The RNA-binding protein Musashi2 regulates asymmetric neural precursor cell divisions of the developing cerebral cortex

by

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ABSTRACT

THE RNA-BINDING PROTEIN MUSASHI2 REGULATES ASYMMETRIC NEURAL PRECURSOR CELL DIVISIONS OF THE DEVELOPING CEREBRAL CORTEX

Kathryn E. Reynolds
University of Guelph, 2016

Advisor: Dr. John P. Vessey

The brain’s complex cortical circuitry is generated through asymmetric divisions of neural precursor cells (NPCs). These divisions produce one NPC plus one differentiated neuron or intermediate progenitor, due in part to localization of fate determinants to a single region of the dividing cell. This research identifies Musashi2 (mMsi2) as a critical component of cortical asymmetric NPC division. Temporal expression profiles and in vitro immunofluorescence detected mMsi2 within NPCs and some neurons of the mouse cortex throughout neurogenesis. shRNA knockdown in cortical primary culture demonstrated that mMsi2 loss increases NPCs and proportionally decreases neurons and intermediate progenitors, while mMsi2 knockdown in utero similarly increased cells within immature layers of the mouse cortex. mMsi2 overexpression in cortical primary culture again increased NPCs, suggesting that developmental mMsi2 expression must be tightly regulated. Together, these results indicate that mMsi2 promotes neuronal formation during asymmetric NPC division, and is therefore essential to cortical development.
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Lastly, I am very thankful for the support of my friends and family – you have been an immense help throughout the past two years.
Author’s Declaration of Work Completed

I, Kathryn E. Reynolds, declare that all work presented in this thesis is my own.
# Table of Contents

ABSTRACT ................................................................................................................................. ii
Acknowledgements .................................................................................................................... iii
Author’s Declaration of Work Completed .................................................................................. iv
Glossary of Abbreviations ........................................................................................................... viii

## CHAPTER ONE: Introduction ................................................................................................. 1
- Development of the cerebral cortex ......................................................................................... 1
- Asymmetric cell division .......................................................................................................... 5
- The Musashi RNA-binding protein family ............................................................................... 8

*Drosophila melanogaster* Musashi (dMsi) represses translation in external sensory organ precursor cells ................................................................................................................................. 9

*Mus musculus* Musashi1 (mMsi1) is a proposed translational repressor found in NPCs .......... 10
- Identification of *Mus musculus* Musashi2 (mMsi2) as a potential candidate for cortical cell fate determination ................................................................................................................................. 13

## CHAPTER TWO: Materials and Methods .............................................................................. 17
- Animals .................................................................................................................................. 17
- Cell culture ............................................................................................................................... 17
- Western blotting ...................................................................................................................... 20
- Transient transfection of cultured cells .................................................................................. 21
- *In utero* electroporation ......................................................................................................... 23
- Immunocytochemistry and immunohistochemistry ................................................................. 24
- Microscopy and quantification ................................................................................................. 26
- Statistics .................................................................................................................................. 27

## CHAPTER THREE: Results .................................................................................................... 28
- mMsi2 is expressed in the mouse cerebral cortex throughout development ......................... 28
- mMsi2 is expressed in NPCs and some neurons of the cerebral cortex .................................. 30

*In vitro* mMsi2 knockdown increases NPCs, decreases neurons, and decreases intermediate progenitors in primary culture ......................................................................................................................... 33

*In vivo* mMsi2 knockdown with shRNA + GFP increases the number of cells expressing GFP in the VZ/SVZ, and decreases the number of cells expressing GFP in the CP .......... 37
**In vitro** mMsi2 overexpression increases NPCs, decreases neurons, and decreases intermediate progenitors in primary culture........................................................................................................40

**CHAPTER FOUR: Discussion** ........................................................................................................... 42
Consistency with previous studies ........................................................................................................... 42
mMsi2-positive neurons likely belong to a specific deep-layer neuronal population.................. 45
mMsi2 promotes asymmetric NPC divisions of the developing cerebral cortex ................ 46
mMsi2 loss *in utero* affects the distribution of cells throughout the cortical layers .......... 48
mMsi2 overexpression mimics the knockdown phenotype ......................................................... 49
Potential functional differences between mMsi2-S and mMsi2-L isoforms......................... 50
mMsi1 and mMsi2 are unique within the embryonic cerebral cortex................................ 51

**CHAPTER FIVE: Future Directions** ........................................................................................................ 53
Identifying changes in cell type ratios and/or neuronal migration upon *in vivo* mMsi2 loss.... 53
Identifying mMsi2 binding partners ................................................................................................. 53
Exploring the translational repressor hypothesis ........................................................................ 54

**CONCLUSION** ................................................................................................................................. 56

**REFERENCES** ....................................................................................................................................... 57
List of Figures

Figure 1.1. Development of the embryonic mouse brain................................................................. 2
Figure 1.2. Asymmetric division of a neural precursor cell (NPC)................................................. 4
Figure 1.3. dMsi mediates translational repression in IIb neural precursor cells of the future

_Drosophila_ external sensory organ.................................................................................................. 11
Figure 1.4. Structural comparison between mMsi1 and mMsi2....................................................... 14
Figure 2.1. Timeline of cerebral cortex development......................................................................... 18
Figure 3.1. mMsi2 is expressed in the mouse cerebral cortex throughout development.............. 29
Figure 3.2. mMsi2 is expressed in NPCs and some neurons of the cerebral cortex...................... 31
Figure 3.3. mMsi2 is expressed from the mouse cerebral cortex VZ to CP throughout

development....................................................................................................................................... 32
Figure 3.4. shMsi2C and shMsi2D successfully knock down mMsi2-FLAG in HEK293T cells.34
Figure 3.5. shMsi2-1 and shMsi2-2 successfully knock down cortical mMsi2 expression in

primary culture.................................................................................................................................... 36
Figure 3.6. mMsi2 knockdown _in vitro_ leads to an increase in NPCs, as well as decreases in both

neurons and intermediate progenitor cells....................................................................................... 38
Figure 3.7. mMsi2 knockdown _in vivo_ leads to an increase in cells in the VZ/SVZ and a decrease

in cells in the IZ and CP.................................................................................................................... 39
Figure 3.8. mMsi2 overexpression _in vitro_ leads to an increase in NPCs, as well as decreases in

both neurons and intermediate progenitors...................................................................................... 41
Figure 4.1. Proposed role of mMsi2 in asymmetric NPC divisions of the developing cortex. .... 47
## Glossary of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACD</td>
<td>asymmetric cell division</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<td>CP</td>
<td>cortical plate</td>
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<td>d</td>
<td>days</td>
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<td>DDX1</td>
<td>DEAD box 1 RNA helicase</td>
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<tr>
<td>dH2O</td>
<td>distilled water</td>
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<tr>
<td>dMsi</td>
<td><em>Drosophila</em> Musashi</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EJC</td>
<td>exon junction complex</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>G</td>
<td>gauge</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>hours</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>IPC</td>
<td>intermediate progenitor cell</td>
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<td>intermediate zone</td>
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<tr>
<td>kDa</td>
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<td>mitogen-activated protein kinase</td>
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<td>nuclear localization signal</td>
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<tr>
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<td>neural precursor cell</td>
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<td>optimal cutting temperature compound</td>
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<tr>
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<tr>
<td>PABP</td>
<td>poly-A binding protein</td>
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</tr>
<tr>
<td>rpm</td>
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<td>RNA-IP</td>
<td>RNA-immunoprecipitation</td>
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<td>ribonucleoprotein</td>
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<td>RNA recognition motif</td>
</tr>
<tr>
<td>SEM</td>
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<tr>
<td>shctrl</td>
<td>nonspecific scrambled control short hairpin RNA</td>
</tr>
<tr>
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<td>short hairpin RNA</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
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<tr>
<td>TTK69</td>
<td>Tramtrack69</td>
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<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween20</td>
</tr>
<tr>
<td>μg</td>
<td>micrograms</td>
</tr>
<tr>
<td>UL</td>
<td>upper layer</td>
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<tr>
<td>μm</td>
<td>micrometres</td>
</tr>
<tr>
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<tr>
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<td>volts</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
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CHAPTER ONE: Introduction

Development of the cerebral cortex

The formation of the cerebral cortex is a critical aspect of embryonic brain development, as this structure is the centre of complex higher-order thinking processes including conscious thought, reasoning, and decision-making (Kandel et al., 2013). Deficits in the cellular and molecular pathways involved in neurogenesis and cortical neuron migration have previously been shown to lead to a variety of neurodevelopmental disorders, including autism spectrum disorder (Wegiel et al., 2010) and schizophrenia (Reif et al., 2007), due to the creation of a disordered cerebral cortex possessing deficits in neuronal populations as well as inefficient neuronal connectivity. It is therefore imperative that the molecular events governing the development of the mammalian cerebral cortex be understood, in order to further comprehend the disease states in which these events go awry.

The development of the cerebral cortex begins during early embryogenesis. Upon the closing of the neural tube during primary neurulation, multipotent neuroepithelial cells of anterior neuroectodermal origin line the newly formed lateral ventricles and subsequently lose their epithelial characteristics (Franco & Muller, 2013). This conversion produces a population of neural precursor cells (NPCs), which are multipotent neural stem cells retaining the ability to differentiate into the mature cells of the cerebral cortex (Florio & Huttner, 2014). The NPC population is located in the ventricular zone (VZ), a cortical region which lies immediately dorsal to the lateral ventricles (Figure 1.1), and extends into the subventricular zone (SVZ), dorsal to the VZ. During early brain development, the limited NPC pool created at the VZ expands through symmetric mitotic divisions in order to facilitate future growth (Florio & Huttner, 2014). Once this precursor pool has expanded, these symmetric cell divisions no longer
Figure 1.1. Development of the embryonic mouse brain. Schematics and immunofluorescently labelled image of an E15 coronally sectioned brain slice, displaying key anatomical locations and cell types within the developing cerebral cortex. Red asterisks indicate the lateral ventricles, and yellow arrows indicate the direction of neuronal migration. Figure modified from Center for Integrative Brain Research (2014), Hutton & Pevny (2014), and Pacary et al. (2012).
prevail. At the onset of neurogenesis, asymmetric cell divisions (ACDs) become prominent, whereby a single NPC divides to both renew its population and also form a new cell with a different identity (Gönczy, 2008). In most asymmetric NPC divisions, one daughter cell retains its mother cell’s NPC identity, while the other differentiates into a daughter cell with either a neuronal or intermediate progenitor identity (Knoblich, 1997) (Figure 1.2). Intermediate progenitors populate the intermediate zone (IZ), the middle zone of the developing cortex which lies basal to the VZ (Figure 1.1). These cells divide symmetrically to produce two neurons, thereby rapidly expanding the growing cortex (Florio & Huttner, 2014).

Terminally differentiated neurons, either formed directly from asymmetric NPC divisions or indirectly from symmetric intermediate progenitor divisions, migrate to the cortical plate along previously laid down projections (Figure 1.1). This neuronal migration is facilitated in part by NPCs, which provide the scaffold upon which neurons migrate basally (Marin et al., 2010) in order to populate and expand the cortex. Migration of neurons occurs both tangentially and radially, with tangential migration expanding the lateral dimension of the cortex while radial migration enlarges the height of the cortex (Marin et al., 2010). Coordinated migration of newly formed excitatory neurons serves to develop the six-layered cerebral cortex in an inside-out manner, as newborn neurons travel past those previously laid down in order to form the most basal cortical layers (Florio & Huttner, 2014). The mature cerebral cortex thus contains the oldest terminally differentiated cells at the innermost, or deep, layers of the cortex, while the youngest terminally differentiated cells make up the outermost, or upper, cortical layers (Marin et al., 2010).

Following the creation of neurons during the neurogenic phase of embryogenesis, a Notch signalling pathway-induced signal switch halts neurogenesis and permits the onset of
Figure 1.2. Asymmetric division of a neural precursor cell (NPC). mRNA (shown in orange) and other localization factors (shown in blue) are first segregated to one pole of the dividing NPC. This takes place, in part, due to RNA-binding protein-mediated mRNA localization along the cytoskeleton, actively transported by motor proteins (shown in black). Following cleavage (A), one daughter cell retains the maternal NPC identity (1), while the other daughter cell inherits the asymmetrically localized content. These factors may either specify the neuronal (2) or intermediate progenitor cell (IPC) (3) identity of the differentiated daughter cell. IPCs later divide symmetrically (B) to produce neurons, thereby populating the developing cerebral cortex. Figure modified from Inaba & Yamashita (2012).
gliogenesis (Zhou, Kumari, Xiao, & Tan, 2010). The transmembrane signalling receptor Notch is an additional effector of neocortical neurogenesis which possesses a unique pattern of regulation in NPCs. Its action is regulated by the Numb homologs Numb and Numb-like, which both act as inhibitors of Notch signalling (Zhou et al., 2010). The Notch-mediated signal switch to gliogenesis allows for the creation of the supporting astrocytes, oligodendrocytes, and microglial cells of the cerebral cortex (Florio & Huttner, 2014), and prevents the formation of excess neurons once an adequate population has been reached. Proper asymmetric division of NPCs, correct migration of newborn cells, and a timely switch from neurogenesis to gliogenesis are thus necessary requirements of successful brain development (Hatten, 1993).

**Asymmetric cell division**

Asymmetric cell divisions are not only important in the formation of cells populating the cortex, but are also a vital process in many other tissues and organisms due to their ability to create cellular diversity while simultaneously regenerating the stem cell population to permit future growth (Gönczy, 2008). There are three key processes which comprise a typical asymmetric cell division: disruption of symmetry in the dividing cell to create polarity, localization of mRNA and proteins to a single pole, and asymmetric alignment of the mitotic spindle to ensure that division creates two cells with distinct contents (Gönczy, 2008).

In the model organism *Caenorhabditis elegans*, the structure of asymmetrically dividing cells has been shown to be reinforced by the action of an actin and non-muscle myosin II network (Gönczy, 2008). Phosphorylation of the myosin light chain MLC-4 leads to initial contractility of this actomyosin network, whereby the posterior pole contracts as the anterior pole expands (Gönczy, 2008). Further creation of polarity is mediated through the reorganization of
the actomyosin network, due to the specific action of Par proteins. A partition defective (Par) protein complex comprised of Par3, Par6, and atypical protein kinase C (aPKC) migrates to the anterior pole of the cell while Par1 and Par2 proteins are segregated to the posterior pole, a phenomenon mediated by the action of Par5 as well as the cytoskeleton-remodelling Rho-GTPase Cdc42 (Cappello et al., 2006; Etienne-Manneville, 2004; Gönczy, 2008). Aided by this anterior-posterior axis determination, the actomyosin cytoskeleton thus further contracts at the anterior pole, but remains non-contractile at the posterior pole (Gönczy, 2008).

The active process of polar mRNA localization involves the creation of a heterogeneous ribonucleoprotein mRNA transport complex within the cytoplasm, followed by kinesin- or dynein-driven movement of this complex toward one pole of the cell through interactions with proteins such as RNA-binding proteins (Holt & Bullock, 2013). RNA-binding proteins are critical components of this complex and serve many functions, such as RNA stability, structural support, and translational regulation. Together, these proteins exert control over NPC maturation and allow for differential inheritance of mRNA (Holt & Bullock, 2013). A number of RNA-binding protein subfamilies which carry out important functions in neural cells have been identified, including the Elav subfamily, which contains the invertebrate Elav and vertebrate Hu proteins and maintains survival of mature neurons (Okano & Darnell, 1997; Yao et al., 1992). Certain RNA-binding proteins may also assist in the maturation of pre-mRNA to produce mRNA, with roles in splicing and polyadenylation (Glisovic et al., 2008). One such example involves the exon junction complex (EJC), which contains RNA-binding proteins Y14 and Barentsz as well as two other core proteins. Following the splicing of pre-mRNA in the nucleus, the EJC is deposited onto spliced mRNAs. Y14 and Barentsz are then involved in the shuttling of spliced mRNAs from the nucleus to the cytoplasm (Le Hir et al., 2001; Macchi et al., 2003), as
well as their asymmetric localization within the cytoplasm. The Staufen2 RNA-binding complex has also been identified as a critical component of NPCs, and is asymmetrically localized to create differential cell fates. Staufen2 is found in complexes with the translational repressor Pumilio2, an RNA helicase DEAD box 1 (DDX1), and prox1 mRNA in NPCs of the apical VZ (Vessey et al., 2012). Knockdown of either Staufen2, Pumilio2, or DDX1 leads to the mislocalization of prox1 mRNA and thus disruption of asymmetric NPC division. As a result of this failure of mRNA localization, NPCs are unable to be renewed and the production of terminally differentiated neurons prevails (Vessey et al., 2012).

The positioning of the mitotic spindle is also a key component permitting the division of the cell into two daughters possessing distinct fates. Both microtubule depolarization and the pulling action of dynein lead to the generation of force from the spindle poles (Gönczy, 2008). The posterior pole typically generates greater force than the anterior pole, leading to asymmetric positioning of the mitotic spindle within the dividing cell. Following the positioning of the mitotic spindle, the mother cell is able to divide to form two daughter cells, often of unequal sizes due to the asymmetric positioning of the spindle (Gönczy, 2008).

Upon completion of these key processes of asymmetric localization, one daughter cell will inherit the asymmetrically localized fate determinants, of which the mRNA components are then translated to form proteins which influence cell fate specification (Gönczy, 2008). Thus, an asymmetric cell division produces two daughter cells of distinct identities (Figure 1.2).

Not only does polar mRNA localization spatially restrict protein synthesis to one daughter cell, it also does so temporally, as mRNA should not be translated to form protein until the cell has divided fully. Following complete cell division, the inhibition of protein synthesis
must be lifted in order for fate-determining proteins to create the distinct identities of differentiated daughter cells. It is well-known that precursor proliferation and differentiation may be regulated transcriptionally through the action of transcription factors (Davis & Reed, 1996; Shimazaki et al., 1999), but these processes may also be controlled post-transcriptionally (Johnstone & Lasko, 2001). In *Drosophila*, mRNA localization-associated translational repression is crucial for the formation of the developing oocyte. A series of body axis-determining genes are segregated during mitotic division. *oskar* and *nanos* mRNA are localized to the posterior pole of the oocyte, while *bicoid* mRNA is localized to the anterior pole (Kugler & Lasko, 2009). Translation of *oskar* mRNA is repressed by the RNA-binding protein Bruno until mid-neurogenesis, at which time this repression is alleviated and *oskar* is permitted to form a specialized cytoplasm containing determinants of body axis patterning (Kugler & Lasko, 2009). Translation of *nanos* and *bicoid* mRNA is repressed until fertilization of the oocyte, when Nanos and Bicoid protein gradients are formed to further determine body segment polarity (Kugler & Lasko, 2009). Such a crucial instance of transient translational repression during embryogenesis suggests that this process is both conserved and highly important across species.

**The Musashi RNA-binding protein family**

Due to the aforementioned instances of asymmetric cell division and translational repression, it is hypothesized that reversible translational control mechanisms function alongside mRNA localization elements during asymmetric NPC divisions (Johnstone & Lasko, 2001). The Musashi (Msi) RNA-binding proteins are potential mediators of translational repression and asymmetric division in the developing cortex; the function of the mouse Msi orthologue mMsi2 is further explored in this research.
The Msi RNA-binding protein family contains members from *Xenopus laevis* (Good et al., 1998), *Caenorhabditis elegans* (Good et al., 1998), *Drosophila melanogaster* (Nakamura, Okano, Blendy, & Montell, 1994), *Mus musculus* (Sakakibara et al., 1996; Sakakibara et al., 2001), *Homo sapiens* (Good et al., 1998), and others; some of which are known to participate in translational repression and/or mRNA localization (Kawahara et al., 2008; Nakamura et al., 1994). These proteins are defined by the presence of two approximately 80-90 amino acid-long tandem RNA recognition motifs (Nakamura et al., 1994) at their N-terminus, termed RRM1 and RRM2, which permit the binding of the Msi protein to mRNA (Gunter & McLaughlin, 2011). Each RRM is comprised of the ribonucleoprotein motifs RNP-1 and RNP-2, which are eight and six amino acids long, respectively (Sakakibara et al., 2001). RNP-1 is very highly conserved, while RNP-2 differs among Msi proteins but maintains an aliphatic and aromatic nature (Sakakibara et al., 2001). These RNP regions are crucial for the binding specificity of the Msi RNA-binding proteins.

**Drosophila melanogaster** *Musashi* (dMsi) represses translation in external sensory organ precursor cells

Significant insight into the function of Msi RNA-binding proteins comes from research investigating the *Drosophila* orthologue, dMsi. dMsi is a significantly longer protein than the other Msi orthologues (Sutherland et al., 2013), and it shares only 36% similarity with mouse orthologues due to differences in amino acids throughout the protein (Good et al., 1998).

dMsi plays an important role in translational repression, as evidenced from research investigating the formation of *Drosophila* external sensory organ mechanoreceptors responsible for hearing, touch, and proprioception (Jarman, 2002). The *Drosophila* external sensory organ is
composed of non-neural mechanical socket and shaft cells as well as sensory neurons and glia (Nakamura et al., 1994). These cells originate from one of two cell types: either non-neuronal IIa precursors or neuronal IIb precursors. In IIa precursors, *tramtrack69 (ttk69)* mRNA is translated into Tramtrack69 (TTK69) protein, which conveys a non-neuronal fate (Figure 1.3). However, in IIb precursors, dMsi binds a poly uridine-rich sequence at the 3’ untranslated region (UTR) of *ttk69* mRNA to inhibit translation (Sutherland et al., 2013), and the absence of TTK69 determines the resultant cells’ neural fate (Nakamura et al., 1994) (Figure 1.3). This dMsi action is permitted by the Notch signalling pathway. Although dMsi is present in both IIa and IIb precursor cells, IIb precursors secrete a Delta ligand, which in turn activates IIa precursors through the Notch transmembrane receptor in order to specifically inhibit IIa dMsi (Gönczy, 2008). In dMsi mutants, the absence of dMsi-mediated translational repression leads to the conversion of IIb precursors into IIa precursors (Figure 1.3). The resulting mutant phenotype lacks neurons and glia due to the absence of sufficient IIb precursors. However, it contains an abundance of socket and shaft cells as a result of excess IIa precursors, leading to the production of excess sensory bristles (Nakamura et al., 1994). dMsi thus acts as a known translational repressor in the *Drosophila* nervous system, alluding to the potential for mammalian Msi-mediated translational repression in NPCs.

*Mus musculus* Musashi1 (mMsi1) is a proposed translational repressor found in NPCs

The mouse orthologue mMsi1 is primarily expressed within the brain, specifically by NPCs within the VZ at the onset of neurogenesis. Its expression gradually decreases with development, vanishing completely once NPCs leave their mitotic state and mature into neurons (Sakakibara et al., 1996). Thus, mMsi1 has been termed a marker of immature and dividing cells.
Figure 1.3. dMsi mediates translational repression in IIb neural precursor cells of the future *Drosophila* external sensory organ. (Wildtype) In wildtype IIa non-neural precursors, *ttk69* mRNA is permitted to be translated into TTK69 protein, as dMsi is blocked by a Notch signal initiated through the Delta ligand of a IIb neural precursor cell. dMsi is active in wildtype IIb cells, and binds to the 3' UTR of *ttk69* mRNA to repress its translation. The presence or absence of TTK69 determines the cells’ fates, as cells possessing TTK69 become neurons or glia while those lacking TTK69 become non-neural socket or shaft cells. (dMsi mutant) IIa cells are unaffected by the loss of dMsi. In IIb cells lacking dMsi, translation of *ttk69* cannot be repressed, and IIb cells adopt the characteristics of non-neural IIa cells, resulting in an excess of non-neural socket and shaft cells. Figure modified from Nakamura et al. (1994).
within the cortex, and its transient expression may point toward a role as a transient translational repressor.

Subsequent research has hinted at mMsi1’s role in the maintenance of NPC self-renewal, which involves the Notch signalling pathway. Proliferative status of a cell is achieved through the actions of Notch, while this immature proliferative status may be turned off through binding of the Numb protein to the intracellular domain of the Notch transmembrane receptor (Okano et al., 2002). In order to maintain the proliferative status of NPCs, mMsi1 is proposed to translationally repress numb by binding to its 3’ UTR (Okano et al., 2002). This binding would allow Notch activation to prevail, promoting a self-renewing state. In this way, mMsi1 is hypothesized to translationally repress a repressor of Notch signalling, while also influencing cell fate.

Recent research has used a mouse embryonic carcinoma model of neurogenesis to provide evidence supporting mMsi1’s capability for translational repression. In postnatal day (P) 19 mouse embryonic carcinoma cells derived from a teratocarcinoma and differentiated into neural cells through retinoic acid treatment, the cyclin-dependent kinase inhibitor p21 was translationally repressed by mMsi1 binding (Battelli et al., 2006). Significantly, knockdown of mMsi1 in this model of neurogenesis also led to a decrease in neurons accompanied by an increase in cells recently committed to a neuronal lineage (Battelli et al., 2006). This finding suggests that mMsi1 participates in neural asymmetric divisions and in fact may mediate the asymmetric localization of mRNA to affect cell fate, similar to the actions of the Staufen2 complex (Vessey et al., 2012).
**Identification of *Mus musculus* Musashi2 (mMsi2) as a potential candidate for cortical cell fate determination**

Given dMsi’s aforementioned role in translational repression, as well as mMsi1’s proposed function in neural asymmetric divisions, the poorly characterized mouse orthologue mMsi2 was chosen as a promising candidate for cortical cell fate determination. Located on chromosome 11, mMsi2 spans 378 886 base pairs and contains 7 exons (Sakakibara et al., 2001). There are two known isoforms of this RNA-binding protein in mammals, namely mMsi2-L and the splice variant mMsi2-S, which differ by a span of 18 amino acids (Sakakibara et al., 2001). *mMsi2* mRNA is known to be present in the brain, but is also found in a wide variety of tissues including the heart, lungs, and skeletal muscle (Sakakibara et al., 2001). Within the brain, cortical *mMsi2* appears to be expressed into adulthood (Sakakibara et al., 2001). Few studies have investigated the role of mMsi2 in the context of brain development prior to this research.

The two mouse Msi proteins share significant structural similarities, including 75% conserved amino acid residues (Sakakibara et al., 2001), while their RRM regions are 78% (RRM1) and 91% (RRM2) similar, respectively (Sakakibara et al., 2001) (Figure 1.4). The RNP regions are completely conserved between mMsi1 and mMsi2 (Sakakibara et al., 2001), indicating highly similar RNA-binding specificity. However, a significant structural difference between these proteins lies in their C-termini, where mMsi1 contains both a protein binding domain (PBD) and an additional sequence of 58 amino acids (Gunter & McLaughlin, 2011) (Figure 1.4). While the role of this 58 amino acid-long region is unknown, it likely serves an additional function not possessed by mMsi2, indicating probable differences in expression and/or function between these mammalian Msi proteins. These probable differences are further...
Figure 1.4. Structural comparison between mMsi1 and mMsi2. Both proteins contain two RNA recognition motifs (RRMs; shown in yellow), which are each composed of two ribonucleoprotein motifs (RNPs; shown in orange). The nuclear localization signal (NLS) found in RRM1 is conserved between mMsi1 and mMsi2. However, an NLS-like sequence is located in RRM2 of mMsi1, but no such sequence exists in mMsi2. Additionally, mMsi1 contains a protein binding domain (PBD; shown in green) and an extended C-terminus. Figure redrawn from Kaneko et al. (2000), with additional information from Sakakibara et al. (2001) and Gunter & McLaughlin (2011).
supported by mMsi2’s expression across tissue types and developmental stages, in contrast to mMsi1’s primarily neurogenic expression (Kaneko et al., 2000).

To date, the majority of mMsi2 research has focused on its role in tumorigenesis, as mMsi2 displays a unique pattern of expression in several types of cancer. mMsi2 has recently been identified as a prospective biomarker of hepatocellular carcinoma. High mMsi2 expression in hepatocellular carcinoma cells is correlated with poor clinical prognosis, and evidence from RNAi knockdown and cell invasion experiments suggests that loss of mMsi2 expression diminishes their invasiveness (He et al., 2014). mMsi2 is also currently being investigated as a potential therapeutic target for acute myeloid leukemia. Similar to hepatocellular carcinoma, high expression of mMsi2 in leukemic cells is correlated with increased prognosis of acute myeloid leukemia and decreased probability of recovery (Zhang et al., 2014). shRNA knockdown of mMsi2 expression in leukemic cell lines led to cell cycle arrest, inactivation of MAPK signalling pathways, and initiation of apoptosis. mMsi2’s upregulation in tumours may point toward functions in maintaining a cell’s proliferative status, or regulating the duration of the cell cycle (Zhang et al., 2014).

While the actions of dMsi and mMsi1 have been thoroughly investigated, the specific contribution of mMsi2 to mammalian cortical development remained unclear prior to this research. The knowledge of Msi protein expression in neural stem cells of different organisms, coupled with its demonstrated ability to repress translation both in Drosophila external sensory organ precursors and a mouse embryonic carcinoma model of neurogenesis, highly suggested to us that mMsi2 is involved in translational repression facilitating asymmetric NPC division. Due to key structural differences between mMsi1 and mMsi2, we hypothesized that mMsi2 would play a unique and critical role in asymmetric NPC divisions contributing to the formation of the
mature cerebral cortex, and furthermore, its absence would lead to disordered NPC differentiation similar to that observed upon mMsi1 knockdown in a mouse embryonic carcinoma model of neurogenesis.

To test this hypothesis, mMsi2 expression in the cerebral cortex was first investigated through the creation of a temporal Western blot expression profile. Following confirmation of mMsi2 expression throughout neurogenesis, with visible enrichment between E14-E15, immunofluorescence (IF) at key neurogenic stages identified mMsi2 as a cytoplasmic component of NPCs and some neurons. shRNA knockdown was then employed to observe potential changes in cell fate determination following mMsi2 loss. Knockdown of mMsi2 in cortical primary culture led to a significant increase in NPCs and decreases in neurons and intermediate progenitors following 3 days (d) in vitro, suggesting that asymmetric NPC divisions are disrupted in the absence of mMsi2. This knockdown was repeated in utero, again demonstrating a significant change in the localization of knockdown cells which favoured the VZ/SVZ at the expense of both the IZ and CP, and indicating an increase in NPCs and/or impaired migration. mMsi2 overexpression in cortical primary culture produced a very similar phenotype to knockdown, with a significant increase in NPCs and reductions in neurons and intermediate progenitors. We therefore conclude that mMsi2 is a crucial component in the division of NPCs, promoting asymmetric division and neuronal formation during early to mid neurogenesis.
CHAPTER TWO: Materials and Methods

Animals

Western blotting and primary cultures were completed using wildtype outbred CD1 mice, purchased timed pregnant ± 12 hours (Charles River Laboratories, Sherbrooke, Quebec) and housed at the University of Guelph Central Animal Facility until dissection. All housing and experiments involving mice complied with the guidelines and regulations outlined by the Canadian Council on Animal Care. Wildtype outbred mice were chosen to minimize potential impairments of cortical development that can arise due to repeated rounds of inbreeding (Crawley et al., 1997). The wildtype CD1 strain in particular was selected due to large litter sizes (Aldinger et al., 2009), ensuring ample cortical tissue and reducing the number of animals sacrificed. Embryonic dissection took place at key stages of cortical development (Figure 2.1), producing primary tissue cultures for transfection and/or immunocytochemistry, lysates for Western blotting, and whole brains for immunohistochemistry. Cortical tissue was extracted and plated at E12.5 during early neurogenesis, then fixed and stained at E15 in order to reflect the typical cell divisions occurring by mid neurogenesis. Western blot lysates were prepared at intervals between E11 and E19 to capture mMsi2 expression throughout all stages of neurogenesis, while E12 and E15 whole brains were extracted to facilitate immunohistochemistry assessing the expression of mMsi2 within major cortical regions (VZ/SVZ, IZ and CP).

Cell culture

Cortical tissue was isolated from E12.5 cortices, dissociated, and plated on 12mm German glass coverslips (Electron Microscopy Sciences) within 24-well plates (Nuclon Delta
Figure 2.1. Timeline of cerebral cortex development. E12 indicates the beginning of neurogenesis, when NPCs are abundant. E15 (corresponding to approximately 3 days in culture) indicates the peak of neurogenesis, where approximately equal proportions of NPCs and neurons are present. E17-18 indicates the completion of neurogenesis.
Surface, Thermo Fisher). Prior to dissection and plating, coverslips were flame sterilized, coated with 4% laminin (Corning) and 2% poly-D-lysine (Sigma Aldrich) in sterile water (GE Healthcare) for 24h, then washed 2x with sterile water and incubated in supplemented neural stem cell media (NeuroCult media plus 10% neural stem cell proliferation supplement, StemCell Technologies) for 2h.

Timed pregnant CD1 mice were euthanized with CO₂ (4 L/min; Linde), then transferred to a sterile dissection hood (HeraGuard Eco, Thermo Fisher) prior to the removal of live embryos for dissection and tissue recovery. Following abdominal sterilization with 75% ethanol, a bilateral transverse incision through the skin and abdominal wall revealed the uterine horns. Uterine horns were detached and immersed in 1X PBS pH 7.4 (Life Technologies) in a 10 cm petri dish (Corning). Embryos were subsequently removed from yolk sacs and transferred to individual petri dishes filled with fresh PBS. Visible under a dissecting light microscope (Nikon SMZ 745T), embryos were affixed to the dish surface using 21G needles (PrecisionGlide, BD) to immobilize and expose the frontal lobes. Skin, skull, and meninges were removed using forceps (Fine Science Tools) to reveal the developing cerebral cortex. The lateral and medial ganglionic eminences are located in the ventral ventricular zone adjacent to the cortex and contribute to inhibitory interneuron formation (Anderson et al., 2001), and were therefore avoided during tissue excision. Cortical tissue was excised with clean forceps, then transferred to ice-cold primary culture media for dissociation and tissue culture (NeuroCult media plus 10% neural stem cell proliferation supplement, 0.2% 10 μg/mL EGF, 0.1% 10 μg/mL FGF, and 1% penicillin/streptomycin (Sigma Aldrich); StemCell Technologies). Cortical tissue was plated at a concentration of 175 000 cells/well, in 1 mL primary culture media, and incubated for 3 days (37°C, 5% CO₂; FORMA Series II Water Jacket CO₂ Incubator, Thermo Scientific).
HEK293T cells were maintained on 10cm tissue culture dishes (Corning), at an average density of 1 000 000 cells/mL, in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F-12 HAM (Sigma Aldrich) with 10% bovine calf serum (Sigma Aldrich) and 1% penicillin/streptomycin (Sigma Aldrich). Plated cells were incubated at 37°C under 5% CO₂ (FORMA Series II Water Jacket CO₂ Incubator, Thermo Scientific) and passaged twice weekly to a 1:10 dilution. For transient transfection experiments, HEK293T cells were replated at a 1:3 dilution into 6-well plates (Nuclon Delta Surface, Thermo Fisher), then transfected following 24h incubation (see Transient transfection of cultured cells).

Western blotting

Cortical tissue was excised from CD1 mice aged E11-E19 (see Cell culture for dissection procedures) and collected in ice-cold PBS, then transferred into ice-cold lysis buffer (7 mL 1X brain extraction buffer, composed of 25 mM HEPES pH 7.3; 150 mM KCl; 8% glycerol; 0.1% NP-40 in milliQ H₂O (Sigma Aldrich): 1 protease inhibitor tablet (Protease Inhibitor Mini Tablets, Thermo Scientific Pierce); used at a ratio of 6 E12.5 hemispheres: 1 mL lysis buffer). Tissue was homogenized by hand in a glass dounce (Wheaton), and the resulting mixture was centrifuged to separate protein lysate from pelleted debris (10 min, 10 000 rpm, 4°C). Following centrifugation, protein was quantified in triplicate using a Micro BCA Protein Assay kit (Thermo Scientific Pierce). Lysates were subsequently mixed and boiled (95°C, 10 min) in sample buffer (2M Tris pH 6.8, glycerol, SDS, pyronin Y, β-mercaptoethanol; Sigma Aldrich) for a final concentration of 1X, then stored at -80°C until use.

Lysates from ages E11 to E19 (10 µg each) were run on 10% resolving, 4% stacking SDS-PAGE gels, wet transferred to nitrocellulose membrane (BioRad), and blocked using 5%
BSA (Sigma Aldrich) in 1X TBST (Sigma Aldrich). Blots were cut between 72 kDa and 52 kDa to facilitate simultaneous probing with both anti-mMsi2 and high molecular weight loading control antibodies, and incubated in primary antibody overnight at 4°C. Primary antibody incubation was followed by 3 x 8min washes in 1X TBST, 1h incubation in horseradish peroxidase secondary antibody (BioRad), an additional 3 x 8min washes in 1X TBST, then treatment with enhanced chemiluminescence (ECL Prime, GE Healthcare) and development on film (Clinicselect blue X-ray film, Carestream; SRX-101A developer, Konica Minolta). Primary antibodies chosen were anti-mMsi2 (Abcam, ab76148, 1:200 dilution), and loading control antibodies against ubiquitously expressed focal adhesion protein Vinculin (Abcam, ab129002, 1:2000 dilution) and RNA helicase DDX1 (Sigma Aldrich, HPA034503, 1:1000 dilution), as previously used in similar studies (Vessey et al., 2012).

**Transient transfection of cultured cells**

shRNA vectors specific to mMsi2 (with no sequence similarities to mMsi1) were purchased from ORIgene and compared to known mMsi2-S and mMsi2-L sequences (BLAST, NCBI) to permit knockdown specificity. These shRNAs were then validated using a HEK293T cell line, as use of this cell line permitted higher transfection efficiency than in cultured NPCs (Thomas & Smart, 2005). Four Kanamycin-resistant shRNA vectors and one Kanamycin-resistant scrambled 29-mer control shRNA vector (ORIgene) as well as one Ampicillin-resistant Msi2-FLAG overexpression construct (Okano et al., 2002), sequence-verified at the Genomics Facility, Advanced Analysis Centre, University of Guelph, were transformed into *E. coli* (subcloning efficiency DH5α competent cells, Invitrogen) and incubated overnight at 37°C. One colony of each preparation was selected to be grown in LB (Sigma Aldrich) overnight at 37°C, with Kanamycin or Ampicillin (1:1000 dilution, Sigma Aldrich) as appropriate. DNA
puriﬁcation (Endofree and High Speed Maxi Kits, Qiagen) and subsequent quantiﬁcation (NanoDrop) and dilution (to a ﬁnal concentration of 5 ug/uL in nuclease-free water, Thermo Fisher) permitted transfection into HEK293T cells using lipid-based transfection (Lipofectamine 2000, Invitrogen). HEK293T cells were co-transfected with shRNA (0.75 μg/well) and FLAG-tagged mMsi2 (0.25 μg/well) and incubated for 24h (37°C, 5% CO2; FORMA Series II Water Jacket CO2 Incubator, Thermo Scientiﬁc). Protein extraction occurred on ice using 1X brain extraction buffer plus protease and RNase inhibitors (7 mL 1X brain extraction buffer composed of 25 mM HEPES pH 7.3; 150 mM KCl; 8% glycerol; 0.1% NP-40 in milliQ H2O (Sigma Aldrich): 1 protease inhibitor tablet (Protease Inhibitor Mini Tablets, Thermo Scientiﬁc Pierce)), and was followed by protein quantiﬁcation, done in triplicate (Micro BCA Protein Assay kit, Thermo Scientiﬁc Pierce). Western blotting revealed successful co-transfection with working shRNAs. These validated shRNAs (shMsi2C and shMsi2D) were thus selected for in vitro knockdown, and were renamed shMsi2-1 and shMsi2-2, respectively.

To facilitate in vitro knockdown, primary tissue cultures were co-transfected with validated shRNAs and nuclear GFP (nGFP; courtesy of Dr. Freda Miller, SickKids, Toronto; used as a positive indicator of transfection) to knock down endogenous mMsi2 expression within primary culture. shRNA (0.75 μg/well) and nGFP (0.25 μg/well) were combined in a ratio which ensured that GFP-transfected cells were highly likely to also receive shRNA (Vessey et al., 2012). Transfection took place 1h after E12.5 tissue culture (as discussed in Cell culture) using the non-liposomal transfection reagent FuGENE 6 (Promega), which was revealed to be optimal for transfection efﬁciency and cell health following comparison of multiple transfection reagents. Following transfection, primary cultures were incubated (37°C, 5% CO2; FORMA Series II Water Jacket CO2 Incubator, Thermo Scientiﬁc) in the presence of growth factors (NeuroCult
primary culture media with 10% neural stem cell proliferation supplement, 0.2% 10 μg/mL EGF, 0.1% 10 μg/mL FGF, and 1% penicillin/streptomycin (Sigma Aldrich; StemCell Technologies) until E15, then were fixed in 4% paraformaldehyde and immunofluorescently labelled (see *Immunocytochemistry and immunohistochemistry*).

**In utero electroporation**

Following successful *in vitro* knockdown, mMsi2 knockdown was repeated *in vivo* using *in utero* electroporation (thanks to Sarah Burns, SickKids, Toronto). shRNA vectors (shMsi2D or shctrl, ORIgene) were combined with nGFP at a ratio of 3 μg shRNA:1 μg nGFP to ensure delivery of shRNA, mixed with 0.05% Trypan Blue (as a dye to facilitate precision of injection; Sigma Aldrich), diluted in dH2O, and preloaded into a glass capillary micropipette. Timed pregnant CD1 mice (Charles River Laboratories, Sherbrooke, Quebec) were anaesthetized with isoflurane (2%-1.5%, Baxter Corporation) plus O2 (0.8L) and N2O (1.2L). A midline incision through the skin and abdominal wall revealed the developing embryos at E13, allowing for shRNA injection through the uterine wall, yolk sac, embryonic skin, skull, and cortical tissue into one embryonic lateral ventricle. Application of a 50V current, delivered in five 40ms pulses separated by 950ms intervals (Vessey et al., 2012), created pores in NPC membranes to allow for shRNA and GFP uptake. Following electroporation, uterine horns were returned to the mother, the abdominal incision was sutured closed, and the mother was treated with a subcutaneous injection of analgesic (0.05-0.1 mg/kg Anafen Ketoprofen, Merial). Following 3d development *in vivo*, the pregnant mother was euthanized using CO2 asphyxiation (4 L/min; Linde) and intact E16 brains were extracted. Brains were immediately drop-fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 24h, dehydrated in 30% sucrose (Sigma Aldrich) in HBSS (Thermo Fisher), and stored at -80⁰C in Optimal Cutting Temperature (OCT) Compound.
(Tissue-Tek). OCT-treated cortices were coronally cryosectioned at -20°C (Leica CM1860 cryostat) into slices of 15 μm thickness and mounted onto gelatin-coated (0.5% gelatin and 0.05% chromium potassium sulphate in milliQ H2O; Sigma Aldrich) microscope slides (Selectfrost, Thermo Fisher), then stored at -80°C prior to immunohistochemistry and imaging (see *Immunohistochemistry and immunocytochemistry* and *Microscopy and quantification*).

**Immunocytochemistry and immunohistochemistry**

Following 3d *in vitro*, E15 primary cultures were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) diluted in HBSS (Thermo Fisher), then permeabilized in 0.2% Nonidet P-40 in HBSS (Sigma Aldrich). Fixed cultures were blocked in 5% BSA (Sigma Aldrich) plus 6% Normal Goat Serum (Jackson ImmunoResearch) in HBSS (Thermo Fisher), and co-stained in half block for 2h with antibodies specific to both i) mMsi2 and ii) NPC, neuronal, or mitotic markers. These include embryonic stem cell marker and transcription factor Sox2 (Thermo Fisher, MA1-014, 1:200 dilution), NPC marker and intermediate filament protein Nestin (R&D Systems, MAB2736, 1:250 dilution), neuronal marker and microtubule element βIII-Tubulin (BioLegend, 801201, 1:2000 dilution), post-mitotic neuronal marker and transcription factor SatB2 (Abcam, ab92446, 1:250 dilution), and intermediate progenitor and transcription factor Tbr2 (Abcam, ab23345, 1:200 dilution). Fluorescent secondary antibodies were Alexa Fluor anti-mouse and anti-rabbit 488 and 555 (Life Technologies, 1:1000 dilution in half block, 1h), and were followed by nuclear stain Hoechst (Sigma Aldrich, 33258, 1:2000 dilution in HBSS, 2min). Coverslips were mounted onto microscope slides (Selectfrost, Thermo Fisher) using Permafluor mounting agent (Thermo Fisher) and allowed to dry for 24h prior to imaging.
Whole E12 and E15 brains were collected in ice-cold PBS, then drop-fixed in formalin (10% neutral buffered with 4% w/v formaldehyde, Sigma Aldrich) for 24h, dehydrated in 30% sucrose in PBS (Sigma Aldrich), and sent to SickKids Hospital, Toronto for paraffin embedding and slicing. Prepared paraffin-embedded slides from E12 and E15 embryos were immunofluorescently labelled with the same anti-mMsi2 and anti-marker antibodies listed above to observe the global cortical distribution of mMsi2. Immunohistochemistry on paraffin-embedded slides included additional dehydration and antigen retrieval steps prior to blocking. Paraffin removal and dehydration took place in sequential dilutions of 100% and 50% xylenes (Thermo Fisher; diluted in 95% ethanol), followed by 95%, 70% and 50% dilutions of ethanol in milliQ H₂O. Immersion of slides in boiling sodium citrate buffer (10 mM pH 6.0; Sigma Aldrich) facilitated antigen retrieval. A modified blocking solution was also used, comprised of 4% BSA (Sigma Aldrich), 6% Normal Goat Serum (Jackson ImmunoResearch), and 0.3% Triton X (Sigma Aldrich) in 1X PBS pH 7 (Life Technologies). Primary antibody, secondary antibody, and Hoechst nuclear stain incubations were completed as described above.

Cryosectioned E16 in utero electroporated brains (see In utero electroporation for preparation) were labelled with anti-GFP primary antibody and Hoechst nuclear stain to visualize the localization of GFP-positive cells. Cryosections were processed in the same manner as primary cultures, with the exception of the modified blocking solution used for paraffin sections. Primary and secondary antibodies, as well as Hoechst nuclear stain, were prepared as described above.
Microscopy and quantification

Epifluorescent light microscopy (Zeiss Axio Observer Z.1) was employed to qualitatively assess characteristics of individual Msi2-positive cells derived from primary culture. Representative images of mMsi2/marker co-stains were taken at 63x magnification (Hamamatsu ORCA-Flash 4.0LT C11440 camera) with the apotome enabled (Zeiss Apotome.2) to minimize background signal from scattered light. Imaging of paraffin sections took place at 20x magnification with the apotome enabled, to qualitatively observe patterns of mMsi2 expression.

Quantification, performed blinded and by a single individual, assessed the proportion of cell types present to identify potential changes in NPC division. Coverslips were each scanned at 20X magnification (Nikon Eclipse 50i epifluorescent microscope) for an average of 200 healthy cells (lacking a condensed or fragmented nucleus; with visible nucleoli present) across random fields of view. For quantification of mMsi2/β3T and mMsi2/SatB2 co-expression, ~200 healthy mMsi2-positive cells were assessed for marker expression. Marker positivity was averaged across 3 biological replicates to produce a mean percentage of cells expressing both the marker and mMsi2. For in vitro knockdown experiments, intensity of mMsi2 expression in ~200 healthy GFP-positive cells was first visually assessed between controls and shMsi2-1/shMsi2-2-transfected cells, using the criteria of highly mMsi2-positive (mMsi2++), moderately mMsi2-positive (mMsi2+), and mMsi absent (mMsi2-). The rate of cell death via apoptosis was also compared between control and knockdown conditions by counting GFP-positive cells displaying condensed nuclei. Following this additional validation of shRNA efficacy, knockdowns were co-stained with anti-GFP plus anti-βIII-Tubulin, Nestin or Tbr2 antibodies (see Immunocytochemistry and immunohistochemistry) and ~200 healthy GFP-positive cells per marker were assessed for marker positivity or negativity. After three separate dissections,
cultures, and transfections, the percentages of GFP-positive/marker-positive cells were averaged. These methods were also used to quantify *in vitro* overexpression.

To analyze the efficacy of *in vivo* knockdown and the cortical localization patterns of cells lacking mMsi2, images of E16 electroporated cortices spanning from the lateral ventricle to the meninges were cropped to isolate a section of cortex containing GFP-positive cells throughout all three cortical layers. Borders between each cortical layer were observed through changes in nuclear density visible within the Hoechst channel, and drawn in ImageJ (National Institutes of Health). GFP-positive cells present in each cortical layer were counted, and averaged with counts obtained from all electroporated slices per cortex, n=3 cortices per treatment.

**Statistics**

Statistical analyses following quantification of *in vitro* and *in vivo* knockdown and overexpression experiments were performed in Microsoft Excel using paired two-tailed t tests for means (n=3; n=4 for *in vitro* knockdown co-stained with Tbr2, and all *in vivo* knockdown experiments). Data were presented as mean + SEM. All differences were considered significant at p<0.05.
CHAPTER THREE: Results

**mMsi2 is expressed in the mouse cerebral cortex throughout development**

In order to permit Western blotting and IF experiments, an antibody specific to mMsi2 was first chosen and verified. Antibody specificity was tested through transfection of FLAG-tagged mMsi1 and mMsi2 overexpression constructs into HEK293T cells and subsequent probing with anti-mMsi2, plus anti-FLAG and anti-Vinculin controls. Two isoforms of mMsi2 are known to exist, namely mMsi2-S and mMsi2-L, and indeed, two bands were present at the correct molecular weights of 35 kDa and 37 kDa, respectively (Sakakibara et al., 2001) (Figure 3.1A). These double bands can be attributed to the presence of both mMsi2-S and mMsi2-L and not to cross-reactivity with mMsi1 (which is very similar in size to mMsi2-L at 39 kDa); while both isoforms are endogenously expressed by HEK293T cells, mMsi2-FLAG was detected solely in the mMsi2-FLAG-transfected cells through the presence of a third band (running slightly higher than endogenous protein due to an increase in molecular weight attributed to the FLAG tag), a band which was absent in mMsi1-FLAG-transfected cells (Figure 3.1A). Positive transfection of FLAG-tagged mMsi-1 and mMsi-2 constructs were verified through probing with anti-FLAG, producing single bands of equal intensity at 46 kDa and 42 kDa, respectively, and corresponding to the weight of the mMsi proteins plus overexpression construct additions. An anti-Vinculin loading control verified consistent protein concentrations between lanes. These results indicate that the mMsi2 antibody chosen is specific to mMsi2, and subsequent results thus solely reflect the expression of mMsi2.

Western blots comparing the key stages of neurogenesis (E11-E19) in the CD1 mouse cerebral cortex were then produced in order to confirm mMsi2’s presence during embryonic brain development. A representative Western blot is shown in Figure 3.1B. mMsi2 expression
Figure 3.1. mMsi2 is expressed in the mouse cerebral cortex throughout development. Representative Western blots revealing mMsi2 expression in the CD1 mouse cortex. (A) The mMsi2 antibody is specific to mMsi2 and does not cross-react with mMsi1. HEK293T cells were transfected with mMsi1-FLAG or mMsi2-FLAG overexpression constructs, and lysed to collect protein after 24h. Upon incubation with anti-mMsi2 antibody, bands at 35 kDa and 37 kDa are endogenously expressed mMsi2 in HEK293T cells (S and L isoforms), while the single band at 42 kDa (marked with an arrow) is FLAG-tagged mMsi2. Upon incubation with control anti-Flag antibody, single bands denote positive transfection with FLAG-tagged constructs, while an anti-Vinculin antibody serves as a loading control. (B) Cortical lysates from E11-E19, probed with an anti-mMsi2 antibody and an anti-Vinculin loading control.
was observed throughout all tested stages of neurogenesis, from E11 just prior to the initiation of neurogenesis, to E19 just prior to birth. Two mMsi2 bands were consistently seen at 35 kDa and 37 kDa throughout neurogenesis, corresponding to the molecular weights of mMsi2’s two known isoforms, mMsi2-S and mMsi2-L (Sakakibara et al., 2001). Darker bands were observed for both isoforms at E14 and E15, indicating an increase in protein during mid-neurogenesis. mMsi2 expression then slightly decreased by E19, following the conclusion of neurogenesis (Figure 3.1B). The Vinculin loading control remained approximately consistent throughout, indicating similar protein concentrations between lanes and allowing for such comparisons to be made.

**mMsi2 is expressed in NPCs and some neurons of the cerebral cortex**

The identity of mMsi2-positive cells was revealed through E15 primary culture co-stains of mMsi2 plus NPC markers Nestin and Sox2, and neuronal markers βIII-Tubulin and SatB2. In all co-stains, mMsi2 was consistently found to primarily localize within the cytoplasm with slight nuclear staining (Figure 3.2). Co-stains revealed that NPCs express mMsi2, as both the NPC markers Nestin and Sox2 were highly co-localized with mMsi2 (Figure 3.2A). Some mMsi2-positive cells expressed neuronal marker βIII-Tubulin; however, the neuronal marker SatB2 was rarely found to co-localize with mMsi2 (Figure 3.2B). Upon quantification, it was found that approximately 34% of mMsi2-positive cells in primary culture were βIII-Tubulin-positive neurons, while SatB2-positive neurons accounted for approximately 16% of the mMsi2-positive population.

mMsi2 co-stains with NPC marker Sox2 and neuronal marker βIII-Tubulin were repeated on E12 and E15 paraffin-embedded brain slices in order to observe mMsi2’s localization within the three major layers of embryonic cortex (VZ/SVZ, IZ, and CP). At E12, the majority of
Figure 3.2. mMsi2 is expressed in NPCs and some neurons of the cerebral cortex. Immunofluorescent labelling of cortical primary culture, revealing the cellular identity and intracellular localization of mMsi2-positive cells. Primary cultures extracted and plated at E12.5, and labelled at E15 with antibodies against mMsi2 (red), (A) NPC markers Nestin and Sox2 (green), and (B) neuronal markers βIII-Tubulin and SatB2 (green). Merges show co-expression of mMsi2 with both NPC markers, but only occasionally with βIII-Tubulin, and rarely with SatB2. Scale bars: 20 μm.
Figure 3.3. mMsi2 is expressed from the mouse cerebral cortex VZ to CP throughout development. Immunofluorescent labelling of cortical slices, revealing the cortical distribution of mMsi2+ cells throughout early (E12) and mid (E15) neurogenesis. E12 and E15 paraffin-embedded coronal slices labelled with antibodies against mMsi2 (red), (A) NPC marker Sox2 (green), and (B) neuronal marker βIII-Tubulin (green). Merges show mMsi2 expression within NPCs and neurons during early and mid neurogenesis. VZ: ventricular zone, CP: cortical plate. Scale bars: 20 μm.
cortical cells from the VZ to CP were NPCs and thus expressed Sox2, with only a thin layer at the CP expressing βIII-Tubulin. The large Sox2-positive layer at this developmental stage was also highly mMsi2-positive (Figure 3.3A); this co-localization was anticipated given the results of primary culture co-stains (Figure 3.2A). By E15, the Sox2-rich VZ layer had significantly diminished, and the βIII-Tubulin-rich CP layer accounted for roughly half of the cortical depth. This change was also coupled with a slight depletion of mMsi2 in the CP, in comparison to the VZ. However, similar to primary culture, some βIII-Tubulin-positive cells were additionally mMsi2-positive, and mMsi2 expression thus persisted in the CP (Figure 3.3B).

**In vitro mMsi2 knockdown increases NPCs, decreases neurons, and decreases intermediate progenitors in primary culture**

Following demonstration that mMsi2 possessed characteristics permitting its involvement in asymmetric NPC divisions, its expression was knocked down using shRNAs in order to study its role in these divisions. In order to observe the effect of mMsi2 loss on the ACD of NPCs, two shRNAs were selected which effectively knocked down mMsi2 expression. To select these shRNAs, four candidate shRNAs and one nonspecific scrambled control shRNA were co-transfected into HEK293T cells along with a Msi2-FLAG expression construct. Upon protein extraction and Western blotting with antibodies against mMsi2 and loading control DDX1, two of these shRNAs, shMsi2C and shMsi2D, visibly knocked down expression of mMsi2-FLAG in comparison to controls (Figure 3.4). This was visualized through decreased protein expression in comparison to untransfected and nonspecific shRNA controls, paired with consistent DDX1 expression. These two shRNAs were selected for primary culture transfections, and were renamed shMsi2-1 and shMsi2-2, respectively.
Figure 3.4. shMsi2C and shMsi2D successfully knock down mMsi2-FLAG in HEK293T cells. shRNA selection for in vitro and in vivo knockdown experiments. Western blot demonstrating shRNA knockdown in HEK293T cells co-transfected with mMsi2 shRNAs 2A-2D/nonspecific scrambled 29-mer control + mMsi2-FLAG and probed with specific antibodies against DDX1 (loading control) and FLAG. shRNAs 2C and 2D were selected due to their ability to knock down mMsi2-FLAG expression, and are hereafter referred to as shMsi2-1 and shMsi2-2, respectively.
To quantify knockdown of endogenous mMsi2, successfully transfected cells (visible through positive GFP staining) were assessed for marker positivity using antibodies against NPC, neuronal, and intermediate progenitor markers. \textit{In vitro}, knockdown with either shMsi2-1 or shMsi2-2 led to significant decreases in the intensity of mMsi2 expression (Figure 3.5A). The percentage of very highly mMsi2-positive (mMsi2++) cells in culture decreased from 36.124\% in controls to 12.477\% (p=0.031, n=3) and 10.320\% (p=0.040, n=3) in shMsi2-1 and shMsi2-2 knockdown conditions, respectively (Figure 3.5B). To ensure these effects were indeed due to an increase in NPCs and concomitant loss of neurons, and not due to cell death, GFP-positive condensed nuclei were quantified to assess the rate of cell death via apoptosis (Tone et al., 2007). Co-transfection with control shRNA + GFP produced 7.031\% condensed nuclei, in comparison to transfection with shMsi2-1 and shMsi2-2, which produced 5.960\% (N.S.; p=0.346, n=3) and 6.174\% (N.S.; p=0.257, n=3) condensed nuclei, respectively (Figure 3.5C). As neither of these changes in nuclear condensation were statistically significant, cell death was not considered a confounding factor of the observed mMsi2 knockdown.

The observed increase in NPCs was subsequently found to be accompanied by an increase in mMsi2-negative cells, from 12.083\% in controls to 24.850\% (N.S. trend; p=0.156, n=3) and 48.126\% (p=0.003, n=3) upon knockdown with shMsi2-1 and shMsi2-2, respectively (Figure 3.5B). However, the percentage of moderately mMsi2-positive cells (mMsi2+) did not significantly change between knockdown and control conditions: 51.793\% of shctrl/GFP-transfected cells were mMsi2+, a percentage which increased to 62.673\% mMsi2+ (N.S.; p=0.366, n=3) upon knockdown with shMsi2-1, but decreased to 41.554\% mMsi2+ (N.S.; p=0.116, n=3) following knockdown with shMsi2-2 (Figure 3.5B).
Figure 3.5. shMsi2-1 and shMsi2-2 successfully knock down cortical mMsi2 expression in primary culture. (A) Knockdown of mMsi2 in cortical primary culture. Cortical tissue excised at E12.5, transfected with shMsi2-1 or shMsi2-2 + nGFP, and labelled after 3d in vitro with antibodies against GFP (positive indicator of transfection) and mMsi2. (B) Quantification revealed a decrease in cells strongly expressing mMsi2 (mMsi2++), and an increase in cells lacking mMsi2 (mMsi2-). Data presented as mean + SEM. *p<0.05, **p<0.01 at t test, n=3. (C) Cell death (nuclear condensation) did not significantly differ between control and knockdown conditions. Data presented as mean + SEM, all N.S. at t test, n=3. Scale bar: 5 μm.
Following successful *in vitro* mMsi2 knockdown with nGFP + shRNAs, primary culture co-transfections were co-stained with GFP (as an indicator of successful transfection) and markers of cortical cell identity, namely Nestin, βIII-Tubulin, and intermediate progenitor marker Tbr2. Quantification of co-stain overlays revealed a phenotype characterized by an increased NPC population at the expense of both neurons and intermediate progenitors. GFP-positive and Nestin-expressing cells were seen to increase from an average of 59.27% in control-transfected cells to 77.20% (p=0.001, n=3) upon knockdown with shMsi2-1, and 71.08% (p=0.034, n=3) upon knockdown with shMsi2-2 (Figure 3.6A,D). mMsi2 knockdown also revealed decreases in the neuronal population, as control-transfected cultures contained an average of 38.08% GFP-positive/βIII-Tubulin-positive cells, compared to 19.68% (p=0.021, n=3) and 24.29% (p=0.012, n=3) upon knockdown with shMsi2-1 and shMsi2-2, respectively (Figure 3.6B,E). The small intermediate progenitor population again decreased upon mMsi2 knockdown, from 8.90% GFP-positive/Tbr2-positive cells upon control transfection to 6.65% (shMsi2-1; p=0.011, n=4) and 6.0% (shMsi2-2; N.S. trend; p=0.127, n=4) (Figure 3.6C,F).

While the sum of these percentages is slightly higher than 100%, some cells at this stage of development are in transition and thus may express multiple markers at the time of fixation and staining.

*In vivo* mMsi2 knockdown with shRNA + GFP increases the number of cells expressing GFP in the VZ/SVZ, and decreases the number of cells expressing GFP in the CP

In order to confirm that the knockdown phenotype seen *in vitro* was biologically relevant, shRNA knockdown was repeated *in vivo* at E13 using *in utero* electroporation, co-transfecting nGFP along with shMsi2-2 and shctrl. Analysis of GFP localization 3d post-electroporation
Figure 3.6. mMsi2 knockdown \textit{in vitro} leads to an increase in NPCs, as well as decreases in both neurons and intermediate progenitor cells. \textit{In vitro} knockdown revealing changes in the proportions of NPCs, neurons, and intermediate progenitors in cortical primary culture upon loss of mMsi2. Cortical tissue was excised at E12.5, transfected with verified shRNAs + nGFP, and immunofluorescently labelled at E15 with antibodies against GFP (positive indicator of transfection) plus (A) NPC marker Nestin, (B) neuronal marker βIII-Tubulin, and (C) intermediate progenitor marker Tbr2. An increase in (D) NPCs was observed following knockdown, accompanied by decreases in (E) neurons and (F) intermediate progenitors (N.S. trend in shMsi2-2, p=0.127) in culture. Data presented as mean + SEM. *p<0.05; **p<0.01 by t test, n=3 (with the exception of (F): n=4). Scale bars: 5 μm.
Figure 3.7. mMsi2 knockdown in vivo leads to an increase in cells in the VZ/SVZ and a decrease in cells in the IZ and CP. In vivo knockdown revealing changes in the ratio of shRNA-treated cells in the three major embryonic cortical layers. (A) Live E13 embryos were electroporated with shMsi2-2 + nGFP, surgically removed after 3d in vivo, coronally sectioned, and labelled with an anti-GFP antibody plus Hoechst nuclear stain. (B) Quantification revealed an increase in GFP-positive cells in the ventricular/subventricular zone (VZ/SVZ), accompanied by decreases in the intermediate zone (IZ) and cortical plate (CP). Data presented as mean + SEM. *p<0.05, **p<0.01, ***p<0.005 by t test, n=4. Scale bars: 100 μm.
showed significant changes in the distribution of cells expressing GFP between the cortical layers in knockdown and control brains, favouring cells of the VZ/SVZ at the expense of those in the IZ and CP. In control-electroporated brains, 33.341% of GFP-positive cells remained in the VZ/SVZ 3d post-electroporation, while 21.449% resided in the IZ and 45.210% migrated to the CP (Figure 3.7). With mMsi2 knockdown (using shMsi2-2), cortical migration diminished significantly, with 58.694% of GFP-positive cells remaining in the VZ/SVZ (p=0.008, n=4), 11.90% residing in the IZ (p=0.002, n=4), and 29.406% migrating to the CP (p=0.048, n=4) (Figure 3.7).

In vitro mMsi2 overexpression increases NPCs, decreases neurons, and decreases intermediate progenitors in primary culture

To further explore the effect of mMsi2 on NPC division, mMsi2 was overexpressed in primary culture via transfection with Msi2-FLAG. Surprisingly, E15 GFP co-stains with Nestin, βIII-Tubulin, and Tbr2 revealed a similar phenotype to that seen with in vitro knockdown, namely an increase in NPCs accompanied by a decrease in neurons and intermediate progenitors 3d post-transfection. Quantification of co-stain overlays revealed an increase in Nestin-positive/GFP-positive cells from 62.885% in GFP-transfected controls to 80.886% Nestin-positive/GFP-positive cells following transfection with mMsi2-FLAG (p=0.011, n=3) (Figure 3.8A,D). A decrease in neurons was also observed, from 37.266% βIII-Tubulin-positive/GFP-positive cells upon control transfection to 19.546% (p=0.013, n=3) upon overexpression (Figure 3.8B,E). Similar to in vitro knockdown, this decrease in neurons was also accompanied by a decrease in intermediate progenitors, from 5.628% Tbr2-positive/GFP-positive control-transfected cells to 2.755% (p=0.028, n=3) upon overexpression (Figure 3.8C,F).
Figure 3.8. mMsi2 overexpression in vitro leads to an increase in NPCs, as well as decreases in both neurons and intermediate progenitors. *In vitro* overexpression of mMsi2-FLAG in cortical primary culture, revealing changes in the proportion of NPCs, neurons, and intermediate progenitors in primary culture. Cortical tissue was excised at E12.5, transfected with a verified mMsi2-FLAG construct and/or nGFP, and immunofluorescently labelled at E15 with antibodies against GFP (a positive indicator of transfection) plus (A) NPC marker Nestin, (B) neuronal marker βIII-Tubulin, and (C) intermediate progenitor marker Tbr2. An increase (D) in the percentage of NPCs in culture was observed following overexpression, accompanied by decreases in (E) neurons and (F) intermediate progenitors. Data presented as mean ± SEM. *p<0.05 by t test, n=3. Scale bar: 5 μm.
CHAPTER FOUR: Discussion

The results presented here demonstrate that mMsi2 is present within the developing cortex and, furthermore, appears to regulate asymmetric NPC division. A temporal protein expression profile revealed that both mMsi2-S and mMsi2-L are present within the cortex between E11-E19, but are both enriched between E14-E15, a time frame corresponding with the midpoint of neurogenesis. Immunocytochemistry and immunohistochemistry revealed that the cells expressing mMsi2 are often NPCs, but some neurons do additionally express mMsi2. Upon in vitro knockdown of mMsi2, the ratio of cell types present in culture changed significantly, indicating that mMsi2 must be a crucial component of NPC division. A higher proportion of NPCs were present upon mMsi2 loss, accompanied by decreases in neuronal and intermediate progenitor populations. Knockdown in vivo also produced changes in the distribution of cells present, leading to an increase in cells residing within the VZ/SVZ and a decrease in cells within the IZ and CP. Finally, overexpression of mMsi2 was attempted in vitro. This produced a similar phenotype to that of in vitro knockdown, with increases in NPCs and corresponding decreases in neurons and intermediate progenitors visible under excess mMsi2, suggesting that the concentration of mMsi2 within a cell must be tightly regulated in order to facilitate correct NPC division. Together, these results indicate that mMsi2 is likely a crucial component of NPC division, and thus cortical development, since its absence and excess both lead to disruptions in neuronal formation.

Consistency with previous studies

The results reported here are consistent with mMsi2 acting as a component of cortical cell fate determination. Firstly, mMsi2 was found to be present within the cortex throughout all stages of neurogenesis (Figure 3.1B), and was furthermore highly expressed in the cytoplasm of
NPCs, as well as within a subset of neurons (Figure 3.2). While these findings alone are not specific evidence supporting a role in cell fate determination, such characteristics permit a potential role in asymmetric NPC division, as any protein involved in asymmetric localization within a dividing NPC must be present throughout ~E12-E15 when neurogenic ACDs are abundant (Figure 2.1).

The primarily cytoplasmic intracellular localization of mMsi2 seen here is also consistent with a potential role in translational repression, similar to that of dMsi (Nakamura et al., 1994) and the proposed role of mMsi1 (Battelli et al., 2006; Okano et al., 2002), as translation occurs in the cytoplasm (Alberts et al., 2002) and thus must also be repressed in the cytoplasm. The specific intracellular localization of mMsi2 had, until now, been poorly reported in scientific literature. In preliminary work characterizing mMsi2 as an RNA-binding protein, mMsi2 was reported to demonstrate exclusively cytoplasmic localization in the NG108 neural cell line (Sakakibara et al., 2001). However, a nuclear localization signal (NLS) is present within RRM1 of mMsi2 (Sakakibara et al., 2001), allowing this protein to enter the nucleus and leading to assumptions that mMsi2 might be localized in the nucleus, at least temporally. Our results, in highly relevant cortical primary cultures, show faint nuclear staining accompanied by strong cytoplasmic staining (Figure 3.2), similar to the localization seen in NG108 cells (Sakakibara et al., 2001). This indicates that while mMsi2 does possess an NLS, it does not reside in the nucleus for extended amounts of time, instead perhaps trafficking mRNA from the nucleus to the cytoplasm where it can be asymmetrically localized prior to NPC division. Nucleocytoplasmic shuttling experiments, using leptomycin B as a blocker of nuclear export protein exportin (Wolff et al., 1997) and, separately, ivermectin as a blocker of nuclear import proteins importin α and
importin β (Wagstaff et al., 2012), were attempted to further elucidate the potential trafficking activity of mMsi2; however, results were inconclusive.

The temporal patterns of mMsi2 expression seen here are also consistent with previous research. Our temporal expression profile (Figure 3.1A) follows that of the seminal paper reporting the presence of mMsi2 (Sakakibara et al., 2001), wherein cortical mmisi2 mRNA was observed throughout all time points of neurogenesis and into adulthood. However, the increased expression in mid-neurogenesis (E14-E15) compared to other time points is a novel observation not seen in the mRNA expression work of Sakakibara et al. (2001). Our Western blots show peak expression of mMsi2 at a time when a substantial NPC layer is present in the VZ/SVZ (Figure 3.3A), but numerous neurons have also been produced through asymmetric divisions and have migrated to the CP (Figure 3.3B). In contrast, by E19, the NPC layer at the VZ/SVZ has been depleted and asymmetric divisions have given way to symmetric divisions (Florio & Huttner, 2014). This peak in expression at E14-E15 therefore suggests that mMsi2 is present in the NPCs undergoing asymmetric division, but is also inherited by some neurons, a conclusion that is consistent with our in vitro mMsi2 expression results (Figure 3.2). Given the characteristics of other known RNA-binding proteins, it is unsurprising to find mMsi2 inherited by a subset of neurons and thus present at some degree throughout the later stages of neurogenesis. Other RNA-binding proteins known to participate in this process also demonstrate similar patterns of expression; the RNA-binding protein Staufen2 is one such example. This RNA-binding protein is expressed by NPCs during early neurogenesis, but is additionally inherited by neurons following asymmetric division. Furthermore, Staufen2 is believed to control the asymmetric localization of prox1 mRNA, leading to inheritance of prox1 by a single daughter cell (Vessey et al., 2012), an event similar to the proposed function of mMsi2.
mMsi2-positive neurons likely belong to a specific deep-layer neuronal population

The differential expression of mMsi2 among βIII-Tubulin-positive and SatB2-positive neuronal populations is an informative but also somewhat expected result. Sakakibara et al. (2001) also previously reported mMsi2 to be present in some but not all neurons, a finding which they did not expand upon. While βIII-Tubulin and SatB2 are both markers of post-mitotic neurons, they provide additional insight into cortical mMsi2 localization due to their differences in expression. During neurogenesis, development occurs inside-out, from the initial formation of deep cortical layers V-VI to the eventual formation of superficial upper layers (Desai & McConnell, 2000; McConnell & Kaznowski, 1991). The transcription factor SatB2 is only expressed within neurons of the upper cortical layers (cortical layers II-IV). Specifically, SatB2-positive neurons are found exclusively within a subpopulation of neurons within these upper layers, a category designated as UL1 (as opposed to the SatB2-negative UL2 category of upper-layer neurons, and deep layers) (Britanova et al., 2008). In contrast, the microtubule protein βIII-Tubulin is expressed by neurons regardless of their position within the cortex (Menezes & Luskin, 1994). The weak co-expression of mMsi2 with SatB2 (Figure 3.2) indicates that mMsi2 is found in neurons of UL2 and deep layers, and may therefore be found primarily in older or firstborn populations of neurons. Neurons also do not immediately begin to express SatB2 following terminal differentiation and mitotic exit; there is a >9h delay between mitotic exit and the initiation of SatB2 expression (Britanova et al., 2008). This may indicate that neuronal mMsi2 expression may be carried over from asymmetric localization within NPCs, but ceases concurrently with their exit from the mitotic cycle to signal neuronal maturation. However, our Western blotting results show that mMsi2 expression persists through to E19 (Figure 3.1B). This indicates that mMsi2 is likely retained in a subset of βIII-Tubulin-positive deep-layer and UL2
neurons past their mitotic exit, as the majority of mMsi2-containing NPCs are absent and most neurons have already exited mitosis by E19 in CD1 mice (Finlay & Darlington, 1995).

**mMsi2 promotes asymmetric NPC divisions of the developing cerebral cortex**

mMsi2 is not only present in the cells which undergo asymmetric divisions to rapidly populate the developing cortex, but its loss also significantly impacts the ratio of cells produced through NPC divisions. Upon mMsi2 loss *in vitro*, the NPC population significantly increased, while the neuronal and intermediate progenitor populations both decreased (Figure 3.6). This increase in NPCs and accompanying decrease in terminally differentiated neurons indicates a premature shift away from neuron-producing ACDs and toward symmetric cell division of NPCs (Florio & Huttner, 2014). This shift suggests that mMsi2 must therefore play a key role in the formation of neurons within the developing cortex, as the neuronal population is significantly diminished when cells are lacking mMsi2. Furthermore, since neurons are generated through asymmetric NPC division during neurogenesis, the process of asymmetric localization appears to be disrupted upon mMsi2 loss (Figure 4.1). mMsi2 must therefore be specifically acting as a component of cell fate determination via asymmetric localization.

A shift in the neurogenic timeline as seen here is not unique to mMsi2; it has also been seen with knockdown of other RNA-binding proteins. shRNA knockdown of Staufen2 and its binding partners Pumilio2 and DDX1 reduced asymmetric NPC division and initiated premature neurogenesis (Vessey et al., 2012), while knockdown of mMsi1 in a mouse embryonic carcinoma cell model of neurogenesis reduced neurogenesis in favour of neural progenitor cell renewal (Battelli et al., 2006). Our results showing that mMsi2 loss alters the timeline of
During neurogenesis, NPCs divide asymmetrically to renew the NPC population as well as to produce neurons. This asymmetric division is thought to be facilitated, in part, by mMsi2, as mMsi2 loss \textit{in vitro} (via shRNA knockdown) leads to an increase in NPCs and a corresponding decrease in neurons and intermediate progenitors. We propose that this phenotype is due to a failure of asymmetric cell division upon mMsi2 loss, wherein cellular contents are distributed equally among daughter cells and result in both daughter cells taking on an NPC identity.
symmetric vs. symmetric cell divisions to favour symmetric NPC divisions therefore
demonstrate a phenotype consistent with previous research.

**mMsi2 loss in utero affects the distribution of cells throughout the cortical layers**

A significant and potentially similar change in cellular distribution was also observed
following mMsi2 loss in utero, with a higher number of GFP-positive cells being retained in the
VZ/SVZ, and fewer GFP-positive cells migrating to the IZ and CP. Ongoing work is aimed at
determining the ratio of cell types present upon in vivo shRNA knockdown, but we hypothesize
that this change in distribution may be explained in one of two ways: 1) since cells in the
VZ/SVZ are likely NPCs, and cells in the CP are likely terminally differentiated neurons, these
results mimic the phenotype observed in vitro; or 2) an impaired ability for cortical migration has
trapped terminally differentiated cells within the VZ/SVZ, thus elevating the proportion of cells
residing in this cortical layer. The first of these scenarios is most likely, given the change in cell
type ratios seen with in vitro knockdown; however, both outcomes have been seen previously in
cortical development research. For instance, shRNA knockdown of FoxP2, a protein involved in
language development and neural plasticity, leads to a decrease in neurogenesis, visible in utero
by significantly higher proportions of cells expressing GFP in the VZ/SVZ and few cells in the
IZ or CP (Tsui et al., 2013). FoxP2 was ultimately found to regulate the formation of cortical
intermediate progenitors, thus promoting neurogenesis. Conversely, shRNA knockdown of
Staufen2 leads to mislocalization of newly formed neurons, a finding which was identified as
either an effect of impaired neuronal migration upon Staufen2 loss, or a byproduct of NPC loss,
since NPCs provide scaffolds upon which newly born neurons migrate to the CP (Vessey et al.,
2012). Future research in the Vessey lab aims to co-stain in utero-electroporated brain slices with
GFP plus antibodies specific to NPC, neuronal, and intermediate progenitor markers, in order to fully understand the changes in localization observed upon in utero electroporation.

**mMsi2 overexpression mimics the knockdown phenotype**

While mMsi2 knockdown both in vitro and in vivo increased NPCs at the expense of neurons and intermediate progenitors, mMsi2 overexpression in vitro also produced a very similar phenotype (Figure 3.8). Taken at face value, the high degree of similarity between these knockdown and overexpression results suggests an environment in which the concentration of mMsi2 must be tightly controlled in order to be effective in asymmetric NPC divisions, and thus mediate cortical development. One possibility requiring further investigation is the potential sequestration of mMsi2 upon overexpression, leaving the cell with insufficient mMsi2 to perform asymmetric localization and thus closely mimicking the mMsi2 knockdown phenotype.

It has been found that cell stress leads to compartmentalization of hnRNP-Q, another RNA-binding protein hypothesized by the Vessey lab to assist in cortical cell fate determination, within cytoplasmic stress granules (Quaresma et al., 2009). While the cells quantified here appeared healthy, it is conceivable that mMsi2 overexpression could be a stressful event for primary cultures, possibly prompting such sequestration. In an attempt to alleviate potential cell stress from overexpression transfections, a series of titrations were attempted with increasingly smaller additions of overexpression construct. However, reducing the concentration of mMsi2-FLAG in vitro did not lead to visible changes in cell health or cell type ratios compared to the results presented here.
Potential functional differences between mMsi2-S and mMsi2-L isoforms

One consideration not addressed in this research involves the individual contribution of each mMsi2 isoform. While our results show that mMsi2-S and mMsi2-L collectively play a key role in the generation of neurons, they do not isolate the specific contribution of each isoform. As the mMsi2 antibody we used recognizes both mMsi2-S and mMsi2-L, our IF experiments did not identify potential differences in localization or expression between the two isoforms. The shRNAs chosen for mMsi2 knockdown also 100% align with a sequence that is conserved between mMsi2-S and mMsi2-L (sequences compared via BLAST, NCBI); therefore, *in vitro* and *in vivo* shRNA knockdown indiscriminately abolished the effects of both mMsi2 isoforms on cell fate determination. It remains unclear from the literature the precise differences between mMsi2-S and mMsi2-L in terms of cortical expression and function. Recent cancer research has attempted to answer this question; for instance, Wuebben et al. (2012) reported that mMsi2-L alone may promote division of embryonic stem cells. However, simultaneous knockdown of both isoforms was unable to be rescued by treatment with a single isoform (Wuebben et al., 2012). It is therefore possible that differences in cortical expression and/or function do exist, or that activity and thus importance to neurogenesis may differ between the two isoforms (*i.e.* one isoform being in an active state, and one inactive). These potential differences between isoforms could be further investigated via the development of shRNAs specific to each isoform, and the completion of subsequent knockdown experiments to compare the phenotype(s) resulting from knockdown of mMsi2-S vs. mMsi2-L.
**mMsi1 and mMsi2 are unique within the embryonic cerebral cortex**

An additional consideration is the potential overlap in expression and function between the two mouse Msi orthologues, mMsi1 and mMsi2. Our research suggests a unique role for mMsi2 in neurogenesis, due to the differences between known mMsi1 expression/function (Kaneko et al., 2000; Sakakibara et al., 1996) and the mMsi2 results both reported here and previously (Sakakibara et al., 2001). We endeavored to isolate the contribution of mMsi2 by selecting antibodies and shRNAs highly specific to mMsi2 alone. The mMsi2 antibody used here was first tested through overexpression in HEK293T cells to ensure that it did not cross-react with mMsi1. An absence of an overexpression band in mMsi1-FLAG-transfected cells in Western blots was observed, in comparison to consistent anti-FLAG and anti-Vinculin loading controls (Figure 3.1A). This mMsi2 antibody specificity confirms that the Western blot expression profile (Figure 3.1B) and results from IF studies (Figures 3.2, 3.3, 3.5-3.8) presented here solely reflect the expression of mMsi2. Additionally, no significant sequence similarities were found between mMsi1 and the shRNAs chosen for *in vitro* and *in vivo* knockdown, indicating that the knockdown phenotypes reported here were entirely due to an absence of mMsi2.

In previous research, mMsi1 has been identified as an NPC marker due to its transient expression, which peaks at the onset of neurogenesis and steadily diminishes throughout development (Sakakibara et al., 1996). Here, both isoforms of mMsi2 reach peak expression during the midpoint of neurogenesis and continue to be expressed past its completion, and IF results show that mMsi2 is present in the cytoplasm of not only NPCs but also a subset of neurons. These different patterns of expression indicate that mMsi1 and mMsi2 must hold distinct functions in asymmetric cell division and/or brain development, since they are expressed...
at different times and in different cell types. This is also supported by the results of shRNA knockdown experiments in which mMsi1 was unable to compensate for the loss of mMsi2 in self-renewal of embryonic stem cells (Wuebben et al., 2012), again suggesting distinct functions for mMsi1 and mMsi2.

An additional experiment to further explore potential compensatory effects between mMsi1 and mMsi2 would involve simultaneous knockdown of both mMsi proteins, followed by a comparison of quantification results to those of mMsi1 and mMsi2 knockdown alone. However, we experienced difficulty optimizing our primary cultures for transfection with a single shRNA plus nGFP, and the added stress to the cells following transfection with two shRNAs plus nGFP may lead to negative effects on cell health which would abolish any mMsi-related differences in NPC division. To resolve these differences, an shRNA could be designed to target a highly conserved region between mMsi1 and mMsi2, which would knock down both mMsi1 and mMsi2 and allow for comparisons of total mMsi knockdown vs. knockdown of mMsi1 or mMsi2 alone.
CHAPTER FIVE: Future Directions

Identifying changes in cell type ratios and/or neuronal migration upon \textit{in vivo} mMsi2 loss

Moving forward, \textit{in utero} electroporation co-stains will be completed using NPC and neuronal markers to elucidate potential differences in either the ratio of NPCs:neurons present, or in defects of neuronal migration. E13 embryos, electroporated at a single lateral ventricle with nGFP plus shRNA and allowed to develop \textit{in vivo} for 3d, will be extracted, fixed, and stained with antibodies against NPC marker Sox2, neuronal marker βIII-Tubulin, or intermediate progenitor marker Tbr2 along with anti-GFP. GFP-positive cells in each cortical region will be counted and assessed for marker positivity or negativity. It is hypothesized that, similar to \textit{in vitro} knockdown experiments, a higher proportion of NPCs will be present in knockdown cultures than in controls, at the expense of both neurons and intermediate progenitors. This result would confirm a failure of asymmetric NPC division upon mMsi2 loss.

Identifying mMsi2 binding partners

Since mMsi2 is an RNA-binding protein, it will be imperative to identify the specific target RNAs of mMsi2. RNA-immunoprecipitation (RNA-IP) will be performed in order to pull down candidate RNAs which successfully bind with mMsi2, and interactions between these candidate RNAs and mMsi2 will be confirmed via gel-shift assays. Gel-shift assays separate out RNA-protein complexes due to the fact that they migrate through a polyacrylamide gel in a slower fashion than RNA or protein alone, and therefore serve as a qualitative indicator of RNA-binding. mMsi2 target RNAs from other tissues, and RNAs that have been previously implicated in neurogenesis in other organisms (as determined through \textit{in silico} searches) will be used as positive controls, alongside nonspecific binding partners as negative controls. It is predicted that
mMsi2 will participate in specific binding interactions with candidate(s) implicated in neurogenesis, due to the confirmed interaction of specific binding between dMsi and ttk69 mRNA in *Drosophila* (Nakamura et al., 1994).

**Exploring the translational repressor hypothesis**

Following the identification of mMsi2 target RNAs, mMsi2’s capacity for translational repression in the asymmetric division of NPCs will be identified. Previous research suggests that mMsi1 binds to the 3’UTR of a target mRNA, where it interacts with the target mRNA’s poly-A binding protein (PABP) at its C-terminal protein binding domain (Kawahara et al., 2008) (Figure 1.4) and can thus repress translation. This interaction would prevent PABP from forming the 80S ribosome translation initiation complex with eukaryotic initiation factor 4G, therefore repressing translation (Kawahara et al., 2008). Thus, mMsi1’s ability to repress translation is thought to hinge on the actions of its protein binding domain. It remains unclear how this binding is regulated, including whether specific factors turn this binding on or off, though a regulator must exist if mMsi proteins indeed function as transient translational repressors to create the differential daughter cells produced through asymmetric NPC division. Despite the structural similarities between the Msi proteins, mMsi1 is the only Msi protein known to contain such a protein binding domain (Figure 1.4). This significant structural difference raises questions regarding the yet unknown mechanism of action of mMsi2; while evidence suggests that multiple Msi proteins repress translation ((Battelli et al., 2006; Nakamura et al., 1994), this lack of a protein binding domain may prevent mMsi2 from acting as a translational repressor. However, dMsi is known to function as a translational repressor in the creation of the *Drosophila* external sensory organ despite its lack of a protein binding domain (Nakamura et al., 1994), indicating that more than one mechanism may fulfil the Msi proteins’ actions. If mMsi2 indeed
acts as a translational repressor similar to dMsi and mMsi1, it may share the same mechanism of action as dMsi, or a yet unknown mechanism, due to its lack of a protein binding domain.

In order to determine whether mMsi2 is capable of repressing translation in NPCs, as an absence of translation is hypothesized to be responsible for the observed changes in NPC behaviour following mMsi2 loss, candidates which successfully bind with mMsi2 will be studied for translational repression using dual-luciferase reporter assays. FLAG-tagged 5’ and 3’ UTRs of verified target RNAs will be cloned upstream and downstream, respectively, of the firefly luciferase gene. Also contained on this plasmid is the control renilla luciferase gene. HEK293T cells will then be co-transfected with this dual-luciferase construct, as well as recombinant FLAG-tagged mMsi2, and fluorescence of both firefly and renilla luciferase will be quantified. In the event that mMsi2 represses translation via interaction with either the 3’ or 5’ UTRs, firefly luciferase fluorescence will be reduced in comparison to renilla luciferase. Two independent control experiments will be utilized. The first will involve a firefly luciferase gene not flanked by the 3’ and 5’ regulatory elements and the second will involve flanking the firefly luciferase gene with 3’ and 5’ regulatory elements of a gene known to not be responsive to mMsi2 regulation. This future research will indicate whether mMsi2 does indeed repress translation, or whether its actions occur through another, yet unidentified, mechanism.
CONCLUSION

This research identifies the RNA-binding protein mMsi2 as a crucial component of NPC division which permits correct cortical development during early to mid neurogenesis through the promotion of asymmetric cell division. A temporal protein expression profile revealed mMsi2 to be expressed in the cortex throughout all stages of neurogenesis, with heightened expression during mid-neurogenesis, while immunocytochemistry indicated a primarily cytoplasmic pattern of localization within NPCs and some neurons; characteristics both consistent with a potential role in asymmetric localization within NPCs. Manipulation of mMsi2 in vitro and in vivo also produced a distinct phenotype, indicating that the presence of mMsi2 is critical in the regulation of neurogenesis. shRNA knockdown in cortical primary culture led to significant increases in NPCs and corresponding decreases in neurons and intermediate progenitors, suggesting a failure of asymmetric division upon mMsi2 loss. mMsi2 knockdown in utero produced similar results, with increased cells residing in the VZ/SVZ and decreased cells within both the IZ and CP, a finding which may again point to an absence of asymmetric division but may also be indicative of impaired cortical migration. Overexpression of mMsi2 in cortical primary culture produced a phenotype of increased NPCs at the expense of neurons and intermediate progenitors, providing evidence that mMsi2 must be tightly controlled within a cell in order to permit correct cortical development. These results will be supplemented with future research investigating mMsi2’s target RNAs within the cortex, as well as its ability to transiently repress translation during asymmetric NPC division, in order to further understand the role played by mMsi2 in NPC division and cortical development.
REFERENCES


