both sexes were tested on the rotarod, elevated plus maze and trace fear conditioning.
**Open-field test** - The test was performed on the first day of the novel object recognition test. Mice were exposed for 5 min to a rectangular arena (46x25x20 cm) and the distance covered was tracked using a Logitech HD Pro webcam. The videos were then analyzed using the TopScan LITE software (Cleversys Inc., Reston, VA, USA).

**Novel object recognition** - The test was performed as described [67]. Briefly, mice were habituated to the empty arena on the first day for 5 min. After 24 hours, two similar objects were presented and the interaction with each object was tracked using a webcam. The pair of objects used in the test was randomized between animals. On day 3, a novel object replaced one of the objects and the mice were video-tracked. The placement of the novel object was randomized between animals. The videos were analyzed using the ObjectScan software (Cleversys Inc.) to estimate interaction times.

**Rotarod** - Mice were placed on a stationary rotarod (AccuRotor Rota Rod Tall Unit, 63-cm fall height, 30-mm diameter rotating dowel; Accuscan, Columbus, OH, USA). The dowel was then accelerated at 60 rpm/min, and the latency to fall (in seconds) was recorded. The procedure was repeated for 4 consecutive trials, which were averaged to give the daily latency to fall for each mouse. The trials were repeated for 2 more days for a total of 3 days.

**Elevated plus maze** – The apparatus consists of four arms (66cm x 6.4cm), an open area in the center (6.4 cm), two opposing open arms and two opposing closed arms (20cm high wall) with sliding doors at the end. The maze is elevated at a height of 60cm from the floor. Mice were placed in the center of the maze and the time spent in each zone was recorded for 10 min using the webcam. The TopScan LITE software analyzed the videos.
Trace fear Conditioning – A modified procedure of the test was performed as described [68]. Mice were trained by exposing them for 3 min to a chamber (34x 28x30 cm) where they received three consecutive pairs of tone (10s) and shock (0.5mA, 1s) with an empty trace interval of 1s and a 3 min break between each tone-shock pairing. Behavior was recorded using a webcam in a new context with the same tone but without shock 48 hours after training (trace fear conditioning). Five days after training, the mice were placed back in the original training chamber without tone or shock and recorded for 6 min (context conditioning). The videos were analyzed using TopScan LITE to measure the level of freezing in both cases.

Venn Diagram for Mov10/Fmrp/Ago2 overlaps

842 genes with Fmrp-CLIP sites from [27] contained NCBI’s Entrez Gene IDs in the Supplementary Table. All but two of the IDs were in the current Bioconductor org.Mm.eg.db database (v 3.4.0) (RRID: SCR_006442). According to NCBI, these two IDs were replaced with different IDs, so we used the updated IDs. Mouse gene symbols for the 842 Entrez IDs were pulled from org.Mm.eg.db. Human genes with Ago2 binding sites were taken from Suppl. Table 3 [28]. There were 7,153 binding sites in 3,416 unique Ensembl Gene IDs. Ensembl Gene IDs were converted to Entrez Gene IDs using the current Bioconductor org.Hs.eg.db database (v 3.4.0); 3,173 Ensembl IDs had perfect 1:1 matches with Entrez IDs. 150 genes had no matches based on Ensembl ID, but we were able to assign Entrez IDs for 109 of them using the Gene Symbol or RefSeq ID listed in Suppl. Table 3. 93 genes had more than one Entrez ID listed for the Ensembl ID, however many of the EntrezID were for miRNA, and 57 genes matched to a single mRNA Entrez ID; an additional 33 genes were matched to a single EntrezID
using the Gene Symbol or RefSeq ID from Suppl. Table 3. We were left with 3,372 unique Ensembl genes, although a few of these were assigned the same Entrez ID, so there were 3,335 unique Entrez Gene IDs that had Ago2 binding sites. Human gene symbols for these Entrez IDs were pulled from org.Hs.eg.db.

Mouse genes with Mov10 binding sites were taken from Additional File 2.11. There were a total of 930 binding sites, although 252 of these were in intergenic/intronic regions and were not assigned to a gene. An additional 2 sites were assigned to two different genes and these were removed. The remaining sites were in 539 unique, current Entrez Gene IDs. Mouse gene symbols for the 539 Entrez IDs were pulled from org.Mm.eg.db. While database IDs like Entrez Gene or Ensembl are much more stable over time than gene symbols, they are species-specific and hence cannot be used to easily map between species. Instead, both human and mouse use similar nomenclature systems for gene symbols, such that gene symbols from different databases at the same point in time should be comparable. The main difference is in capitalization, so symbols were matched by converting both to all capital letters. While only using genes with identical symbols between human and mouse will miss some true homologs, it simplifies the comparison by removing any many-to-one or many-to-many relationships that would be impossible to assess statistically. The two Bioconductor databases from the same release, org.Hs.eg.db_3.4.0 and org.Mm.eg.db_3.4.0, share 16,760 gene symbols in common. This gene set was used as the background to assess whether Fmrp, Ago2 and Mov10 tend to have binding sites in the same genes. After removing genes not in the background, we compared 821 Fmrp genes, 3,130 Ago2 genes and 502 Mov10 genes for overlaps (Figure 2.5B). First, we compared the pairwise overlaps
using a one-sided Fisher’s exact test and all 3 sets were highly significant (Fmrp & Mov10, 77 genes in common, \( p = 2.15 \times 10^{-19} \), Fmrp & Ago2, 490 genes in common, \( p = 4.85 \times 10^{-159} \), Mov10 & Ago2, 193 in common, \( p = 5.57 \times 10^{-26} \)). If we test the pairwise overlap between Fmrp and Mov10 using the full mouse background of 23,294 genes, their overlap of 80 genes is even more significant (\( p = 2.50 \times 10^{-27} \)), indicating that the common background is actually more conservative. We also used a permutation approach to test whether the amounts of overlap in the Venn diagram were more than expected by chance by randomly selecting gene sets of 821, 3,130 and 502 from the 16,760 background and counting the numbers of genes in common. We repeated this 50,000 times and used the resulting distributions of overlap values to empirically derive one-tailed \( p \)-values for our four observed overlaps: 1) Fmrp and Mov10 only (30 genes), 2) Fmrp and Ago2 only, (443 genes), 3) Mov10 and Ago2 only (146 genes) and 4) all 3 binding sites (47 genes). The Fmrp and Mov10 only overlap, had a \( p \)-value = 0.01116, which while significant is not nearly as significant as the other three overlap gene sets, which all had \( p = 0 \), meaning a larger overlap was not seen in 50,000 random samplings. R version 3.3.3 (R project for statistical Computing-RRID: SCR_001905), using a custom script to randomly pull out subsets of genes of the correct sizes and count overlaps was used.
### Table 2.1

**Number of pups from heterozygote mating**

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<td>44 (28%)</td>
<td>112 (72%)</td>
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**Screening of embryos from E9.5**

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<td>5 (36%)</td>
<td>9 (64%)</td>
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**Screening of embryos from E12.5**

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<tbody>
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<td></td>
<td>0 (0%)</td>
<td>21 (100%)</td>
<td>0 (0%)</td>
<td>21 (100%)</td>
</tr>
</tbody>
</table>
Figure 2.1. Mov10 is significantly elevated in young mouse brain and is both nuclear and cytoplasmic. A) Brain extract (25ug) from C57BL/6 at ages indicated was immunoblotted for Mov10 and eIF5α as a loading control (top panel). Bar graph of the three independent experiments is shown in the bottom panel. Spearman’s rank-order correlation ($\rho$ (70)= -0.371, ***$p=0.001$). B and C) 3-3’ Diaminobenzidine (DAB) stain of P1 brain (sagittal section) and counterstained with Hematoxylin.
**Figure 2.1 (cont.)** - CTX-cortex, HC-hippocampus, CB-cerebellum, HB-hindbrain, MB-midbrain. (B) No primary antibody; (C) Mov10 antibody. Images obtained using the Hamamatsu Nanozoomer slide scanning system. Scale bar = 1mm D-I) Mov10 immunohistochemistry of P2 brain (D, E, F) and adult hippocampus (G, H, I) Scale bar = 10um.
Figure 2.2. Mov10 binds repeat element RNA and mRNA in P2 brain and blocks retrotransposition. A) Results of RNA-IP followed by sequencing. Left pie-chart shows the distribution of all immunoprecipitated RNAs. Right pie-chart shows the classification of the repeat elements.
Figure 2.2 (cont.) - B) RT-PCR of Mov10 or Irrelevant (IRR) IP from P2 brains for Mov10 iCLIP target mRNA (Prrc2b) and an active mouse L1 RNA (mL1TF) and for the mRNA Grin2A, which does not bind Mov10 [9]. C, D) q-PCR of DNAse I and RNAse H treated genomic DNA isolated from P3 heterozygote (HET) or WT littermate brain (n=3) amplified with ORF2 primers and 5S rDNA for normalization. Values plotted relative to adult genomic DNA content. Error bars represent SEM, *p <0.05 (Student’s t-test, two-tailed). E) Representative gel images of the reverse transcriptase assay set up as shown in the table, SSRTIII was preincubated with the indicated concentrations of purified Mov10 or human purified recombinant FMRP as a control, followed by RT-PCR of RNAs bound by Mov10 (Prrc2b or L1TF) or not (Gapdh). F) RT-qPCR of Prrc2b, mL1TF and Gapdh with indicated ratios of Mov10 and SSRTIII. Biological replicates are shown and the fold change was compared to the RT-only reaction of each gene. Error bars represent SD, *p <0.05 (Student’s t-test, two-tailed). G) Representative gel images for the RT assay using equimolar amounts of the Mov10 helicase-deficient mutant and SSRTIII. H) Capture assay with WT, C-terminal and N-terminal of Mov10 and reverse transcriptase (SSRT III) or BSA covalently coupled to beads. I) Mov10 or irrelevant (IRR) IP from P2 brains immunoblotted for L1-ORF2 (representative image of n=3). The averages from biological replicates of the ratio between ORF2 and Mov10 for each lane are indicated below, the P-values of which are not significant.
Figure 2.3. Mov10 binds mRNAs encoding proteins involved in neuron projection and cytoskeleton by RIP, iCLIP.
Figure 2.3 (cont.) - **A**) GO analysis of RIP mRNAs from postnatal brain. Y axis-GO terms-Cellular Compartment; X axis- negative log (base 10) of the ten lowest P values (See Additional file 2.5). **B**) GO analysis of iCLIP mRNAs from postnatal brain. Y axis-GO terms for Cellular Compartment and Molecular function; X axis- negative log (base 10) of the P values, only showing terms with p-values of $10^{-6}$ or lower. (Right) Autoradiograph of Irr and Mov10 IP from P0/P1 brains from iCLIP (See Additional file 2.7).
Figure 2.4. Mov10 is required for neurite outgrowth and neuronal morphology.
Figure 2.4 (cont.) - A-C) Brightfield images of N2a (WT), Mov10 knockout N2a (KO) and Mov10 transgene rescue of KO (Rescue). Scale bar represents 200um. D) Quantification of neurite length of WT, KO and rescue, analyzed by one-way ANOVA (F(2,19) = 32, p=0.000, p-values, ** 0.03, *** 1.60484E-06). Error bars represent SEM. 500-800 differentiated neurons were counted from triplicate experiments and a total of 11 images were counted per condition. Lower panel is Mov10 immunoblot of WT, KO and Rescue; eIF5α is loading control. E) Schematic of significantly changed genes between WT undifferentiated and differentiated N2a. The number of differentially expressed genes as determined by CuffDiff (p-value < .005 and FPKM > 1) under both conditions is displayed (top). Venn diagram of genes differentially expressed in the KO versus WT. Orange (813)- genes identified from comparison between undifferentiated WT and KO; Green (781)- genes identified from comparison between differentiated WT and KO; Purple (513)- Mov10-regulated genes (bottom). F) Enrichment map of top Gene Ontology terms for the 513 Mov10 regulated genes (DAVID, p value < 0.025) showing enrichment for genes related to nervous system development, axon guidance, and neuron projection. Fraction of genes up (blue) and down (orange) regulated in KO cells. G) Map1b immunoblot from WT, Mov10 KO and rescue. Gapdh is the loading control. H) Significantly changed genes between WT differentiated and undifferentiated (324). 64 of those genes significantly changed in the opposite direction of WT in the KO.
Figure 2.5. Mov10, Fmrp and Ago2 bind mRNAs enriched for neuronal genes. A)

Top panel shows graph of mean neurite lengths between WT N2a cells treated with Irr siRNA, Fmr1 siRNA and Mov10 KO N2a cells treated with Fmr1 siRNA (n=3) Error bars represent SEM. Statistic- one-way ANOVA, (F (2,64)=28, p<0.001, p-values = *** 6.01E-06, NS= 0.13). Bottom panel shows a representative Fmrp western blot of the three conditions. eIF5α is the loading control.
**Figure 2.5 (cont.) - B** Venn diagram showing the overlap between brain-derived iCLIP targets of Fmrp, Mov10 and Ago2. All three proteins in brain commonly bound 47 mRNAs and the overlap was highly significant (see Methods). **C** GO analysis of the 47 overlapped genes from panel B. Y-axis is GO terms for Cellular Compartment and the X-axis is the negative log$_{10}$ of the P-values (see Additional file 2.12).
Figure 2.6. Normal levels of Mov10 are required for normal neuronal morphology.

A) 25ug of total brain extract from P2 mice (genotypes shown above: HET is the Mov10 heterozygote, missing one copy of Mov10) immunoblotted for Mov10 and eIF5α. Immunoblot quantification (n=3), Error bars represent SD, *p <0.05 (Student’s t-test, two-tailed). B and C) Map2 immunostaining of hippocampal neurons from DIV14 WT (B) and Mov10 heterozygous (C) neurons. D) Dendritic morphology analysis. Confocal z-stacks of Map2-stained WT or Mov10 heterozygote DIV 14 neurons were analyzed using Sholl. Statistics were calculated using Two-Way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM and *p < 0.05. (n = 56 neurons for WT, and n = 94 neurons for Mov10 HET).
Figure 2.7. Normal levels of Mov10 are required for normal behavior. A) Activity in an open field of WT and Mov10 heterozygous (HET) littermates (n=17) plotted as Distance travelled (mm). Error bars represent SEM, *p < 0.05 compared to WT (Student’s t-test, two-tailed). B) Percent time spent in open arms in the elevated plus maze by WT and Mov10 heterozygotes (n=10). Both sexes were used because no significant difference was observed between sexes (WT, p 0.71, Mov10 HET, p 0.33; Student’s t-test, two-tailed). Error bars represent SEM and *p <0.05 compared to WT (Student’s t-test, two-tailed).
Additional file 2.1. Mov10 levels are elevated in FVB mice and are independent of sex. A) FVB brain (25ug) at ages indicated, immunoblotted for Mov10 and eIF5α (loading control). Quantification of three independent experiments performed as shown below. Error bars represent SD and P-value indicates **p<0.01 compared to adult.

B) Top panel- 25ug of P0 brains from 2 male and 2 female mice were immunoblotted for Mov10. 25ug of adult brain lysate was used for comparison. Bottom panel- Genomic DNA was isolated from the P2 brain lysates of each mouse and PCR was performed using SRY primers. Actin was used as a PCR control.
Additional file 2.2. Mov10 is nuclear in P0 cortex and hippocampal cultures compared to adult.
Additional file 2.2 (cont.) - A) DAB staining of P0 (left) and adult hippocampi (right) with Mov10. Inset shows the cellular localization of Mov10. Images obtained using the Hamamatsu Nanozoomer slide scanner. Scale bar = 250um. B) Immunofluorescence staining of Mov10 in P0 (left) and adult (right) cortex. Images obtained using the Nanozoomer slide scanning system. Scale bar = 100um. C) Immunofluorescence of endogenous Mov10 (red) at DIV1 (top panel), and DIV14 (bottom panel) in cultured primary hippocampal neurons. Nuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI). Scale bar = 20 um for top panel and 10um for bottom panel. D) Immunohistochemistry of Mov10 in mouse testes sections from a WT male. Scale bar = 20um. E) Representative immunoblot from the nuclear fractionation of P2 brain (n=3). 25ug of purified nuclei preparations and cytoplasmic lysate was loaded and the proportion of Mov10 in the Supernatant (S) and Pellet (P) were determined using immunoblotting. TFIID and Histone were used as controls for fractionation.
Additional file 2.3. Schematic of gene trap insertion into murine Mov10 gene to generate a knockout allele. Domain structure of Mov10 corresponding to exon sequence of murine Mov10 (NM_008619.2). Exons are shown as black vertical lines and the gene trap vector is shown as inserting (red arrow) 3' of start (ATG) in that exon. The resulting targeted allele is shown at the bottom. Gene trapping strategy is described [111]. C57BL/6 ES cell (Clone IST13267G7sE6, RRID:IMSR_TIGM:IST13267G7) from the Texas Institute of Genomic Medicine (TIGM) was used to generate the Mov10 heterozygote.
Additional file 2.4. Mov10 binds mRNAs involved in actin cytoskeleton by RNA-seq. **A)** Screen shot from the sequencing of Mov10 exon2 in Mov10 KO N2A clone. Top panel is from WT Neuro2a. The bottom panel shows the mutant clone with the insertion generated by CRISPR-Cas9 mediated gene targeting. The mutation is boxed out.
Additional file 2.4 (cont.) - B) Gene ontology analysis for Cellular compartments from undifferentiated and differentiated WT Neuro2a. (See Additional File 9 online)
Additional file 2.5. Behavior testing of Mov10 heterozygotes. A) Rotarod testing was performed on both WT and Mov10 heterozygous littermates (HET) of both sexes (n=11). No significant difference was found between sexes (WT, p = 0.44, Mov10 HET, p=0.81; Student t-test, two-tailed). Latency to fall (millisecond) was calculated by averaging four trials per animal over 3 days. Error bars represent SEM. B) WT and Mov10 HETs (n=10) of both sexes were used in the elevated plus maze and the percent time spent in the open and closed arms are plotted. Error bars represent SEM.
Additionale file 2.5 (cont.) - C) Trace fear conditioning memory test- the level of freezing (percentage) in a new context with tone was assessed for WT and Mov10 HETs (n=11). Both sexes were tested and no significant difference was found (WT, p=0.33, Mov10 Het, p=0.34; Student t-test, two-tailed). Error bars represent SEM. D) Context fear memory test. The level of freezing (percentage) was measured on re-exposure to training context and is plotted for both WT and Mov10 HETs (n=11). Both sexes were tested and no significant difference was found (WT, p=0.97, Mov10 Het, p=0.38; Student t-test, two-tailed). Error bars represent SEM. E) Percent novelty preference was calculated from interaction times \( \{100 \times (\text{time spent with novel object}/\text{time spent with both objects}) \} \) and is plotted for WT (n=10) and Mov10 HET (n=12) males in the novel object recognition test. Error bars represent SEM. Student t-test, one-tailed.
2.8 References


CHAPTER 3

RNA HELICASE MOV10 IS ESSENTIAL FOR GASTRULATION AND CNS DEVELOPMENT

(This work has been submitted to Developmental Dynamics)

3.1 Abstract

Background: Mov10 is an RNA helicase that modulates access of Argonaute 2 to microRNA recognition elements in mRNAs. We examined the role of Mov10 in Xenopus laevis development and show a critical role for Mov10 in gastrulation and in the central nervous system during embryonic and postnatal development. Results: Knockdown of maternal Mov10 in Xenopus embryos using a translation blocking morpholino led to gastrulation defects, failed convergent extension and a failure to neurulate. RNA sequencing of the Mov10 knockout embryos showed significant upregulation of mRNAs compared to controls at Stage 10.5. Additionally, the degradation of the miR-427 target mRNA, cyclin A1, was affected in the Mov10 knockouts. These defects suggest that Mov10’s role in miRNA-mediated regulation of the maternal to zygotic transition could lead to pleiotropic effects that cause the gastrulation defect. Additionally, the knockdown of zygotic Mov10 showed that it was necessary for normal head, eye and brain development in Xenopus consistent with a recent study in mouse. Conclusions: Mov10 is essential for gastrulation and normal CNS development and function.
3.2 Introduction

The RNA helicase Mov10 was originally described as a cofactor for RNA-induced silencing complex (RISC) component Argonaute 2 (Ago2) that was required for microRNA (miRNA)-guided cleavage of a reporter [1]. Mov10 binds to G-rich secondary structures in mRNAs and unwinds RNA in a 5′-3′ direction in an ATP-dependent manner [2, 3]. Mov10 also associates with nonsense-mediated decay factor UPF1 [2]. In addition, Mov10 suppresses viral RNAs and retrotransposition in cultured cells [4, 5] We recently showed that Mov10 is required during embryonic development because the Mov10 knock out mouse is embryonic lethal; however, we were unable to identify the early developmental defects associated with this lethality [6].

Here, we demonstrate a conserved role for Mov10 during embryonic development in *Xenopus laevis*. Blocking translation of maternal Mov10 in *Xenopus* embryos leads to a severe gastrulation defect and failure of the embryo to undergo neurulation, possibly due to the misregulation of the maternal to zygotic transition (MZT) where one proposed mechanism involves the degradation of maternal mRNAs by RISC to allow zygotic transcription to begin [7]; [8]. Loss of zygotic Mov10 in *Xenopus* leads to defects in the differentiation of the retina and abnormalities in brain development. These data agree with our findings in mice where in addition to being essential for early development, Mov10 expression was found to be significantly elevated in the brain shortly after birth through adolescence [6]. We propose that Mov10 plays a vital role in gastrulation and normal CNS development.
3.3 Results

Mov10 is required for normal gastrulation and neural tube formation

Based on our finding that Mov10 knockouts show early embryonic lethality in mice, we decided to use Xenopus laevis to study the cause of this lethality because of its accessible, external mode of development. We introduced either a control- or a Mov10 translation blocking morpholino into one-cell Xenopus embryos, which targeted maternal as well as zygotic mRNA transcripts of Mov10 to prevent them from being translated. In contrast to the control embryos (Fig. 3.1A-F), the translation blocking morpholino (m-MO) disrupted the completion of gastrulation, leading to extrusion of central yolk cells (Fig.3.1G-L). Time-lapse imaging of the m-MO treated embryos showed that gastrulation is initiated but invagination appeared to proceed in a more uniform, symmetrical fashion around the blastopore, as indicated by a complete ring of dark pigmentation, when compared to the asymmetrical appearance found in control embryos (Supplemental videos 3.1 and 3.2, Fig.3.1A-L). Additionally, there is no distinct formation of Brachet’s cleft at the blastopore lip (compare Fig. 3.1K to 3.1E) indicating that vegetal rotation is delayed and limited in extent. As the blastopore closes, which is driven primarily by convergent thickening, it subsequently reopens in the m-MO treated embryos to generate a central mass of exposed endodermal cells (compare Fig.3.1, M-P, Supplemental videos 3.1 and 3.2)

During normal development, cells of the notochord undergo convergent extension (CE) and the notochord shears posteriorly along the anterior-posterior axis with respect to the adjacent paraxial mesoderm. This does not appear to occur in the m-MO treated embryos, where late involution of the axial and paraxial mesoderm is
prevented [9] (see Fig. 3.1B-H, Q-R). In normal embryos, tissue separation occurs to permit involution of marginal zone tissues, and the notochord acts as a zipper to pull together the paraxial somitic files at the midline. The location of these cells can be visualized by immunofluorescence using the Tor70 and 12-101 antibodies, which stain the notochord and somites respectively (Fig.3.1Q) [9, 10]. In contrast, in the m-MO treated embryos mesodermal tissues fail to undergo the initial CE, and the notochord and somitic mesoderm remain separated and subsequently undergo later CE to reopen the blastopore, where these tissues come to lie at the lateral sides of the open blastopore (Fig.3.1R). As a result, the defective embryos exhibited a “ring” or “boat-shaped” phenotype (Fig.3.1N, P). Together, these findings indicate that Mov10 is required early during development for normal gastrulation and neural tube formation. We were able to rescue these defects by co-injecting Mov10 mRNA designed to be resistant to m-MO action (Fig.3.2). We saw a dose dependent increase in the rescued phenotype, as we injected a greater amount of Mov10 mRNA (Fig.3.2A) suggesting that the gastrulation defect is a direct result of the absence of Mov10.

The timing of Mov10’s role in *Xenopus* development (Stage 10) coincides with the Maternal to Zygotic Transition (MZT)—the process where maternal mRNAs are degraded—to allow continued development now driven by zygotic transcripts [11]. Elimination of maternal transcripts occurs at the start of MZT and continues until after Early Gastrula Transition (EGT) [7, 8]. Early zygotic transcription is coupled with the rapid miRNA-mediated degradation of maternal targets, as exemplified in *Xenopus* by miR-427-mediated degradation of cyclin A1 [12]. Since Mov10 and Ago2 cooperate to regulate transcripts through the miRNA pathway [13], we examined whether Mov10 may
be involved in MZT using the Hydroxyurea (HU) assay. HU induces apoptosis in embryos after the maternally-encoded apoptotic inhibitors are degraded at MZT [14] (Fig.3.3A). Thus, if a protein involved in the degradation of maternal mRNAs is absent, the embryo is protected from HU-induced apoptosis at Stage 9.5 (EGT), as shown previously for B56-epsilon mRNA [15] (Fig.3.3B-D). In the case of Mov10, m-MO injected into one cell of a 2-cell embryo showed a normal pigmented appearance in the viable progeny of the injected cell (green arrow), suggesting that the maternal apoptotic inhibitors were not degraded, thus, MZT was blocked. In contrast, the uninjected side (white arrow) underwent HU-induced apoptosis (Fig.3.3E-G). The apoptotic-resistance of the m-MO and B56-epsilon injected embryos showed that MZT is delayed and implicates Mov10 in MZT. We verified this by examining the effect of m-MO on cyclin A1 mRNA, a target that contains a single miR-427 binding site that is normally degraded at the onset of zygotic transcription [12]. In m-MO treated embryos, cyclin A1 mRNA levels remained high at Stages 8-11 compared to controls, where the levels decreased drastically as zygotic transcription begins (Fig.3.3H). We conclude that Mov10 appears to be required for MZT, possibly by mediating RISC activity.

To examine the global effect of Mov10 loss on mRNAs at this stage, we isolated total RNA from Stage 10.5 control and m-MO treated embryos for RNA-seq. As expected for a miRNA-mediated regulatory event, Mov10 knockout embryos showed a significant upregulation of mRNAs compared to controls both at a 1.5-fold and a 10-fold cut-off (Fig.3.3I). Gene ontology analysis of the upregulated genes included RNAs involved in cellular movements, extracellular matrix, actin-filament based processes and cell adhesion under the category of “Biological processes” (Fig.3.4). Under the category
“Molecular function,” RNAs for calcium binding and actin filament binding are also significantly increased. Since activation of the egg at the onset of fertilization causes an increase in intracellular calcium levels, perturbation of these RNAs should be expected to affect signaling cascades necessary for multiple events including cytoskeletal rearrangements [8]. The misregulated genes could thus contribute to the defects seen in gastrulation. These data suggest that Mov10 affects MZT through the RISC pathway, which subsequently cause pleiotropic effects leading to defective gastrulation and neurulation during embryonic development.

**Zygotic Mov10 is required for normal CNS development**

We next examined the effect of zygotic Mov10 knockdown using a splice-blocking morpholino that targets the 5’ splice junction between the third and fourth exon resulting in a downstream frameshift and degradation of the zygotic pre-mRNA (hereafter, z-MO) (Fig 3.5E). *Xenopus* embryos were injected with control morpholino or z-MO at the one-cell stage, which allows them to progress past MZT, and then examined at the tadpole stage, where the effect of a zygotic knockdown might be seen (stage 36; Fig.3.5A-D). Mov10 targeting by this morpholino was confirmed by RT-PCR (Fig.3.5E). The z-MO treated embryos hatch normally and show muscle contractility. Interestingly however, Mov10 z-MO treated embryos showed a significantly smaller eye diameter compared to control-morpholino injected embryos (Fig.3.5 A,B,F). This may be relevant to Mov10’s role in the CNS since retinal tissue is derived from the diencephalon, which is a component of the forebrain. In a recent study we showed that Mov10 plays a role in the development of the CNS [6]. Our data agree with that study.
where whole-mount *in-situ* hybridization of Mov10 in *Xenopus* tail-bud stage shows expression in the eye anlage and nervous system [16]. In addition to these defects, we also observed that the z-MO treated embryos had a significantly smaller anterior-posterior body length compared to controls (Fig. 3.5G).

To further characterize the eye size, as well as query for internal brain defects, we sectioned and stained control-morpholino and Mov10 z-MO-treated embryos. Normal differentiation and layering of the retina was not typically observed in the z-MO treated embryos (compare Fig. 3.6A to 3.6D). Though the overall brain morphology was not affected, we observed a thickening of the ventricular zone (vz) (compare Fig. 3.6B to 3.6E). The ventricular zone is a transient embryonic tissue layer immediately surrounding the ventricle that contains neural stem cells [17, 18]. As the neural stem cells become depleted through their differentiation into neurons, the ventricular zone becomes diminished [19]. To examine the identity of the cells in the *Xenopus* ventricular zone, we examined the cells for expression of Sox3 {SRY (sex determining region Y)-box 3}, a marker for neural precursor cells [20-22], and for Myt1 (myelin transcription factor 1), a marker for primary differentiated neurons [21, 23]. In control MO-injected *Xenopus*, we observed Sox3 staining in ventricular cells while Myt1-staining excluded the ventricular zone and was seen in the marginal zone (Fig 3.7A-C, J-L). In contrast in the z-MO treated embryos, there is more extensive staining with Sox3 in ventricular cells including in the more ventral regions (Fig 3.7D-I) when compared to controls (Fig 3.7A-C). The staining with Myt1 appears similar between the control and z-MO treated embryos (compare Fig 3.7J-L with Fig 3.7M-O). The difference in Sox3 expression could suggest abnormal patterns of proliferation of the neural precursors when Mov10 is
absent. These findings support our conclusion that Mov10 is required for normal CNS development as observed in mice [6]. We also saw reduced parachordal cartilages surrounding the notochord in the z-MO treated embryos (Fig. 3.6C to 3.6F) [24, 25]. Parachordal cartilages expand and form the basal plate of the chondrocranium and are important for craniofacial development [26].

3.4 Discussion

The embryonic lethality observed in *Xenopus* is consistent with the developmental increase in Mov10 mRNA levels seen in both *X. laevis* and *X. tropicalis*, suggesting an important role for Mov10 during development [27]. Elimination of maternal Mov10 leads to gastrulation defects and a failure of the embryo to undergo normal CE, which leads to a failure in neurulation (Fig. 3.1). Like other RISC components--Dicer, Drosha and Ago2--loss of Mov10 causes embryonic lethality in mouse [6] [28-30]. The regulation of MZT by Mov10 suggests a role similar to that of Ago2 at the two-cell stage in mouse embryos [31]. Additionally, our data shows that the absence of Mov10 at the onset of development results in an overall increase in mRNA levels around MZT consistent with RISC function (Fig. 3.3I). This increase in mRNA levels could be due to misregulation of multiple maternal transcripts or a few key regulatory transcripts that then leads to large-scale defects at gastrulation. Mov10 has low expression levels in the adult mouse brain [6] similar to what is reported in the Allen brain atlas [32, 33]; however, there is an approximately 40-fold increase in Mov10 levels in P0-P3 mouse brain [6], suggesting an important role in brain development. Furthermore, the knockdown of zygotic Mov10 in *Xenopus* leads to eye defects, as well
as abnormal organization of the ventricular zone in the brain, which we confirmed with a neuronal precursor marker (Fig. 3.6 and 3.7). These data suggest a conserved role for Mov10 in the normal development of the brain.

In summary, we show a key role for Mov10 in gastrulation and in the central nervous system during embryonic development in *Xenopus*, which supports our earlier study in mouse [6]. Knockdown of maternal Mov10 in *Xenopus* embryos leads to gastrulation defects and a failure to neurulate possibly due to pleiotropic effects. The large-scale disorganization of cytoskeletal mRNAs could be due to Mov10’s role in miRNA-mediated regulation of MZT. In addition, zygotic Mov10 is necessary for normal head and eye development in *Xenopus* consistent with its function in the mouse [6].

**3.5 Experimental Procedures**

**Microinjection of *X. laevis* embryos**

Morpholinos were dissolved in RNase-free H2O to a stock concentration of 2mM to facilitate co-injection with RNA at various concentrations. Depending on the experiment, either zygotes or embryos at the two-cell stage (stage 2, all stages follow [34] were immersed in 5% Ficoll solution (diluted with 1/20X Normal Amphibian Medium; Slack, 1984) and immobilized in rounded pits made in clay-lined petri dishes [35, 36]. Graded concentrations of morpholinos or RNAs were injected using glass microinjection needles (World Precision Instruments, Inc, Sarasota, FL; #TW100F-4) with a Narishige micromanipulator (Narishige USA, East Meadow, NY) and Harvard Apparatus into either fertilized eggs or unilaterally into single cells at the two-cell stage for the hydroxyurea assay (described below in Hydroxyurea Assay). Following injections,
embryos were transferred to 1/20X NAM solution to recover and cultured at room temperature or 16°C with daily 1/20X NAM changes until various time points were reached for analysis. The experiments were carried out with embryos from at least 6 biological replicates, i.e., six mating pairings.

**Morpholino Oligonucleotide Design**

The Mov10 RISC complex RNA helicase sequence is publicly available from Xenbase in the *Xenopus laevis* J-strain version 9.1 genome data [37]. Following analysis, this sequence data was used to design a translation blocking morpholino (m-MO, Gene Tools, LLC) that targets bases -17 to 9 of the *Xenopus laevis* Mov10 transcript. Splice blocking morpholinos (z-MO) were also designed to target the intron/exon 4 region. The design also includes an incorporated 5’- fluorescein tag (green) to allow imaging of cells expressing the morpholino. Additionally, a standard fluorescein-tagged random control morpholino (Con) was used as a negative control (targeting the human globin intron). This control morpholino is not known to target any *X. laevis* sequences and has been used in previous experiments to assay for potential non-specific effects from the injections or possible toxicity [38].

**Generation of *X. laevis* Mov10 mRNA**

An altered full-length synthetic RNA of *X. laevis* Mov10 was generated for injection into *X. laevis* embryos. The sequence immediately downstream of the Mov10 start site was altered by changing the third/wobble amino acid while preserving the original protein coding sequence to prevent Mov10 translation blocking Morpholino hybridization (IDT gBlocks). The full-length altered cDNA was synthesized in two pieces (fragment 1 =
1221nt; fragment 2 = 1987nt) and Gibson assembly (New England Biolabs, Ipswich, MA) was used to directionally ligate the gBlocks into the pCS2+ vector between the ClaI and XhoI restriction sites. Sequences were verified by the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, IL). Rescue RNA was made from PCR-amplified template (SP6 and T3 primers). The SP6 mMessage mMachine kit (Thermo/Ambion) was used to transcribe RNA, followed by purification of the RNA with the RNease MinElute Cleanup Kit (Qiagen).

**Xenopus histology**

Stage 36 tadpoles were fixed in 4% Paraformaldehyde in 1X PBS for 1 hour at RT, followed by three washes in 1X PBS for 15 min each. The tadpoles were serially dehydrated in 30%, 50% and 70% ethanol for an hour each at RT and stored in 70% ethanol. Body length and eye diameter were measured using ImageJ software. For whole mount histology, the tadpoles were incubated in 95% ethanol for 15 min followed by three 100% ethanol washes for 15 min each. The tadpoles were then moved to xylene and incubated for 30 min with fresh changes of xylene every 10 min. This was followed by a 6 hour incubation in 50% Paraplast plus xylene solution at 60 deg. The tadpoles were then moved to fresh Paraplast plus twice for 6 hours each at 60 degrees before embedding in plastic boats. Sections were prepared using a Spencer 820 rotary microtome, affixed to slides and dried overnight. The sections were deparaffinized and stained with Hematoxylin & Eosin staining. The staining was performed using Hematoxylin 7211 and Eosin Y reagents (ThermoScientific, Waltham, MA) according to the manufacturers protocol. The images were captured using a Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). A biological replicate of 25 – 30
Stage 36 tadpoles from three different matings were scored.

**Xenopus embryo IHC**

Embryos were devitellinized and fixed in 4% paraformaldehyde in PBS for 15 min at RT, followed by three washes of 1X PBS for 15 min each. The embryos were sequentially dehydrated using 25%, 50%, 75%, 95% and 100% ethanol for 15 min each. The embryos were prepared for paraffin embedding and sectioned as described in the *Xenopus* histology methods. For, IHC, the sections were deparaffinized using xylene and rehydrated before Heat-induced Antigen retrieval in 1X citrate buffer (pH-6.0) [39]. The slides were stained using the following primary antibodies at 0.5ug/ml- Tor-70 (a kind gift from Prof. Ray Keller), 12-101 (Developmental Studies Hybridoma Bank, Iowa), at 1:500 dilution for Sox3 and Myt1 (kind gift from Prof. Klymkowsky and Prof. Papalapoulo respectively) [40]. The secondary antibodies were Alexa Flour 488 (Jackson Immunoresearch, West Grove, PA) and RITC-conjugated IgM specific antibody (Jackson Immunoresearch) used at a concentration of 1:200. The experiment was done using 6 biological replicates, i.e., six mating pairs for each condition.

**Hydroxyurea Assay**

Two-cell *Xenopus* embryos were rinsed in 1X MMR and injected using glass microinjection needles with a Narishige micromanipulator and Harvard Apparatus microinjector. The embryos were transferred to 0.25X MMR containing a final concentration of 30mM hydroxyurea (Sigma Aldrich, St. Louis, MO) and incubated at 16 degrees. The embryos were monitored closely till they reached Stage 9.5 and imaged. The experiment was conducted thrice using three biological replicates.
RNA- Sequencing and Analysis of *Xenopus* total RNA

Total RNA was isolated from three biological replicates of Stage 10.5 embryos from WT and m-MO injected embryos using the TRIZOL (Ambion, Thermo Fisher Scientific, Waltham, MO) reagent. The samples were sequenced at the High-Throughput Sequencing and Genotyping Unit of the Carver Biotechnology Center at the University of Illinois. Strand-specific single-end libraries were prepared using the TruSeq Stranded mRNAseq Sample Prep kit (Illumina, San Diego, CA). The libraries were quantitated by qPCR, pooled in equimolar amounts and sequenced on 2 lanes of a HiSeq 4000 (Illumina; sequencing kit version 1), generating over 780 million 100 bp single-end reads. Fastq files were generated and demultiplexed per sample with the bcl2fastq v2.17.1.14 Conversion Software (Illumina), which also trims Illumina adapters from the reads and removes any resulting sequences shorter than 35 bp. All bases across the reads showed quality scores greater than Q30 (FASTQC version 0.11.4) so quality trimming was not performed. The *Xenopus laevis* reference genome version 9.1 was downloaded from Xenbase [37] along with the version 1.8.3 gene models containing 45,099 genes (as of August 9th, 2016, this appears to be renamed to version 1.8.3.1). The gene models were converted from gff3 to gtf format using the gff_read program from cufflinks (version 2.2.1) [41]. Reads were aligned to the genome using STAR (version 2.5.2a) [42] using parameters --sjdbGTFfeatureExon CDS, --sjdbGTFtagExonParentGene gene_id and --quantMode GeneCounts, the last of which outputs read counts per gene_id, summing over all coding sequences (CDS) of the gene. The read counts were input into R 3.3.1 [43] for data pre-processing and statistical analysis using packages from Bioconductor [44], as indicated below. Initial quality
control analysis indicated that one of the three Mov10 morpholino (m-MO) treated replicates had many fewer reads and looked very different and therefore it was removed from the analysis. Genes without 1 Count Per Million (CPM) mapped reads in at least two of the 5 samples were filtered out; 19,368 of the 45,099 genes passed this filter and were analyzed using edgeR 3.16.0.[45] using the quasi-likelihood pipeline [46, 47] that also accounted for the total library size for each sample and an extra TMM normalization factor [45] for any biases due to changes in total RNA composition of the samples. Due to the large number of genes with very small fold-changes, we tested for differential expression of at least 1.5 fold and at least 10 fold change up or down, using the TREAT method [48] adapted for quasi-likelihood F-tests [46]. Adjustment for multiple testing was done using the False Discovery Rate method [49].

Additional annotation information for the X. laevis genes was pulled from 2 sources that were published concurrently: blast mappings to human genes done by [50] and gene symbols assigned by Xenbase in gene model annotation 1.8.3, which was a major update to the annotated gene symbols by merging 2 databases and human curation by three experts [51]. Supplemental Table S1 deposited by Ding et al. (2016) in NCBI’s Gene Expression Omnibus (GSE75278) contains X. laevis IDs from “genome assembly JGI9.1”, human protein IDs, human gene symbols and descriptions. These X. laevis IDs are actually the Xenopus Gene Nomenclature Committee’s symbols, but for the Xenbase version 1.8.0 gene models (Name attribute in Xlaevisv1.8.Named.gene.gff3.gz). The major annotation update to Xenbase version 1.8.3 only changed the symbols, not the number or locations of genes, so the internal ID attribute is the same for all 1.8 gene model sets and was used to link the human gene
symbols to our differential expression results. Xenbase recently published a small version upgrade (1.8.3.2) to the gene model set we had used (1.8.3.1), which corrected gene symbols for five gene models. We used the gene symbols from 1.8.3.2, which are based on human gene nomenclature [37]. Both sets of gene symbols, Ding et al. (2016) and Xenbase, needed minor correcting to remove leading/trailing white spaces, *X. laevis*’ sub-genome extensions and to fix capitalization differences with the symbols in Bioconductor’s org.Hs.eg.db_3.4.0 annotation package. Both sets of gene symbols also contained older symbols that org.Hs.eg.db_3.4.0 listed as retired aliases; we updated the older symbols to current symbols if possible, also resolving a few aliases that mapped to more than one current symbol by inspecting the gene description from Ding et al. (2016). Comparisons of the Ding et al. (2016) and Xenbase annotations showed overwhelming agreement between the two with 73.8% of genes annotated to the same symbol or no symbol (Supplemental Table 3.1). Only 2.6% of genes were annotated to different symbols by the two sources, and many of these were just different members of the same gene family (e.g., DOCK6 vs DOCK7). In these cases we preferentially used the Ding et al. (2016) annotation because it annotated more genes overall and had descriptions listed as well. The 23.6% of genes annotated with a symbol in only once source were assigned that symbol. The selected gene symbols were then used to pull NCBI Entrez Gene IDs, gene names and Gene Ontology terms from org.Hs.eg.db_3.4.0.

Of the 19,368 *X. laevis* gene models that were expressed in our samples, only 1,470 were not annotated to a human symbol and the rest were annotated to only 10,935 unique human genes due to the tetraploid genome of *X. laevis*. To do over-
representation testing on GO term pathways based on human annotations, each unique human symbol was deemed “significant” if at least of the X. laevis genes mapping to it had FDR p < 0.05 for the > 1.5 FC test. 4,596 human genes were called significant under these criteria, irrespective of direction of change. When directionality is taken into account, 140 human genes had significant X. laevis genes changing in both directions; this is not terribly surprising given the nature of mapping to human genes and both results could be true if the gene has multiple splice variants. We did not try to resolve these discrepancies but instead counted the genes as both significantly up (2,826 genes) and down (1,883) when conducting GO testing separately based on direction. Over-representation testing was done separately for the all-significant, up- significant and down- significant gene sets using the 10,935 unique genes as the background. The GOstat package’s (v 2.40.0) conditional hypergeometric testing was performed to reduce the redundancy of related GO terms. Comparison of GO terms’ raw p-values between the all, up and down gene sets was done to see which GO terms were specific to the direction of change and which included genes changing in both directions. Heatmaps of –log10 (p-values) across the 3 gene sets were made separately for BP, MF and CC categories; GO terms with raw p-values < 0.001 in any gene set were included for MF and CC, but due to the larger number of BP terms, only those with raw p-values < 0.0001 in any gene set were included in the heatmap.

All of the R codes necessary for the above analyses are in Supplemental File 3.3 (Skariah_GSE86382_Bioc3.4.txt). No additional files are needed because the codes show how to download the necessary sample counts and annotations from GEO and Xenbase. The codes produce the results presented in Skariah et al. when run with R
3.3.1 / Bioconductor 3.4. There are currently reviewer links Reviewer / collaborator link to *Xenopus* RNAseq **GSE86382**
### 3.6 Figures

**Figure 3.1. Maternal Mov10 is required for gastrulation and neural tube formation.**

**A-F)** Whole-mount images of control embryos undergoing normal gastrulation. **G-L)** Whole-mount images of maternal Mov10 knockdown (m-MO) embryos that have failed to complete gastrulation. **M-P)** Dorsal, whole-mount images from Control (Con) (M) and m-MO treated embryo (N), Stage 10.5, Lateral view of a Con embryo (O), View from anterior end of m-MO treated embryo showing boat-shaped phenotype (P). d-dorsal, v-ventral, a-anterior, p-posterior, yc-yolk cells, cg-cement gland. The dotted lines show the plane of section in Q and R. **Q)** Section through a control (Con) showing notochord and somites joined along the AP axis, stage 10.5. Antibody to Tor70 recognizes notochord {in red}; Antibody 12-101 recognizes somatic mesoderm {in green}; DAPI in blue. **R)** Section through a typical m-MO embryo showing failure to undergo normal convergent extension and form a nervous system.
Figure 3.1 (cont.) - Bl-blastopore lip, bp-blastopore, cg-cement gland, nt-neural tube, a-anterior, p-posterior. Sm-somite, nc-notochord, nt-neural tube. Scale bar in R = 120um (A-L), 450um (M-N), 440um (O-P), 80um (Q-R).
Figure 3.2. Co-injection of Mov10 mRNA rescues the gastrulation defect. A) Rescue of m-MO by introduction of 125pg and 375pg of Mov10 mRNA. Error bars represent SEM, p value < 0.001 (Student’s t-test, two-tailed). B-D) Whole-mount images of Stage12 Con (B), m-Mo (C) and rescue embryos co-injected with Mov10 mRNA (250pg) (D). np- neural plate, bp-blastopore, yp- yolk plug. Scale bar in D = 120um.
Figure 3.3. Mov10 regulates MZT through RISC. A) Schematic of the Hydroxyurea assay. EGT- Early Gastrula Transition, HU-Hydroxyurea.
**Figure 3.3 (cont.)- B-D** Whole mount images of two-cell embryos, where only one cell was injected with morpholino targeting a positive regulator of MZT (B56ε). A total of 22 embryos were injected and 21 of those showed the expected phenotype. **E-G** Images of two-cell embryos, where only one cell was injected with m-MO. Scale bar- 300um. A total of 22 embryos were injected and all of them showed the expected phenotype. Green arrow- MO-injected side, white arrowhead- uninjected side. **H** qRT-PCR of cyclin A1 levels in control and m-MO embryos at indicated stages. Error bars represent SEM. **NS**- Not significant, p *<0.05 (Student’s t-test, two-tailed). **I** Differential expression results for Mov10 morpholino treated (Mov10) vs. control (C), X-axis-average expression value (TMM-normalized Counts Per Million, log2 scale) of each A. laevis gene and y-axis is log2 (Mov10/C). Each point is a single gene: groups of genes colored red or blue had significantly greater than or less than 1.5 FC difference (unlogged), respectively. Groups of genes colored pink or light blue also had significantly greater than or less than 10 FC difference (unlogged), respectively. The numbers of genes listed at the +/- 1.5 FC level include the genes with +/- 10 FC.
Figure 3.4. Mov10 regulates mRNAs involved in cytoskeletal function in *Xenopus*.

**A)** Heat maps of $-\log_{10}$ (p-values) showing the comparison of all, up and down gene sets from *X.laevis* for Biological Process (BP) category. Due to the larger number of BP terms, only those with raw p-values < 0.0001 in any gene set were included in the heatmap. **B)** Heat maps of $-\log_{10}$ (p-values) showing the comparison of all, up and down gene sets from *X.laevis* for Molecular Function (MF) category. GO terms with raw p-values < 0.001 in any gene set were included for MF. The arrows point to relevant GO categories. Arrows indicate GO terms of interest. (see text) See Table S1
Figure 3.5. Knockdown of zygotic Mov10 causes decrease in eye and body size.

A) Whole-mount images of control morpholino (Con) injected tadpoles at Stage 36. A total of 46 tadpoles were analyzed.
Figure 3.5. (cont.) - B) Whole-mount images of zygotic Mov10 knockdown (z-MO) tadpoles at Stage 36. A total of 48 tadpoles were analyzed and 45 of them showed a small eye phenotype. (C, D) Fluorescein images of the tadpoles from A and B. E) RT-PCR from control or z-MO treated embryos using Mov10 primers. ODC (Ornithine Decarboxylase) was used as a control. F) Graph showing eye diameter in um from Con injected and zygotic Mov10 knockout (z-MO) embryos. Error bars represent SEM, p **<0.05 (Student’s t-test, two-tailed). G) Graph showing overall anterior-posterior (AP) length in mm from Control and zygotic morpholino treated embryos measured at Stage 36. A total of 67 tadpoles were analyzed. Error bars represent SEM, p **<0.05 (Student’s t-test, two-tailed). Scale bar in D = 800um
Figure 3.6. Knockout of zygotic Mov10 leads to defects in the eye and brain structure. A) H&E stain of Con MO injected embryos showing sections of eye (A), brain (B) and notochord (C). (D-F) H&E stain of z-MO injected embryos showing sections of eye (D), brain (E) and notochord (F). Many of the z-MO injected embryos did not exhibit well-defined layers in the retina. gc-ganglion cell layer, ip-inner plexiform layer, bc-bipolar cell layer, op-outer plexiform layer, pr-photoreceptor, rp-retinal pigment epithelium, mz-marginal zone, vz-ventricular zone, pc-parachordal cartilage, nc-notochord, “dl”-disorganized layering in D. Scale bar in F = 100um (A-B and D-E), and 170um (C-F).
Fig 3.7. Knockout of Mov10 shows abnormal staining of neuronal precursors in the ventricular zone. **A-C)** Control section shows the junction of the forebrain and midbrain and a few Sox3 positive precursors associated with the beginning of the midbrain at the bottom of the figure. A) Sox3 positive neuronal precursor cells surrounding the ventricle. B) DAPI staining of the same region. C) Merge of Sox3 and DAPI stains. **D-I)** Sections from a similar region as shown in (A) from two representative z-MO treated tadpoles stained with Sox3 antibody. D,G) Sox3 positive neuronal precursors, E, H) DAPI staining, F, I) Merge of Sox3 and DAPI. Notice the enhanced staining of Sox3 in the more ventral regions compared to the control embryo. **J)** A stage 36 tadpole showing the plane of sectioning for A through P. **K-M)** Myt1 staining for differentiated neurons in a control midbrain region. K) Myt1 positive differentiated neurons, L) DAPI staining, M) Merge. **N-P)** Sections from midbrain in z-MO treated tadpoles. N) Myt1 positive differentiated neurons. O) DAPI staining. P) Merge. vn-ventricle, vz-ventricular zone, rp-roof plate, fp-floor plate. Scale bar in P = 40um.
3.7 References


CHAPTER 4
UNDERSTANDING THE ROLE OF FMRP IN THE miRNA PATHWAY

4.1 Abstract

The Fragile X Mental Retardation Protein (FMRP) is critical for normal cognition and its absence leads to Fragile X Syndrome (FXS), the most common form of inherited intellectual disability. FMRP is an RNA binding protein that binds ~4% of brain mRNAs and regulates their translation. We have previously shown that FMRP controls the translation of neuronal mRNAs through the microRNA (miRNA) pathway and this regulation is based on its association with Mov10 and Ago2. This study attempts to answer some key questions about the nature of FMRP’s interaction with the RNA components of the miRNA pathway. Here, we show that immunoprecipitated FMRP and the phosphorylated forms of FMRP associate with a neuronal precursor miRNA 125b (pre-miRNA125b) in a northwestern assay. However, neither the full length FMRP nor the K-Homology RNA binding domains of FMRP can directly bind to either pre-miRNA125b or miR125b in a filter binding assay. We also see differential binding of pre-miRNA125b to heterogeneous nuclear Ribonucleoprotein A1 (hnRNPA1) depending on whether hnRNPA1 bound to the phosphorylated or unphosphorylated form of FMRP. These findings show conclusively that FMRP does not directly bind precursor or miRNAs. Additionally, along with evidence from published studies and our observation of differential precursor binding to hnRNPA1, we propose a possible model for FMRP and hnRNPA1 in neurons.
4.2 Introduction

Fragile X Mental Retardation Syndrome (FXS) accounts for almost 20% of all X-linked mental retardation pathologies. The most significant clinical features of FXS are developmental delays and intellectual disability [1]. FXS is caused by the transcriptional silencing of the FMR1 gene, which encodes an RNA-binding protein called the Fragile X Mental Retardation Protein (FMRP). FMRP binds to ~4% of neuronal mRNAs and regulates their activity-dependent local translation at synapses. Consequently, the absence of FMRP affects local protein synthesis leading to spine abnormalities and aberrant synaptic plasticity as seen in the Fmr1 knockout mouse model [2-4]. FMRP contains two tandem RNA binding KH (K-homology) domains, KH1 and KH2, and a C-terminal RGG box domain. In vitro RNA selection and mapping experiments have shown that the RGG domain binds G-quadruplex containing mRNAs while the second KH domain (KH2) binds an RNA structure called a loop-loop pseudoknot or “kissing complex” (Fig.4.3) [5, 6]. The KH2 domain alone is sufficient to bind kissing complex RNAs and suggests that the KH1 domain is not involved in this interaction. Thus there is no identified RNA target for the KH1 domain of FMRP yet [6].

The miRNA pathway regulates ~60% of expressed genes employing small, non-coding RNAs that are largely repressors of translation [7]. miRNAs are genomically encoded, small (~20-24 nt) RNAs that base pair to sequences in the 3’ UTR of mRNAs to regulate their translation [8]. miRNAs are synthesized as primary microRNAs (pri-miRNA) that are then processed into 70-80 nt precursor miRNAs (pre-miRs), exported to the cytoplasm and further processed into miRNAs by specific protein complexes acting at each step [9]. The first studies to show a connection between FMRP and the
miRNA pathway opened up the possibility of diseases arising from defective miRNA mediated regulation [10-12]. More recent studies have also shown evidence that FMRP associates with precursor miRNAs and miRNAs and that the phosphorylation of FMRP mediates this association [13-15]. Perhaps the most relevant supporting study that motivated this work was the finding of pre-miRs and miRs cross-linked to FMRP in iCLIP studies [16], suggesting that Fmrp might directly bind these important RISC factor components. However, none of these studies have specifically addressed if FMRP is a key player that binds directly to miRNAs or pre-miRs to affect translation or if it is an auxiliary protein that associates with the components of the miRNA pathway and uses this as a flexible platform to guide itself to its targets. This question is even more relevant since it is becoming increasingly evident that RNA binding proteins like FMRP have intrinsically disordered protein regions that coordinate RNA binding besides their canonical RNA binding domains [17]. Additionally, it has been known that FMRP is present in cellular substructures like messenger Ribonucleoprotein (mRNP) complexes and neuronal RNA granules [18, 19]. These structures are proposed to form from the interactions between the disordered regions of proteins, alternately called prion-like or Low Complexity (LC) domains that undergo reversible phase-transitions [20-22]. FMRP contains LC domains in the C-terminal half of the protein and can assemble into complexes with other LC domain containing proteins like FUS [20]. Thus it is conceivable that FMRP might be indirectly associating with miRNA components in the RNA-induced Silencing Complex (RISC).

In this study we show conclusively that FMRP’s role in the miRNA pathway does not involve direct binding to either pre-miRNAs or miRNAs. Additionally, the WT FMRP
complex also contains hnRNPA1 based on Liquid Chromatography-Mass Spectrometry (LC-MS) analysis (our data) and hnRNPA1 binds to pre-miRNA as well in our northwestern assay. Based on the size of hnRNPA1 protein that is bound to pre-miR, it appears that it might be phosphorylated. More interestingly, there is a differential binding of pre-miRNA between hnRNPA1 and the phosphorylated and unphosphorylated forms of FMRP. These data show that the role of FMRP is to assist in miRNA-mediated regulation and also suggests that FMRP and hnRNPA1 might interact through their LC domains and have a phosphorylation-dependent role in this pathway.

4.3 Results

**Immunoprecipitated FMRP and its phosphomimics bind pre-miR125b.**

It has been widely reported that miRNAs and pre-miRs are found associated with FMRP in cell culture studies as well as in brain [14, 16]. Additionally, miR125b was enriched in FMRP immunoprecipitates and was found to target the 3'UTR of the NR2A subunit of NMDA receptors thereby regulating its translational levels. However, the interaction might be an indirect one since FMRP also associates with many components of the miRNA pathway including an RNA-dependent interaction with the miRNA effector protein AGO2 [23]. Additionally, the phosphorylation status of FMRP was shown to regulate the miRNA-mediated suppression of PSD-95 mRNA at synapses [15]. In order to query this association, we over expressed FLAG-tagged WT FMRP along with its phosphomimics (referred to as ALA and ASP hereon) in HEK293T cells and immunoprecipitated using an antibody to FMRP. The ALA and ASP-substituted FMRP represent the constitutively unphosphorylated (ALA) and phosphorylated (ASP) forms of
FMRP since they carry a single Serine to Alanine (S499A) or a Serine to Aspartate (S499D) mutation at the highly conserved phosphorylation site in the C-terminus of FMRP [24]. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membrane and probed with $^{32}$P-labeled pre-miR125b, a brain-specific precursor miRNA [14]. This is referred to as a Northwestern assay because a labeled RNA is used to probe immobilized proteins to determine if they can directly bind the RNA [25]. All the three forms of FMRP bound pre-miR125b probe (Fig. 4.1, top panel). The binding between the three proteins was not significantly different as determined by the Western blot (Fig. 4.1, bottom panel) and densitometry. We also tested $^{32}$P-labelled miR125b in the northwestern assay but did not find any signal, possibly due to the degradation of the 22nt miRNA probe. As controls to confirm the specificity of the northwestern signal, we performed the same assay using SC1 RNA, a known interactor of both FMRP and its paralog FXR1 along with BSA and the KH domains of FMRP, which do not bind SC1 [26]. We find that SC1 RNA bound to recombinant FXR1P with increasing concentrations of protein but as expected it did not bind either BSA or the KH domains of FMRP (Fig. 4.2). Thus the signal seen in the northwestern assay shows specificity for genuine RNA protein interactions only. This data supports the possibility that FMRP could directly bind pre-miRs or miRNAs as seen in other studies [14, 16].

The KH1/KH2 domains of FMRP do not bind either miR125b or pre-miR125b.

Of the three RNA binding domains of FMRP, the KH1 domain does not have an identified RNA substrate while the KH2 domain binds an in vitro- selected RNA ligand termed “kissing complex” RNA [6]. Pre-miRs have a stem-loop structure that resembles
part of the kissing complex loop-loop pseudoknot structure (Fig. 4.3). Also, FMRP was found to recognize the loop region of the kissing complex since disruption of the loop abrogated the binding of FMRP. We wanted to test if the naturally occurring pre-miR structure could be a substrate for the KH domains of FMRP. In order to check for direct binding, human FMRP KH1/ KH2 domains were expressed as His-tagged fusion proteins and purified as described in the methods section for use in the filter-binding assay. The purified protein was passed through a second heparin column and quantified using BSA standards on a Comassie gel (Fig. 4.4). The RNA substrates were generated by in vitro transcription reaction and end labeled with $^{32}$P-gamma-ATP. Both the recombinant FMRP KH1/KH2 and the radiolabeled RNA substrates were used in a nitrocellulose filter-binding assay to test for direct binding. Our results clearly show that miR125b and pre-miR125b2 do not bind the KH1/KH2 domains of FMRP as compared to kissing complex RNA (kc2) and a mutated form (kc2m) where the loop-loop interaction is disrupted. The result also shows that pre-miRs are not a preferred substrate of either the KH1 or KH2 domains of FMRP.

**Full length FMRP does not bind pre-miR125b or miR-125b directly.**

Having established that the pre-miRs or miRNA is not a substrate for the KH domains, we tested the full length FMRP in filter binding assays to see if other domains might participate in direct binding to precursor or miR125b. We used G-quadruplex containing SC1 and SC1 mutant (SC1m) as controls in this assay. SC1 RNA binds to the RGG box of FMRP with very high affinity [5, 27, 28]. As shown in Fig. 4.5, we did not see binding of the full-length recombinant FMRP to either substrates while the control RNAs...
behaved as expected. These results show that none of the other domains of FMRP can interact with either precursor miRNA or miRNA substrates and suggest that FMRP is indirectly associated with miRNAs via complexed proteins.

**WT FMRP and its phosphomimcs show differential hnRNPA1 binding.**

To further investigate the association of FMRP within the complex, we analyzed the second protein that also bound pre-miR125b in the Northwestern assay at approximately 38kDa in all the three immunoprecipitates (Fig.4.6, top panel). This protein was identified as hnRNPA1 by LC-MS and confirmed by Western blot using an antibody specific for hnRNPA1 (Fig.4.6, bottom panel). This suggests that FMRP is in a complex with hnRNPA1 both of which are recognized and bound by labeled pre-miR. The association of FMRP and hnRNPA1 has been shown before and is RNA-mediated [29]. Here we see that the constitutively phosphorylated and unphosphorylated forms of FMRP also associate with hnRNPA1. hnRNPA1 associated with unphosphorylated FMRP (ALA) shows an approximately 4-fold increase in signal for pre-miR125b binding than the WT and ASP FMRP based on densitometry (Fig.4.6, top panel). The signals between WT and ASP are not significantly different and the western blot of FMRP and hnRNPA1 shows that the loading is equal for all lanes (Fig.4.6, bottom panel). Additionally, the precursor bound hnRNPA1 band migrated slightly slower than the expected size of 34kDa for hnRNPA1 (compare bottom panel western with the northwestern) and is the right size for hnRNPA1 phosphorylated by Casein Kinase II (CKII) *in vitro* [30]. We hypothesize that unphosphorylated FMRP preferentially associates with phosphorylated hnRNPA1, which has bound more readily by pre-miR.
Phosphorylation of hnRNPA1 is required for its nuclear export [31-33]. In addition, nuclear hnRNPA1 is involved in the processing of primary miRNA 18a (pri-miR18a) and in vivo crosslinking and immunoprecipitation showed that it bound to the precursor of this miRNA as well [34]. Primary miRNA processing happens in the nucleus and it is likely that the phosphorylated hnRNPA1 bound to the precursor is exported out of the nucleus and forms a complex with the unphosphorylated FMRP via interactions between the LC domains. Both FMRP and hnRNPA1 contain LC domains in their C-terminal regions and are capable of forming hydrogels using the biotinylated-isoxazole (b-isox) chemical [20, 21]. These chemically induced hydrogels emulate the cellular aggregates called RNA granules, which are classified based on their composition. The neuronal granules contain FMRP and mRNAs along with other translational initiation factors and ribosomal subunits and are transported to the synapse for activity-mediated translation [35]. More recently, it was shown that pre-miRs are present at the base of spines at synapses and are rapidly processed on stimulation [36]. Interestingly, this study used pre-miR18a, which is bound by hnRNPA1 [34]. Based on these observations and our findings we propose a model wherein FMRP and phosphorylated hnRNPA1 accumulate in neuronal granules with a specific precursor and are transported along the dendrite to synaptic spines. Dicer that is present at the synapse might rapidly process the pre-miR to miRNA releasing it to AGO2 for miRNA Recognition Element (MRE) binding. Additionally, FMRP might become phosphorylated at the same time to cause the dissociation of both Dicer and hnRNPA1 [13] (Fig.6). The complex is now held in a translationally repressed condition awaiting an activation signal that will cause dissociation of FMRP and AGO2 and activate translation of the mRNA (Fig.4.7) [15].
4.4 Discussion

Fragile X syndrome is caused by the absence of FMRP, which is a selective RNA binding protein. The loss of FMRP leads to aberrant translation of its mRNA targets, which further leads to the functional deficits that characterize this syndrome [37, 38]. One of the key regulators of translation in the cell is the miRNA and the effector proteins associated with this pathway. Despite the growing evidence of an association of FMRP with the miRNA pathway, there is no definite mechanism of how this key regulator of translation might be operating within this pathway. In order to dissect out the role of FMRP in the miRNA pathway, we carried out Northwestern assays to establish whether immunoprecipitated FMRP binds to the brain specific pre-miR 125b. Since the phosphorylation status of FMRP has been implicated in miRNA-mediated regulation, we also tested the constitutively phosphorylated and unphosphorylated mimics of FMRP in this assay. We find that all three forms of FMRP bound pre-miR125b (Fig.4.1). This data confirms earlier findings and suggests that FMRP may be associating with miRNA or pre-miRs. However, the interaction might be indirect since FMRP associates with various components of the RISC pathway [10, 11, 14]. We addressed this question by carrying out a direct binding assay using the minimal KH1/KH2 RNA binding domains of FMRP and in vitro transcribed brain-specific miRNA to begin with. In the same assay, we also tested for pre-miRNA as a possible cellular substrate for the uncharacterized KH1 domain of FMRP. Our results show conclusively that the tandem KH domains of FMRP do not bind either miRNAs or pre-miRNA and suggests that FMRP does not bind miRNAs or pre-miRNAs via its KH domains (Fig.4.4). However, it is possible that the binding may be occurring through other parts of the protein and so we performed the
same assay using full length FMRP synthesized in Sf9 cells. Again, we find that both pre-miR125b and miR125b do not associate with full-length FMRP (Fig.4.5), showing that FMRP is not a direct interactor of the RNA components of the RISC pathway. Thus our data suggests that since FMRP is an mRNA binding protein and present in neuronal or transport RNA granules, its role in the complex is that of an auxiliary protein that is bound to the mRNA cargoes within the complex. The question of how it becomes a part of this complex is perhaps answered by the observation that the only other protein that bound pre-miR125b and was seen at detectable levels in the FMRP immunoprecipitate was hnRNPA1 (Fig.4.6). There is evidence of such an association in astrocytic GW-182 containing Bodies (GWB), which are reported as both nuclear and cytoplasmic structures that also contain components of the RISC pathway [39, 40]. Such structures are hypothesized to form from phase transitions occurring between LC domain containing proteins within cells [20, 21]. It is conceivable that FMRP-containing bodies found in neurons may exhibit heterogeneity in their components and may interact with GW bodies as well. Based on this finding, we propose a possible model for the interaction of FMRP and phosphorylated hnRNPA1 in precursor transport and processing in neurons (Fig.4.7).
4.5 Methods

Purification of recombinant KH1/KH2 domains of FMRP

The pET21b encoding the KH1/KH2 domains of FMRP (a gift from Dr. Jen Darnell) was transformed in BL21DE3 and cultured at 37°C, 250 rpm in Luria-Bertani media (LB, Fischer Scientific) containing 200ug/mL ampicillin (Amp, Fisher Scientific). The clone was frozen at -80°C as 100uL aliquots. For purification, the 100uL aliquot was added to a 5ml LB (Amp) broth and grown overnight at 37°C, 250 rpm. The next day 2.5ml of the overnight culture was inoculated into 250mL LB (Amp) and grown for 3hrs at 37°C to an O.D$\text{600}$ of 0.6. The culture was then induced with 1mM IPTG and grown at R.T for 2hrs. The induced cells were then pelleted at 5000rpm for 10min and stored at 4°C on ice and processed the next day. The pellet was resuspended in 2X lysis buffer (20mM HEPES, 600mM LiCl, 10mM imidazole, 10% glycerol, pH-7.5) along with 1mg/ml benzamidine, 1mM PMSF, 5ug/ml DNase I and one complete™ mini protease inhibitor cocktail tablet (Roche Diagnostics). The cells were sonicated (Cole-Parmer Ultrasonic processor) thrice for 30 seconds each in 10 sec pulses with 10 sec on ice. The crude lysate was spun at 11000rpm for 60min at 4°C and the supernatant was mixed in a tube with 5ml of Ni-NTA resin (Invitrogen) that had been pre-equilibrated with Binding buffer (10mM HEPES, 300mM LiCl, 5% glycerol, 20mM Imidazole, 1mg/mL Benzamidine, pH – 8.0). The protein was bound to the resin by gently rotating the tube at 4°C for an hour. The resin was then packed in a column and allowed to settle as the lysate was collected in a tube. The resin was washed with 10 column volumes of buffer 1 (10mM HEPES, 300mM LiCl, 5% glycerol, 10mM Imidazole, 1mg/mL Benzamidine, pH – 8.0) at a flow rate of 0.5ml/min. This was followed by 10 column volumes of Buffer II (10mM HEPES,
300mM LiCl, 5% glycerol, 20mM Imidazole, 1mg/mL Benzamidine, pH – 8.0) and 10 column volumes of Buffer III (10mM HEPES, 300mM LiCl, 5% glycerol, 25mM Imidazole, 1mg/mL Benzamidine, pH – 8.0) at a rate of 0.5ml/min. A final wash was done with 5 column volumes of Buffer IV (10mM HEPES, 300mM LiCl, 5% glycerol, 50mM Imidazole, 1mg/mL Benzamidine, pH – 8.0) at 1ml/min. The protein was then eluted at the same rate with the Elution buffer (10mM HEPES, 300mM LiCl, 5% glycerol, 100mM Imidazole, 1mg/mL Benzamidine, pH – 8.0) in 3ml fractions. The elutions were analyzed on a 12% SDS gel and the fractions containing the purified KH1/KH2 domain (24kDa) were pooled and cleaned over a heparin column (GE healthcare) and exchanged into 1X SBB buffer (200mM potassium acetate, 5mM Magnesium acetate and 50mM Tris acetate, pH 7.4). The fractions were then quantified by using BSA standards on a Commassie gel with the Image J software.

**Purification of recombinant full-length FMRP**

Sf9 cells from log phase (1.5-2.5x10^6 cells/ml) with >95% viability was used for transfection in unsupplemented Grace Insect cell media (Invitrogen). Briefly, 8x10^5 cells were plated in 6-well plates and allowed to attach to the dishes for 15min. Plating media containing 1.5ml of 10% FCS supplemented Grace and 8.5ml of unsupplemented Grace was prepared and added to the cells after they attached completely. Cellfectin reagent (Invitrogen) was used to transfect 1-2ug of purified FMRP-Bacmid DNA. The cells were incubated at 27 degrees for 4 hours and the media was replaced with supplemented Grace media. The cells were incubated for 72hrs and observed for signs of viral infection. After 72hrs, the cells were transferred to a 15ml conical and spun down. The
supernatant was filtered through a 0.2u filter to collect the P1 (passage1) viral concentrate. The P1 stock was then amplified by infecting Sf9 cells at Multiplicity of Infection (MOI) of 0.1 for 72hrs in spinner flasks. The P2 supernatant is collected and assayed using a Viral Plaque assay to determine the viral titer. Based on the assay, the virus can be amplified once more to reach a titer of 10^7-10^8 pfu/ml for protein purification. FMRP purification was done from 500ml Sf9 cells using an MOI of 1 in spinner flasks and buffers described in [16]. The purification was done using Cobalt-TALON (Clontech) columns and FMRP was eluted in 300mM imidazole and dialyzed with glycerol containing buffer before freezing in aliquots. The fractions were quantified by using BSA standards on a Comassie gel with the Image J software.

**In vitro transcription of ^32^P- labeled RNA ligands**

Transcription templates for pre- miRs125b1 and b2 were generated by PCR using the pED368 FhSynRedW mir-125b1 and pED249 FhSynRedW mir-125b2 constructs respectively (gift from Edbauer). The primers used for pre-miR125b2 are:

TAATACGACTCACTATAGGGCCTAGTCCCTGAGACCCTAAC

GCCTAGGTCTCCAAGAGCCTG. The primers TAATACGACTCACTATAGGG and TCACAAGTTAGGGTCTCAGGGACCCCTATAGTGAGTCGTATTA were annealed to generate the mir125b template. The DNA templates were purified over G-25 (GE healthcare) columns. In vitro transcription reactions were done using 1ug of DNA template in a 25uL reaction with 1mM dNTPs (Roche), 1uL of T7 RNA polymerase (Roche) and 1uL of RNAsin (Promega). The reactions were incubated at 37°C for 2hrs and treated with 1uL of RQ1 DNase (Promega) for 10min at the same temperature. The
RNA templates were dephosphorylated using Shrimp Alkaline Phosphatase (USB) at 37°C for 90min and deactivated at 65°C for 15min. The dephosphorylated RNA templates were end labeled using \(^{32}\)P-gamma-ATP and 1uL of T4 PNK (NEB) and cleaned on a G-25 column (GE healthcare) before Phenol chloroform and ethanol precipitation. The RNA was precipitated overnight with 100% ethanol and 10% Sodium acetate (0.5M) at -80°C. The pellet was washed with 70% ethanol, dried and resuspended in DEPC treated water.

**Northwestern Assay**

Immunoprecipitated proteins were separated on a 4-20% gradient gel and transferred to a PVDF membrane overnight at 45mV /4°C. The membrane was then blocked with 5% fat-free milk containing 20ug/ml yeast tRNA (Sigma-Aldrich) for 1hr at room temperature (RT). The membrane was subsequently washed with EB buffer (10mM HEPES- pH-7.6, 3mM MgCl\(_2\), 40mM KCl, 1mM DTT, 0.2% Nonidet P-40/ IGEPAL, 1mM PMSF) for 1 hour at RT. \(^{32}\)P labeled probe for the sense strand of pre-miR125b was added at 2ng/ml (5x10^6 counts per million/cpm) along with 20ug/ml yeast tRNA in EB buffer to the membrane and incubated for 3hrs at RT. The buffer was removed and the membrane was washed extensively with 3-4 buffer changes for 15min each at RT till the counts read roughly 1000 cpm or less on a Geiger counter. The membrane was briefly air-dried and exposed to an autoradiograph for 18hrs before imaging.
Filter Binding Assay

The Filter-binding Assay was carried out as per the protocol described in [26]. Briefly, 10ul of 10,000cpm (1-5 fmol) of heat-renatured, $^{32}$P-labeled in-vitro transcribed RNA was added to serial dilutions of either full length FMRP or KH1/KH2 –FMRP and incubated at room temperature for 10min. Samples were filtered through MF nitrocellulose HAWP-02500 membranes (Merck-Millipore, Billerica, MA, USA) on Millipore vacuum manifold and washed thrice before quantifying by scintillation counting. Total RNA counts were estimated by spotting 10ul of RNA on a filter disc. A background control (without protein) was also used in the filtration assay. Data are expressed as percentage of the Total RNA and plotted using Excel (Microsoft).

Immunoprecipitation of WT FMRP and phosphomimics

HEK293T cells were plated at a density of 5x10$^6$ cells and transfected after 24hrs with plasmids bearing WT-FMRP, ALA and ASP phosphomimics using lipofectamine in Opti-MEM (Gibco). The cells were recovered with Trypsin after 24hrs growth in complete DMEM media and lysed in lysis buffer (50mM Tris-pH 7.6, 150mM NaCl, 30mM EDTA, 0.5% Triton X 100). The cells were spun down to remove nuclei and the supernatant was transferred to tubes containing 50ul of 50% Protein A Sepharose beads coupled to 7G 1-1 antibody to FMRP. The lysate was immunoprecipitated for 2 hours at 4°C. After 2 hrs, the tubes were spun and the lysated removed. The beads were washed thrice with lysis buffer for 5 min each and boiled in Laemmli buffer.
4.6 Figures

Figure 4.1. Northwestern of FMRP and FMRP phosphomimic (Asp) and mutant (Ala) shows binding to pre-miR125b2. (Top) Northwestern assay with pre-miR125b. The first two lanes are controls with the beads used for immunoprecipitation alone (Beads) and an irrelevant antibody coupled to the beads (Mock). The remaining three lanes show FLAG-tagged WT, ALA and ASP FMRP, which was pulled down with an FMRP-specific antibody and probed with $^{32}$P-labeled pre-miR125b. The bottom panel is a Western blot using an antibody specific to FMRP.
Figure 4.2. Control experiment showing the specificity of Northwestern assay.

The top panel is a northwestern assay with SC1 RNA. The first three lanes contain recombinant FXR1 (rFXR1) protein at 1ug (1), 2ug(2) and 3ug(3) concentrations. The next three lanes contain 1ug, 2ug and 3ug of BSA as a control and the last three lanes contain recombinant KH1/KH2 domains of FMRP at 1ug, 2ug and 3ug concentrations. The blot was probed with $^{32}$P-labeled SC1 RNA. The bottom panels show a Commassie gel loaded in the same manner as the northwestern gel.
Figure 4.3. Structure of the Kissing complex RNA and pre-miRNA 124 - Structure of the Kissing complex RNA [156] and a pre-miR with the microRNA sequence boxed (from TargetScan).
Figure 4.4. The KH1/KH2 domain of FMRP does not bind precursor or mature miRNAs. Graph depicting the results of a filter-binding assay using kc2, kc2m and precursor and mature 125b RNA with increasing amounts of recombinant KH1/KH2 domain of FMRP (n=3). Error bars represent SD (Student's t-test, two-tailed).
Figure 4.5. Full length FMRP does not bind precursor or mature miRNA- A) Coomassie gel showing varying concentrations of recombinant full-length FMRP and BSA as a standard for protein estimation. B) Graph showing representative data from a filter-binding assay using SC1, SC1mut and precursor and mature miR125b RNA with increasing concentrations of FMRP. This experiment was done twice with two separate protein preparations.
Figure 4.6. Northwestern assay showing the co-bound FMRP and hnRNPA1 with pre-miR125b – Top panel shows the northwestern assay using $^{32}$P labeled premiR125b. The first lane is a control with beads used for conjugating the antibody (Beads), the second lane is an immunoprecipitation using an irrelevant antibody (Mock) and the next three lanes are immunoprecipitation using an FMRP-specific antibody to WT, ALA and ASP-FMRP. The bottom panels show the western blots using FMRP and hnRNPA1 antibody.
Figure 4.7. Proposed model for the FMRP-hnRNPA1 complex in precursor transport and processing – 1) Phosphorylated hnRNPA1 binds to precursor in the nucleus and is transported out into the cytoplasm [31]. 2) FMRP with its bound mRNA cargo and transport proteins associates with phosphorylated hnRNPA1 and precursor miRNA. 3) The complex is transported down the dendrite to the base of synaptic spines. 4) Dicer present at the spines associates with the precursor-containing complex and processes the precursor to miRNA in an activity dependent manner [186]. 5 and 6) At the same time, FMRP becomes phosphorylated and dissociates from both Dicer [13] and hnRNPA1 and moves to a repressed complex containing AGO2 bound to the mRNA-miRNA complex. 7) Dephosphorylated hnRNPA1 is transported back to the nucleus and phosphorylated to repeat the cycle.
4.7 References


CHAPTER 5

CONCLUSION

This thesis is the first systematic study of the RNA helicase Mov10 both in the developing embryo and in the postnatal brain. The introductory chapter outlines the pertinent studies that have been done to understand the function of Mov10 and concludes that this is a multifunctional RNA helicase that has multiple important functions in the cell. It also points to the gaps in the field regarding the role of Mov10 in brain, especially its crucial function in suppressing retroelements which are active in differentiating neurons as well as its function in regulating neuronal mRNA transcripts through the miRNA pathway. In Chapter 2 of this work, I have attempted to address these gaps by studying Mov10 in the developing postnatal brain and show that nuclear Mov10 is indeed critical for suppressing retroelements. More interestingly, cytoplasmic Mov10 binds and regulates cytoskeletal mRNAs in the developing brain providing the first report of a role for Mov10 in cytoskeletal dynamics in the developing brain. These findings along with the behavioral defects seen in the Mov10 heterozygote mice lends precedence to a possible role for Mov10 in diseases of the brain that have underlying cytoarchitectural deficits. Additional hints to what the exact molecular nature of this function might be comes from the established role that Mov10 has in the miRNA pathway. In support of this, preliminary data is provided in Chapter 2 and Appendix A that strongly suggests that Mov10, FMRP and Ago2 could function together to regulate translation of cytoskeletal mRNAs in brain. Future work in this direction would require a systematic target-based approach to identify key transcripts that might contribute to
formation of normal neuronal cytoskeleton such as Map1b using cell and neuronal cultures. Additionally, conditional knockout of Mov10 in brain followed by structural mapping and behavioral studies would be required to confirm the function of Mov10 in the development of normal brain circuitry.

An evolutionarily conserved role for Mov10 in the developing embryo was discovered in the studies described primarily in Chapter 3 and partly in Chapter 2 of this thesis, where the absence of Mov10 in both mouse and Xenopus led to early embryonic lethality. This is yet another novel role for Mov10 at a completely different stage in organismal development. However, there is a common theme of cytoskeletal defects observed at this stage just as in the developing brain. These defects may arise as a result of the misregulation of miRNA-mediated degradation of transcripts at the Maternal to Zygotic Transition (MZT) leading to indirect effects later in gastrulation or could also be a result of the direct misregulation of key cytoskeletal transcripts by Mov10 at gastrulation. In order to delineate these possibilities a systematic study of the effect of Mov10 knockdown at specific stages in embryonic development would be necessary. Thus, the generation of a conditional Mov10 knockout mouse will be crucial to study the exact molecular roles that Mov10 plays both at the embryonic and postnatal stages of development.

Chapter 4 of this thesis attempts to address an outstanding question in the field about the nature of the interaction of FMRP and the RNA components of the RISC pathway. This is answered by using recombinant proteins and in vitro transcribed precursor and miRNA molecules in a filter-binding assay. The data shown here proves conclusively that FMRP does not bind either precursor miRNA or miRNA directly but
acts within the complex as an accessory protein. Additional data points to a possible role for FMRP and its phosphomimcs that are bound to hnRNPA1 in assisting with the transport and processing of precursor miRNAs. Mov10 is also a part of the RISC complex and associates with FMRP and is found at synapses. It would be interesting to dissect out the composition of these different complexes that work together and determine how their dynamics change in a spatial and temporal manner. Future work would also require confirming the different steps of the proposed model using primary neuronal cultures from Fmr1 knockout mice and activity-mediated stimulation to show the role of FMRP in precursor processing.
APPENDIX A

N-TERM OF MOV10 RESCUES NEURITE DEFECT

Mov10 is required for neurite outgrowth in N2a cells (Fig.2.4) and FMRP and Mov10 may participate in the miRNA pathway to regulate mRNA targets involved in neurite outgrowth (Fig.2.5). In support of this finding, we have data showing that the N-terminus of Mov10 associates with FMRP and that the N-terminus of FMRP associates with Ago2. Both these interactions are RNA-dependent (Kenny PJ, unpublished data). Additionally, the N-terminal half of Mov10 has a proposed role in perhaps being a platform for interaction with other proteins as shown in viruses [1]. We hypothesized that the N-terminal half of Mov10 might be interacting with FMRP bound to Ago2 to regulate the common cytoskeletal targets that we find in our bioinformatics analysis (Fig.2.5B). To test this we transfected Mov10 knockout N2a cells with the N-terminal half and the C-terminal half of Mov10 along with the helicase mutant, where a mutation of the conserved Lysine to Alanine in Motif I (K531A in mouse) abrogates the helicase activity [2]. We find that the N-terminal half of Mov10 alone can rescue the neurite length phenotype to WT levels (Fig A.1- compare Mov10 KO to Mov10 KO+ N-term). The helicase-dead mutant also shows recovery of neurite length to WT levels suggesting that the helicase activity is not required for this function (Fig.A.1- compare Mov10 KO and Mov10 KO+K531A) rather the presence of the N-terminal half in this construct is responsible for the rescue. This is further supported by the observation that the helicase domain containing C-terminus of Mov10 also does not rescue the neurite length (Fig.A.1- compare Mov10 KO to Mov10 KO+ C-term). This data shows that the
interaction of the N-terminus of Mov10 with FMRP that is bound to Ago2 might be modulating the translation of the common cytoskeletal mRNAs. This is being tested by ongoing investigations at the mRNA and protein levels for these targets in the lab.

Figure A.1. The N-terminus of Mov10 is required for regulating neurite outgrowth.

The top panel is a graph showing the mean neurite length in um for the various conditions described on the X-axis. The bottom panel shows the Western blot of Mov10 under the different conditions. The HA-tagged C-term and N-term Mov10 blots were probed using an antibody to HA. elf5α was used as a loading control. A total of 800 or more differentiated neurons were counted from six experiments and at least 20 images were counted per condition. Statistics used was one-way ANOVA.
A.1 Methods

Counting of neurite length for N2a

Neuro2a wild type and Mov10 knockout cells were plated in a 24 well plate at a density of 1.5x10⁴ cells per well and incubated for 24 hours at 37 degrees in DMEM (with 10% FCS). One set of the Mov10 knockout wells were transfected with the K531A, HA-C-term Mov10 and HA-N-term Mov10 plasmids using Lipofectamine (2000) before differentiating with DMEM (2% FCS) and 20uM Retinoic Acid (Sigma-Aldrich) a day later. The cells were allowed to differentiate for 48 hours and imaged under transmitted light using an EVOS cell-imaging microscope. The images were anonymized and analyzed by an experimenter blinded to the conditions using the Axiovision Image analysis software. A total of 800 or more differentiated neurons were counted from six experiments and at least 20 images were counted per condition.

A.2 References
