INVESTIGATING THE ROLE OF RNA-BINDING MOTIF PROTEIN 8A (RBM8A, ALSO KNOWN AS Y14) IN SCHIZOPHRENIA

A Thesis in

Genetics

by

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ABSTRACT

Schizophrenia is a severe mental illness which affects approximately one percent of the world’s population. While the exact cause remains unknown, we do know that it results from a combination of both genetic and environmental factors. With advancements in DNA sequencing technology, several individual genes in addition to larger chromosomal regions have been identified as schizophrenia-associated. Despite the identification of these candidate genes, the complexity of the disease has prevented the elucidation of a clear molecular mechanism. Identifying the risk factors is only the first step towards building a stronger understanding of the disease model. Given the polygenic nature of schizophrenia, it is also necessary to continue the search for additional potential risk genes.

This thesis will focus on the RNA-binding motif protein 8A gene (RBM8A), commonly referred to as Y14. Both names appear in the literature, but for simplicity I will solely refer to it as Y14. Although Y14 has not yet been linked to schizophrenia, our group is working to suggest that this gene is an important factor in helping to understand the disease. For this purpose, we looked at several ways in which we could manipulate the gene. First, we were able to achieve effective Y14 overexpression *in vitro*, which led to *in vivo* studies indicating that Y14 can regulate hippocampal neural development and anxiety-like behaviors in mice. Additionally, we designed a Y14 conditional knockout mouse which recently resulted in the production of eight male chimeras. Overall, the techniques employed to investigate the potential role of Y14 have provided exciting new ideas and will serve as a platform for the future study of schizophrenia and other neurological disorders.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Antibiotic-Antimycotic</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variation</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DISC1</td>
<td>Disrupted-in-Schizophrenia 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNBP1</td>
<td>Dysbindin-1</td>
</tr>
<tr>
<td>EJC</td>
<td>Exon-junction complex</td>
</tr>
<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense-mediated decay</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PPI</td>
<td>Pre-pulse inhibition</td>
</tr>
<tr>
<td>PTC</td>
<td>Premature termination codon</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rbm8a</td>
<td>RNA-binding motif 8a</td>
</tr>
<tr>
<td>RIP</td>
<td>RNA-binding protein immunoprecipitation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TAR</td>
<td>Thrombocytopenia with absent radii</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>Upf</td>
<td>Up-frameshift proteins</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus G</td>
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Chapter 1

Introduction.

“One of the most elusive diseases known to man and unknown to medicine.” – James Joyce

Schizophrenia is a severe brain disorder which affects approximately one percent of the world’s population, contributing to about 2.2 million Americans. It has been described as the most debilitating form of mental illness and those who suffer from this disease are susceptible to a wide range of symptoms and cognitive deficits. The etiology remains unknown, although concordance rates have provided some insight. Monozygotic twins show a concordance value of 48 percent, indicating that schizophrenia must arise from some combination of both genetics and environmental factors (Gottesman & Wolfram, 1991). This led to the formation of several hypotheses, including the neurodevelopmental model and the neurodegenerative model.

Schizophrenia is truly an all-encompassing disease in that it can affect a person’s thought process, dictate their actions, and manipulate their general outlook on life. It can affect both men and women, although it is more commonly observed in men. It can also affect people from all age groups and is classified as either “Child-Onset”, meaning signs of schizophrenia emerge in childhood or adolescence, or “Adult-Onset”, when a person develops symptoms later in life and shows no indication of the disease up until that point. Often psychiatrists face difficulties in diagnosing young children, but it is the most frequent psychotic disorder occurring in adolescents and adults (Starling & Feijo, 2012). The diagnostic criterion for schizophrenia has been well defined and applies to both onset types, but any individual may display a different combination of symptoms with varying degrees of severity.

The symptoms can be divided into three main categories: positive, negative, and cognitive. The positive symptoms are the classic traits which tend to be perceived as stereotypical schizophrenic behavior- such as hallucinations, delusions, and disorganized
thinking. These hallmark symptoms make it difficult for people with schizophrenia to distinguish reality from imagination. Negative symptoms refer to the lack of normal social skills and emotions, which make it difficult to function in society and establish a career, thus preventing an affected individual from becoming self-sufficient. Lastly, the cognitive category refers to deficits in memory, focus, and learning (Starling & Feijo, 2012).

Despite being well characterized, the question researchers are concerned with remains unanswered. What is causing schizophrenia? The neurodevelopmental hypothesis suggests that a brain malformation developed early on in life can eventually lead to Adult-Onset schizophrenia. This coincides with the etiology of autism and attention-deficit hyperactivity disorder (ADHD). Alternatively, the neurodegenerative hypothesis suggests that the brain developed normally but prematurely deteriorates. While histopathology does not support this hypothesis, it correlates with results of clinical and biochemical tests (Gupta & Kulhara, 2010). While researchers agree that schizophrenia is linked to a developmental defect in the brain, it leads us to the next question- which genes are responsible?

**The complex genetics of schizophrenia.**

Recent advancements in DNA sequencing technology have allowed researchers to search for a genetic explanation for schizophrenia. Given the complexity of the disease and the variation among patients, it can be deduced that schizophrenia operates on a multifactorial level. Initial studies focused on specific genes and led to the identification of *Dysbindin-1* (DTNBP1) and *Neuregulin 1* (NRG1) as the main associating factors (Harrison & Owen, 2003), while also indicating the implication of several other genes as well, such as *Disrupted-in-Schizophrenia 1* (DISC1). In addition to individual genes, researchers have also focused on the molecular study of copy number variations (CNVs). Interesting results were discovered from a genome-wide association study (GWAS) for CNVs published in a 2008 issue of Nature. The study
was conducted on a large population to identify *de novo* CNVs before testing for association with schizophrenia. They identified three microdeletions at chromosome regions 1q21.1, 15q11.2 and 15q13.3 to be schizophrenia-associated (Stefansson et al., 2008).

**Y14 is a key factor in the Exon Junction Complex (EJC).**

Y14 is located within the 1q21.1 region, which was previously identified in the GWAS study as a schizophrenia-associated deletion. While this provides some basis for a potential implication in schizophrenia, understanding the function of Y14 reveals additional evidence for connecting the gene to the disorder. Y14 is one of the core proteins constituting the EJC, which is the machinery employed by nonsense-mediated decay (NMD). NMD is a specialized RNA surveillance mechanism serving as an important regulator of eukaryotic gene expression. It functions to identify and degrade mRNA transcripts possessing a premature termination codon (PTC) to prevent the expression of aberrant or truncated proteins. The key to NMD is to recognize how a PTC is differentiated from a normal stop codon, and this is where Y14 and the EJC come into play.

Eukaryotic gene expression is regulated at all levels. The process of pre-mRNA splicing has specific regulatory mechanisms working to ensure that the proper formation of mature mRNA leads to the translation of the correct protein. One of these “quality control mechanisms” involves depositing an EJC approximately 20-24 nucleotides upstream of exon-exon junctions. The EJC is comprised of several core proteins including Y14, Magoh, elf4AIII and Barentsz/MLN51. Following traditional mRNA processing, the EJCs will remain on the mature mRNA as it is exported from the nucleus to the cytoplasm.

Once in the cytoplasm, translation can begin as normal with the recruitment of a ribosome to the 5’ end of mRNA. NMD is coupled with the pioneer round of translation, specifically within the termination step, in attempt to immediately distinguish PTCs from normal
stop codons. It works by exploiting the location of a stop codon, which would normally be found within the final exon. Thus, there would be no additional exon-exon junctions downstream of the stop codon, meaning no additional EJCs left on the mRNA. Alternatively, if an EJC still remains downstream of the stop codon, it marks the stop codon as premature and signals for NMD to occur (Hwang et al., 2012).

The EJC proteins serve as a binding platform for NMD-associated proteins, specifically the Up-frameshift proteins (Upf) 2 and 3b. When a ribosome encounters a PTC, the release factors eRF1 and eRF3 will stop translation and recruit Upf1 and SMG1 to the stalled ribosome. Together, they form a SURF complex which can then bridge the ribosome to the downstream EJC. The SURF complex then interacts with Upf 2 and 3b. This interaction results in the phosphorylation of Upf1 by SMG1, marking the aberrant mRNA transcript for degradation (Le Hir et al., 2008). Without Y14 and the other core EJC proteins, this process would be unable to occur, eliminating NMD as a secure form of regulation.

Implications of EJC factors in brain function.

Interestingly, several other EJC and NMD-associated factors have been implicated in neurodevelopment and/or mental illness. Magoh, one of the core EJC components, has been linked to neurodevelopment through its impact on neural stem cell division regulation. Researchers generated a Magoh haploinsufficient mouse line which displayed a specific phenotype. Not only was brain volume significantly reduced, but the brains displayed structural disorganization and fewer neurons, indicating that Magoh deficiency results in microcephaly. Microcephaly is a well-defined neurodevelopmental disorder marked by reduced brain size and cognitive deficits (Silver et al., 2010).

The translation initiation factor eIF4AIII, which functions as a DEAD-box RNA helicase, is another of the core EJC proteins (Chan et al., 2004). After its identification in the EJC, a novel
role emerged for eIF4AIII as a regulator of neuronal function and synaptic strength (Giorgi et al., 2007). This finding provides further evidence for linking the EJC to neurodevelopment.

The EJC-associated protein Upf3b has been repeatedly linked to mental illness. Large-scale sequencing of the X chromosomes from families with a history of X-linked mental retardation led to the identification of a mutated form of Upf3b (Tarpey et al., 2007). Another group further elucidated the function of Upf3b, noting its prevalence in dendritic spines and confirming its association with mental retardation and autism (Laumonnier et al., 2010). Perhaps most strikingly, Upf3b was recently directly linked to schizophrenia by a group who performed a study on a set of brothers, one with Child-Onset schizophrenia and the other with autism. Both brothers were also diagnosed with ADHD, yet the mother was unaffected by any of these disorders. Their work provided convincing evidence showing that autism spectrum disorders and schizophrenia were attributed to a mutation in Upf3b (Addington et al., 2011). The involvement of other EJC and NMD associated proteins in mental illness and neurodevelopment merits the consideration that Y14 may be involved in these types of disorders, and a potential connection to schizophrenia should be further addressed.

**Y14 also plays a role in Thrombocytopenia-Absent Radius (TAR) syndrome.**

TAR syndrome is marked by major physical abnormalities and a low platelet count. Recently, researchers at the University of Cambridge uncovered the cause of TAR syndrome. It was known that a deletion in the 1q21.1 region (the same CNV found to be associated with schizophrenia) is evident in the majority of cases, but researchers were still searching for the additional causative factor. To investigate this, they genotyped all affected individuals and were able to identify two SNPs which were prevalent in the sample population. Additionally, further sequencing of the Y14 exons revealed a mutation giving rise to a null allele. With this, they were able to conclude that TAR syndrome arises from the combination of inheriting a null allele and
one of the SNPs in Y14. The null allele can be a result of a frameshift mutation, deletion, or PTC within the Y14 exons (Albers et al., 2012). This finding verifies the importance in the full functionality of Y14 and its potential to contribute to disease. Of additional interest to our study, approximately seven percent of TAR syndrome cases are associated with mental retardation (Skórka et al., 2005). While this is a relatively low percentage, it is consistent with our idea that Y14 has the potential to contribute to a mental disorder. Now that I have provided the reasoning behind our project and why we believe it warrants further investigation, I will discuss the ways in which we manipulated Y14 in hopes to elucidate a clear role for this gene and its associated protein in schizophrenia.
Chapter 2

Overexpression Construct.

Introduction.

With the constant progression of molecular cloning strategies, there are multitudes of ways for a geneticist to manipulate their gene of interest. Generally, the goal is to reveal the function of a gene through the establishment of an observable mutant phenotype. Perhaps the most intuitive way to gain insight to gene function is to observe what happens in its absence. This is referred to as a “loss-of-function” application and can be successful in showing mutant phenotypes following a knockdown or knockout technique. Alternatively, there is insight to be gained by observing what happens when the gene is expressed in excess, rather than a deficiency. The common theme of these molecular cloning techniques focuses on gene dosage. By definition, gene dosage refers to “the number of copies of a given gene present in a cell of an organism” (Gene Dosage, 2013). When a gene is overexpressed, it correlates to an excess of the gene product.

This chapter will focus on one of my sub-projects, specifically the analysis of Y14 overexpression. This investigation was a joint effort among our lab members and collaborators. My primary research goal was to create a stable cell line and verify Y14 overexpression with the Western blot technique. In turn, this molecular preparation led to investigating the effects of Y14 overexpression in vivo. Behavioral and neurodevelopmental analysis in a mouse model was documented by my colleagues and will provide relevance to the topic of linking Y14 to schizophrenia.
DNA construct.

The creation of the Y14 overexpression construct was completed by Dr. Yingwei Mao. Human Y14 cDNA was provided by Dr. Wilkinson (UCSD), which was then PCR amplified and cloned into the lentiviral vector pLV-3FLAG7HA-T2A-GFP provided by Dr. Wenyuan Wang (MIT).

Figure 2-1. Schematic representation of lentiviral Y14 expressing construct.

Viral vectors as a mode of gene delivery.

To express our plasmid in vitro, we needed to deliver the Y14 overexpression construct (Figure 2-1) to the cell. A common molecular technique, referred to as transduction, is to infect living cell cultures with viruses to aid in delivering target DNA to the original genome of the cell. Several options exist for such delivery, and each option has a specific set of criterion and resulting outcomes. The term “viral vectors” refers to the collection of options for gene delivery. As mentioned previously, there are several factors to be considered before choosing the appropriate viral vector.

Two of the main factors that need to be considered when choosing a delivery method are the target cell type and the desired expression pattern. For this specific experiment, 293T cells were used. This cell line is a variant of the Human Embryonic Kidney (HEK) cell line which has been modified for increased transfection efficiency. Furthermore, 293T cells are stable and relatively easy to grow. For the purpose of this experiment, the desired outcome was to achieve integration of the Y14 overexpression plasmid into the genome of the host 293T cell. Additionally, we wanted the ability to transfect both dividing and non-dividing cells. These
criteria called for a lentiviral vector. A retroviral vector is another viable option for genome integration and long-term expression, but unlike a lentivirus, retroviruses can only infect dividing cells (Varmus et al., 1977). Once the appropriate vector is chosen, the next step is to perform a transfection for virus production.

**Virus Production: Materials and Methods.**

Polyethylenimine (PEI) is a commonly used, commercially available reagent for DNA delivery. It works by binding to condensed plasmid DNA so that the resulting PEI-DNA complex can be delivered to the nucleus while the DNA remains protected. The lentiviral vector contains GFP, allowing for visualization of transfection efficiency. The following section describes the technical procedure of PEI transfection for virus production.

**PEI transfection.**

293T cells were grown in uncoated 10cm culture dishes in regular cell culture medium (DMEM supplemented with 10% FBS and 1% AA). For each construct from which virus production is desired, the protocol requires six dishes of cells grown to approximately 70% confluency. Table 2-1 describes the reagents required for PEI transfection. Prior to beginning this experiment, I performed several maxi preps to yield abundant quantities of the required DNA. As mentioned before, DMEM and PEI are commercially available.
Table 2-1. Transfection reagents. Summary of each reagent and its significance. The required amount was established in the “PEI Transfection for Virus Production” protocol used in our lab. DNA concentration was read with a photospectrometer and the calculated amount indicates the volume required for 6 plates (allowing for excess).

Two samples were prepared, each containing the full list of components from the Reagent column of Table 2-1. In a 15mL Falcon tube, the reagents were combined and incubated at room temperature for 5 minutes. The difference between the two samples was in the plasmid DNA. One sample contained Y14 overexpression plasmid (LV-Y14) and the other contained a control plasmid (LV-linker). Following the incubation, the total volume in each sample tube was divided equally into six parts. The transfection mixture was then added drop-wise to the 10cm dishes containing 293T cells (in 10mL of regular culture media). Equal distribution of the mixture among the plate was obtained by gently shifting the plates. The resulting dishes (6 of which were transfected with LV-Y14 and 6 transfected with LV-linker) were placed in the cell culture incubator set to 37°C and 5% CO₂. Following a 5 hour incubation, the medium was removed and replaced with 5mL of fresh regular culture media to each dish. The cells were placed back in the incubator. Transfection efficiency was monitored periodically by checking for GFP, and the dishes were incubated for a total of 48 hours before collecting the viral supernatant.
Precipitation of constructed virus.

The supernatant from all dishes for each sample was collected and pooled in a 50mL conical tube. This resulted in 30mL of viral supernatant for both samples, LV-Y14 and LV-linker. The supernatant was centrifuged at 1000xG for 5 minutes, and then filtered using a .45µm filter and a 30mL syringe into a clean centrifuge tube. Each tube was covered with parafilm before being balanced and loaded into the rotors of the Optima L-90K Ultracentrifuge. Samples were centrifuged at 25,000rpm for 90 minutes at 4°C. Following centrifugation, the viral medium was discarded and the pellet was resuspended in 100µL of PBS. The lentiviruses were then incubated in the cold room (4°C) with steady shaking overnight and subsequently aliquoted for experimental use.

Western Blot: Materials and Methods

The first application of the lentivirus was to infect 293T cells and confirm that the construct had integrated into the genome. Infection was achieved by adding the lentivirus in combination with a 1:1000 dilution of protamine sulfate directly to 293T cells in regular culture medium. Aside from GFP visualization, another method for checking the efficiency is to assess Y14 expression with a Western blot. Given that lentiviral delivery results in genome integration, the cells should be able to be expanded after the initial infection, resulting in a stable overexpression line. The following section summarizes the technique and results of the Western blot.

Collecting lysate.

One 10cm dish of each 293T-Y14 and 293T control was used for lysate collection. The cells were washed twice with PBS and placed on ice. Cell lysis buffer was supplemented with protease inhibitors (see Appendix for complete list) and 1mL of supplemented buffer was added to each dish. The cells were incubated on ice for 10 minutes before using a cell scraper and
transferring the lysate to a 1.5mL Eppendorf tube. The tubes were then incubated for an additional 10 minutes on ice, with vortexing every 2 minutes. They were then centrifuged at 14,000rpm for 10 minutes at 4°C. The supernatant was transferred to a clean 1.5mL Eppendorf tube and the concentration was read with a photospectrometer.

_Gel Electrophoresis and Transfer._

Samples were prepared by adding the appropriate amount of 6X protein loading dye to the lysate and incubating on the 100°C heating block for 5 minutes. They were then loaded onto a 12% polyacrylamide gel next to Precision Plus Protein™ Kaleidoscope Standards (Bio-Rad). The gel was run at 200V for 1.5 hours. Following electrophoresis, the gel was equilibrated in transfer buffer for 30 minutes with gentle shaking. The PVDF membrane was briefly treated with methanol and then equilibrated in transfer buffer as well. The transfer was performed at low voltage (40V) overnight in the cold room (4°C).

_Immunoblotting._

The following day, the blot was allowed to dry and then briefly treated with methanol (20 seconds). The blocking step was performed with 5% milk for 1 hour at room temperature. The blot was incubated with primary antibody, Rabbit anti-Y14 (Millipore) diluted 1:1000 in 5% milk, overnight in the cold room (4°C). The following day, the blot was washed three times with TBST for 10 minutes each, followed by incubation with secondary antibody, Anti-Rabbit-HRP diluted 1:1000 in milk, for 1 hour at room temperature. Washes were performed as previously described followed by ECL detection. To verify the Rabbit antibody, I also repeated this procedure with Mouse anti-Y14 (Santa Cruz) diluted 1:500 in 5% milk. Figure 2-2 shows how both antibodies clearly indicate Y14 overexpression in the 293T-Y14 cell lysate as compared to the control. I also probed with β-Actin, as shown in Figure 2-3, which serves as an additional control to show that the amount of protein used in the Western was consistent among samples.
This also validates the observation of Y14 overexpression by eliminating loading discrepancy as a possible confound.

**Western Blot: Results**

*Figure 2-2. Y14 overexpression confirmation.* Both samples show the expected band for Y14 at 20kD. The overexpression line shows a specific band at approximately 37kD, which represents the HA-tag of the overexpression construct. **A)** Mouse-anti-Y14 antibody  **B)** Rabbit-anti-Y14 antibody

*Figure 2-3. β-Actin expression in control and overexpression lysate.* The band at 43kD correlates to the expected molecular weight of β-Actin. The band intensity for each sample is relatively equal, indicating equal protein lysates to verify the previous blot showing Y14 overexpression.
Discussion.

The goal of this work was to investigate a potential connection between Y14 and schizophrenia through the overexpression of Y14. More specifically, our lab was interested in the \textit{in vivo} effects of Y14 overexpression on behavior and neurodevelopment. The following section was adopted from a manuscript that our lab recently submitted on the ability of Y14 to regulate hippocampal neural development and anxiety behaviors, which is useful for putting the molecular work that I described into context.

Adult mice were stereotaxically injected with the Y14 overexpression lentivirus into the dentate gyrus (DG) of the hippocampus. Injections were performed by Dr. Yingwei Mao and Dr. Amal Alachkar using the following coordinates: +2.06 mm antero-posterior, ±1.4 mm lateral from the bregma, and 2.2 mm ventral to skull from bregma. The final volume of injection was 2µL per site. The mice were then given three weeks to recover before their use in any tests (Alachkar et al., 2012).

Following their recovery, the mice were subjected to a variety of behavioral tests performed by Dr. Alachkar including open field, forced swimming, and social interaction. Interestingly, Y14 overexpression mice showed abnormal behaviors for each behavioral test in comparison to the control mice. Collectively, two behavioral phenotypes were observed in the Y14 overexpression mice. One of these phenotypes was an increase in anxiety-related behavior. For example, in the social interaction test where the “test mouse” (with Y14 overexpression in the DG) was given the option of interacting with a “stranger mouse” or not, the test mouse spent less time interacting with the stranger mouse and preferred isolation. This could indicate a potential social deficit, reminiscent of the negative symptoms of schizophrenia. Alternatively, other behavioral tests such as the forced swim test indicated a manic phenotype, rather than depressive, coinciding with the positive symptoms of our disease model.
In addition to observing the behavioral effects of lentiviral injection, Alachkar et al. also looked at electrophysiology and the effect of overexpression on neurogenesis. It was found that Y14 overexpression results in increased adult neurogenesis, which the authors correlated to the observance of a manic phenotype deduced as the suppression of depressive behaviors (Alachkar et al., 2012). This was the first type of study where Y14 was closely examined as a potential link to cognitive deficits and abnormal neurodevelopment. Ideally, the molecular cloning strategy executed in my work will lead to more in-depth investigations of a role for Y14 in schizophrenia.
Chapter 3

Conditional knockout construct.

Introduction.

This chapter will discuss the process of creating a conditional Y14 knockout mouse in attempt to provide in vivo insight to the function of our gene of interest. Our goal was to design a mouse model where Y14 could be inactivated in specific cells or tissues, which is a unique benefit of using conditional gene targeting. In a traditional germline knockout, the gene is inactivated in every cell. Alternatively, a conditional strategy works by using a Cre/LoxP system to maintain cell type-specific gene expression (Freidel et al., 2011).

Cre is a DNA recombinase which has been extremely well studied and has even been referred to as “the universal reagent to genome tailoring” (Nagy, 2000). No additional factors are necessary, which make it the most widely used and trusted form of gene targeting. Cre will catalyze recombination between two of its highly specific recognition sequences, called loxP sites. This allows any gene of interest to be excised from the genome when in the presence of Cre, as long as it is “floxed” - a term used to describe being flanked by two loxP sites. It should be noted that depending on the orientation of the loxP sites on the DNA molecule, the recombinase system can yield different outcomes. When loxP sites are in the same orientation, Cre will delete the floxed sequence. Conversely, when the sites are in opposite directions Cre will invert the floxed sequence. For our study, we are interested in observing the effects of Y14 inactivation so identical loxP site orientation had to be incorporated into our cloning strategy.

To make this a “conditional” knockout, two strains of mice are necessary. In one, the gene of interest will be floxed. In the other, Cre will be tied to a cell type-specific promoter. Crossing the Cre line with the loxP line will then provide the Cre/loxP recombination system, where Cre can excise the floxed sequence only in the cell types where Cre expression is turned
on (Nagy, 2000). Cre lines are commercially available - for example, Jackson laboratory has an extensive list of Cre strains where recombinase expression is limited to certain tissues or cell types. The next section will describe the strategy we used to create a conditional knockout construct.

**Designing the targeting vector.**

The first step in preparing a conditional knockout (cKO) is to design the targeting vector. This work was done by Dr. Mao, and I will briefly describe the gene targeting strategy he designed to knock out Y14 based on the sequence of the C57/B6 mouse model. To be able to devise this sort of strategy, it is necessary to obtained a detailed map of Y14 including all of the restriction enzyme sites and the structure of the gene, as well as to simultaneously develop a strategy for screening the clones. The goal was to strategically introduce loxP sites so that we can selectively inactivate Y14 in specific cells with the Cre recombinase system. As shown in Figure 3-1, Y14 consists of 6 exons although the final exon is largely non-coding.

![Figure 3-1. Y14 gene summary.](image)

Red blocks represent the exons of Y14.

LoxP sites were inserted in the same direction so that the floxing of exons 2 through 4 will lead to their excision. As a result, the loss of these critical exons will lead to inactivation of the gene. As a positive selection marker, a neomycin-resistance cassette (PGK-neo) was inserted downstream of Exon 4 inside the loxP sites. PGK-neo is flanked by separate recognition sites for a different recombinase system for their specific removal, referred to as the FLP/FRT recombination system. Inserting PGK-neo inside of the loxP sites is a common form of positive
selection, as resistance to the antibiotic neomycin allows us to identify which cells successfully underwent homologous recombination to contain the target vector. This is a vital step leading to the screening of ES cells, which I will discuss in greater detail in a later section. The final targeting vector is shown in Figure 3-2.

![Figure 3-2. Y14 Targeting vector.](image)

Verifying the targeting vector.

Once the construct was cloned into the vector, it was necessary to do some preliminary tests before continuing. First, we needed to establish a screening protocol for effective primers. We adopted a protocol from The KCC Transgenic and Gene Targeting Facility at Thomas Jefferson University for quantifying transgene copy number. It should be noted that for the cKO project, we are not concerned with testing copy number, as there will only be two copies of the endogenous gene to knock out. However, this protocol is useful for our experiment in that it can indicate which primers are most effective at detecting the Y14 cKO construct. Based on the information that the DNA content of a haploid mouse is $3 \times 10^6$ kb, calculations were performed to determine how much of the Y14 cKO DNA needed to be diluted into control DNA to result in a dilution series of known copy number. For this experiment, the control DNA was wild-type genomic mouse DNA.
Preparation of genomic DNA.

Tails were snipped from wild-type C57/B6 mice and transferred to a 1.5mL Eppendorf tube. I added 500µL of Tail Lysis Buffer (100mM Tris.Cl pH8.0, 5mM EDTA, 0.2% SDS, 200mM NaCl) and 10µL of 20mg/mL Proteinase K to each tube and samples were incubated overnight in the 37°C shaker. The following day, the tubes were centrifuged at 13,000rpm for 10 minutes to pellet any undigested hair. The supernatant was transferred to a clean 1.5mL Eppendorf tube and 500µL of isopropanol was added. Tubes were inverted continually until the appearance of white strands signified the precipitation of DNA. Tubes were then centrifuged at 13,000rpm for 10 minutes at 4°C. The supernatant was discarded and the DNA was washed with 1mL of 70% ethanol. Residual ethanol was removed and the DNA was dissolved in 100µL of dH₂O. The genomic DNA was now ready to be used as the control for the quantification of Y14 cKO copy number.

Copy Number Quantification

First, I created a dilution series of our cKO construct in WT genomic DNA ranging from 0.5 to 30 copies. This dilution series was then used in PCR to detect the Y14 cKO construct with primers designed by Dr. Mao. Several controls were used including a positive control (5ng of cKO construct DNA) and a negative control (5ng of WT genomic DNA). An additional negative control using dH₂O is not shown but is also necessary. The reaction mixture was prepared as follows: 4µL 5X Phusion Buffer, 0.4µL dNTPs, 0.2µL Phusion DNA Polymerase, 0.5µL forward primer, 0.5µL reverse primer, 13.4µL dH₂O, and 2µL of the specific DNA. The samples were then run in a thermocycler on a touchdown program with annealing temperatures ranging from 65°C to 57°C. For analysis, PCR samples were run on a 2% agarose gel at 110V for 20 minutes next to a 10KB DNA ladder. Figure 3-3 shows that the primers successfully PCR out the construct at the
level of a single copy number, with the amount gradually increasing in the dilution series as expected.

![PCR detection of Y14 cKO construct](image)

**Figure 3-3. PCR detection of Y14 cKO construct.** A single copy refers to the Y14 cKO construct diluted into WT genomic DNA. Each well contains 10µL of Phusion PCR samples. The controls include 5ng of Y14 cKO DNA without any genomic DNA, and 5ng of WT genomic DNA without any cKO construct.

**A collaboration for Embryonic Stem (ES) cell targeting.**

With the genotyping technique in place, the next step was to send the Y14 cKO construct out for ES cell targeting. We collaborated on this project with Dr. Lin Gan at the University of Rochester Medical Center. While we were able to design and confirm the constructs on site, ES cell targeting is a specialized technique and was performed at their facility. The aim is to introduce our Y14 cKO target vector to mouse ES cells by electroporation such that homologous recombination can take place. For this process, their facility requires at least 100µg of DNA. Invitrogen PureLink® HiPure Plasmid Filter Purification Kit was used for the maxi preparation of the construct. Additionally, they require that we provide them with linearization instructions since DNA must be in linear form for recombination to work. Analyzing the sequence of the vector tells us that Xho1 will linearize the Y14 cKO construct. Prior to sending
out the samples, a test linearization was performed. In addition to a PCR screen, a Southern blot technique is a common way to screen clones based on restriction enzyme digestion patterns. Once we were confident with the PCR approach, we did not optimize the Southern strategy. However, in addition to the Xho1 linearization, Figure 3-4 shows the patterns that would be expected if the construct was cut with specific enzymes.

**Restriction Enzyme Digestions**

The maxi prep yielded a concentration of 1.41µg/µL. We wanted to cut 300ng of DNA so the original stock was diluted to 100ng/µL. The digestion recipe was as follows: 3µL Y14 cKO DNA, 0.5µL restriction enzyme, 2µL of respective NEB buffer and 14.5µL dH2O. The sample was gently mixed and incubated in the 37°C water bath for one hour. For analysis, 10µL of the digestion sample was run on a 1% agarose gel at 150V for 20 minutes next to a 10KB DNA ladder. The results are shown below in Figure 3-4.

![Figure 3-4. Restriction Enzyme Digestion Pattern of Y14 cKO.](image)

Rbm8a refers to the Y14 cKO construct obtained from the maxi prep. The next lane shows a 10KB ladder. Digestion with Xho1 results in linearization of the construct, and the subsequent lanes represent restriction enzyme digestion patterns that verify the construct design.
After sending the samples, we received confirmation that the ES cell targeting was complete. This is the stage in which the neomycin cassette was crucial as it allowed their team to assess which ES clones may be positive, meaning the DNA underwent homologous recombination with the cKO construct, based on resistance to neomycin. They identified 192 ES cell clones as potentially positive and sent us the ES cell DNA samples and their duplicates. The next step was to screen these clones with our PCR genotyping technique to identify those that truly contained the target vector. ES cells are highly unstable, thus it was imperative that the genotyping was completed within one month of receiving the DNA.

**PCR confirmation of Y14 cKO construct in ES cell DNA.**

The ES cell DNA was sent in a 96 well plate. To each well, we added 30µL of digestion cocktail (which consisted of TE buffer to dissolve the DNA) and incubated the plate overnight in the 37°C shaker. Our collaborators agreed to expand six cell lines pending the PCR confirmation of positive products. After extensive genotyping with different primer sets, we decided on the six clones that showed the most convincing results of containing the cKO gene. These six samples were expanded and used for the microinjection which I will discuss in the next section.

**PCR screening of ES cell clone DNA.**

PCR recipes were prepared as follows: 4µL 5X GC Buffer, 0.4µM 10mM dNTPs, 0.5µL forward primer, 0.5µL reverse primer, 0.2µL Phusion DNA Polymerase, 10.4µL dH₂O, and 4 µL DNA. The positive control was one copy of the Y14 cKO construct used in the dilution series previously described and the negative control was water. We experimented with several combinations of primers to see which gave the most convincing results. The following figures show the PCR results and identification of the Y14 cKO construct, indicating the six clones we chose to expand for the microinjection.
Figure 3-5. Identification of ES clones with F4 R1 primers. 8µL of Phusion PCR samples were run on a 0.8% agarose gel at 200V for 25 minutes. The positive control shows the cKO construct at 3000bp. (A) The red box around Lane 4 (sample C3) indicates a positive result with this primer set. (B) The red box around Lanes 2 (sample E11) and 4 (sample F8) indicates a positive result with this primer set.
Figure 3-6. Identification of ES clones with 5’arm primers. 8µL of Phusion PCR samples were run on a 0.8% agarose gel at 200V for 15 minutes. The positive control shows the cKO construct at 220bp. The red box around Lanes 2 through 4 indicates a positive result with this primer set. The lane numbers correspond to the samples as following: 1: E1 (plate 1). 2: E1 (plate 2). 3: D3 (plate 1). 4: D3 (plate 2).

Figure 3-7. Identification of ES clones with F4 R4 primers. 8µL of Phusion PCR samples were run on a 0.8% agarose gel at 200V for 25 minutes. The positive control shows the cKO construct at 3000bp. The red box around Lane 7 (sample F11) indicates a positive result with this primer set.
Production of chimeras.

The technical details of the ES cell microinjection are beyond the scope of this thesis, but the goal is for E3.5 blastocysts to be injected with cKO DNA and then implanted in the uterus of psuedopregnant female mice. If the process is successful, the female mouse will give birth to a litter containing chimeric mice. “Chimera” is the term used to refer to an individual organism which contains two distinct cell populations. (In this case, the host ES cells and the modified ES cells from injection). Chimeric mice are a widely used research tool as they can be easily recognized due to the exploitation of coat color. When ES cells come from mice with two different coat colors, a chimera will then show a patchy or striped coat color indicating the possession of both cell types. For this study, the Y14 cKO construct was injected into ES cells from C57/B6 mice (black coat) and implanted in the uterus of albino C57/B6 mice (white coat). When the litter is born, it takes approximately one week before chimeric offspring can be identified by a mixed black and white coat color. Production of chimeras is the first step in successful gene targeting, but must be followed by selective breeding to ensure that germ-line transmission is obtained.

The most recent update on this project came from Gan’s team at University of Rochester in late January. Four litters were born resulting in a total of eight male chimeras. This update validates our strategy and screening process and can be considered a significant accomplishment in the Y14 conditional knockout project. When the chimeras reached the appropriate weaning-age of approximately twenty-one days, they could be separated from the mother and sent to our laboratory. It should be noted that chimeras can be either male or female, but males are the preferred sex for this project. Not only do they reach reproductive maturity sooner than females, but they are associated with a higher rate of germ-line transmission (Chimera, 2012). Figure 3-8 shows photographic representation of the chimeras.
upon arrival to our laboratory, where they are currently in quarantined conditions as per the protocol of receiving new mice. When the weaned chimeras are approximately eight weeks old, they will be bred to B6 albino females to assess germ-line transmission. (If a resulting pup has black coat color, germ-line transmission was obtained).

Figure 3-8. Chimeric mice. The observance of patchy coat color indicates successful chimera production for the Y14 conditional knockout project.
Discussion of future direction.

Projects revolving around gene targeting and animal breeding tend to occur over an extended period of time. The creation of a tissue-specific conditional knockout requires the additional step of crossing with a Cre line. Several Cre lines have been established that are linked to a specific tissue or cell type, and the Jackson Laboratory website is a valuable resource as it lists the recombinase expression data for each strain. For this project, the strains leading to Y14 inactivation in the neurons or blood would be of significant interest. The immediate next step for this project is to confirm germ-line transmission as stated in the previous section. Those individuals can then be backcrossed to C57/B6 mice to obtain pure homozygotes. Upon validation, the Cre line matings can be set up depending on the tissue of interest. The resulting litter of conditional Y14 knockouts can then be used in future studies.

The key for the continuation of this project is to elucidate an interesting phenotype. This in itself may very well provide the basis for a future student’s thesis work. A common and critical phenotype is for the Y14 knockout genotype to be lethal, indicating that the gene is imperative for developmental success. Alternatively, knockouts may develop as normally and not possess any obvious physical abnormalities. In this case, there are several directions for future study to determine the effects of Y14 deletion in specific tissues.

Analyzing schizophrenia-associated behavior in a Y14 cKO mouse model.

Recalling our hypothesis that Y14 plays a role in schizophrenia, certain behavioral tests can be performed to identify schizophrenia-related behaviors in the conditional knockouts. The behaviors associated with schizophrenia and their respective tests have been well categorized for the mouse model. Typical behavior tests include social interaction, pre-pulse inhibition (PPI), forced swim test, open field test, Y-maze evaluation of spatial recognition, and latent inhibition test (Vuillermot et al., 2011). These tests are proficient at assessing the behavioral and cognitive
deficits typical of schizophrenic patients. Even in a mouse model, gating measures can provide valuable input. “Gating” is a term used to refer to the inability to process information, or more specifically lacking the ability to filter or “gate” stimuli and thoughts from conscious thinking. This is a significant contributing factor to the positive symptoms of schizophrenia, such as hallucinations and delusions. PPI is the most frequently used test for gating analysis (Geyer & Moghaddam, 2002).

**Analyzing morphological changes in the brain of a Y14 cKO mouse model.**

In addition to behavioral phenotypes, one can examine the effect of Y14 on brain pathology. Lateral ventricle enlargement is known to be associated with schizophrenia and can be easily assessed by magnetic resonance imaging (MRI). In fact, it is the earliest and most frequently reported structural change observed in schizophrenia (Gaser et al., 2004). In addition to enlargement of the ventricles, other morphological features can suggest a schizophrenic phenotype. The gray matter of the brain, comprised of neuronal cell bodies, shows an overall volume reduction in schizophrenia patients. Even more specifically, the term “cortical thinning” refers to the gradual decrease in thickness of the cerebral cortex and corpus callosum (Hatayama et al., 2011). Cortical thinning has been well characterized in Alzheimer’s disease but has also been linked to schizophrenia. Consistent with the volume reduction of gray matter observed in affected individuals, an overall decrease in the size of neuronal cell bodies may also serve as a morphological marker for schizophrenia (Harrison, 1999).

**Analyzing Y14 cKO mouse model for signs of TAR syndrome.**

As stated in the introduction, Y14 has recently been linked to TAR syndrome. It was found that a Y14 deficiency in combination with a low-frequency SNP results in this human disorder. However, it has not yet been portrayed in an animal model. Therefore it would be of exceptional interest to see if any of the TAR phenotypes are found in the Y14 cKO mouse.
TAR phenotypes are those for which it was named after, Thrombocytopenia with Absent Radii. Thrombocytopenia refers to a decrease in platelets, or the clotting cells found in blood. One interesting idea would be to cross our Y14 cKO mouse to a Cre-line where recombinase activity is specific to the blood and look for signs of thrombocytopenia. Alternatively, the absence of the radius in the forearm could also coincide with TAR association. These types of analyses will be of great interest to our group while working towards the goal of linking Y14 to schizophrenia in the context of behavior, cognitive abilities, and/or neuropathology. It is important to analyze one target at a time and aim for clear results indicating a schizophrenia-related phenotype.
Chapter 4

RNA-Binding Protein Immunoprecipitation.

Introduction.

RNA-binding protein immunoprecipitation (RIP) is an offshoot of basic immunoprecipitation (IP), a widely used protein isolation technique that employs the specificity of antibodies to “pull down” proteins from a lysate mixture. The difference is in the ability of the RIP technique to identify the types of RNA which are bound to the precipitated protein. Y14 has a pseudonym of Rbm8a, which stands for RNA-binding protein 8A, stemming from the tendency of Y14 to bind mRNA. The preferential binding of Y14 has been well characterized due to its presence in the EJC, and it is known to associate with mRNAs arising from splicing events, regardless of location (meaning it will interact with nuclear mRNAs or exported cytoplasmic mRNAs). Conversely, it will not associate with pre-mRNAs or introns. As for the duration of interaction, Y14 remains bound to associated mRNAs until translation begins (Dostie & Dreyfuss, 2002). In this chapter, I will discuss the RIP technique and how it can contribute to clarifying the potential role of Y14 in schizophrenia.

The first step in this experiment is to isolate the RNA from tissue or cell extract. The technique I used was adapted from a 2006 Nature protocol by Keene et al. describing RIP-Chip, where the term “Chip” refers to subsequent microarray analysis. (This is not to be confused with ChIP, which refers to chromatin immunoprecipitation to observe the interaction of proteins and DNA.) The goal of this experiment is to identify specific RNA’s that are bound by Y14 in the brain.
Materials and methods.

Preparation of buffers.

Two buffers are used in this experiment: Polysome Lysis Buffer and NT2 Buffer. They were prepared in RNase-free water as described in the Nature protocol. The Polysome Lysis Buffer consisted of 100 mM KCl, 5 mM MgCl$_2$, 10 mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT, 100 units/mL RNase Out, 400 μM VRC, and necessary protease inhibitors (See Appendix for complete list of inhibitors used). The NT2 buffer consisted of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl$_2$, and 0.05% NP40.

Preparation of lysate.

For this experiment I used one adult wild-type C57/B6 mouse brain and one post-natal wild-type C57/B6 mouse brain. A dounce homogenizer was prepared by rinsing several times with RNase-free water. For each sample, the entire brain was homogenized in 1mL of Polysome Lysis Buffer, first by hand and then by subsequent pipetting. Homogenized lysate was then incubated on ice for 5 minutes before overnight storage in -80°C. The following day, lysates were thawed on ice and centrifuged at 15,000rpm for 15 minutes to remove any un-homogenized pieces. The supernatant of the lysate contains the ribonucleoproteins and was used for the next portion of the experiment.

Preparation of sepharose-G beads.

In order to identify specific proteins, I used sepharose-G beads conjugated to the appropriate antibodies. This allows for the protein of interest to indirectly bind to the beads, while centrifugation and washes can then isolate the protein along with any associated mRNAs. The first step in bead preparation was to pre-swell the beads by combining 500μL of the bead slurry with 2.5mL NT2 buffer supplemented with 5% BSA in a 15mL Falcon tube and allowing the tube to tumble in the cold room at 4°C for one hour. Two samples of beads were prepared: one
for use with Y14 antibody and the other for use with a control IgG antibody. After one hour, 500µL of each bead mixture was transferred to 1.5mL Eppendorf tubes. Pulse centrifugation yielded a bead pellet of approximately 50µL in each tube. Excess NT2 buffer was removed and the appropriate antibody (Santa Cruz Mouse-anti-Y14 or Mouse-anti-IgG) was added such that there was 1 µg of antibody per sample. The tubes were incubated in the cold room (4°C) with constant tumbling overnight. The following day, the beads were washed with 1mL of cold NT2 buffer for a total of five washes. Following the final wash, the pellet was resuspended in 850µL of cold NT2 buffer supplemented with protease inhibitors. At this point, the beads were ready for use in immunoprecipitation.

**Immunoprecipitation.**

With the beads conjugated to their respective antibodies (Y14 and IgG), each bead sample was divided equally and 100µL of homogenized brain lysate was added directly to the beads. This was done to account for all combinations of antibodies and brain types, resulting in four samples: IgG adult, IgG post-natal, Y14 adult, and Y14 post-natal. After mixing the beads and lysate, the tubes were pulse centrifuged to re-pellet the beads. Additional supernatant was removed and the samples were tumbled for 2 hours at room temperature. After the incubation, the beads were washed with 1mL NT2 buffer for a total of five washes before resuspending the pellet with 100µL NT2 buffer. To isolate RNA from the bead mixture, 1 added 1mL of TRIzol reagent to each tube. After vortexing, the tubes were incubated at room temperature for 5 minutes prior to the addition of 200µL of chloroform under a fume hood. The tubes were shaken by hand for 15 seconds and incubated at room temperature for 2 minutes. The samples were then centrifuged at 12000xG for 15 minutes to result in phase separation. The aqueous RNA-containing layer was transferred to a clean 1.5mL Eppendorf tube, and 500µL of isopropanol was added before incubating at room temperature for 10 minutes. Samples were
centrifuged again at 12000xG for 10 minutes at 4°C, supernatant was discarded, and 1mL of ethanol was added to wash the precipitated RNA. The resulting RNA pellet was allowed to air dry for approximately 10 minutes before resuspending in 50µL RNase-free water. At this point, both tubes were incubated in the 65°C heat block for 10 minutes before storage in -80°C.

**Analysis of isolated RNA.**

The concentration of the isolated RNA was read using a photospectrometer. Samples were collected at two different time points- total cellular mRNA and final mRNA after TRIzol separation. Tables 4-1 and 4-2 show the results of the RIP experiment.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Adult</td>
<td>4.7</td>
</tr>
<tr>
<td>IgG Post-Natal</td>
<td>8.3</td>
</tr>
<tr>
<td>Y14 Adult</td>
<td>8.4</td>
</tr>
<tr>
<td>Y14 Post-Natal</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Table 4-1. Total Cellular mRNA.* Samples were collected prior to immunoprecipitation.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Adult</td>
<td>-1.7</td>
</tr>
<tr>
<td>IgG Post-Natal</td>
<td>-1.6</td>
</tr>
<tr>
<td>Y14 Adult</td>
<td>-1.5</td>
</tr>
<tr>
<td>Y14 Post-Natal</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

*Table 4-2. Final mRNA product.* Samples were collected after TRIzol separation of immunoprecipitation reaction.
Discussion.

The negative readings for concentration of final RIP products are indicative of the first source of error. When using a photospectrometer to read concentrations, it is necessary to first provide a blank sample to account for any buffers that exist in the experimental samples. For this experiment, the blank for total cellular mRNA would be different than the blank for final RNA product. In the first set of samples, the total cellular mRNA was collected by homogenizing the brain in Polysome Lysis Buffer. In the second set of samples, referred to as “Final RNA products”, the RNA was resuspended in water. Failure to recognize this difference resulted in negative concentration readings. However, these readings can provide some insight for optimizing the protocol. It seems that we are able to isolate RNA post homogenization of the tissue, but we lose the RNA somewhere in the immunoprecipitation process. Potential sources of error include insufficient antibody-bead conjugation or RNA degradation at any step in the process.

The future direction of this work requires initial optimization of the protocol. When we establish a method for accurately and efficiently isolating RNA from lysate, there are several parameters to be investigated. First, the mRNA can be converted to cDNA and sent for sequencing and subsequent analysis. Then, it would be interesting to experiment with different tissues such as the various developmental stages of the brain, in addition to performing RIP analysis using tissues subjected to Y14 overexpression or knockdown. The ability to identify the entire set of mRNA binding targets of Y14 would provide unique insight not only to the structure and function of the protein, but could also shed light on post-transcriptional regulation processes exhibited by Y14.
CONCLUSION

There were three main projects completed within the scope of this thesis. While there are numerous ways to investigate the function of a gene and its associated product, my work focused on the methods of overexpression, conditional knockout, and RIP. Each sub-project independently contributed to the overarching theme of elucidating a role for Y14 in schizophrenia. The completion of the overexpression project is a useful tool for both in vitro and in vivo work. We were able to establish and maintain stable cell lines and reproducibly verify Y14 overexpression with Western blotting. In addition to the stable cell lines currently existing, future work can be done to overexpress Y14 in other cell types, such as neural progenitor cells and mature neurons. Importantly, the in vivo work described was able to provide preliminary results for linking Y14 to schizophrenia by signifying its involvement in hippocampal neurodevelopment and anxiety behaviors. In addition to overexpression, the production of chimeras from the conditional knockout project was a significant achievement in the course of this investigation. It verified the success of our cloning strategy and screening process. The ability to target the knockout of Y14 in specific cell types or tissues will be an extremely valuable tool for examining the effect the gene may have on disease and/or neurodevelopment. Finally, the RNA-immunoprecipitation work still requires optimization. By working on establishing an effective protocol, current and future lab members can benefit from the unique insight that RIP can provide when working with any RNA-binding protein. In summary, this work provides the basis for continuing to explore any unknown potential functions of Y14, and will aid in the ability of our lab to make strides towards developing a novel therapy or cure for schizophrenia.
Appendix.

List of Protease Inhibitors.

<table>
<thead>
<tr>
<th>Common abbreviation</th>
<th>Protease inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Aprotinin</td>
</tr>
<tr>
<td>B</td>
<td>Bestatin</td>
</tr>
<tr>
<td>β-GPS</td>
<td>BETA-glycerophosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>Na$_2$VO$_4$</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>P</td>
<td>Pepstatin</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
</tbody>
</table>


