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FUNCTIONAL STUDY OF CMYA5, A CANDIDATE GENE FOR SCHIZOPHRENIA

Anting Hsiung
Virginia Commonwealth University

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FUNCTIONAL STUDY OF CMYA5, A CANDIDATE GENE FOR SCHIZOPHRENIA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Anting Hsiung
B.S. Biological Sciences: Cell Biology and Genetics
University of Maryland, College Park, 2009

Director: Rita Shiang, Associate Professor
Department of Human and Molecular Genetics

Virginia Commonwealth University
Richmond, Virginia
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<td>aa</td>
<td>amino acid</td>
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<tr>
<td>AD</td>
<td>Activation domain</td>
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<td>AP-3</td>
<td>Clathrin-adaptor complex 3</td>
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<tr>
<td>BCCP</td>
<td>Biotin Carboxyl Carrier Protein</td>
</tr>
<tr>
<td>BD</td>
<td>Binding domain</td>
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<tr>
<td>BLOC-1</td>
<td>Biogenesis of lysosome-related organelles complex 1</td>
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<tr>
<td>CER</td>
<td>Cerebellum</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CNV</td>
<td>Copy-Number Variation</td>
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<td>CTX</td>
<td>Cortex</td>
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<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
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<td>DLPFC</td>
<td>Dorsolateral prefrontal cortex</td>
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<td>DRD2</td>
<td>Dopamine receptor D2</td>
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<td>GWAS</td>
<td>Genome- wide association study</td>
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<td>HB</td>
<td>Hindbrain</td>
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<td>HPC</td>
<td>Hippocampus</td>
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<td>IBs</td>
<td>Inclusion bodies</td>
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<td>IF</td>
<td>Intermediate filament</td>
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<td>Acronym</td>
<td>Description</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MOC</td>
<td>Mander's overlap coefficient</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>MSN</td>
<td>Medium spiny neuron</td>
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<td>MZ</td>
<td>Monozygotic twins</td>
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<td>NF</td>
<td>Neurofilament</td>
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<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
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<td>ONPG</td>
<td>ortho-Nitrophenyl-β-galactoside</td>
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<tr>
<td>PCC</td>
<td>Pearson's correlation coefficient</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>STR</td>
<td>Striatum</td>
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<tr>
<td>TCF4</td>
<td>Transcription factor 4</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>Y2H</td>
<td>Yeast two-hybrid</td>
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ABSTRACT

FUNCTIONAL STUDY OF *CMYA5*, A CANDIDATE GENE FOR SCHIZOPHRENIA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Anting Hsiung
B.S. Biological Sciences: Cell Biology and Genetics
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Virginia Commonwealth University
Richmond, Virginia
May 2017

*CMYA5* is a candidate gene for schizophrenia because of the association of variant rs10043986 (Pro4063Leu). Studies of *CMYA5* and its gene product, myospryn, in brain and neuronal cells have not been previously reported. We examined the neuronal expression of myospryn and its binding partner, desmin intermediate filament (IF), and investigated the difference in binding and colocalization of the two alleles of myospryn to IFs. Myospryn and desmin are expressed in brain regions. Using yeast
two-hybrid and surface plasmon resonance, the T allele (Leu) is found to have higher binding affinity to desmin than the C allele (Pro). Myospryn localizes to the cytoplasm and nucleus and is weakly to moderately colocalized with desmin in myoblast, neuroblastoma, and glioblastoma cell lines. Peripherin and vimentin, brain-related IFs, have similar degrees of colocalization. rs10043986 does not affect the colocalization of myospryn to IFs, but it affects the colocalization of myospryn to F-actin. Dysbindin, another schizophrenia candidate gene, is found to weakly colocalize with myospryn in myoblast, neuroblastoma, and glioblastoma cell lines. The expression of myospryn in the brain suggests functions that are relevant to schizophrenia. rs10043968 is a functional variant that results in differential binding of myospryn to desmin. We hypothesize that the interaction between myospryn to IFs provides structural support and efficient rearrangement of the cytoskeleton network during early neuritogenesis. Myospryn might also be involved in intracellular trafficking affecting synaptic function through dysbindin in conjunction with the BLOC-1 complex and IFs. Myospryn might also play important roles in neurotransmission based on a literature search of its binding partners PKA, calcineurin, and α-actinin.
Schizophrenia

Introduction

Schizophrenia (OMIM 181500) is a chronic, severe, and disabling brain disorder with a prevalence of approximately 1%. It affects more than 21 million people worldwide and is more common in males than females. The clinical features are characterized by positive symptoms (e.g., delusions, hallucination, and disorganized thinking), negative symptoms (e.g., flat affect, loss of pleasure, interest, and speech), and cognitive symptoms (e.g., poor executive functioning, trouble focusing, and memory problems). The age of onset typically begins in adolescence or early adulthood however men tend to develop schizophrenia earlier than women (Rajji et al., 2009). People with schizophrenia have 2- to 3-fold increased mortality, often due to suicide during the early phase of the disorder, and later from cardiovascular, metabolic, and infectious diseases (McGrath et al., 2008). The diagnosis of schizophrenia relies on clinical observation and the criteria in American Psychiatric Association’s fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5); no diagnostic laboratory tests or biomarkers are available. Treatments focus on eliminating the symptoms, such as antipsychotic medication, along with psychosocial support. Although treatments are available for psychotic symptoms, the pathways or biological mechanisms that cause schizophrenia remain unknown.
Causes of Schizophrenia

Schizophrenia is a complex disorder with both genetic and environmental factors contributing to the risk for developing the disease. Many genes are involved, and each gene confers only a small effect on the phenotype. The interactions between genes and environment also play important roles.

Genetics

Family, twin and adoption studies provide evidence for the genetic basis of schizophrenia. First-degree relatives of schizophrenia patients have a higher morbidity risk (2-9%) for schizophrenia, compared to the risk in relatives of controls (0-1%) (reviewed in Shih et al., 2004). Studies also show that second- and third- degree relatives have risk estimates of 3% and 1.5%, respectively (Maier et al., 2002). Overall, the child of a parent with schizophrenia has a ten-fold increased risk over the general population (Gejman et al., 2010). The concordance rates of schizophrenia for monozygotic (MZ) twins are approximately 40%–50%, compared with 6%–10% in dizygotic (DZ) twins (Gejman et al., 2011; Cardno & Gottesman, 2000). Twin studies also reveal that schizophrenia is highly heritability with a point estimate of heritability in liability to schizophrenia at 81% (Sullivan et al., 2003). Adoption studies show that the risk travels with the biological rather than the adoptive relationship, and biological relatives of affected adoptees have a higher risk for schizophrenia than the adoptive relatives (Shih et al., 2004; Gejman et al., 2011).
Genome-wide association studies (GWASs)

Before the availability of GWAS, candidate gene and linkage studies have attempted to identify genetic variations that are involved with schizophrenia, but the genes identified could not be replicated due to the small sample size lacking statistical power (Sullivan 2008). A comprehensive study of 14 candidate genes with 1,870 cases and 2,002 controls of European ancestry found no association of schizophrenia with single nucleotide polymorphisms (SNPs) in these 14 candidate genes (Sanders et al., 2008). An association study is a linkage disequilibrium-based study that identifies common genetic variations, such SNPs with allele frequencies >1%, by comparing the individuals with and without the disease. A significant difference in allele frequency between the two populations identifies the association with the phenotype, and a GWAS surveys the whole genome for these associations. An early GWAS (Stefansson et al., 2009) of 2,663 schizophrenia cases and 13,498 controls found associations with the Major Histocompatibility Complex (MHC) on chromosome 6, transcription factor 4 (TCF4), and the neurogranin gene (NRGN). Another GWAS by the International Schizophrenia Consortium, 2009, using 3,322 schizophrenia cases and 3,587 controls found associations with myosin XVIIIB (MYO18B), the MHC region, the 22q11.2 deletion region, and zinc finger protein 804A (ZNF804A). An additional GWAS used the Molecular Genetics of Schizophrenia case-control sample followed by meta-analysis of the data sets mentioned above found association of a region on chromosome 6p22.1 that contains the MHC region (Shi et al., 2009); the MHC finding remains the most significant and consistent. In 2011, a GWAS of 21,856 discovery samples, and 29,839 replication samples uncovered five new loci and two that have previously implicated
(MHC and TCF4) (Ripke et al., 2011). The strongest finding of this study was with rs1625579 located within an intron of a putative primary transcript for MIR137, a regulator of adult neurogenesis, neuronal maturation, and brain development. Four genes, TCF4, CACNA1C (calcium channel, voltage-dependent, L type, α 1C subunit), CSMD1 (CUB and Sushi multiple domains 1), and C10orf26 (WBP1L; WW domain binding protein 1-like) achieved genome-wide significance and contain predicted MIR137 target sites (Ripke et al., 2011). A 2013 GWAS found thirteen new loci, as well as the replication of several others, including MHC, C10orf26, DPYD (dihydropyrimidine dehydrogenase)-MIR137, SDCCAG8 (serologically defined colon cancer antigen 8) and MMP16 (matrix metallopeptidase 16) (Ripke et al., 2013). A 2014 GWAS of 36,989 cases and 113,075 controls reported 128 genome-wide significant SNPs in 108 loci, 83 of which were new, and most of the genes are expressed in brain and not in tissues less likely to be relevant to schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The study found an association of DRD2 (dopamine receptor D2), which is a target of antipsychotic drugs, and many genes involved in glutamatergic neurotransmission and synaptic plasticity. Chen et al. (2015) summarized the top 25 common variants identified by GWASs, which showed a large number of common risk alleles associated with schizophrenia and the genetic risk for schizophrenia is highly polygenic: many genes contribute to development of the disorder but each gene only has a small effect. Many risk loci identified have unknown function and not located in protein-coding regions of genes; therefore, it is still unclear how those common variants contribute to the etiology of schizophrenia.
Copy-Number Variation (CNV)

CNVs are chromosomal deletions and duplications that range in size from kilobases to megabases of DNA sequence, and genomic studies have identified 11 rare, but recurrent CNVs that individually confer a relatively high risk for schizophrenia: 1q21.1, 2p16.3 (NRXN1; neurexin 1), 3q29, 15q11.2, 15q13.3 and 22q11.2, and duplications at 1q21.1, 7q11.23, 15q11.2-q13.1, 16p13.1 and proximal 16p11.2 (reviewed in Kirov, 2015). Most recurrent pathogenic CNVs are large (>400 kb), typically involving dozens of genes, and are individually rare (frequency <0.1%) (Morrow, 2010). The largest genome-wide analysis of CNVs recently confirmed the association of 1q21.1, 2p16.3 (NRXN1), 3q29, 7q11.2, 15q13.3, distal 16p11.2, proximal 16p11.2 and 22q11.2 (CNV and Schizophrenia Working Groups of the Psychiatric Genomics Consortium, 2017). The study also showed a global enrichment of CNV burden in schizophrenia, specifically in genes associated with synaptic function. Overall, CNVs are relative rare but account for substantially higher risk for schizophrenia.

Rare de novo Mutations

De novo mutations can be both large chromosomal copy number changes such as CNVs or small mutations affecting one or a few nucleotides such as single nucleotide variations (SNVs). Exome sequencing technology is used for identifying small, rare de novo mutations associated with schizophrenia. Fromer et al. (2014) found an excess of small deleterious de novo mutations in schizophrenia affecting the components of activity-regulated cytoskeleton-associated protein (ARC) and N-methyl-D-aspartate
receptor (NMDAR) complexes, as well as mutations in the targets of the fragile X mental retardation protein (FMRP) complex. Another large case-control exome sequencing study also found an enrichment of rare mutations in the ARC complex of the postsynaptic density, targets of FMRP, and voltage-gated calcium ion channels (Purcell et al., 2014). Together, those large-scale genetic studies of schizophrenia suggest common pathways are involved in risk for schizophrenia.

Environment

Despite the evidence for high heritability, MZ concordance is only 40%–50%, suggesting that genetic risk factors do not entirely explain schizophrenia and that environmental risk factors are also important. Some environmental factors that affect early neurodevelopment during pregnancy, such as maternal stress (Khashan et al., 2008), maternal infections (Khandaker et al., 2013), maternal malnutrition (Xu et al., 2009), and obstetric complications (Brown 2011), are found to contribute to schizophrenia. Paternal age (McGrath et al., 2014), season of birth (Davies et al., 2003), urban birth (McGrath & Scott 2006), cannabis use (Casadio et al., 2011), and migration (Selten et al., 2007) have also been associated with schizophrenia. There is accumulating evidence that environmental exposure, as early as the periconceptional period of pregnancy and potentially as late as adolescence or adulthood, could play an important role in the susceptibility to schizophrenia. Thus, the study of environmental factors may have important implications for the identification of causes and prevention.
**Pathophysiology**

Many brain imaging and neuropathological studies have attempted to relate the manifestations of schizophrenia to altered structure and function of particular brain regions and circuits, but the exact pathophysiological of schizophrenia remains poorly understood. Two of the most influential hypotheses concerning the neurobiology underlying the disorder involve dopamine and glutamate.

**Brain Abnormalities**

Schizophrenia is associated with subtle differences in brain structures, identified using imaging techniques such as MRI (magnetic resonance imaging). A decrease in whole brain volume has been found in patients with schizophrenia compared with healthy subjects, with a global decrease (2%) in cerebral volume and an increase (26%) in total ventricular volume (Wright *et al*., 2000). Gray matter loss (2%) is more prominent than white matter loss (1%) (Wright *et al*., 2000). The cerebral regions with the lowest volume are the amygdala and hippocampus (Wright *et al*., 2000). Enlarged ventricles in patients are associated with the reduction in the thalamus, which is adjacent to the body of lateral ventricles (Gaser *et al*., 2004). A recent study by ENIGMA (Enhancing Neuro Imaging Genetics through Meta Analysis) Consortium using brain MRI scans from 2,028 schizophrenia patients and 2,540 healthy controls has found smaller hippocampus, amygdala, thalamus, accumbens, and intracranial volumes, as well as larger pallidum and lateral ventricle volumes (van Erp *et al*., 2016). van Erp *et al*., 2016, also found that the enlargements of putamen and pallidum are associated with duration of illness and age, and the hippocampal deficits are more severe in
unmedicated patients. This replicates previous findings and suggests that treatment with predominantly second-generation (atypical) antipsychotics may ameliorate hippocampal volume deficits (van Erp et al., 2016).

**Dopamine**

Dopamine is a major catecholamine neurotransmitter in the central nervous system that is involved in the regulation of a variety of functions. Two dopaminergic pathways, the mesolimbic and mesocortical, are critical for affect, emotion, attention, and motivation that are implicated in schizophrenia. The mesolimbic pathway transmits dopamine from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens in the ventral striatum, hippocampus, and amygdala and correlates with pleasant feelings, reward, and desire (incentive salience) (Meisenzahl et al., 2007). The mesocortical pathway transmits dopamine from the VTA to the frontal cortex and is involved in cognitive function, e.g. working memory (Meisenzahl et al., 2007). Once released from presynaptic terminals, dopamine activates members of a family of G protein-coupled dopamine receptors named D1 to D5. The D1 receptor is expressed at the highest level in the brain, and the D2 and D3 receptors are expressed both pre- and post-synaptically in the brain (Beaulieu & Gainetdinov, 2011). A presynaptically located, specific monoaminergic membrane bound transporter molecule, the dopamine transporter (DAT), regulates both duration and magnitude of dopamine neurotransmission at the synapse by removing dopamine from the synaptic cleft through active reuptake (Meisenzahl et al., 2007).

The dopamine hypothesis proposes that hyperactivity of dopamine transmission...
is responsible for schizophrenia symptoms – it was first formulated by Rossum (1966) based on the observation that antipsychotics block dopamine receptors and supported by the correlation between clinical doses of antipsychotic drugs and their potency to block dopamine D2 receptors (Baumeister & Francis, 2002). In 1991, Davis et al. proposed that the positive symptoms of schizophrenia are the results of striatal D2 receptor hyperstimulation due to hyperactive mesolimbic dopamine projections, whereas the negative symptoms and cognitive impairments are due to prefrontal cortex D1 receptor hypostimulation caused by reduced mesocortical dopamine projections (Davis et al., 1991). The development of Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) imaging techniques allow for the in vivo examination of dopamine function, such as the dopamine synthesis, release, and availability of post-synaptic dopaminergic receptors and transporters (Kim et al., 2013). The dopaminergic abnormality in schizophrenia is presynaptic with elevation of presynaptic striatal dopamine synthesis capacity (Howes et al., 2012). The release of dopamine can be determined indirectly by measuring the reduction of radiotracer binding after pharmacological challenge (e.g., amphetamine), which provides evidence of significantly increased dopamine release in patients compared with control subjects (Howes et al., 2015). Meta-analyses found no evidence for a difference in striatal DAT level in patients with schizophrenia (Howes et al., 2012; Chen et al., 2013). Increased D2/3 receptor density was observed in patients but the alteration was inconsistent and small (Howes et al., 2012). Together, the existing evidence suggests that presynaptic striatal dopaminergic function is elevated in patients with schizophrenia who have more dopamine availability and dopamine release. However, pharmacological and other
studies show that dopaminergic dysfunction is unlikely to explain the full range of clinical features of schizophrenia.

Glutamate

Glutamate is the most abundant amino acid in the brain and is the primary excitatory neurotransmitter. The glutamate hypothesis is based on the fact that phencyclidine (PCP), ketamine, and other antagonists of the N-methyl-d-aspartate (NMDA) subtypes of glutamate receptors induce schizophrenia-like, positive, negative, and cognitive symptoms (Coyle, 1996). The NMDA receptor is a heterotetramer composed of two obligatory NR1 subunits and two of a family of four NR2 subunits. The NR1 subunit mRNA and protein expression and NR2C mRNA expression are decreased in the dorsolateral prefrontal cortex (DLPFC) of postmortem brain from people with schizophrenia, and a genetic variation in the NR2B gene is associated with significantly lower reasoning ability in schizophrenia (Weickert et al., 2013). In addition, many genes (e.g. Neuregulin 1 (NRG1)) that are associated with schizophrenia are directly or indirectly involved in glutamatergic transmission via NMDA receptors (Harrison & Owen, 2003; Greenwood et al., 2011). NRG1 is a trophic factor that signals through the activation of receptor tyrosine kinase ErbB4 to modulate the NMDA receptor signaling and plays crucial roles in neurodevelopment (Hahn et al., 2006). A hypoactive glutamate system can severely impede the proper formation of neural circuits during brain development, cause excessive pruning, and affect the number of synapses retained during adolescence (Konradi & Heckers, 2003).
**CMYA5 (Myospryn)**

A large collaborative group of investigators found strong evidence that CMYA5 is a candidate gene for schizophrenia in a two-stage study with more than 33,000 subjects (Chen et al., 2011). The study was first conducted using data mining analyses of two GWA datasets of European Americans and found three candidate variants in the CMYA5 gene, rs3828611 (H3358Q), rs10043986 (P4063L) and rs4704591 in the 3′ region. In the second-stage, additional replication samples in multiple European and one African American populations using standard meta-analyses showed that rs10043986 and rs4704591 were significantly and independently associated with schizophrenia. Many follow-up studies have also confirmed the association between the CMYA5 gene and schizophrenia in East Asian populations (Li et al., 2011; Furukawa et al., 2013; Zhang et al., 2013; Watanabe et al., 2014; Han et al., 2015).

Some studies found rs3828611 is associated with schizophrenia in Chinese samples (Li et al., 2011; Watanabe et al., 2014; Han et al., 2015), but conflicting results are observed in the Japanese population (Furukawa et al., 2013) and another Chinese Han population (Zhang et al., 2013), likely due to genetic heterogeneity. Rs10043986 is not polymorphic in East Asian populations (Li et al., 2011; Furukawa et al., 2013; Zhang et al., 2013). Rs4704591 is found significantly associated with schizophrenia in the Caucasian samples (Chen et al., 2011) but not in East Asians (Li et al., 2011; Furukawa et al., 2013; Zhang et al., 2013; Watanabe et al., 2014; Han et al., 2015), suggesting the existence of ethnic heterogeneity. These studies provide evidence that the CMYA5 gene is a potential common schizophrenia-related gene. However, CMYA5 is not in the
108 schizophrenia-associated genetic loci identified from the largest molecular genetic study (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

The CMYA5 gene encodes for the protein myospryn, identified as a muscle-specific, tripartite (TRIM)-related protein (Benson et al., 2004), and is associated with Duchenne muscular dystrophy (Tkatchenko et al., 2001) and hypertension (Nakagami et al., 2007). Current studies of CMYA5 focus on its role in skeletal and cardiac muscles, but the precise functions of myospryn remain unknown. No studies of CMYA5 in the brain and neuronal cells have been reported. rs10043986 in CMYA5 is one of a few missense polymorphisms that are associated with schizophrenia, and it changes the highly conserved Pro to Leu. This SNP is located at the C-terminus and carboxyl of the SP1A and RYanodine receptor (SPRY) domain, a region that is involved in binding to self (Benson et al., 2004), α-actinin (Durham et al., 2006), desmin (Kouloumenta et al., 2007), dystrophin (Reynolds et al., 2008), M-band titin (Sarparanta et al., 2010), and calcineurin (Keilbasa et al., 2011) (Figure 1). Myospryn also interacts with protein kinase A (PKA) (Reynolds et al., 2007), protease calpain 3 (Sarparanta et al., 2010), and dysbindin (Benson et al., 2004), a schizophrenia susceptibility gene (Straub et al., 2002). These interactions suggest that myospryn is involved in vesicular trafficking and intracellular signaling (Sarparanta, 2008; Tsoupi & Capetanaki, 2013).
Figure 1: Myospryn structure and its binding partners.

A major portion of the 449-kDa protein consists of repetitive sequence without predictable domains, whereas the C-terminus of 570 amino acids has a TRIM-like domain structure. Close-up of the TRIM-like region shows the B-Box' zinc finger, coiled-coil (BBC), two fibronectin 3-like (FN3) and SPRY (SP1A and RYanodine receptor) domains. Bars indicate binding sites for reported interaction partners: PKA RIIα (Reynolds et al., 2007), desmin (Kouloumenta et al., 2007), dysbindin (Benson et al., 2004), self-association (Benson et al., 2004), dystrophin (Reynolds et al., 2008), α-actinin (Durham et al., 2006), calpain 3 (Sarparanta et al., 2010), titin (Sarparanta et al., 2010), and calcineurin (Keilbasa et al., 2011). rs10043986 (Pro4063Leu) is located at the C-terminus, 3' to the SPRY domain. Figure adopted from Sarparanta (2008). J Muscle Res Cell Motil. 29(6-8):177-80.
CMYA5 (Myospryn) and DES (Desmin)

The DES gene encodes protein desmin, a type III intermediate filament (IF). Desmin is one of the earliest myogenic markers in skeletal muscle and heart and plays essential roles in striated muscle development and the maintenance of structural and functional integrity of the muscle (reviewed Capetanaki et al., 2015; Hnia et al., 2015). Desmin interacts with other members of the IF family, cytolinkers bridging organelles and cytoskeleton, chaperones and adaptor proteins and proteins that have been implicated in proteolysis, posttranslational modifications and signaling important for proper skeletal and cardiac muscle functions (Capetanaki et al., 2007 & 2015). Desmin is also important for the positioning, distribution, and function of mitochondria (Milner et al., 2000). Kouloumenta et al., 2007, found that the amino terminal of desmin (aa 58-103) interacts with the last 24 aa of myospryn. Both proteins colocalize at the periphery of the nucleus in mouse neonatal cardiomyocytes and at intercalated disks and costameres of the adult heart muscle (Kouloumenta et al., 2007). Myospryn also colocalizes with the KDEL receptor marker for endoplasmic reticulum (ER) (Kouloumenta et al., 2007). Desmin is required for the proper perinuclear localization of myospryn and the proper positioning of lysosomes (Kouloumenta et al., 2007). In addition, desmin co-immunoprecipitates with dysbindin and pallidin, components of the biogenesis of lysosome-related organelles complex 1 (BLOC-1), and this association is likely through myospryn (Kouloumenta et al., 2007). The findings suggest a role for desmin IFs in vesicle trafficking and organelle biogenesis and/or positioning, and that proper BLOC-1 formation might require proper myospryn-desmin association (Tsoupri & Capetanaki, 2013; Hnia et al., 2015). Global proteomic analysis of postmortem DLPFC samples
from schizophrenia patients and controls showed that desmin is 1.42x upregulated (Martins-de-Souza et al., 2009), suggesting the involvement of cytoskeleton assembly in schizophrenia.

**CMYA5 (Myospryn) and DTNBP1 (Dysbindin)**

DTNBP1 encodes protein dysbindin and was initially found as an evolutionary conserved 40-kDa coiled-coil-containing protein that binds to α- and β-dystrobrevin in muscle and brain (Benson et al., 2001). Myospryn was first identified as a binding partner of dysbindin (Benson et al., 2004), a subunit of the BLOC-1 complex. Dysbindin was reported to associate with schizophrenia by Straub et al., 2002, followed by positive association in other patient cohorts (Schwab et al., 2003; Van Den Bogaert et al., 2003; Tang et al., 2003; Williams et al., 2004; Kirov et al., 2004). However, inconsistent association of the DTNBP1 locus and schizophrenia was also observed (Morris et al., 2003; Mutsuddi et al., 2006; Peters et al., 2008; Strohmaier et al., 2010). Nevertheless, the reduction of presynaptic dysbindin was found in the hippocampal formation sites of postmortem schizophrenia brains (Talbot et al., 2004), as well as reduced mRNA expression in the DLPFC (Weickert et al., 2004). Dysbindin is located in presynaptic axon terminals of glutamatergic pathways and that the reduction may contribute to the changes in synaptic connectivity and glutamate neurotransmission (Talbot et al. 2004; Weickert et al., 2008). A study in a primary cortical neuronal culture shows that overexpression of dysbindin induces expression of presynaptic proteins and increases extracellular basal glutamate and potassium-evoked exocytotic glutamate release, whereas the knockdown of endogenous dysbindin protein has the reverse effect
(Numakawa et al., 2004). The overexpression of dysbindin protects cortical neurons against neuronal death due to serum deprivation (Numakawa et al., 2004). Dysbindin plays important roles in additional synaptic functions, such as affecting kinetics of transmitter release (Chen et al., 2008) and functioning presynaptically in adaptive homeostatic modulation of vesicle release in Drosophila (Dickman & Davis, 2009). These studies are a necessary first step in suggesting a mechanism by which DTNBP1 contributes to disease pathophysiology. However, dysbindin does not function in isolation; instead, dysbindin is part of a heterooctamer BLOC-1 complex. This complex has been related to multiple cellular functions including membrane protein sorting, membrane fusion, and is required for targeting of synaptic vesicle proteins to the nerve terminal (Newell-Litwa et al., 2010; Newell-Litwa et al., 2009) (reviewed in Mullin et al., 2011; Ghiani et al., 2011). Myospryn might be a part of BLOC-1-like complex in muscle (Benson et al., 2004), and the majority of brain dysbindin, if not all, exists as part of BLOC-1 (Ghiani et al., 2010). It is possible that myospryn and dysbindin works together in the brain through BLOC-1.

The goal of this study is to determine the expression of myospryn and desmin in brain regions and characterize the differential binding between the two different alleles of CMYA5 to desmin. We will focus on determining the consequences of rs10043986 by examining the changes in subcellular location and distribution of myospryn and desmin. This functional study will provide a mechanism to explain how a missense SNP leads to association with schizophrenia, which will lead to a better understanding of the pathophysiology of schizophrenia.
CHAPTER 2: MATERIALS AND METHODS

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Tissues (whole brain, striatum, hindbrain, hippocampus, cerebellum, and cortex) were homogenized in TRIzol Reagent (Invitrogen) using dounce homogenizers and cells were lysed in TRIzol Reagent by pipetting up and down. Total RNA was extracted from tissues and cells using TRIzol Reagent according to the manufacturer’s protocol. To generate first-strand cDNA, 2 µg of total RNA with 100 ng of both oligo dT and random primers were incubated at 70°C for 10 minutes. A mix of 1X M-MLV RT Buffer, 10 mM DTT, 1 mM dNTPs, 10 units RNasin (Promega) were added, followed by the addition of 200 units of M-MLV Reverse Transcriptase (Promega) or 1 µL DEPC ddH₂O (-RT control) in a total reaction volume of 20.25 µl. The reaction was incubated at 37°C for one hour and heat-inactivated at 95°C for 5 minutes. PCR was performed in 6.25 µL reactions with 1.25 µL from cDNA synthesis, 1X PCR Reaction Buffer, 0.2 mM dNTPs, 0.5 pmol/µL of each primer, and 0.05 µL of Phire Hot Start DNA polymerase (Finnzymes). PCR cycling conditions were denaturation of 1.5 minutes at 94°C, followed by cycles of 94°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 7 minutes. PCR products were separated on 6% polyacrylamide gels and stained with ethidium bromide for visualization. The sequence, the expected product size, annealing temperature, and number of cycles for each primer pair are listed in Table 1. RT-PCR was used to determine the expression of CMYA5, Desmin, and Dtnbp1 in various mouse brain regions and cell lines.
<table>
<thead>
<tr>
<th>Gene (species)</th>
<th>Sequence (5’ → 3’)</th>
<th>Size (bp)</th>
<th>Annealing Temp / Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMYA5 (mouse &amp; rat)</td>
<td>F: TGTACTGGAGCGTGAAACAAGG</td>
<td>468</td>
<td>55°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: CATTGGTGCTCTCACATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMYA5 (human)</td>
<td>F: ACTGACAAAGGTGGTTGGCAC</td>
<td>325</td>
<td>65°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: TCTGCAGAAAGTGGACCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmin (mouse)</td>
<td>F: ATGGCCTTGGATGGAGATC</td>
<td>215</td>
<td>50°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: TGTTGTTGCTGTGTAGCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmin (mouse &amp; rat)</td>
<td>F: CTCAGGCAGCAATAAGAAGC</td>
<td>451</td>
<td>60°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: CCGGTCTCAATGGTGTTGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmin (human)</td>
<td>F: AAGCTGAGGAGTGGTACAAG</td>
<td>111</td>
<td>65°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: TCTGGTGTCGGTATCCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dtnbp1 (mouse &amp; rat)</td>
<td>F: CACTTCACAGAGAGCCAAAGG</td>
<td>250</td>
<td>60°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: AACTCAACTGCCCACACAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTNBP1 (human)</td>
<td>F: ATACATGGGCTGCACTTCAC</td>
<td>202</td>
<td>65°C / 30 cycles</td>
</tr>
<tr>
<td></td>
<td>R: CTCGCCCTCAAATGAGTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH (human)</td>
<td>F: ACAGTCAGGGCGCTTCTCTTT</td>
<td>94</td>
<td>60°C / 20 cycles</td>
</tr>
<tr>
<td></td>
<td>R: ACGACCAAAATCCGGACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gapdh (mouse)</td>
<td>F: TGGCAACAAATCTCCACTTTTG</td>
<td>111</td>
<td>60°C / 18 cycles</td>
</tr>
<tr>
<td></td>
<td>R: AGCCTCGTCCGTGGACAAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gapdh (rat)</td>
<td>F: ACGACCCCTTCATTGACC</td>
<td>589</td>
<td>63°C / 22 cycles</td>
</tr>
<tr>
<td></td>
<td>R: CAGTGAGCTCCCCGTTCAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Protein Extraction and Western Blotting Analysis

Tissues were homogenized and lysed in HENTS buffer (250mM HEPES-NaOH pH 7.7, 1mM EDTA, 1X protease inhibitor cocktail (Sigma-Aldrich), 1% Triton X-100, 0.1% SDS). Cells were harvested by washing with 1X PBS and briefly treated with 0.25% trypsin-EDTA (Gibco) at room temperature until cells dissociated from the flask. Cells were centrifuged at 1,000 rpm for 1 minute, followed by 1X ice-cold PBS wash. Cell pellets were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1% NP-40, 0.25% Na-deoxycholate) with 1X protease inhibitor cocktail. Tissue lysates were incubated on ice for 20 minutes, and cell lysates were incubated on ice for 10 minutes. Lysates were cleared by centrifuge at 14,000 rpm for 20 minutes at 4°C, twice. The supernatant contained the soluble proteins, and the insoluble pellet was further denatured with Buffer 1 (8M Urea, 10mM sodium phosphate buffer, pH 7.4, 0.1% β-mercaptoethanol) for the detection of desmin and other IFs (Leung & Liem, 2006). The lysate was mixed with SDS Sample Buffer (2X) (0.125M Tris-HCl, pH 6.8, 0.4% SDS, 2% β-mercaptoethanol, 20% glycerol, and 0.01 mg/mL bromophenol blue) in a 1:1 ratio and denatured at 95°C for 5 minutes or 75°C for 10 minutes for the detection of myospryn. The lysates were separated on 7.5% SDS-polyacrylamide (PAGE) gels or on NuPAGE® Novex 3-8% Tris-Acetate Gel (Invitrogen) for the detection of myospryn and electroblotted onto a PVDF membrane (BioRad) at 100 volts for approximately 2 hours. The membrane was blocked for 4 hours at room temperature or overnight at 4°C in 5% nonfat dry milk in TBST (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween-20) for the detection of myospryn. The membrane was then incubated with the primary
antibody overnight at 4°C, diluted in 5% milk in TBST, or one hour at room temperature for the UT266, myospryn antibody.

Primary antibodies that were used include rabbit anti-myospryn UT266 (Durham et al., 2006), a gift from Dr. Francisco Naya (Boston University) (1:2000), mouse anti-α-Tubulin (1:1000, Sigma-Aldrich), rabbit anti-desmin (1:1000, Abcam), goat Dysbindin D-20 (1:100-250, Santa Cruz Biotechnology), mouse Vimentin (1:1000, Novus Biologicals), and mouse Peripherin (1:1000, Novus Biologicals). The appropriate HRP secondary antibody was then added at a dilution of 1:10,000 for anti-rabbit and anti-mouse antibodies and 1:2,000 for anti-goat antibody in 5% milk in TBST for 30-45 minutes at room temperature. Visualization of the immunoreactive proteins was performed using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer, Inc.).

**Yeast Two-Hybrid Assay (Y2H)**

The specific alleles of the known interacting regions of *CMYA5* (NM_153610, nt 12205 to 12279), desmin (NM_001927, nt 258 to 395), alleles of a larger fragment of *CMYA5* (NM_153610, nt 11648 to 12279) that contains the minimum binding region to titin, and titin (NM_133378, nt 99776 to 100510) were first cloned in pBluescript SK+ (pBSSK+) via PCR and TA cloning. The primers have restriction endonuclease sites incorporated in them, which were then used for directional cloning into Y2H vectors. The primer sequence, the expected size, annealing temperature, and the restriction endonuclease of each primer pair are listed in Table 2. DNA of a subject who is heterozygous for that *CMYA5* allele was obtained from Dr. Xiangning Chen and was
used as the template for PCR. PCR reactions using CMYA5-RI-R primers can amplify both alleles, so the identity and integrity of each clone was determined by sequencing.

Full-length human peripherin and vimentin fragment were obtained from plasmids mEmerald-Peripherin-N-18 (Addgene Plasmid #54227) and mCherry-Vimentin-N-18 (Addgene Plasmid #55158), created by Michael Davidson. The mEmerald-Peripherin-N-18 plasmid was digested with Nhel and BamHI, followed by 5’-overhang fill-in and cloned into the pBSSK+ plasmid digested with EcoRV. The orientation of peripherin/pBSSK+ was determined by digesting with Ncol and EcoRI, with the correct orientation yielded 4.2kb and 262bp fragments and the wrong orientation yielded 3.2kb and 1.2kb. The peripherin/pBSSK+ was then subcloned to pAS2-1 using EcoRI & Sall. The mCherry-Vimentin-N-18 plasmid was digested with Nhel and AgeI, followed by 5’-overhang fill-in to clone into the pBSSK+ plasmid digested with EcoRV. The orientation of vimentin/pBSSK+ was determined by digesting with EcoRI and StyI, with the correct orientation yielded 4.0kb and 439bp fragments and the wrong orientation yielded 3.4kb and 1.0kb fragments. The vimentin fragment then cut out of the pBSSK+ plasmid using BamHI and subcloned into pAS2-1. The orientation of vimentin/pAS2-1 was determined by digesting with StyI, with the correct orientation yielded 9.4kb and 478bp and the wrong orientation yielded 8.8kb and 1.0kb fragments.
<table>
<thead>
<tr>
<th>Product Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Size (bp)</th>
<th>Annealing Temp</th>
<th>Restriction endonuclease</th>
</tr>
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<tbody>
<tr>
<td>CMYA5</td>
<td>CMYA5-Nco-F: CCATGGTTAATGAGGGTGTCACCCCT CMYA5-Ri-R: GAATTCTGCTGCTACGAGATCC</td>
<td>107</td>
<td>60°C</td>
<td>Nco</td>
</tr>
<tr>
<td>CMYA5T</td>
<td>CMYA5-Nco-F: CCATGGTTAATGAGGGTGTCACCCCT CMYA5-Ri-Ra: GAATTCTGCTGCTACGAGATCC</td>
<td>107</td>
<td>60°C</td>
<td>Nco</td>
</tr>
<tr>
<td>Desmin</td>
<td>Des-Ri-F3: GAATTCTGAGTTCGCCGGGTGTTTC Des-Sal-R: GTGCACGGTCAGAAACTCCTGTTCA</td>
<td>234</td>
<td>58°C</td>
<td>EcoRI</td>
</tr>
<tr>
<td>CMYA5tnC</td>
<td>CMYA5tn-NcoF: CCATGGGACTCCAGCTGAAAGTAAAC CMYA5-Ri-R: GAATTCTGCTGCTACGAGATCC</td>
<td>644</td>
<td>55°C</td>
<td>Nco</td>
</tr>
<tr>
<td>CMYA5tnT</td>
<td>CMYA5tn-NcoF: CCATGGGACTCCAGCTGAAAGTAAAC CMYA5-Ri-Ra: GAATTCTGCTGCTACGAGATCC</td>
<td>644</td>
<td>55°C</td>
<td>Nco</td>
</tr>
<tr>
<td>Titin</td>
<td>TTN-NcoF: CCATGGGCTCCAGAAATGTATACTCC TTN-Ri-R: GAATTGCACAGGCCCTTAAATGG</td>
<td>746</td>
<td>58°C</td>
<td>Nco</td>
</tr>
</tbody>
</table>
Both alleles of \textit{CMYA5} were cloned into a GAL4 activation domain (AD) vector (pACT2, Clontech Laboratories), and the corresponding binding fragment of desmin, titin, full-length peripherin and vimentin were cloned into the DNA-binding domain (BD) vector (pAS2-1, Clontech Laboratories). The Matchmaker Two-Hybrid System 2 (Clontech Laboratories) was used according to the manufacturer’s protocols. A reporter, β-galactosidase, was used to screen for positive interaction in solid colony-lift filter assay and liquid culture assays using X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and ONPG (ortho-Nitrophenyl-β-galactoside) as a substrate, respectively. Prior to the Y2H experiment, all clones in yeast vectors were transformed into yeast strain Y187 separately to ensure no activation for the reporter gene in the Colony-Lift Filter Assay. Then, \textit{CMYA5} C or T in pACT2 was transformed into Y187 that already contained Desmin/pAS2-1. The pACT2 vector alone was used as a negative control, and the pCL1 vector that encodes the full-length, wild-type GAL4 protein was used a positive control for β-galactosidase assays. For the Liquid Culture Assay, four or five separate transformant colonies were used and assayed in triplicate for each experiment, and the average of the triplicate is $n = 1$. The experiment was performed three times. A two-tailed $t$-test was used to determine if there was a difference in β-galactosidase units between the \textit{CMYA5} C allele and the T allele using JMP software. For other interactions, both co-transformation and sequential transformation were performed. For the sequential transformation, the AD vectors were transformed into yeast Y187 that already contained BD vectors/titin, peripherin or vimentin. The Colony-Lift Filter Assay was then performed to determine the interaction.
Protein Isolation

The two alleles of CMYA5, the same region as the Y2H experiments, were cloned into the pENTR3C vector (Invitrogen) via directional cloning. The CMYA5 C allele in pBSSK+ were digested with Smal and Xhol and ligated into the pENTR3C digested with XmnI and XhoI. The CMYA5 T allele fragment from pBSSK+ was digested with EcoRI and first cloned into CMYA5/pBSSK+ digested with EcoRI, and then cloned into CMYA5/pENTR3C using Ncol and Xhol. Both alleles were subsequently cloned into the Gateway expression vectors pDEST15 (GST tag) and pDEST17 (6X-HIS tag) (Invitrogen) via LR reaction using the Gateway LR Clonase II kit (Invitrogen). Desmin, the same region as the Y2H experiments, was cloned into the Biotin Carboxyl Carrier Protein (BCCP) vector (Kumar et al., 2011) (a gift from Dr. Qinglian Liu). BCCP was removed from pTrc·BCCP·DnaJ (106) and cloned into pBSSK+ using BamHI. The pTrc vector was then self-ligated. The stop codon in BCCP/pBSSK+ was removed by digesting with Agel and Spel and replaced with annealed oligonucleotides 5’-CCGGTAGAATTTGACGAGCCGCTG-3’ and 5’-CTAGCTCGATGACGACCAGCGCCGCTC-3’ that had Agel and Spel overhangs. The resulting vector (BCCPnX/pBSSK+) was then digested with Xbal and AleI, and Desmin/pBSSK+ was digested with Xbal and HindIII 5’ overhang fill-in to create BCCP-Desmin/pBSSK+. It was then digested with EcoRV and Sacl and cloned into pTrc vector digested with Sacl and Ncol 5’ overhang fill-in.

The fusion proteins were expressed in BL21(DE3) competent cells. The CMYA5-GST proteins were purified according to Smith & Johnson (1988) using Glutathione Agarose (Sigma-Aldrich). The Desmin-BCCP and CMYA5-HIS proteins were isolated
from denatured inclusion bodies (IBs) and then refolded, as described in Burgess (2009). Briefly, after lysing the bacteria that contained BCCP-Desmin, the IBs were solubilized using 0.3% Sarkosyl (N-Lauroylsarcosine) (Sigma-Aldrich). The denatured protein was slowly dripped, diluted 30-fold, into Base Protein Refolding Buffer (50mM Tris, 19mM NaCl, 0.8mM KCl, pH 8.2) contained 8 µM Biotin for one hour at room temperature. The refolded protein was then filtered through low protein binding 0.22 µM membrane filter (Corning) and concentrated using Amicon Ultra Centrifugal Filter Unit (Millipore). The IBs of CMYA5 C-HIS protein was solubilized using 0.5% Sarkosyl, and the IBs of CMYA5 T-HIS protein was solubilized using 6M guanidine hydrochloride (GuHCl) and 8M Urea. The denatured protein was refolded by flash diluted 20-fold into Base Protein Refolding Buffer for one hour at room temperature. The CMYA5-HIS proteins were further purified using HisPur Ni-NTA Resin according to the manufacturer’s Batch Method (Thermo Scientific). All proteins were checked by separating on a SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

**Surface Resonance Plasmon (SPR)**

SPR experiments were performed using Biacore T200 (GE Healthcare) with a Series S sensor chip CM5. The BCCP-Desmin ligand was immobilized using standard amine-coupling chemistry with a flow rate of 10µL/min in HBS-EP+ Buffer (GE Healthcare). The surface was activated with a 7 minutes pulse of EDC/NHS. The BCCP-Desmin protein, 15 µg/ml in 10mM sodium acetate pH 4.0, was injected for 420 seconds in one of the flow cells; flow cell 1 had buffer injected to serve as a reference
surface. A pulse of 1M ethanolamine pH 8.0 was injected to quench the reaction and remove non-specifically bound protein. The analytes (CMYA5 C, CMYA5 T, and GST or 6X-His) were injected over the flow cells at concentrations of 0, 0.25, 0.5, 1, 2, 4, 4, 8, and 16 µM (in random order) at a flow rate of 30 µL/min and at 25°C. The complex was allowed to associated and dissociated for 700 and 500 seconds, respectively. The surfaces were regenerated with two 40 seconds injection of 10 mM glycine-HCl pH1.5. Data were first analyzed by Biacore T200 Evaluation Software v.1.0, where the response is subtracted from a blank flow cell (Fc1), normalized response RU to 0 based on the injection start time, and subtracted from blank injection (0 µM), and the response for every 0.1 second was exported to Microsoft Excel. Response in resonance units versus time in seconds for various concentrations injected was graphed together as a sensorgram. The highest concentration injected (16µM or 8µM) was graphed separately, and the response for CMYA5 C and CMYA5 T was subtracted from the tag negative control (GST or 6X-HIS). The fold-change was determined by dividing the response of CMYA5 C over the response of CMYA5 T for every 0.1 second and then calculated the average.

Cell Culture

N18TG2 mouse neuroblastoma (a gift from Dr. Dana Selley), U87, U251 T98G human glioblastoma cell lines (gifts from Dr. Paul Fisher) were maintained in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with GlutaMAX™ (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technologies or Serum Source International). SH-SY5Y human neuroblastoma (a gift from Dr. Patricia
Trimmer) cell line was maintained in a 1:1 mixture of Eagle’s minimum Essential Medium (Sigma-Aldrich) and Ham's F-12 Nutrient Mix (Life Technologies) supplemented with 10% FBS. C2C12 mouse myoblast (ATCC) cell line was maintained in DMEM (high glucose, pyruvate) (Life Technologies) supplemented with 10% FBS. All cell lines were maintained in 5% CO₂ at 37 °C.

**Expression Vector Construct and Transfection**

The larger fragment of both CMYA5 alleles were cloned into pENTR3C by digesting CMYA5tnC&T/pBSSK+ with Ncol and EcoRI followed by 5’ overhang fill-in by DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) and digesting pENTR3C with Dral and EcoRV; then the excised CMAY5tnC&T fragments were ligated to pENTR3C via blunt-end ligation. The two alleles of CMYA5 were cloned into the N-terminus FLAG tag pDEST 26 vector (pD26 N-FLAG) (Amr et al., 2007), via LR reaction. The pD26 N-FLAG without ccdB gene vector was constructed by digesting pD26N-FLAG vector with BglII and SalI, followed by 5’ overhang fill-in and self-ligation. The pD26N-FLAG(-ccdB) vector was used as a positive control for transfection experiments.

mEmerald-Desmin-N-18 was created by Michael Davidson (Addgene plasmid # 54060).

The mEmerald-Desmin-N-18 plasmid was transfected into T98G using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. T98G cells (1 x 10⁶ cells) were grown on coverslips in a CELLSTAR® Tissue Culture 6-well plate (Greiner Bio-One) overnight. Each well was transfected using 7.5 µL Lipofectamine 2000 (Invitrogen) in 125 µL serum free DMEM/F12 mixed with 5 µg of mEmerald-
Desmin-N-18 in 125 µL serum free DMEM/F12, incubated the DNA-lipid complex for 5 minutes, and added them to the cells and incubated for 24 hours.

For other transfections, 1 x 10^6 cells were grown on Poly-L-Lysine (Sigma-Aldrich) coated coverslips in a 6-well plate overnight. Each well was transfected using 5 µL Lipofectamine 2000 (Invitrogen) in 125 µL serum free DMEM/F12 mixed with 5 µg of CMYA5tnC or CMYA5tnT in pD26N-FLAG vector in 125 µL serum free DMEM/F12, incubated the DNA-lipid complex for 5 minutes, and added them to the cells and incubated for 48 hours.

For serum starvation experiments, 1 x 10^6 cells were grown on Poly-L-Lysine coated coverslips in a 6-well plate overnight. The cells were then maintained in DMEM supplemented with 0.5% FBS for overnight. The cells were then transfected as described above.

**Immunocytochemistry and Confocal Laser Scanning Microscopy**

To determine endogenous expression of myospryn, desmin, dysbindin, vimentin in N18TG2, U251, SH-SY5Y, T98G, and C2C12 cell lines, 2 x 10^6 cells were grown on Poly-L-Lysine coated coverslips in a 6-well plate overnight. Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature, methanol for 10 minutes at -20°C for dysbindin and peripherin staining, and methanol for 10 minutes and acetone for 1 minute at -20°C for rabbit anti-FLAG staining. After washing three times with cold PBS, cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes at room temperature, or no permeabilization for methanol fixed cells. Cells were blocked with 5% donkey serum (Sigma-Aldrich) and 0.3% Triton X-100 in PBS for
secondary antibodies raised on donkey or 1% bovine serum albumin (BSA) in PBST (PBS + 0.05% Tween-20) for 1 hour at room temperature for other secondary antibodies, or both for double staining. Cells were then incubated with primary antibodies diluted in blocking solution for 1 hour at room temperature. Primary antibodies used include UT266 myospryn ab (1:200), mouse Desmin D33 (Thermo Scientific) (1:100), rabbit Desmin (Abcam) (1:200), Dysbindin D20 ab (1:100), Vimentin ab (1:100), Peripherin ab (1:250), mouse anti-FLAG M2 (Sigma-Aldrich) (1:250), and rabbit anti-FLAG (Sigma-Aldrich) (1:100). After washing three times in PBST, cells were incubated with Alexa Fluor 568 donkey anti-rabbit, Alexa Fluor 647 donkey anti-goat, Alexa Fluor 488 chicken or donkey anti-mouse (Molecular Probes) at dilution of 1:250 in 5% BSA for 1 hour at room temperature. Cells were washed three times in PBST and counterstained with 0.1 µg/mL of DAPI (Sigma-Aldrich) for 30 minutes at room temperatures. Coverslips were then mounted to Fisherbrand Superfrost Plus microscope slides using SlowFade Diamond Antifade Mountant (Life Technologies).

To study the colocalization between the FLAG signal and F-actin, cells were fixed with 4% PFA/PBS + 1X Cytoskeleton Buffer (10mM MES pH 6.1, 138mM KCl, 3mM MgCl, 2mM EDTA) + 10% sucrose for 10 minutes at room temperature. After washing twice with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Cells were blocked with 1% BSA in PBST for 1 hour at room temperature. Cells were incubated with mouse anti-FLAG M2 (1:250) and Alexa Fluor 594 Phalloidin (a gift from Dr. Babette Fuss) at 4°C overnight. Next day, cells were incubated with secondary antibody and followed the steps described above. To study the colocalization between the FLAG signal and acetylated α-Tubulin, cells were fixed
with 4% PFA/PBS + 1X Cytoskeleton Buffer+ 10% sucrose for 10 minutes at room temperature. After washing twice with PBS, cells were permeablized with methanol for 3 minutes at -20°C. Cells were block with 5% donkey serum + 0.3% Triton X-100 in PBS + 1% BSA in PBS for 1 hour at room temperature. Cells were incubated with rabbit anti-FLAG (1:100) and mouse acetylated α-Tubulin (1:100; a gift from Dr. Babette Fuss) for 1 hour at room temperature. Cells were incubated with secondary antibodies and followed the steps described above.

Images were obtained using Zeiss LSM 700 Confocal Laser Scanning Microscopy with Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Zeiss Zen Software (Black Edition) was used to determine the colocalization, and the degree of colocalization is categorized according to Zinchuk & Grossenbacher-Zinchuk (2014).
CHAPTER 3: RESULTS

Myospryn and Desmin Expression

Mouse *Cmya5* and *Desmin* transcripts are shown to express in whole brain, striatum, hindbrain, hippocampus, cerebellum, cortex, and skeleton muscle using RT-PCR (Figure 2). Myospryn transcript is also expressed in N18TG2, primary mouse mixed glial cultures, primary mouse cortical neurons, SH-SY5Y, U87, U215, and T98G cell lines; desmin is expressed in N18TG2, C2C12, primary mouse mixed glial cultures, primary mouse cortical neurons, U251, and T98G cell lines (Figure 3). Western blotting analysis using the anti-myospryn antibody UT266 (Durham *et al*., 2006) confirmed the expression of myospryn in the same rat brain regions (Figure 4A). Desmin, which is a type III IF, is highly insoluble in conventional extraction buffer, so the protein extraction is performed under denaturing conditions using chaotropic reagents, such as 8M urea (Leung & Liem, 2006). However, such reagents cause distortion of the polyacrylamide gel during electrophoresis and bleed-through into another lanes. Nonetheless, the result showed that desmin is expressed in the insoluble fraction of the same mouse brain regions (Figure 4B).
Figure 2: Myospryn and desmin mRNA expression in mouse brain regions. Using RT-PCR, *myospryn* and *desmin* transcripts are shown to express in mouse brain regions including whole brain, striatum, hindbrain, hippocampus, cerebellum, and cortex. Skeleton muscle is used as a positive control. -RT is the negative control for the cDNA synthesis to ensure no genomic DNA contamination, and the ddH$_2$O is the negative control for PCR.
Figure 3: Myospryn and desmin mRNA expression in cell lines.
A) Using RT-PCR, the expression of *myospryn* and *desmin* in mouse cell lines (N18TG2 and C2C12), mouse mixed glia and cortical neuron, and rat mixed glia are shown. Mouse brain and skeletal muscle are used as positive controls for RT-PCR, and Gapdh is used as the positive control for the cDNA synthesis. B) Using RT-PCR, the expression of *myospryn* and *desmin* in various human cell lines (SH-SY5Y, U87, U251, and T98G) are shown. Human frontal cortex and skeletal muscle are used as positive controls for RT-PCR, and GAPDH is used as the positive control for the cDNA synthesis. -RT is the negative control for cDNA synthesis to ensure no genomic DNA contamination, and the ddH₂O is the negative control for PCR.
Figure 4: Western blot analysis of myospryn and desmin in brain regions.
A) Myospryn is expressed in rat striatum (STR), hindbrain (HB), hippocampus (HPC), cerebellum (CER), and cortex (CTX). B) Desmin is expressed in the insoluble fraction of mouse STR, HB, HPC, CER, and CTX. *Lysates were extracted under denaturing condition. α-Tubulin is used as a loading control.
**Binding property of rs10043986 to desmin**

rs10043986 changes the conserved amino acid 4063 from Pro to Leu, carboxyl to the SPRY domain of myospryn. Since the binding of myospryn to desmin takes place in this region and the binding sequence is short and precisely mapped, Y2H was used to investigate whether the two variants of rs10043986 would change myospryn binding to desmin. The desmin/BD vector was first transformed into the yeast strain Y187, and then CMYA5 C, CMYA5 T, and pACT vectors were sequentially transformed into the yeast. White colonies in the Colony-Lift Filter Assay using X-Gal as substrate indicates the two proteins do not interact, whereas blue colonies indicates the two proteins interact. The result shows that both alleles of myospryn bind to desmin in the Colony-Lift Assay, as indicated by the blue colonies (Figure 5). The result showed that both alleles of myospryn interact with desmin and the amino acid change does not abolish the binding. To further investigate if the two alleles have differential binding to desmin, a quantitative assay using liquid cultures and ONPG as a substrate was used. The hydrolysis of ONPG by β-galactosidase produces a yellow color that can be quantified using colorimetric absorption. In three independent experiments where each experiment assayed four or five colonies, the major allele (Pro or C allele) shows weaker binding compared to the minor allele (Leu or T allele), as shown in Figure 6 ($t = 3.47$, $df = 25$, p-value = 0.0019).
Figure 5: Y2H Colony-Lift Filter Assay.
Both CMYA5 C allele and T alleles bind to desmin, which resulted in the colonies turned blue. The CMYA5 C, CMYA5T, and pACT2 activation domain vectors were transformed into yeast Y187 that already contain desmin-DNA-binding vector. pACT2 vector contains no insert that serves as a negative control. pCL1 has full-length GAL4 that serves as a positive control for the assay.
Figure 6: Y2H Liquid Culture Assay using ONPG as the substrate.
The average β-galactosidase activity for CMYA5 C allele is 1.10 units (SE = 0.063), and the average β-galactosidase activity for CMYA5 T allele is 1.42 units (SE = 0.066). The observed means are significantly different (t = 3.47, df = 25, p-value = 0.0019). One unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of ONPG to o-nitrophenol and D-galactose per minute.
Next, proteins were expressed and purified from bacteria to perform the SPR experiment. The two alleles of *CMYA5*-GST with a GST tag were isolated using the Glutathione pull-down procedure, and the purity of the fusion proteins were checked on a SDS-PAGE gel and stained with Coomassie Blue. Figure 7A indicated that the protein that will be used for the SPR experiment was not completely pure. The identities of the fusion proteins were confirmed using western blot analysis with anti- *CMYA5* and anti-GST (Figure 7B). BCCP-Desmin protein was insoluble and mostly expressed in the IBs. The IBs were denatured using three different denaturants, 6M GuHCl, 8M Urea, and 0.3% Sarkosyl, and the proteins in IBs were then refolded in the presence of biotin. The purity of the proteins was checked by Coomassie stain (Figure 8A). The BCCP tag on desmin is biotinylated, which can be pull-down by Streptavidin beads, and the pull-down protein was checked by western blotting (Figure 8B). The results showed that only the IBs denatured with 0.3% Sarkosyl contained BCCP-Desmin that can be pulled-down with Streptavidin, indicating 0.3% Sarkosyl denaturant works best.
Figure 7: Coomassie staining and Western Blot Analysis of GST fusion proteins.  
A) The purified GST fusion proteins were run on SDS-PAGE and stained with Coomassie Blue to check the purity of the proteins. The red box highlighted the protein at the correct predicted size.  
B) The proteins were then checked with western blotting with anti-CMYA5 (top) and anti-GST (bottom). The GST tag is a relative large and is located at the N-terminal. The multiple bands observed in the anti-GST blot is likely due to the bacterial did not translate the complete transcript or due to degradation of the fusion protein.
Figure 8: Coomassie staining and Western Blot Analysis of BCCP-Desmin.
A) The IBs from bacteria expressed BCCP-Desmin were denatured using 6M GuHCl, 8M Urea, or 0.3% Sarkosyl, and the proteins were then refolded. The proteins were run on SDS-PAGE and stained with Coomassie Blue to check the purity of the proteins. The red box highlighted the protein at the correct predicted size. B) The BCCP tag was biotinylated, which can be pulled-down by streptavidin beads. Western blot analysis using anti-Desmin was performed on the pull-down proteins, and the result showed only 0.3% Sarkosyl denatured IBs can refold properly to be pulled-down by streptavidin.
SPR using BiaCore T200 was used to verify the differential binding observed. Figure 9A showed sensorgrams (response in resonance units versus time in seconds) for injection of CMYA5C-GST, CMYA5T-GST, and GST negative control over a CM5 sensor chip surface immobilized with BCCP-Desmin. The analytes were allowed to associate with ligand for 700 seconds and then dissociate for 500 seconds. However, the response never reached a steady-state, where the all the ligands were occupied by analytes and the response would stay constant at the end of injection phase. Overall, CMYA5 T (Leu allele) has higher RU than CMYA5 C (Pro allele), and the GST tag negative control had relatively high response, indicating high non-specific binding. Comparing the 16 µM injection of each analyte after GST response subtraction, it shows that CMYA5 T has 1.5 and 1.6-fold higher response than the CMYA5 C (Figure 9B). However, the relative high response for the GST alone negative control revealed a potential problem with using this protein tag. This is likely due to its large molecular weight and its ability to dimerize. Therefore, a different tag, 6X-His, was used to repeat the experiment.

The CMYA5-HIS proteins were insoluble and expressed in the IBs fraction. Thus, the IBs were denatured proteins extracted, followed by refolding and purifying using Ni-NTA Resin. The purified proteins were then checked on SDS-PAGE and stained with Coomassie blue, as shown in Figure 10. The 6X-HIS negative control proteins were soluble, so the soluble lysates were purified using Ni-NTA Resin. However, other proteins that contained HIS were also pulled-down, as shown in Figure 10.
Figure 9: SPR with injections of *CMYA5 C*, *CMYA5 T*, and GST.

A) Sensorgrams for injection of *CMYA5 C*, *CMYA5 T*, and GST negative control over a sensor chip surface immobilized with desmin (top = 1778 RU; bottom = 1833 RU). The proteins are allowed to associate for 700 seconds and dissociate for 500 seconds, and the concentrations used are, 0.5, 1, 2, 4, 8, and 16 µM. B) Sensorgrams for comparing 16 µM inject of all three analytes (top) and after 6X-GST subtraction (middle). Fold-change (bottom) is determined by *CMYA5 T* RU divided by *CMYA5 C* RU. *CMYA5 T* has about 1.5-fold (left) and 1.6-fold (right) higher RU than the *CMYA5 C*. 
Figure 10: Coomassie staining of CMYA5-HIS, CMYA5T-HIS and 6X-HIS proteins. The CMYA5 proteins were extracted from the IBs, refolded, and purified using Ni-NTA resins. The 6X-HIS proteins were purified from the soluble fraction using Ni-NTA resins. The purified proteins were run on SDS-PAGE and stained with Coomassie Blue. The predicted molecular weights of CMYA5-HIS and 6X-HIS are 10.3kDa and 3.0 kDa, respectively and indicated by the red boxes.
The SPR experiment was repeated using $CMYA5$-HIS and 6X-HIS negative control. Again, the analytes were injected over a CM5 sensor chip surface immobilized with BCCP-Desmin. The result also shows that $CMYA5$ T allele has higher responses than the $CMYA5$ C allele (Figure 11A). The 6X-HIS negative control had low response, suggesting the protein tag does not interfere the binding and have low binding to BCCP-Desmin. The highest injected concentration 8 $\mu$M has 2.4-fold and 1.8-fold higher RU over two experiments (Figure 11B). The Y2H and SPR experiments showed the amino acid change results in differential binding.
Figure 11: SPR with injections of *CMYA5 C*, *CMYA5 T*, and 6X-HIS.
A) Sensorgrams for injection of *CMYA5 C*, *CMYA5 T*, and 6X-His negative control over a sensor chip surface immobilized with desmin (top = 570 RU; bottom = 1400 RU). The proteins are allowed to associate for 700 seconds and dissociate for 500 seconds, and the concentrations used are 0.25, 0.5, 1, 2, 4, and 8 µM. B) Sensorgrams for comparing 8 µM inject of all three analytes (top) and after 6X-HIS subtraction (middle). Fold-change (bottom) is determined by *CMYA5 T* RU divided by *CMYA5 C* RU. *CMYA5 T* has about 2.4-fold (left) and 1.8-fold (right) higher RU than the *CMYA5 C* after 6X-HIS subtraction.
Subcellular Localization of Myospryn and Desmin

Using the C2C12 cell line as a positive control, myospryn was localized in the cytoplasm and nucleus (Figure 12A). A similar localization pattern was observed in the N18TG2 mouse neuroblastoma and T98G human glioblastoma cell lines (Figure 9A). Different organisms (mouse and human) and different cell types (neuroblastoma and glioblastoma) show similar results, suggesting the localization is not organism- or cell type-specific. Desmin was localized in the cytoplasm of the C2C12 cells, but the endogenous expression in N18TG2 and T98G was too weak to visualize. A full-length human desmin was transfected to determine its localization. Transfected desmin was localized in the cytoplasm, similar to the endogenous localization of desmin in C2C12, and only localized in the perinuclear region (Figure 9A).

Next, the colocalization of myospryn and desmin was determined using Zeiss Zen Software (Black Edition), where the white color in far right panels showed pixels that contained both red and green signals (Figure 12A). Figure 12B describes the colocalization coefficients, Mander’s overlap coefficient (MOC), and Pearson’s correlation coefficient (PCC). The degrees of colocalization can be categorized as very weak (MOC = 0 to 0.49; PCC = −1.0 to −0.27), weak (MOC = 0.50 to 0.70; PCC = −0.26 to 0.09), moderate (MOC = 0.71 to 0.88; PCC = 0.1 to 0.48), strong (MOC = 0.89 to 0.97; PCC = 0.49 to 0.84), and very strong (MOC = 0.98 to 1.0; PCC = 0.85 to 1.0), as described in Zinchuk & Grossenbacher-Zinchuk (2014). Colocalization coefficients describe the contribution of each one of two selected channels to the pixels of interest. The degree of colocalization for colocalization coefficients is categorized as very weak (0 to 0.54), weak (0.55 to 0.77), moderate (0.78 to 0.94), strong (0.95 to 0.98), and very
strong (0.99 to 1.0), as described in Zinchuk & Grossenbacher-Zinchuk (2014). For example, the numbers 0.570 and 0.959 in the first row of Figure 12B mean that 57.0% of myospryn (red) pixels colocalize with desmin (green) pixels and 95.9% desmin (green) pixels colocalize with myospryn (red) pixels. Pearson’s correlation coefficient describes the correlation of the intensity distribution between channels. It ranges from -1 to 1, where 0 indicates no significant correlation and -1 indicates complete negative correlation. Overlap coefficient according to Manders indicates an overlap of the signals and is insensitive to differences in the signal intensity between the two channels. Its value ranges from 0 to 1.0 (e.g. 0.5 means 50% of both pixels overlap).

Although previous studies have shown that myospryn physically interacts with desmin in muscle (Kouloumenta et al., 2007), the quantitative colocalization analysis shows that there is only weak to moderate colocalization between myospryn and desmin in C2C12. Myospryn and transfected desmin in N18TG2 are weakly (MOC = 0.61) to moderately (PCC = 0.12) colocalized, similar to the degree of colocalization in C2C12 (MOC = 0.64; PCC = 0.12), while T98G has a stronger degree of colocalization (MOC = 0.72; PCC = 0.38) (Figure 12B). Interestingly, greater than 90% of desmin colocalized with myospryn and only about 50% of myospryn colocalized with desmin, suggesting that myospryn might have additional binding partners and diverse functions.
Figure 12: Subcellular localization of myospryn and colocalization with desmin.
A) Images of DAPI, myospryn, and desmin (or transfected desmin) staining in C2C12 (top), N18TG2 (middle), and T98G (bottom) show myospryn is localized in both cytoplasm and nucleus and desmin is localized in cytoplasm. Merged images were analyzed for colocalization, where the white color in far right panels showed pixels that contained both red and green signals. Scale bar = 20µm. B) Colocalization coefficients, overlap coefficient, and correlation coefficient for myospryn and desmin. Number of images (N) was obtained using Zeiss LSM 700 Confocal Laser Scanning Microscopy with the same setting within a cell line and analyzed for colocalization. Colocalization analysis was done using Zeiss Zen Software (Black Edition). For C2C12, the analysis was performed on whole image, whereas the analysis for N18TG2 and T98G was performed only on cells expressed transfected desmin. All coefficients numbers were mean ± 95% confidence interval. The degree of colocalization is categorized below each coefficient, as described in Zinchuk & Grossenbacher-Zinchuk (2014).
Desmin, Peripherin, and Vimentin – Type III Intermediate Filaments

Desmin is a muscle-specific type III intermediate filament, and other members of type III IF include syncoilin, vimentin, peripherin and glial fibrillary acidic protein (GFAP) (Hnia et al., 2015). Since desmin is a muscle-specific type III IF and endogenous expression could not be detected in neuroblastoma and glioblastoma cell lines by immunohistochemistry, we reasoned that other IFs might be binding to myospryn in those cells. Kouloumenta et al., 2007, found that the amino terminal of desmin (aa 58-103) interacts with the last 24 aa of myospryn. Performing protein BLAST from the NCBI website using GenBank™ accession number P31001, from aa 58 to 103 against mouse and human protein databases revealed peripherin and vimentin are highly similar to this region of desmin. This region of mouse desmin shares 91% (43/47) identity with human desmin, 61% (23/38) and 58% (23/40) identity with mouse and human peripherin, and 69% (11/16) identity with mouse and human vimentin (Figure 13).

Both peripherin and vimentin are expressed in similar mouse brain regions, as well as neuroblastoma, glioblastoma, and myoblast cell lines (Figure 14). The peripherin and vimentin protein is mostly expressed in the insoluble fraction of lysates, but some soluble expression was also observed.

Peripherin is expressed in the cytoplasm of N18G2 cells (Figure 15A) and is weakly colocalized with myospryn (MOC = 0.55; PCC = 0.09) (Figure 15B). No expression of peripherin was observed in U87 and U251 cells using immunocytochemistry (data not shown) even though the expression was observed using western blotting analysis (Figure 14B).
Vimentin is also expressed in the cytoplasm (Figure 15C) and is moderately colocalized with myospryn in SH-SY5Y (MOC = 0.76; PCC = 0.42) and weakly colocalized in T98G (MOC = 0.65; PCC = 0.06) (Figure 15D). The colocalization of myospryn with vimentin or peripherin is very similar to the colocalization of myospryn and desmin, suggesting the possible association of vimentin and peripherin to myospryn in neuronal and glial cell types.
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<tbody>
<tr>
<td>desmin [Mus musculus]</td>
<td>NP_034173</td>
<td>RTSGGAGGLGSLRSSLGT-----TRAPS-YGAG-----ELLDFSADAVNQEFLAT</td>
</tr>
<tr>
<td>desmin [Homo sapiens]</td>
<td>AAA99221</td>
<td>RTSGGAGGLGSLRASRLGT-----TRTPSsYGAG-----ELLDFSADAVNQEFLT</td>
</tr>
<tr>
<td>peripherin [Mus musculus]</td>
<td>CAA42500</td>
<td>SSA------RLGSPRPRAGA------LRLPS----------ERLDFSMAEALNQEFLAT</td>
</tr>
<tr>
<td>peripherin [Homo sapiens]</td>
<td>NP_006253</td>
<td>SSA------RLGSPRPRAGA------LRLPS----------ERLDFSMAEALNQEFLAT</td>
</tr>
<tr>
<td>vimentin [Mus musculus]</td>
<td>BAA19834</td>
<td>SRSYSSSPGAYVTRSSA------VRGSR--SVPGvrllqDSVDFSADAINTEFKNT</td>
</tr>
<tr>
<td>vimentin [Homo sapiens]</td>
<td>NP_003371</td>
<td>SRSYASSSPGAYATRSSA------VRGSR--SVPGvrllqDSVDFSADAINTEFKNT</td>
</tr>
</tbody>
</table>

**Figure 13: Mouse and human desmin, peripherin, and vimentin alignment.**
Using COBALT Constraint-based Multiple Protein Alignment Tool, mouse desmin, aa 58 to 103, was aligned to mouse and human desmin, peripherin, and vimentin. The red color indicates highly conserved columns and blue indicates less conserved one; this is determined using 2 Bits Conservation Setting.
Figure 14: Western blotting analysis of peripherin and vimentin protein expression.
A) Western blotting analysis showed that peripherin and vimentin are expressed in both soluble and insoluble brain regions (STR, HB, HPC, CER, and CTX). The predicted size for peripherin was 57kDa and vimentin was 50kDa. For the insoluble brain regions, the 100kDa band is likely a dimer. B) Western blotting analysis showed that peripherin and vimentin are expressed in neuroblastoma (SH-SY5Y, N18TG2), glioblastoma (U87, U251, T98G), and C2C12 myoblast cell lines. Multiple bands were observed around the predicted size, likely due to post-translational modifications. * means the lysates were extracted under denaturing condition using 8M urea. α-Tubulin is used as a loading control.
Figure 15: Colocalization of peripherin and vimentin to myospryn.
A) Images of DAPI, myospryn, and peripherin staining in N18TG2 showed peripherin is localized in the cytoplasm. Merged images were analyzed for colocalization, where the white color in far right panel showed pixels that contained both red and green signals. Scale bar = 20 µm. B) Colocalization coefficients, overlap coefficient, and correlation coefficient for myospryn and peripherin. Peripherin is weakly colocalized with myospryn in N18TG2. C) Images of DAPI, myospryn, and vimentin staining in SH-SY5Y (top) and T98G (bottom) showed vimentin is localized in the cytoplasm. Merged images were analyzed for colocalization, where the white color in far right panel showed pixels that contained both red and green signals. Scale bar = 20 µm. D) Colocalization coefficients, overlap coefficient, and correlation coefficient for myospryn and vimentin. Vimentin is moderately colocalized with myospryn in SH-SY5Y and weakly colocalized in T98G. All coefficients numbers are mean ± 95% confidence interval.
Colocalization between two alleles of myospryn and IFs

A region of CMYA5 that harbors both SNP alleles fused to N-terminus FLAG tag was transfected into C2C12 cells to determine if the polymorphism affects its colocalization to desmin. The colocalization of FLAG signal and desmin in C2C12 was determined and no difference was observed (Figure 16A). The representative images of CMYA5-FLAG (green), desmin (red), and DAPI (blue) were shown in Figure 16B. The transfected CMYA5-FLAG is mainly expressed in the nucleus with some cytoplasmic staining; only cells contained the FLAG signal were used for colocalization analysis. The experiment was repeated two more times to ensure that the rs10043986 does not affect the colocalization between CMYA5-FLAG and desmin. The total number (N) of images analyzed, the weighted average, and the 95% confidence interval of all the coefficients were summarized in Table 3, and no difference was observed. Overall, both alleles have a very weak to weak colocalization with desmin, in comparison with a weak to moderate colocalization between endogenous myospryn and desmin.
Figure 16: Colocalization of Desmin and CMYA5-FLAG in C2C12.
A) No significant difference is observed between the C and the T allele in all coefficients, and both alleles have a very weak to weak degree of colocalization to desmin in C2C12. Error bar indicates 95% confidence interval. B) A representative merge image of CMYA5-FLAG (green), desmin (red), and DAPI (blue). The left image showed that CMYA5-FLAG is expressed in the cytoplasm and nucleus, whereas desmin is expressed in the cytoplasm. The white color on the right image showed pixels that contained both red and green signals, and the red line indicates the area that colocalization is being analyzed. Scale bar = 20µm.
Table 3: Colocalization of Desmin and CMYA5-FLAG in C2C12

<table>
<thead>
<tr>
<th>N</th>
<th>Colocalization Coefficient Desmin</th>
<th>Colocalization Coefficient CMYA5-FLAG</th>
<th>Overlap Coefficient (Manders)</th>
<th>Correlation Coefficient (Pearson’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>54</td>
<td>0.737 ± 0.13 Weak</td>
<td>0.583 ± 0.17 Weak</td>
<td>0.46 ± 0.11 Very Weak</td>
</tr>
<tr>
<td>T</td>
<td>51</td>
<td>0.688 ± 0.15 Weak</td>
<td>0.623 ± 0.09 Weak</td>
<td>0.42 ± 0.14 Very Weak</td>
</tr>
</tbody>
</table>
The same experiment was also performed on N18TG2 cells to determine the colocalization between \textit{CMYA5-FLAG} and peripherin (Figure 17), as well as on SH-SY5Y and T98G cells for the colocalization between \textit{CMYA5-FLAG} and vimentin (Figure 18). No difference in colocalization between two alleles of myospryn to IFs, vimentin and peripherin, in neuroblastoma and glioblastoma cell lines was observed (Figures 17-18).

As previously noted, the transfected \textit{CMYA5-FLAG} is mainly expressed in the nucleus with some cytoplasmic staining, whereas the endogenous myospryn has a different expression pattern – mainly in the cytoplasm and low expression in the nucleus. To account for this difference, only the cytoplasm of cells that expressed \textit{CMYA5-FLAG} were used to analyze the colocalization between the two alleles of \textit{CMYA5-FLAG} and vimentin in T98G (Table 4). The analysis was performed on the same set of images as Figure 18C&D, and the result showed the percent of \textit{CMYA5-FLAG} colocalizes with vimentin increased from 0.424 to 0.629 for the C allele and 0.395 to 0.625 for the T allele (Table 4). This increase is expected - as vimentin is only expressed in cytoplasm, excluding the FLAG signal from the nucleus would increase the proportion of the FLAG signal colocalized with vimentin. However, no difference in the other coefficients was observed, as well as no difference in the cytoplasmic colocalization between two alleles to vimentin was observed.

In addition, to determine if other conditions would affect the colocalization, the C2C12 cells were serum starved for overnight prior to the transfection. No difference in colocalization between the two alleles of \textit{CMYA-FLAG} to desmin with starvation and between starved and non-starved cells was observed, as summarized in Table 5.
Figure 17: Colocalization of *CMYA5-FLAG* and peripherin in N18TG2.
A) No significant difference is observed between the C and the T allele in all coefficients, and both alleles have a weak to moderate colocalization to peripherin in N18TG2. Error bar indicates 95% confidence interval. B) A representative merge image of *CMYA5-FLAG* (red), peripherin (green), and DAPI (blue). The left image showed that *CMYA5-FLAG* is expressed in the cytoplasm and nucleus, whereas peripherin is expressed in the cytoplasm. The white color on the right image showed pixels that contained both red and green signals, and the red line indicates the area that colocalization is being analyzed. Scale bar = 20µm.
Figure 18: Colocalization of \textit{CMYA5-FLAG} and Vimentin in SH-SY5Y and T98G.
A) No significant difference is observed between the C and the T allele in all coefficients, and both alleles have a weak colocalization to vimentin in SH-SY5Y. B&D) A representative merge image of \textit{CMYA5-FLAG} (red), vimentin (green), and DAPI (blue). The left image showed that \textit{CMYA5-FLAG} is expressed in the cytoplasm and nucleus, whereas vimentin is expressed in the cytoplasm. The white color on the right image showed pixels that contained both red and green signals, and the red line indicates the area that colocalization is being analyzed. Scale bar = 20µm. C) No significant difference is observed between the C and the T allele in all coefficients, and both alleles have a very weak to weak colocalization to vimentin in T98G. Error bars indicate 95% confidence interval.
Table 4: Colocalization of CMYA5-FLAG and Vimentin in cytoplasm of T98G cells

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Colocalization Coefficient CMYA5-FLAG</th>
<th>Colocalization Coefficient Vimentin</th>
<th>Overlap Coefficient (Manders)</th>
<th>Correlation Coefficient (Pearson’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (cytoplasm)</td>
<td>16</td>
<td>0.629 ± 0.09 Weak</td>
<td>0.706 ± 0.12 Weak</td>
<td>0.52 ± 0.05 Weak</td>
<td>-0.07 ± 0.06 Weak</td>
</tr>
<tr>
<td>T (cytoplasm)</td>
<td>16</td>
<td>0.625 ± 0.09 Weak</td>
<td>0.654 ± 0.11 Weak</td>
<td>0.49 ± 0.05 Very Weak</td>
<td>-0.20 ± 0.06 Weak</td>
</tr>
<tr>
<td>C (whole cell)</td>
<td>16</td>
<td>0.424 ± 0.08 Very Weak</td>
<td>0.719 ± 0.11 Weak</td>
<td>0.50 ± 0.06 Weak</td>
<td>-0.09 ± 0.06 Weak</td>
</tr>
<tr>
<td>T (whole cell)</td>
<td>16</td>
<td>0.395 ± 0.10 Very Weak</td>
<td>0.662 ± 0.11 Weak</td>
<td>0.48 ± 0.05 Very Weak</td>
<td>-0.20 ± 0.06 Weak</td>
</tr>
</tbody>
</table>
## Table 5: Colocalization of Desmin and CMYA5-FLAG with serum starvation

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Colocalization Coefficient Desmin</th>
<th>Colocalization Coefficient CMYA5-FLAG</th>
<th>Overlap Coefficient (Manders)</th>
<th>Correlation Coefficient (Pearson’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (starved)</td>
<td>19</td>
<td>0.671 ± 0.11 Weak</td>
<td>0.455 ± 0.09 Very Weak</td>
<td>0.48 ± 0.06 Very Weak</td>
<td>0.01 ± 0.05 Weak</td>
</tr>
<tr>
<td>T (starved)</td>
<td>13</td>
<td>0.718 ± 0.08 Weak</td>
<td>0.650 ± 0.11 Weak</td>
<td>0.46 ± 0.07 Very Weak</td>
<td>-0.02 ± 0.10 Weak</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>0.764 ± 0.08 Weak</td>
<td>0.560 ± 0.06 Weak</td>
<td>0.48 ± 0.05 Very Weak</td>
<td>-0.02 ± 0.05 Weak</td>
</tr>
<tr>
<td>T</td>
<td>17</td>
<td>0.678 ± 0.12 Weak</td>
<td>0.581 ± 0.07 Weak</td>
<td>0.45 ± 0.06 Very Weak</td>
<td>-0.02 ± 0.06 Weak</td>
</tr>
</tbody>
</table>
Colocalization between two alleles of myospryn and cytoskeleton

Desmin, peripherin, and vimentin are all type III intermediate filaments, which is a component of the cytoskeleton. In addition to IFs, the cytoskeleton is also comprised of microtubules (tubulin) and microfilaments (actin). Proteomics research in schizophrenia has revealed changes in cytoskeletal components (Davalieva et al., 2016). Therefore, the colocalization between alleles of myospryn and cytoskeletal markers was also investigated. Two different alleles of CMYA5-FLAG were transfected into SH-SY5Y, and the transfected cells were stained for anti-FLAG and one of the two cytoskeletal markers, acetylated α-Tubulin and Phalloidin for F-actin. No difference in colocalization between two alleles of myospryn to acetylated α-Tubulin in SH-SY5Y cells was observed (Figure 19). The colocalization between the two alleles of myospryn to F-actin showed a significant difference (Figure 20). A significantly higher percentage of CMYA5 T-FLAG colocalizes with F-actin (p = 0.004). The colocalization between CMYA5 C-FLAG and F-actin is very weak (MOC = 0.45; PCC = -0.27), whereas the colocalization between CMYA5 T-FLAG and F-actin is weak (MOC = 0.54; PCC = -0.16). The amino acid change significantly increases the degree of colocalization to F-actin, suggesting the involvement of actin cytoskeleton in myospryn’s function.
Figure 19: Colocalization of CMYA5-FLAG and acetylated α-Tubulin in SH-SY5Y.
Top: No significant difference is observed between the C and the T allele in all coefficients, and both alleles have a weak colocalization to acetylated α-Tubulin in SH-SY5Y. Error bars indicate 95% confidence intervals. Bottom: A representative merge image of CMYA5-FLAG (red), acetylated α-Tubulin (green), and DAPI (blue). The left image shows that CMYA5-FLAG is expressed in the cytoplasm and nucleus, whereas acetylated α-Tubulin is expressed in the cytoplasm. The white color on the right image showed pixels that contained both red and green signals, and the red line indicates the area that colocalization is being analyzed. Scale bar = 20µm.
Figure 20: Colocalization of Phalloidin (F-actin) and CMYA5-FLAG in SH-SY5Y.  
Top: A T-test was performed in JMP to determine if there is a significant difference in colocalization between the two alleles of myospryn to F-actin. No significant difference is observed in the colocalization coefficient of phalloidin, whereas a significantly higher colocalization coefficient of CMYA5-FLAG, MOC, and PCC were observed in the T allele. The p-values were listed on the top of the bar graph. Error bars indicate 95% confidence intervals.  
Bottom: A representative merge image of CMYA5-FLAG (green), Phalloidin (red), and DAPI (blue). The left image show that CMYA5-FLAG is expressed in the cytoplasm and nucleus, whereas acetylated α-Tubulin is expressed in the cytoplasm. The white color on the right image showed pixels that contained both red and green signals, and the red line indicates the area that colocalization is being analyzed. Scale bar = 20µm.
Y2H – Interaction between myospryn and its binding partners

Since the interaction between myospryn and IFs cannot be determined using co-immunoprecipitation due to the IF insolubility, Y2H was used to look for direct binding. The interaction between myospryn and titin was investigated since the amino acid change (Pro4063Leu) also falls within the titin binding region (Figure 1). The corresponding human regions of the genes were cloned into yeast vectors, where CMYA5 was cloned into GAL4 activation AD vector and the binding partners were cloned into DNA-binding domain (BD) vector. The colony-lift filter assay result is summarized in Table 6. The result showed that desmin interacts with myospryn (first row, Table 6), confirming previous report (Kouloumenta et al., 2007). Despite the high amino acid homology, the result showed that peripherin and vimentin do not directly interact with myospryn (Table 6). Sarparanta et al, 2011 found that the human titin is7–isoform, spanning the last 34 amino acids of M9 domain and 132 C-terminal amino acids of M10 domain, interacts with the myospryn from aa 3860 to the C-terminal. However, the Y2H results did not show that myospryn interacts with titin, unable to reproduce the results from the previous study.
Table 6: Summary of Y2H Colony-Lift Filter Assay

<table>
<thead>
<tr>
<th></th>
<th>CMY A5 C</th>
<th>CMY A5 T</th>
<th>CMY A5tnC</th>
<th>CMY A5tnT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmin (aa 30 – 103)</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Peripherin (full-length)</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Vimentin (full-length)</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Titin (aa. 33185 – 33423)</td>
<td>X</td>
<td>X</td>
<td>White</td>
<td>White</td>
</tr>
</tbody>
</table>

* Blue indicates positive interaction, white indicates negative interaction, and X indicates the interaction is not determined.
Myospryn and Dysbindin: Expression and Localization

Since dysbindin is another candidate gene for schizophrenia and interacts with myospryn in muscle, the expression and the colocalization between dysbindin and myospryn were examined. Using western blotting analysis, dysbindin was shown to express in rat brain regions with a splice variant only present in the brain and not in the heart (Figure 21A), and the mRNA expression in mouse brain regions was also confirmed using RT-PCR (Figure 21B). Dysbindin mRNA was also expressed in N18TG2, C2C12, mouse and rat mixed glia, mouse cortical neurons (Figure 22A), as well as SH-SY5Y, U87, U251, and T98G cells (Figure 22B).

Dysbindin is expressed mostly in the cytoplasm with little nuclear staining (Figure 23A) and is weakly colocalized with myospryn in C2C12, N18TG2, and U251 cells (Figure 23B). All three cell lines showed similar degree of colocalization. Interestingly, 85 – 96 % of dysbindin is localized to myospryn in these cell types.
Figure 21: Dysbindin protein and mRNA expression in brain regions.
A) Dysbindin is expressed in rat STR, HB, HPC, CER, CTX, and mouse heart (H), with a brain-specific splice variant. α-Tubulin is used as a loading control.
B) Using RT-PCR, Dtnbp1 mRNA is shown to express in mouse brain regions including striatum, hindbrain, hippocampus, cerebellum, and cortex. Skeleton muscle is used as a positive control. -RT is the negative control for cDNA synthesis to ensure no genomic DNA contamination, and ddH₂O is the negative control for PCR.
Figure 22: *DTNB1P1* mRNA is expressed in various cell lines.
A) The mRNA expression of dysbindin in various mouse cell lines (N18TG2 and C2C12), mouse mixed glia and cortical neuron, and rat mixed glia are shown. Mouse brain and skeletal muscle are used as positive controls for RT-PCR, and Gapdh is used as the positive control for cDNA synthesis. B) The expression of dysbindin in various human cell lines (SH-SY5Y, U87, U251, and T98G) is shown. Human frontal cortex and skeletal muscle are used as positive controls for RT-PCR, and GAPDH is used as the positive control for cDNA synthesis. -RT is the negative control for cDNA synthesis to ensure no genomic DNA contamination.
Figure 23: Colocalization of dysbindin and myospryn in C2C12, N18TG2, and U251.
A) Images of DAPI, myospryn, and dysbindin staining in C2C12 (top), N18TG2 (middle), and U251 (bottom) showed that myospryn and dysbindin are localized in the cytoplasm and nucleus. Merged images were analyzed for colocalization, where the white color in far right panel showed pixels that contained both red and green signals. Scale bar = 20µm. B) Colocalization coefficients, overlap coefficient, and correlation coefficient for myospryn and dysbindin. Dysbindin is weakly colocalized with myospryn. All coefficients numbers are mean ± 95% confidence interval.
CHAPTER 4: DISCUSSION

Myospryn and Intermediate Filaments (IFs)

*Myospryn and desmin are expressed in brain*

The *CMYA5* gene is identified as a candidate gene for schizophrenia in a two-stage study design with more than 33,000 subjects (Chen *et al.*, 2011). Myospryn has been extensively studied in skeleton and cardiac muscles due to its association with muscular dystrophy and cardiomyopathy. Since schizophrenia is a mental disorder, it is important to characterize myospryn expression in the brain. Although a previous study did not show expression of myospryn in brain tissue using northern blot and western analysis (Benson *et al.*, 2004), the current results show that myospryn and desmin transcripts and proteins are expressed in brain regions including striatum, hindbrain, hippocampus, cerebellum, and cortex. This provides the first evidence that myospryn is expressed in brain regions, suggesting functions in brain that might be relevant to schizophrenia pathophysiology. In addition, myospryn is expressed in primary cortical neuron and mixed glia culture, as well as neuroblastoma and glioblastoma cell lines, suggesting myospryn has function in both neurons and glia. Desmin is known as a muscle-specific intermediate filament in cardiac, skeletal and smooth muscles (Paulin & Li, 2004), but its expression in the insoluble fraction of the brain regions has also been shown. IF protein extraction needs to be performed under denaturing conditions, so the interaction between myospryn and desmin in brain cannot be determined using co-immunoprecipitation. Nonetheless, their expression provides evidence that they play roles in the brain.
An RNA-Seq transcriptome database of the mouse brain provides the gene expression information in various cell classes, including astrocytes, neurons, oligodendrocyte precursor cells (OPCs), newly formed oligodendrocytes (NFOs), myelinating oligodendrocytes (MOs), microglia, and endothelial cells (Zhang et al., 2014). The expression of Cmya5 (myospryn), Des (desmin), Vim (vimentin), and Prph (peripherin) genes in those cells were described in Figure 24. CMYA5 has the highest expression in astrocytes; Des has the highest expression in endothelial cells; Vim has the highest expression in endothelial cells and also highly expressed in astrocytes; Prph has the highest expression in neurons (Figure 24). The CMYA5 gene expression from the database confirmed the mRNA expression in mouse primary cortical neurons and mixed glia, which comprised of astrocytes, oligodendrocytes, and microglia (Figure 3A). Astrocytes play important roles in brain development and function, such as structural and functional support for neurons, establishment of blood-brain barrier, maintenance of extracellular ion balance, and modulate neurotransmitters (Barres, 2008). Astrocytes not only take up neurotransmitters, such as glutamate, but also release gliotransmitters (Hamilton & Attwell, 2010). Thus, astrocyte dysfunction may contribute to the pathology of schizophrenia as suggested by the hypothesis of neurotransmitter dysfunction in schizophrenia (Wang et al., 2015).
Figure 24: *Cmya5, Des, Vim, and Prph* gene expression from an RNA-Seq transcriptome database.

The gene expression of *Cmya5* (A), *Des* (B), *Vim* (C), and *Prph* (D) expression in various purified cell classes of the brain from an RNA-Seq transcriptome database (https://web.stanford.edu/group/barres_lab/brain_rnaseq.html) (Zhang *et al.*, 2014). FPKM = fragments per kilobase of transcript sequence per million mapped fragments.
**rs10043986 changes the binding property of myospryn to desmin**

rs10043986 (C>T) changes the 4063rd amino acid of myospryn from Pro and Leu, and the GWAS shows that the Leu allele has a protective effect (Chen et al., 2011). This proline residue is a highly conserved across mammals, so this amino acid change is very likely to affect protein binding. A quantitative Y2H assay shows that the major allele (Pro or C allele) has weaker binding to desmin compared to the minor allele (Leu or T allele). This differential binding is also confirmed by SPR, showing that the minor allele has about two-fold higher response. Identifying functional consequences of rs10043981 variants that leads a protective effect will provide better understanding of the disease.

**Myospryn is localized to the cytoplasm and nucleus and is weakly and moderately colocalized with desmin**

Previous studies have demonstrated that myospryn is localized in the cytoplasm and perinuclear region of muscle cells (Durham et al., 2006; Kouloumenta et al., 2007). The results showed that myospryn is localized in the cytoplasm and nucleus in C2C12, N18TG2, and T98G cell lines. Despite the fact that myospryn physically interacts with desmin in muscle (Kouloumenta et al., 2007), the quantitative colocalization analysis only showed a weak to moderate colocalization. Greater than 90% of desmin colocalized with myospryn but only about 50% of myospryn colocalized with desmin, suggesting that myospryn might have additional binding partners and diverse functions.

Next, the difference in colocalization between the two alleles of myospryn to desmin in C2C12 was investigated. No difference in colocalization between the two
alleles of myospryn to desmin is observed. Colocalization studies may not be sensitive enough to detect subtle differences in binding. Other techniques, such as fluorescence resonance energy transfer (FRET), may be more sensitive and could determine if the two alleles of myospryn has differential interactions with IFs, as well as the spatiotemporal distribution of proteins and their dynamics. Another limitation of our colocalization studies is the inability to use the full-length myospryn protein due to its large molecular weight. The CMYA5-FLAG protein fragment used in these studies represents only 5% of the whole myospryn protein (aa. 3859 – 4069). Endogenous myospryn is mainly expressed in the cytoplasm with weak nuclear staining, whereas the transfected CMYA5-FLAG protein has higher expression in the nucleus. Although differential binding is observed between myospryn (aa. 4039 – 4069) and desmin and only this small region of myospryn is required for the interaction with desmin to occur in vitro, other interactions at different parts of myospryn may also affect the binding.

Desmin minimum binding region to myospryn is highly similar to peripherin and vimentin

Desmin is known as a muscle-specific type III IFs (Paulin & Li, 2004) though it is also found to express in the brain in the present study. However, it is not visible under immunocytochemistry, which raises the possibility that myospryn might bind to other type III IFs in neuronal cells. Two other type III IFs, peripherin and vimentin, have high protein similarity to desmin within the binding region to myospryn and are shown to be expressed in brain, neuroblastoma and glioblastoma cell lines in the current study. Peripherin is known to be expressed mostly in neurons of peripheral nervous system (PNS) and at low levels in neurons of central nervous system (CNS) that extend axons
toward peripheral structures (Portier et al., 1983; Escurat et al., 1990; Margiotta & Bucci, 2016). Vimentin is widely expressed in mesenchymal cells and is also expressed in almost all CNS neurons early in development (Cochard and Paulin, 1984; Nixon and Shea, 1992). Both peripherin and vimentin are expressed in embryonic brain and at the highest levels during early postnatal stages; their expression declines somewhat before the expression of neurofilament (NF) triplet proteins sharply rises (Kost et al., 1992). However, no interaction between the c-terminus region of myospryn (aa. 3859 – 4069) and peripherin or vimentin was observed using Y2H, which is known for high false-positive and false-negative rates (Huang et al., 2007). As previously mentioned, these interaction cannot be determined using co-immunoprecipitation due to the insolubility of IFs and their need to be extracted under denaturing conditions. It is still possible that myospryn forms complexes with those neuronal IFs but does not directly interact.

Peripherin and vimentin have a similar degree of colocalization to myospryn, as what was observed between desmin and myospryn, suggesting the possible association of vimentin and peripherin to myospryn in neuronal and glial cell types.

**Schizophrenia and Intermediate Filaments**

The intermediate filament is a component of the cytoskeleton, together with microtubules and microfilaments. A recent review of proteomics research in schizophrenia has revealed changes in cytoskeletal components (Davalieva et al., 2016). Specifically, five IFs with disturbed abundance in schizophrenia were observed, desmin, vimentin, glial fibrillary acidic protein (GFAP), and neurofilament medium and light chains (NFM and NFL). An upregulation of vimentin and desmin in schizophrenia
brains is observed, providing evidence that the disruption of cytoskeleton and its associated signal transduction proteins are involved in schizophrenia (English et al., 2009; Martins-de-Souza et al., 2009; Saia-Cereda et al., 2015; Davalieva et al., 2016). Since the colocalization coefficients of desmin, peripherin, and vimentin showed approximately 90% of IFs in the study colocalizes with myospryn, one hypothesis is that the protective allele of myospryn to IFs provides more efficient rearrangement of the cytoskeletal network during early neuritogenesis.

Although we only focus on peripherin and vimentin in the current study, several intermediate filament proteins are expressed in the nervous systems, including nestin, vimentin, peripherin, α-internexin, and neurofilament triplet proteins. Their expression is dependent on the developmental timing and localization within the nervous system (Perrot & Eyer, 2009). Nestin and vimentin are detected earliest during embryonic development, around the 12-somite stage (Cochard & Paulin, 1984; Lendahl et al., 1990). Peripherin is also expressed during early axonal outgrowth in the PNS (Gervasi et al., 2000). Neuronal differentiation is triggered by the expression of NF subunits, NFL (light, 68 kDa), NFM (medium, 160 kDa) and NFH (heavy, 205 kDa) (Shaw & Weber, 1982; Carden et al., 1987; Nixon & Shea, 1992). NFL is expressed at the beginning of neuronal differentiation; NFM is expressed shortly after with the emergence of neurite formation whereas NFH is expressed later during neuronal differentiation (Carden et al., 1987). α-internexin expression precedes NFL and is highest after neurite initiation and during axon elongation in the CNS (Kaplan et al., 1990); its expression persists into adulthood (Yuan et al., 2006). GFAP is the IF expressed in mature astrocytes of adult brain and play roles in astrocyte motility and migration, mitosis, astrocyte-neuron
interactions, and blood-brain barrier and myelination (reviewed in Middeldorp & Hol, 2011). Schizophrenia is a neurodevelopmental disorder (Rapoport et al., 2012) and those IFs play important roles during development; disruption in those genes will contribute to the risk of schizophrenia.

The developmental expression of myospryn is not completely known though its transcript is expressed in the mouse brain at E15.5, specifically in the superficial stratum of dorsal pallium/isocortex (Figure 25). Excitatory glutamatergic neurons originate from the proliferative zone of the dorsal pallium, the forerunner of the neocortex, and migrate to form the cortical plate. The inhibitory GABAergic neurons originate in the ganglionic eminences of the subpallial telencephalon, the forerunner of the basal ganglia. Disruption of those two interneurons during the development is linked to schizophrenia (Levitt et al., 2004; Levitt, 2005). In desmin-null neonatal cardiomyocytes, myospryn loses its distinct localization at the perinuclear region and is more diffused in the cytoplasm, showing the important of the interaction between myospryn and IF for the proper localization and function (Kouloumenta et al., 2007). Thus, characterizing the roles myospryn and IFs play during the neuron development will provide better understanding schizophrenia as a neurodevelopmental disorder.
Figure 25: Expression of myospryn transcript in mouse at E15.5.
A) Figures are from Durham et al. J. Biol. Chem. 2006;281:6841-6849; Fig. 4B, where they identified the myospryn transcript expression using radioactive in situ hybridization of mouse embryo sagittal sections at E10.5 (left) and E15.5 (right). They found that myospryn is expressed in the heart and skeletal muscle at both time points. It also showed that myospryn is expressed in the brain at E15.5 but not at E10.5. B) Images were obtained from the Allen Developing Mouse Brain Atlas at E15.5, sagittal. The reference image (left) showed the overall anatomic structural of the brains, and the region where myospryn transcript is expressed is highlighted. It corresponds to the superficial stratum of dorsal pallium/isocortex (DPall) (right).
**Myospryn and Cytoskeleton**

The cytoskeleton network is comprised of three distinct but highly intertwined filamentous structures, microfilaments (MFs), intermediate filaments (IFs), and microtubules (MTs), that play an important role in maintaining neuronal morphology – its highly asymmetrical shape and structural polarity (Kevenaar & Hoogenraad, 2015). MFs consists of polymers of actin, and MTs are consists of \( \alpha \)- and \( \beta \)-tubulin subunits. Schizophrenia is a neurodevelopmental disorder, and abnormalities in the neuronal cytoskeleton, which plays roles in neurite outgrowth and axonal transportation in the developing nervous system, can have long-term effects on learning and memory (Arnold, 1999; Lewis & Levitt, 2002). In the current study, rs10043984 affects the colocalization of myospryn to actin cytoskeleton with the T allele (Pro) has a stronger degree of colocalization. Although no evidence has shown that myospryn interacts with actin, myospryn interacts with actin-binding protein, \( \alpha \)-actinin. \( \alpha \)-actinin is a crosslinking protein that can arrange F-actin into distinct networks, such as actin bundles, and regulates the length and density of dendritic spines (Cingolani & Goda, 2008). Therefore, it is possible that two different alleles of myospryn differentially bind to \( \alpha \)-actinin, which then leads to differential colocalization with actin cytoskeleton network. Rearrangement of actin cytoskeleton is important for neuronal development and neuroplasticity, such as extension of axon and dendrites, neurite branching, axonal navigation, and synapse formation (Auer et al., 2011). Actin is involved in the synaptic vesicle cycle to regulate neurotransmission release at the presynaptic terminals and the organization and trafficking of postsynaptic receptors at the postsynaptic terminals (Cingolani & Goda, 2008). Dysregulation of actin cytoskeleton pathway is associated
with schizophrenia (Zhao et al., 2015; Yan et al., 2016). Therefore, it is possible that the protective allele of myospryn provides more efficient actin polymerization/depolymerization that is important for structural plasticity.

**Myospryn and Dysbindin**

*Dysbindin is expressed in various brain regions*

Dysbindin (*DTNBP1*) is initially identified as a dystrobrevin-binding protein and is expressed in muscle and brain (Benson et al., 2001). Dysbindin is expressed in neurons and localized in axon bundles and in certain axon terminals, notably mossy fiber synaptic terminals in the cerebellum and hippocampus (Benson et al., 2001). Dysbindin has three major isoforms, named dysbindin-1A, dysbindin-1B, and dysbindin-1C (Oyama et al., 2009; Tang et al., 2009). The full-length human dysbindin-1A has 351 amino acids; dysbindin-1B isoform has 303 amino acids that lacks the C-terminal proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) domain; dysbindin-1C has 270 amino acids that lacks the N-terminal region in front of the coiled-coil domain (Tang et al., 2009). Dysbindin-1A is associated almost exclusively with postsynaptic densities; dysbindin-1B is associated almost exclusively with synaptic vesicles; dysbindin-1C is associated with small amount of synaptic vesicles but mostly with postsynaptic densities (Talbot et al., 2011). In the current study, dysbindin protein is found to be expressed in various mouse brain regions and heart, and a brain-specific isoform, likely dysbindin-1C, is observed. Dysbindin mRNA expression in the brain, skeletal muscle, myoblast, neuroblastoma, and glioblastoma cell lines is also shown, confirming the previous studies that dysbindin is expressed in muscle and brain.
Dysbindin is weakly colocalized with myospryn

A previous study has shown that dysbindin isoforms A and B are localized in the cytosol and nucleus, and isoform C is localized exclusively in the cytosol (Oyama et al., 2009). In our localization studies, a similar result was observed – dysbindin is localized mostly in the cytoplasm with some nuclear localization. Dysbindin is found to weakly colocalize with myospryn in myoblast, neuroblastoma, and glioblastoma cell lines. Interestingly, 85 – 96% of dysbindin is localized to myospryn in these cell types, suggesting the majority of dysbindin functions at the same location as myospryn.

Dysbindin and schizophrenia

Dysbindin is first reported to associate with schizophrenia by Straub et al., 2002, followed by many independent case-control studies that support the association, but no large-scale GWAS has confirmed the association (reviewed in Ghiani & Dell'Angelica, 2011). Nonetheless, reduction of dysbindin expression in brains of patients with schizophrenia has been observed, including the hippocampal formation (Talbot et al., 2004; Weickert et al., 2008), the dorsolateral prefrontal cortex (Weickert et al., 2004), and the cerebral cortex (Bray et al., 2005). Isoform-specific reduction of dysbindin has also been observed in different brain regions of patients with schizophrenia (Tang et al., 2009; Talbot et al., 2011). Studies of sandy mice with a dysbindin-1 null mutation (Dtnbp1<sup>sdy</sup>) have shown dysbindin deficiency leads to cognitive and social behavioral impairments (reviewed Talbot, 2009; Ghiani & Dell'Angelica, 2011), such as deficits in working memory (Karlsgodt et al., 2011) and social interaction (Hattori et al., 2008).
Those studies in sandy mice have showed that the mutation affects adrenal neurosecretion and pre- and postsynaptic aspects of dopaminergic, glutamatergic, and GABAergic transmission (Talbot, 2009). Together, they suggest that dysbindin is a strong candidate gene for schizophrenia susceptibility.

**Myospryn, Dysbindin, IFs in vesicular trafficking**

Myospryn was first identified as a binding partner of dysbindin (Benson et al., 2004), a subunit of the biogenesis of lysosome-related organelles complex 1 (BLOC-1), and BLOC-1 is found to associate with desmin, likely through myospryn in the heart (Kouloumenta et al., 2007). Dysbindin is expressed at a higher level embryonically, and the majority of brain dysbindin exists as a subunit of BLOC-1 (Ghiani et al., 2010). BLOC-1 plays an important role in endosomal trafficking that is involved in synaptic transmission and neurodevelopment, specifically the regulation of a cell-surface D2 dopamine receptor (DRD2), the biogenesis and fusion of synaptic vesicles, and neurite outgrowth (Ryder & Faundez, 2009; Ghiani & Dell'Angelica, 2011; Mullin et al., 2011). Postmortem brains of patients with schizophrenia have significantly reduced expression of dysbindin, and the loss of one BLOC-1 subunit triggers downregulation of other complex subunits (Mullin et al., 2011). Thus, downregulation of dysbindin affects BLOC-1 function in trafficking, and schizophrenia has been proposed as a disorder of membrane and receptor trafficking (Ryder & Faundez, 2009; Schubert et al., 2012). Downregulation of dysbindin compromises the ability for BLOC-1 to traffic D2DR to degradation, which results in increases in surface levels of DRD2 and decreases DRD2 internalization, which results in enhanced dopaminergic neurotransmission (Iizuka et al.,
2007). *DRD2* is also found to associate with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), and the dopaminergic system plays an important role in schizophrenia (Howes *et al.*, 2015). BLOC-1 regulates membrane protein sorting via its association with clathrin-adaptor complex 3 (AP-3) (Salazar *et al.*, 2006; Di Pietro *et al.*, 2006; Newell-Litwa *et al.*, 2009), which is essential for the sorting and exocytosis of synaptic vesicles. Brain BLOC-1 interacts with SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), a superfamily of proteins that control membrane fusion, and regulates neurite outgrowth (Ghiani *et al.*, 2010).

Myospryn has been suggested to play roles in vesicular trafficking and intracellular signaling (Sarparanta, 2008; Tsoupri & Capetanaki, 2013), and IFs are also involved in the regulation of intracellular trafficking (Margiotta & Bucci, 2016). Overall, IFs in neurons are important for the motility of late endosomes and lysosomes between the cell body and the distal regions of the cell (axonal transport) and the regulation of exocytosis and vesicular trafficking during axonal regeneration (Margiotta & Bucci, 2016). Specifically, vimentin, peripherin, and α-internexin interact with the AP-3 complex, and lack of vimentin affects the subcellular distribution of AP-3 and lysosomes and late endocytic/lysosome compartments (Styers *et al.*, 2004). Peripherin interacts with SIP30, a neuronal protein involved in SNARE-dependent exocytosis, suggesting a role of in regulating exocytosis of synaptic vesicles (Gentil *et al.*, 2014). Therefore, it is possible that myospryn works with dysbindin in BLOC-1 and IFs to control neurite outgrowth, the biogenesis and release of synaptic vesicles at presynaptic terminals as well as the downregulation of neurotransmitters receptors at postsynaptic terminals, and
the protective myospryn allele provides more efficient and proper neurotransmission via vesicular trafficking.

**Myospryn and other binding partners**

The current known binding partners of myospryn in skeletal and cardiac muscles include desmin (Kouloumenta et al., 2007), dysbindin (Benson et al., 2004), α-actinin (Durham et al., 2006), PKA (Reynolds et al., 2007), dystrophin (Reynolds et al., 2008), M-band titin (Sarparanta et al., 2010), protease calpain 3 (Sarparanta et al., 2010), and calcineurin (Keilbasa et al., 2011) (Figure 1). Many of those binding partners have roles in brain and have been associated with schizophrenia.

α-actinin is a ubiquitous cytoskeletal actin-binding protein (Oikonomou et al., 2011). While one of the four isoforms, α-actinin-2 (ACTN2), is mainly expressed in the skeletal muscle fibers, it is also expressed in the brain especially in the grey matter (Oikonomou et al., 2011). α-actinin-2 interacts with both NR1 and NR2B subunits of the NMDA receptor (Wyszynski et al., 1997), colocalizes with the NMDA receptor in the postsynaptic densities of excitatory synapses (Wyszynski et al., 1998), and plays roles in the Ca^{2+}/calmodulin-dependent inactivation of the NMDA receptor (Krupp et al., 1999). As mentioned in the Introduction, impaired glutamatergic transmission and reduced NMDA receptor activity underlie the pathogenesis of schizophrenia (Konradi & Heckers, 2003). Thus, α-actinin-2 might have indirect pathogenetic role via NMDA receptor in schizophrenia. Myospryn (mouse aa. 2072 – 3739) interacts with α-actinin-2 though myospryn’s BBC domain, two FN3 domains, and the SPRY domain alone or together in any combination can also interact with α-actinin-2, suggesting the ability to form
numerous interactions with other proteins (Durham et al., 2006). In the same study, myospryn is found to be a transcriptional target of MEF2A (myocyte enhance factor 2A), suggesting myospryn acts as a protein-protein interaction interface to function directly downstream of the MEF2A pathway (Durham et al., 2006). Although MEF2A is known as a muscle-specific related gene, it has been found to associate with schizophrenia (Crisafulli et al., 2015), perhaps through the regulation of myospryn.

The interaction between myospryn and PKA in muscle has identified myospryn as an A-kinase anchoring protein (AKAP), a scaffold protein that recruit kinases to distinct subcellular regions (Reynolds et al., 2007). AKAPs are involved in the regulation of excitatory synaptic plasticity (Sanderson & Dell'Acqua, 2011), and AKAP5 is found to associate with schizophrenia (Wilson et al., 2006; Sutrala et al., 2007). Dopamine receptors function through the regulation of cAMP and PKA via G protein-mediated signaling pathways (Beaulieu & Gainetdinov, 2011). A substrate of PKA, dopamine- and cAMP-regulated neuronal phosphoprotein 32kDa (DARPP-32), is predominately expressed in medium spiny neurons (MSNs) of the striatum and has been implicated in the pathogenesis of schizophrenia due to its role in mediating actions of dopaminergic neurons (Greengard et al., 1999). D1 receptors are found postsynaptically on dopamine-receptive cells such as GABAergic MSNs; D2 receptors are found both presynaptically on dopaminergic nerve terminals and postsynaptically on GABAergic MSNs (Beaulieu & Gainetdinov, 2011). In general, D1-class dopamine receptors stimulate the production of cAMP and increase PKA activity, resulting in phosphorylation of DARPP-32 at Thr$^{34}$ (Svenningsson et al., 2004). The Thr$^{34}$-phosphorylated DARPP-32 becomes a potent inhibitor of protein phosphatase-1 (PP1),
which in turn regulates the phosphorylation of many downstream physiological effectors, including various neurotransmitter receptors, ion channels, and transcription factors (Svenningsson et al., 2004). The enhanced dopaminergic transmission via D1 receptors also results in decreased phosphorylation of Thr$^{75}$-DARPP-32, which reduces inhibition of PKA (Svenningsson et al., 2004). This effect is counteracted by D2-class receptors, which negatively regulate the production of cAMP to inhibit PKA activity and stimulate the PP-2B (also known as calcineurin) signaling cascade (Svenningsson et al., 2004). Therefore, DARPP-32 plays a bidirectional role in dopaminergic neurotransmission and has been implicated in schizophrenia.

Dystrophin is a cytoplasmic protein that links the cytoskeleton and extracellular matrix to provide structural support and mutations in dystrophin are known to cause Duchenne muscular dystrophy (Hendriksen et al., 2015). In the brain, dystrophin is primarily expressed in the hippocampus, prefrontal cortex, and cerebellum (Hendriksen et al., 2015). Dystrophin has many isoforms, ranging in molecular mass from 71 to 427 kDa. Dp427 localizes in the postsynaptic neurons and colocalizes with γ-aminobutyric acid type A (GABA$_A$) receptors (Knuesel et al., 1999). Dp71 is expressed in both neurons and glia and is the most abundant isoform in the brain (Austin et al., 2000). Deficiency of dystrophin results in cognitive dysfunction, deficits in synaptogenesis and channel clustering at synapses, and deficits in learning and memory (Rae & O'Malley, 2016).

Titin ($TTN$) is a scaffold protein aiding myofibrillar assembly during myogenesis and spans from the Z-disk to the M-band within the sarcomere (Savarese et al., 2016). The titin protein consists of four main modular structures: the amino-terminal Z-disc
region, the I-band and A-band regions, and the carboxyl-terminal part spanning the M-band (Savarese et al., 2016). Myospryn is known to interact with M-band titin in muscle (Sarparanta et al., 2010). Mutations in the TTN gene cause different neuromuscular disorders, such as tibial muscular dystrophy and limb-gridle muscular dystrophy (Savarese et al., 2016). Titin is not found to be associated with schizophrenia, but titin expression is increased in the thalamus of [+]-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cycloheptene-5,10-iminehydrogenmaleate (MK-801)-treated rats (Paulson et al., 2004). MK-801, dizocilpine, is an antagonist for NMDA receptors, and MK-801-treated rodents are used as a animal model for schizophrenia (Paulson et al., 2004).

Calpains are calcium-dependent proteases involved in cell survival, plasticity and motility (Ono et al., 2016). Calpain 3 (CAPN3) was initially identified as a muscle-specific calpain but is also expressed in brain, specifically in astrocytes but not in neurons (Konig et al., 2003). Calpain is known to be involved in neuronal apoptosis (Raynaud & Marcilhac, 2006). Myospryn is a substrate of calpain 3 and may protect calpain 3 from autolysis (Sarparanta et al., 2010). So far, no evidence of association between calpain 3 and schizophrenia has been found.

Calcineurin is a calcium/ calmodulin-dependent serine/threonine protein phosphatase and is involved in synaptic plasticity (Groth et al., 2003). A forebrain-specific knockout mouse of calcineurin displays multiple abnormal phenotypes related to schizophrenia, such as impaired working memory, increased locomotor activity, decreased social interaction, and impairments in prepulse inhibition and latent inhibition (Zeng et al., 2001; Miyakawa et al., 2003). A genetic association between a variant ofPPP3CC, the gene encoding the calcineurin gamma subunit, and schizophrenia has
been found, which supports that the calcineurin malfunction contributes to schizophrenia pathogenesis (Gerber et al., 2003). Furthermore, the expression of calcineurin is decreased in the hippocampus of schizophrenia patients (Eastwood et al., 2005). In addition, calcineurin dephosphorylates DARPP-32 in neostriatal neurons, showing its involvement in dopaminergic neurotransmission (Nishi et al., 1999).

In addition to desmin, the amino acid change also falls within the binding region to self-association (Benson et al., 2004), α-actinin (Durham et al., 2006), dystrophin (Reynolds et al., 2008), M-band titin (Sarparanta et al., 2010), and calcineurin (Keilbasa et al., 2011). In the current study, Y2H was performed to determine if the amino acid change affects the binding of myospryn to titin. However, we were unable to reproduce the Sarparanta et al., 2010 study. As already mentioned, Y2H is known for the high false-positive and false-negative rates (Huang et al., 2007). Nonetheless, it will be interesting to see if the amino acid affects the binding to other partners of myospryn.

Taken all together, myospryn’s binding partners fall into three broad categories: 1) cytoskeleton proteins (desmin, α-actinin, dystrophin, and titin), 2) trafficking proteins (IFs and dysbindin), and 3) signaling proteins (PKA, calpain-3, calcineurin). Based on previous studies on binding partners and their relevance to schizophrenia, the likely interactions are summarized in Figure 26. The most plausible function of myospryn is a scaffold protein due to its identification as an AKAP and its large molecular weight for the potential to interact with multiple proteins. The interaction between cytoskeleton proteins and myospryn is likely to provide structural support for proper positioning of organelles, proper localizations of its binding partners, and proper functions of downstream effectors. The interaction with dysbindin/BLOC-1 provides evidence for
myospryn’s involvement neurotransmission, specifically the biogenesis and release of synaptic vesicles at presynaptic terminals and the downregulation of neurotransmitters receptors at postsynaptic terminals. The interaction with signaling proteins suggests myospryn is involved in the dopaminergic neurotransmission, specifically through the cAMP/PKA/DARPP-32 signaling pathway. In addition, the direct interaction between α-actinin-2 that binds to subunits of NMDA receptors also suggests that myospryn might be involved in glutamatergic neurotransmission. We hypothesized that myospryn interacts with cytoskeleton proteins and acts as an AKAP to direct the signaling proteins PKA and calcineurin to proper cellular location for efficient dopaminergic neurotransmission, as well as for the proper formation, release, and recycling of synaptic vesicles through BLOC-1. It is unlikely that myospryn is involved in signaling transduction at the same time as the vesicle trafficking, so it will be important to characterize myospryn’s location and the protein complex that myospryn is involved at specific cell types and brain regions.
Myospryn interacts with dysbindin in the BLOC-1 complex and is likely involved in vesicular trafficking, specifically the synaptic vesicles and neurotransmission. Myospryn also interacts with PKA that is regulated by dopamine receptors, which can then lead to phosphorylation of a PKA substrate, DARPP-32. Myospryn can also interact with calcineurin, which dephosphorylates DARPP-32. Myospryn acts as an AKAP anchoring protein to tether PKA or calcineurin at the proper cellular location for their downstream signaling pathways. Myospryn also interacts with α-actinin, a cytoskeletal actin-binding protein that interacts with NMDA receptor and is involved in glutamatergic neurotransmission. The interaction between myospryn and IF provides structural support.
Future Directions

*Myospryn binding partners in the brain*

The subcellular localization of desmin, one of myospryn’s binding partners, is not visible in neuroblastoma and glioblastoma using immunocytochemistry, despite the mRNA being expressed in those cell lines. We hypothesized that myospryn might bind to other IFs, but the Y2H results showed the c-terminus region of myospryn does not interact with peripherin and vimentin. Therefore, it raised the question what proteins does myospryn interacts in brain and neuronal/glial cell lines. As a starting point, I would test the interaction between myospryn and its other known binding partners in the muscle and see if such interactions also occur in the brain by immunoprecipitation. In addition, the interaction between actin and other actin-binding proteins will also be investigated since the difference in colocalization between two alleles of myospryn to F-actin was observed. Next, I would perform yeast two-hybrid screening using myospryn as the bait and brain cDNA library as the prey. However, as already mentioned, one limitation of Y2H is the high false-positive and false-negative rates. Alternatively, immunoprecipitation of myospryn in brain or neuronal cell lines followed by mass spectrometry might yield more useful information. Identifying myospryn’s binding partners in the brain will help better understand its roles and functions that are relevant to schizophrenia.

*Additional testing of in vivo differential binding*

The insolubility nature of IFs prevents using immunoprecipitation to identify the interaction between myospryn and IFs, desmin, peripherin, and vimentin, *in vivo*. As
already mentioned, the colocalization study may not be sensitive enough to detect the subtle difference in binding between two different alleles of myospryn to desmin, as well as the inability to use the full-length myospryn. Therefore, another technique, such as fluorescence resonance energy transfer (FRET) might be more useful. FRET is based on the nonradiative (dipole-dipole) energy transfer from donor fluorophore (fused to the “bait”) upon excitation to acceptor fluorophore (fused to the “prey”) (reviewed in Zhou et al., 2016). The energy transfer only occurs when the bait and the prey are in close proximity, and the distance between the two molecules is inversely proportional to the signal intensity. Combined with microscopy imaging technologies, FRET can also be used to visualize and quantify protein-protein interactions in real time in living cells (Zhou et al., 2016). This technique can provide information about whether the two alleles of myospryn physically interact with IFs in cells, the distance between them, as well as their spatiotemporal distribution and dynamics.

In the study, we used neuroblastoma and glioblastoma cancer cell lines due to their immortality and providing unlimited supply of cells, which is more cost effective. However, primary cells are more biological relevant and more closely mimic the physiological state of cells in vivo. Alternatively, the breakthrough of cellular reprogramming technology, which turns differentiated cells such as human skin fibroblasts into induced pluripotent stem cells (iPSCs), can generate more suitable cell system for study psychiatric disorders (Takahashi & Yamanaka, 2006; Takahashi et al., 2007). Such technology of reprogramming human iPSCs into neurons and neural progenitor cells has been used as a model for schizophrenia (Wen, 2017). Thus, iPSCs from patients and healthy subjects with the CMYA5 C/C or C/T genotype would provide
a great cell system to study the endogenous myospryn Pro or Leu alleles, as well as cell analysis of neurodevelopment, cytoskeletal dynamic and synaptogenesis. It can provide insights into the pathogenesis of psychiatric disorders at the molecular, cellular, and neuronal circuit levels.

**Developmental expression of myospryn**

Schizophrenia is a neurodevelopmental disorder with the age of onset typically in adolescence or early adulthood (Rapoport et al., 2012). Therefore, events that have happened prenatally and any subtle abnormalities during the early brain development in regions involved in motor, cognitive, and social and emotional functioning could increase the risk for schizophrenia. Thus, as a potential candidate gene for schizophrenia, it's important to identify the expression of myospryn during development. As shown by Durham et al., 2006, myospryn is expressed in the mouse brain at E 15.5 (Figure 19). However, a comprehensive analysis using *in situ* hybridization at more time points will help determine the developmental expression of myospryn. Western blotting analysis on brain at those time points will also support the study.

**Generation of myospryn knockout mouse**

A knockout study is crucial for the understanding of a gene’s function. The recent development of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-9 genome editing technology provides a simple and efficient method to knockout a gene in the cells and to create a knockout mouse (Ran et al., 2013). Knocking out the gene first in mouse neuroblastoma cell lines should be performed to
test the efficiency of the guide RNA created. In addition, these myospryn-null cells can be used to study the effect of lack myospryn on the localization of its binding partners. In the case of desmin-null cells, the localization of myospryn becomes more diffused in the cytoplasm – it will be interesting to see myospryn-null also affects the localization of cytoskeleton markers. Next, a knockout mouse will be generated. Since myospryn is expressed during embryonic development, it is possible myospryn knockout might be embryonic lethal. If that is the case, a brain conditional knockout mouse will generate instead (Shinmyo et al., 2016). Behavior studies on the myospryn knockout mice will be performed to see if they have any schizophrenia-related phenotypes, such as the open field test for locomotor activity and social interaction in home cage test (Miyakawa et al., 2003). Since we hypothesized that myospryn is involved in the glutamatergic neurotransmission, I would study the dopamine synthesis, release, and availability of post-synaptic dopaminergic receptors and transporters using PET/SPECT imaging techniques.

**Summary**

Taken together, the study has shown that *CMYA5*, a candidate gene for schizophrenia, is expressed in brain and neuronal cells and is associated with IFs and dysbindin. The *CMYA5* associated SNP also has differential binding of one of its binding partners, desmin, *in vitro* but a study of the entire protein containing the different alleles will likely be necessary to determine if differential binding *in vivo* occurs. Myospryn still has a wide-range of other binding partners, and their interactions and functions in brain will provide more understanding of myospryn's roles in brain and the
pathways involved in schizophrenia pathophysiology. In addition, identifying functional consequences of rs10043981 variants that leads a protective effect will provide better understanding of the disease.
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Anting Hsiung was born on June 23, 1988, in Taipei, Taiwan and is currently an American citizen. She graduated from the University of Maryland, College Park, in 2009 with a Bachelor of Science in Biological Sciences: Cell Biology and Genetics. She earned the citation in College Park Scholars, Earth, Life, and Time Program, in 2008 at the University of Maryland, College Park. She joined the Department of Human and Molecular Genetics in 2010 at the Virginia Commonwealth University, School of Medicine.