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The role of RNA helicases in neuromuscular development and diseases

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THE ROLE OF RNA HELICASES IN NEUROMUSCULAR DEVELOPMENT AND DISEASES

by

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I would like to sincerely thank Dr. Vandana Gupta for her guidance, instruction, and mentorship throughout this project. Additionally, I would like to thank Dr. Mahasweta Girgenrath for reviewing this thesis and providing constructive feedback.
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ABSTRACT

RNA helicases are enzymes that bind or remodel RNA and RNA-protein complexes. They are involved in numerous cellular functions including RNA metabolism, transcription, translation, and mRNA decay. Defects in helicase function or disregulated expression, can cause diseases. DEAD-box (DDX) RNA helicases are highly conserved and are known to be involved in muscle development and disease, by interacting with muscle specific transcription factors and genes in humans. The Gupta Lab is currently studying zebrafish (an established and reliable model to study muscle diseases) with a mutation in *ddx27*. These fish have impaired motility behavior, skeletal muscle hypotrophy, and extensive central nucleation. They also exhibit disorganization of skeletal muscle, abnormalities in the brain, eyes, and heart. These phenotypes mimic the abnormalities seen in human myotonic dystrophy. It is known that *ddx27* is necessary for regulation of rRNA maturation. Recent studies have pointed to its non-ribosomal roles of nucleolar genes. *IGHMBP2*, another RNA helicase, is known to result in spinal muscular atrophy (SMARD1) or Charcot-Marie Tooth disease when mutated. We used zebrafish and patient myoblast cells to determine the role of *ddx27* in myogenesis and diseases. As a basis for future studies, the 43 known human DDX genes were outlined for their functions.
Immunofluorescence studies in *ddx27* mutant zebrafish showed drastic skeletal muscle and nucleolar assembly defects with large numbers of cells with transcriptionally active euchromatin, suggesting altered gene regulation. In addition, IF with Pax7 (a marker for satellite cells) and MF20 (a marker for myosin heavy chain antibodies) showed a significant increase in the number of Pax7 positive cells that suggest perturbed satellite cell regulation. Nucleolar defects were also seen in cells isolated from myotonic dystrophy patients. While the cause of these defects is not known, the results lead us to believe that *ddx27* may be involved in cell cycle regulation or apoptosis events. Finally, while this study also attempted to develop a zebrafish model of *IGHMBP2* deficiency in order to study and develop therapies for SMARD1, a consistent phenotype was not observed and further work is required to characterize this model.

More than one million Americans suffer from neuromuscular disorders, however many of these conditions have no known treatments. By studying the molecular pathways involved we can attempt to develop therapies for these diseases.
TABLE OF CONTENTS

TITLE........................................................................................................................................i

COPYRIGHT PAGE..................................................................................................................ii

READER APPROVAL PAGE.......................................................................................................iii

ACKNOWLEDGMENTS ........................................................................................................... iv

ABSTRACT............................................................................................................................... v

TABLE OF CONTENTS............................................................................................................. vii

LIST OF TABLES..................................................................................................................... x

LIST OF FIGURES .................................................................................................................. xi

LIST OF ABBREVIATIONS......................................................................................................... xiii

INTRODUCTION ..................................................................................................................... 1

Inherited Disorders of Muscle ................................................................................................. 1

RNA Helicases ......................................................................................................................... 1

DEAD-box (Superfamily 2) RNA Helicases ............................................................................. 2

Myotonic Dystrophy ............................................................................................................... 3

Effects of CTC Repeats on CUGBP1 and MBNL1................................................................. 5

P68/DDX5 ................................................................................................................................ 6

DDX6 ......................................................................................................................................... 6

DDX27 ....................................................................................................................................... 7
Spinal Muscular Atrophy with Respiratory Distress Type I (SMARD1)........ 8
Charcot-Marie Tooth Disease (CMT).............................................................. 8
Ighmbp2 .................................................................................................. 8
Zebrafish as a Model Organism .............................................................. 9

SPECIFIC AIMS ...................................................................................... 12

METHODS ............................................................................................. 14

DDX27 Zebrafish .................................................................................... 14
Zebrafish Myofiber Culture and Immunofluorescence ...................... 15
Zebrafish Whole-Mount Immunofluorescence .................................. 16
In-situ Hybridization and Immunofluorescence ................................ 17
Study of DEAD box (DDX) polypeptides ........................................... 18
Primer Design for DDX genes ............................................................... 19
C2C12 Cell Growth ................................................................................ 21
RNA Extraction and cDNA Synthesis and PCR .............................. 21
IGHMBP2 Zebrafish ............................................................................. 22

RESULTS ............................................................................................... 23

Immunofluorescence of Cultured Zebrafish Myofibers ...................... 23
Whole Mount Immunofluorescence with Acetyl-Histone H3 Antibody .. 24
DDX27 deficiency results in an increase in muscle satellite cells ....... 26
Role of DDX27 in disease pathology in Myotonic dystrophy .............. 26
DDX Expression in skeletal muscle cells ............................................. 30
IGHMBP2 .............................................................................................. 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype of ighmbp2 fish</td>
<td>38</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>41</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>46</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>51</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Antibodies and the associated dilutions used for myofiber culture and IF</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Antibodies and the associated dilutions for whole-mount IF</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Antibodies and the associated dilutions used for in-situ hybridization and IF</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>DDX Primer Sequences</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>DDX RNA Helicases involved in ribosomal biogenesis or translation/initiation processes</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>Predicted genotype versus actual genotype of embryos obtained from <em>ighmBp2</em> heterozygous matings</td>
<td>39</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenotype and genetic mapping of $ddx27$ mutant fish</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Immunofluorescence of Cultured Zebrafish Myofibers</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Wild-type and $ddx27$ mutant fish stained with DAPI and Acetyl-H3</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Whole Mount IF with Pax7, MF20, and DAPI in Wild-type and $ddx27$ mutant fish</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>$DDX27$ forms abnormal protein aggregates in myoblasts of myotonic dystrophy patients</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Fibrillarin forms abnormal protein aggregates in myoblasts of myotonic dystrophy patients</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>C2C12 Cells at different developmental stages</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>Expression of DDX genes in C2C12 Cells at Different Stages of Differentiation</td>
<td>36</td>
</tr>
<tr>
<td>9</td>
<td>$ighmBp2$ Heterozygous Mutation 1</td>
<td>37</td>
</tr>
<tr>
<td>10</td>
<td>$ighmBp2$ Homozygous Mutation 1</td>
<td>37</td>
</tr>
<tr>
<td>11</td>
<td>$ighmBp2$ Heterozygous Mutation 2</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>$ighmBp2$ Homozygous Mutation 2</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Phenotypes of <em>ighmBp2</em> embryos observed on day 3</td>
<td>38</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>13</td>
<td>Phenotypes of <em>ighmBp2</em> embryos observed on day 5</td>
<td>39</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
<td></td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie Tooth Disease</td>
<td></td>
</tr>
<tr>
<td>CNBP</td>
<td>Cellular nucleic acid-binding protein</td>
<td></td>
</tr>
<tr>
<td>DDX</td>
<td>DEAD-box RNA Helicase</td>
<td></td>
</tr>
<tr>
<td>DM1</td>
<td>Myotonic dystrophy type 1</td>
<td></td>
</tr>
<tr>
<td>DM2</td>
<td>Myotonic dystrophy type 2</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td></td>
</tr>
<tr>
<td>DMPK</td>
<td>Dystrophia myotonica protein kinase</td>
<td></td>
</tr>
<tr>
<td>Dpf</td>
<td>Days post fertilization</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>HGNC</td>
<td>HUGO Gene Nomenclature Committee</td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
<td></td>
</tr>
<tr>
<td>MBNL1</td>
<td>Muscleblind-like protein 1</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
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<tr>
<td>PBST</td>
<td>PBS buffer with 0.1% Tween</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformeldehyde</td>
<td></td>
</tr>
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<td>RNP</td>
<td>Ribonucleoprotein</td>
<td></td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>Superfamily</td>
<td></td>
</tr>
</tbody>
</table>
SMARDI ........................................... Spinal muscular atrophy with respiratory distress
SSC ................................................................................................................. Saline sodium citrate
WT ......................................................................................................................... Wild-type
INTRODUCTION

Inherited Disorders of Muscle

Inherited muscle disorders are classified as non-dystrophic myopathies or muscular dystrophies. Both myopathies and dystrophies present with clinical symptoms including weakness, motor delay, and respiratory and bulbar dysfunction. Myopathies are caused by genetic defects that result in changes in the contractile apparatus of the muscle without significant regenerative changes in skeletal muscle. They can be characterized by specific histochemical or ultrastructural changes detectable in a muscle biopsy. Muscular dystrophies are diseases of the muscle membrane or supporting proteins, and are distinctive in their pathological evidence of continuous cycles of muscle degeneration and regeneration (Cardamone, Darras, & Ryan, 2008). Genetic advances in recent years have identified over a hundred neuromuscular disease genes. Surprisingly however, despite the large number of known neuromuscular disease genes, specific therapies remain unavailable due to a lack of clear understanding of the biological functions and pathological pathways regulated by disease-causing genes.

RNA Helicases

RNA helicases are enzymes that utilize ATP to bind or remodel RNA and RNA-protein complexes (ribonucleoprotein or RNP complexes). They are involved in many different aspects of RNA metabolism including transcription, translation, and mRNA decay. RNA helicases are classified into families and superfamilies based on their structure and function (Linder & Jankowsky, 2011). There are six superfamilies, and all
eukaryotic RNA helicases belong to superfamilies (SF) one and two making them relevant for human disease research (Jankowsky, 2011). SF1 and SF2 helicases have similarly structured catalytic cores. Unique enzymes within each of the superfamilies execute a wide array of distinct functions on different substrates. Many biological processes involving DNA or RNA use one or more helicases. Defects in helicase function, as well as disregulated expression of these helicases have been shown to lead to numerous diseases including cancers, developmental defects, and neurodegenerative diseases (Fairman-Williams, Guenther, & Jankowsky, 2010, p. 1).

**DEAD-box (Superfamily 2) RNA Helicases**

DEAD-box (DDX) proteins make up the largest superfamily (superfamily2) of RNA helicases. There are 43 different DEAD-box RNA helicases in humans. These are characterized by their Aspartic acid-Glutamic acid-Alanine-Aspartic acid (DEAD) motif. These proteins are often part of the spliceosome and are involved in eukaryotic translation initiation. They are involved in disassembly of RNPs (proteins associated with RNA involved in a wide array of processes), chaperoning during RNA folding, and stabilization of protein complexes on RNA. DEAD box proteins have a highly conserved core containing RNA and ATP binding sites. They also contain variable domains that surround the conserved core, which may serve as an explanation for the wide variety of functions that each member of this family performs. Mutations in DEAD-box RNA helicases have been linked to a number of diseases (Linder & Jankowsky, 2011). In skeletal muscles, several DDX proteins play an important role in muscle development.
and disease by interacting with muscle specific transcription factors and disease causing genes (Caretti et al., 2006).

**Myotonic Dystrophy**

Myotonic dystrophy is an autosomal dominantly inherited muscular dystrophy (Cardamone et al., 2008). Myotonic dystrophy is the most common form of muscular dystrophy affecting 1 in 8,000 people worldwide. Myotonic dystrophy is classified as type 1 (DM1) or type 2 (DM2) on the basis of genetic mutations. In most populations, type 1 is the most common (“Myotonic dystrophy,” 2015). Currently there are no specific treatments or cures for this disease (“Learning About Myotonic Dystrophy,” n.d.).

DM1 has four clinical forms: congenital, early childhood, adult-onset, and oligosymptomatic late-onset. Adult-onset, or “classic DM1” is the most common presentation diagnosed patients. DM1 presents with myotonia (prolonged muscle contractions) and skeletal muscle weakness and wasting (“Myotonic dystrophy,” 2015). In addition to skeletal muscle, DM1 can also affect cardiac muscle, causing fibrosis in the conduction system and sinoatrial node, resulting in conduction defects and tachyarrhythmia (Meola, Jones, Wei, & Timchenko, 2013). The congenital form presents with severe cognitive abnormalities and a delay in myogenesis (Jones et al., 2015). DM1 also causes patients to develop cataracts. In MRI scans, patients tend to have diffuse white matter in the brain. Additionally, DM1 affects endocrine functions, causing patients to suffer from insulin resistance, which leads to susceptibility to diabetes and hypothyroidism (Meola et al., 2013). The genetic defect in DM1 is caused by an amplified trinucleotide repeat to the dystrophia myotonica protein kinase gene (DMPK).
The expansion of the repeats is often related to the disease severity in affected patients. Normal individuals have between five and thirty-seven repeats, mildly affected individuals have 50-150 repeats, patients with classic DM1 have 100-1,000 repeats, and those with congenital onset have more than 2,000 repeats (“OMIM Entry - # 160900 - MYOTONIC DYSTROPHY 1; DM1,” n.d.).

DM2 typically presents as a late-onset disease and does not include clinical subtypes. The clinical phenotype presented by DM2 is variable. Patients can have disabilities beginning in their 40’s, ranging from early cardiac death to mild proximal weakness or slightly elevated creatine kinase levels (particularly in elderly patients). DM2 may present with muscle symptoms such as proximal or lower limb weakness or myalgic pain. Myotonia is not always present in this disease. DM2 may occasionally be associated with severe muscular atrophy and disability, and patients may have severe myalgic pain. This pain is often the major cause of dysfunction and hindrance to daily activities (Meola et al., 2013). DM2 is caused by an abnormal expansion of repeats in intron 1 of the zinc finger protein-9 gene (ZNF9). Normal individuals have up to 30 repeats, while affected individuals can have between 70 to 11,000 repeats (“OMIM Entry - # 602668 - MYOTONIC DYSTROPHY 2; DM2,” n.d.).

**Mechanism by which CTG/CCTG repeats cause DM1 and DM2**

DM1 is caused by mutations to the dystrophia myotonica protein kinase (*DMPK*) gene. The mutation is located in the 3’ untranslated region, and consists of an expansion of CTG triplet repeats. DM2 is caused by expanded CCTG repeats in intron 1 of the
cellular nucleic acid-binding protein (CNBP; also called ZNF9) (Jones et al., 2015).
Previous studies showed that these mutations result in disease by RNA gain of function mechanisms (Jones et al., 2015). In myotonic dystrophy, the mutant RNA accumulates in cells and results in the disease pathology (Jones et al., 2015). Expanded transcripts form aggregates in the nucleus and cytoplasm of the cell. Expanded CTG repeats in these RNAs form a hairpin structure that interferes with the normal functions of RNA binding proteins, and therefore negatively impacts normal cellular function (Laurent et al., 2012). Studies have identified a number of RNA binding proteins that are sequestered or mis-regulated in myotonic dystrophy such as CUG binding protein 1 (CUGBP1), muscleblind-like protein 1 (MBNL1) Stafuen1, and two members of the DEAD-box RNA helicase family; DDX5 and DDX6 (Jones et al., 2015).

Effects of CTC Repeats on CUGBP1 and MBNL1

CUGBP1 is involved in splicing, as well as a number of functions in the cytoplasm including regulation of RNA translation, RNA deadenylation and RNA stability. CUGBP1 functions as both an activator (act) and repressor (rep) of these processes. Whether CUGBP1 is an activator or repressor depends on phosphorylation at S302. The phosphorylated protein is an activator, while the unphosphorylated form is a repressor. CUGBP1act increases the translation of mRNAs important for skeletal muscle myogenesis, liver proliferation and differentiation, cancer development, and aging. These mRNAs are also important in DM1 and DM2 pathogenesis. In normal cells, the activator and repressor versions of these proteins are balanced, however in DM1 cells both forms are increased. This leads to a misbalance in protein synthesis.
Additionally, interactions of different RNA binding proteins with the mutant CUG RNA might change the normal functions, therefore altering RNA processing in the nucleus and cytoplasm of DM1 cells (Meola et al., 2013).

MBNL1 is a splicing factor that binds RNA through its zinc-finger domains. MBNL1 is sequestered within the nuclear foci of expanded CUG repeats and this results in a loss of function of the protein resulting in mis-splicing of many target pre-mRNAs (Laurent et al., 2012) (Pettersson et al., 2014).

**P68/DDX5**

DDX5 is a member of the DEAD-box RNA helicase family is sequestered in the RNA foci expressing expanded CUG repeats in myotonic dystrophy patients. A previous study showed that DDX5 acts as a modifier by increasing the binding of MBNL onto CUG repeats (Laurent et al., 2012).

**DDX6**

DDX6 is a DEAD-box helicase mainly located in the cytoplasm. It is needed for many steps in regulated mRNA turnover and translation. In mammalian cells specifically it is needed for assembly of processing bodies. These processing bodies contain repressed mRNPs, mRNA decay factors, and proteins important for miRNA-machinery. In a study done by Pettersson et al., over expression of DDX6 was shown to partially rescue missplicing events specific to DM1. This study showed that DDX6 interacts with CUG repeats in DM1 patient fibroblasts and with CUG RNA in-vitro. Overexpression of DDX6 results in a decrease in the amount of nuclear DMPK messenger ribonucleoprotein
foci (DMPK-mRNA). Concordantly, knockdown of endogenous DDX6 results in a significant increase in the number of DMPK-mRNA foci in addition to increased sequestration of MBNL1 in the nucleus. Lastly, this study was able to show that DDX6 can unwind CUG-repeat duplexes in vitro in an ATP dependent manner. This suggests that DDX6 might have the ability to release nuclear DMPK-mRNA foci, and reestablish normal splicing events (Pettersson et al., 2014).

**DDX27**

DDX27 is a member of the DEAD-box helicase family. In a study done by Gupta et al., a forward genetics approach was taken using the zebrafish as a model organism. This study resulted in 13 unique zebrafish mutants with defective skeletal muscle. One of these mutants, named “osoi”, was found to have impaired motility behavior. They also exhibit skeletal muscle hypotrophy and extensive central nucleation. Genetic mapping of this fish led to the discovery of a novel mutation in DEAD-box protein encoding gene ddx27. In yeast, mutations of ddx27 orthologue rs1 result in 25s rRNA maturation defect and 60S ribosomal subunit deficiency. Preliminary data shows that zebrafish and mammalian DDX27 is necessary for regulating the maturation of rRNAs. This would complement data that shows muscle hypertrophy is accompanied by increased ribosomal biogenesis. However, more recent studies have pointed to non-ribosomal roles of nucleolar genes, and that impairment of these functions may lead to cell cycle defects (Tsai & Pederson, 2014).
Spinal Muscular Atrophy with Respiratory Distress Type I (SMARD1)

SMARD1 is a rare autosomal recessive disorder. It is generally characterized by respiratory failure due to diaphragmatic paralysis and presents within the first two months of life. Severe infantile axonal neuropathy is also characteristic of SMARD1. The disease is often life threatening in the first year of life, but it is possible for patients to live into adolescence and early adulthood with ventilator support. (Wagner et al., 2015) Currently, the only treatment involves managing symptoms. There is no specific cure available, making this disease a good candidate for drug screens (“Learning About Spinal Muscular Atrophy,” n.d.).

Charcot-Marie Tooth Disease (CMT)

CMT is a common neuromuscular disorder with a prevalence of 1 in 2500. Progressive length-dependent weakness, muscle atrophy, sensory loss, and areflexia are characteristic of CMT. Until recently, mutations in IGHMBP2 have been associated exclusively with SMARD1, but a study by Cottenie et al. identified patients with clinical features of CMT without respiratory involvement had autosomal recessive mutations in IGHMBP2 (Wagner et al., 2015). Currently there is no cure for CMT, and treatment consists of physical and occupational therapy (“Charcot-Marie-Tooth Disease Fact Sheet,” n.d.).

Ighmbp2

IGHMPB2 is an immunoglobulin µ-binding protein and in humans, it is composed of 993 amino acids. It is classified as an SF1 RNA helicase, and contains an
RNA/DNA helicase domain, an R3H single-stranded nucleic acid-binding domain and a zinc finger domain. IGHMBP2 mRNA is ubiquitously expressed. In vitro studies have shown that IGHMBP2 is involved in transcriptional activation, immunoglobulin class switching and pre-mRNA splicing. The RNA helicase domain of IGHMBP2 suggests its involvement in RNA processing, regulation, or metabolism (Planell-Saguer, Schroeder, Rodicio, Cox, & Mourelatos, 2009). Additionally, IGHMBP2 has been shown to associate with ribosomes, tRNA, and it regulates ribosomal biogenesis. Mutations in the gene result in degeneration of anterior horn cell alpha-motor neurons (Wagner et al., 2015). This results in respiratory distress caused by diaphragmatic palsy and distal motor weakness (Schottmann et al., 2015). Individuals with mutations in IGHMBP2 can present with spinal muscular atrophy with respiratory distress (SMARD1) or with Charcot-Marie Tooth disease (Wagner et al., 2015).

Zebrfish as a Model Organism

The zebrafish, *Danio rerio*, is a small freshwater fish native to inland streams and rivers of India. They are an average of 25 mm in length and have a number of properties that make them a good model organism for research (Markowski, n.d.). Zebrafish are small and robust, allowing them to be kept in a lab in large numbers at a low cost. The fish breed throughout the entire year, with females able to spawn every two to three days. A single clutch may have several hundred embryos. Generation time for zebrafish is on average three to four months, which is relatively short for a vertebrate animal. This makes it a good model for selection experiments. The embryos of zebrafish are also relatively large in comparison to other fish species. When fertilized, the embryos are 0.7
mm in diameter. They are fertilized externally and are also optically transparent. This particular feature allows the embryos to be manipulated and monitored under a dissection microscope through all stages of development. The embryos develop quickly, with precursors to all major organs developing within 36 hours of fertilization ("Zebrafish - Danio rerio - Overview," n.d.). Most importantly, orthologues of most of the human genes are present in zebrafish that share a high sequence homology, thus making them an ideal system to model human diseases.

Zebrafish serve as a particularly good model for studying muscular dystrophy. Zebrafish and humans share a high degree of sequence homology. They contain many similar dystrophy associated proteins as humans. Mutations in these genes can cause phenotypes similar to those seen in human patients. This suggests experimental findings from zebrafish studies will be transferable to mammals (Guyon et al., 2007).

The Current Study

Gupta lab had previously identified a zebrafish mutant (osoi) with hypotrophy and disorganization of skeletal muscle and phenotypic abnormalities in the brain, eyes and heart. The “osoi” mutant fish has a 20 base pair deletion in exon 18 of the ddx27 gene. The phenotypes observed in this mutant are similar to the abnormalities seen in human myotonic dystrophy. It is evident that RNA helicases are critical for ribosomal biogenesis and muscle growth in human disease. With this knowledge, we hope to achieve a better understanding of the biological and molecular pathways regulated by RNA helicases and related RNA binding proteins in skeletal muscle development diseases. This insight may
allow us to develop an approach to identifying potential therapies for neuromuscular diseases.
SPECIFIC AIMS

By understanding the molecular pathways that contribute to neuromuscular disorders, diagnosis and treatment of patients suffering from these disorders can be improved. The objective of this project is to understand the role of RNA helicases in neuromuscular development and diseases. Inherited disorders of muscle include both dystrophies and non-dystrophic myopathies. These conditions are characterized by muscle weakness and impaired movement, and they form a wide array of inherited disease affecting both adults and children. Studies of these diseases in humans are difficult due to the clinical and genetic heterogeneity, variable penetrance, and early mortality. Therefore zebrafish, *Danio rerio*, will be used as an animal model for this study due to their high degree of sequence homology with humans, vertebrate skeletal muscle structure, and rapid ex vivo development. In a previous study performed in our lab, 13 unique zebrafish mutants were identified with defective skeletal muscles. One of these mutants, named osoi, was found to have a loss of function mutation in a novel RNA binding protein, DEAD-box helicase *ddx27*. This helicase is part of a highly conserved family of DEAD-box RNA helicases. Within this family of DEAD-box proteins, there are currently 43 members that have been found in humans. They are involved with a diverse array of functions related to RNA metabolism. Several studies have shown that ribosomal regulation is important for muscle growth. In several models of dystrophies, a decrease in ribosomal function is observed. Earlier studies in our lab suggest there is a defect in ribosomal biogenesis in the skeletal muscle of the *ddx27* mutant fish and mammalian cells. More recent studies suggest non-ribosomal functions of these nucleolar
genes. Therefore, the proposed study is aimed at understanding the roles of ddx27 and related proteins in skeletal muscle development and neuromuscular diseases. DEAD box RNA helicases are involved in RNA metabolism and regulate biological processes that involve RNA function. The focus of this work is on regulation of protein translation processes in skeletal muscle development by DDX proteins.

The specific aims of this study are:

1. To determine the role of ddx27 in myogenesis and diseases
2. To identify DDX genes that are expressed in mammalian muscle cells and regulate ribosomal biogenesis or translation initiation/elongation processes
3. Develop a zebrafish model of ighmBp2 deficiency to study disease pathology and develop therapies for SMARD1 in zebrafish

Through this study, we hope to better understand the molecular mechanisms of RNA binding DEAD-box helicase proteins and their functions in muscle development and disease pathology. Through a series of immunofluorescence studies using multiple markers, we will investigate the role of ddx27 in myogenesis and disease. We aim to understand the role of ddx27 in myotonic dystrophy through in-situ hybridization and immunofluorescence (IF) in patient and control muscle cells. We want to identify additional DDX genes that are expressed in mammalian muscle cells and involved in ribosomal biogenesis or translation as a starting point for future studies. Finally we hope to characterize the phenotype and pathologies associated with IGHMBP2 mutations in zebrafish. Through increased understanding of the molecular mechanisms involved with skeletal muscle hypotrophy and disease, improved corrective therapies can be developed.
METHODS

**DDX27 Zebrafish**

Prior to this study, the Gupta Lab performed ENU F₂ mutagenesis screening to identify mutant zebrafish with skeletal abnormalities. This led to the discovery of a fish with highly impaired swimming behavior, eye defects, pericardial edema, and reduced birefringence indicative of structural muscle defects. These fish were found to have a 20 base pair deletion in exon 18 of the *ddx27* gene resulting in a frameshift mutation. Homozygous fish die at 6 or 7 dpf. The heterozygous fish are fully viable, fertile, and apparently unaffected. The phenotype and genetic mapping of this fish can be seen in Figure 1.

*Figure 1: Phenotype and genetic mapping of *ddx27* mutant fish. *ddx27* mutant fish have leaner muscle and pericardial edema. They also show reduced birefringence in comparison to the wild-type (WT), indicative of disorganized myofiber structure. (WT, top; mutant, bottom). Sequencing shows a 20 base pair deletion in the *ddx27* gene.*
**Zebrafish Myofiber Culture and Immunofluorescence**

Poly-L-lysine was plated in 8 well chamber slides and kept at 37°C for 1 hour. Zebrafish embryos were collected at 4 days post fertilization (dpf). Approximately 40 wild-type and 40 mutant embryos were placed in eppendorf tubes and the water was removed. 900 µl of CO₂ independent media and 150 µl of collagenase type II (3.125 mg/ml) were added. The tubes were rotated on an orbital shaker for approximately 2 hours and checked every 30 minutes to prevent over digestion. The tubes were then centrifuged at 1500 rpm for 5 minutes to pellet the cells. The supernatant was removed, and the cells were washed two times with CO₂ independent media. 1 ml of fresh CO₂ independent media was added to resuspend the cells, and the suspension was passed through a 70 µm filter to remove debris. 200 µl of the suspension was added to each well of the poly-l-lysine coated slide, and the myofibers were allowed to settle on the slide for one hour at RT. The media was removed, and the myofibers were fixed in either PFA for 20 minutes or PFA for 10 minutes followed by -20°C methanol for 10 minutes depending on the antibody to be used. Following fixation, the myofibers were washed 3 times with 1xPBS. Blocking solution (1xPBS, 2 mg/ml bovine serum albumin, 1% sheep serum and 0.25% TritonX-100) was added for 1 hour. The blocking solution was removed, and primary antibody diluted in blocking solution was added and the slides were kept at 4°C overnight. The following day, the primary antibody solution was removed and the myofibers were washed 3 times (5 minutes each wash) with 1xPBS. The secondary antibody diluted in 1xPBS was added for 1 hour at RT. DAPI was added to the secondary antibody solution (1:1000, D1306, Thermofisher Scientific). The secondary antibody was
then removed and the slides were washed 3 times (5 minutes each wash) at RT. Slides were visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope. The antibodies used, dilutions, and fixation conditions can be seen in Table 1.

**Table 1: Antibodies and the associated dilutions used for myofiber culture and IF.**

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Fixation Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinin (Sigma-Aldrich)</td>
<td>1:100</td>
<td>Anti-Mouse</td>
<td>1:100</td>
<td>PFA</td>
</tr>
<tr>
<td>RYR1 (Sigma-Aldrich)</td>
<td>1:100</td>
<td>Anti-Mouse</td>
<td>1:100</td>
<td>PFA</td>
</tr>
<tr>
<td>Lamin A (Sigma-Aldrich)</td>
<td>1:50</td>
<td>Anti-Mouse</td>
<td>1:100</td>
<td>PFA + Methanol</td>
</tr>
<tr>
<td>Fibrillarin (Santa Cruz Biotechnology)</td>
<td>1:50</td>
<td>Anti-Rabbit</td>
<td>1:100</td>
<td>PFA + Methanol</td>
</tr>
<tr>
<td>B23 (Santa Cruz Biotechnology)</td>
<td>1:50</td>
<td>Anti-Rabbit</td>
<td>1:100</td>
<td>PFA + Methanol</td>
</tr>
<tr>
<td>UBF (Santa Cruz Biotechnology)</td>
<td>1:50</td>
<td>Anti-Rabbit</td>
<td>1:100</td>
<td>PFA + Methanol</td>
</tr>
</tbody>
</table>

**Zebrafish Whole-Mount Immunofluorescence**

Mutant and wild-type embryos were fixed in methanol at 2 dpf. The methanol was removed and replaced with 50% methanol/50% PBS for 10 minutes. The embryos were washed two times (5 minutes each wash) in 1xPBS. The PBS was removed and PBS 1% TritonX-100 was added for 1 hour. The embryos were washed in 1xPBS for 5 minutes. Blocking solution (5% goat serum in PBST) was added for 1 hour. The antibodies were diluted in the blocking solution and the embryos were kept at 4°C overnight. The following day the embryos were washed in PBST two times (15 minutes each wash). The secondary antibody was diluted in PBST and added for 1 hour at RT. DAPI was added to
the secondary antibody solution (1:1000, D1306, Thermofisher Scientific). The embryos were washed twice in PBST and mounted on slides. Slides were visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope. The antibodies used and dilutions can be seen in Table 2.

Table 2: Antibodies and the associated dilutions for whole-mount IF

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-H3 (abcam)</td>
<td>1:50</td>
<td>Anti-Rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>Pax7 (DHSB)</td>
<td>1:50</td>
<td>Anti-Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>MF20 (DHSB)</td>
<td>1:50</td>
<td>Anti-Mouse</td>
<td>1:100</td>
</tr>
</tbody>
</table>

In-situ Hybridization and Immunofluorescence

In-situ hybridization and IF experiments were conducted on control and DM1 affected patient myoblasts. The cells were grown in culture and on a chamber slide in human skeletal muscle growth media. The media was removed and the cells were fixed in 2% paraformaldehyde (PFA) for 10 minutes at 4°C. The cells were then washed with 1x phosphate buffered saline (PBS) 5 times (two minutes each wash) at room temperature (RT). Following this step the cells were incubated in pre-chilled (-20°C) methanol for ten minutes. The cells were then incubated in pre-hybridization buffer for ten minutes, followed by incubation in hybridization buffer containing 10 µl of 100/ng/µl stock of DM1 probe. The sequence for the DM1 probe is 5’ - /56-FAM/mCmAmG mCAmG CmAG mCAG CmAG mCAmG CAmG CmAmG CmAmG CmAmG-3’. The “m” in the sequence is a modification of 2’O-methyl RNA bases in order to prevent degradation of the probe. The cells were incubated in hybridization buffer for 3 hours at 37°C. The hybridization buffer was replaced with post-hybridiation buffer and the cells were
incubated for 30 minutes at 45°C. This was followed by a wash in 1 x Saline Sodium Citrate (SSC) buffer for 30 minutes at RT. The cells were washed with PBS buffer with 0.1% Tween (PBST) 2 times (5 minutes each wash). This was followed by blocking in 5% goat serum in 1xPBS for 30 minutes. The cells were incubated overnight at 4°C in the primary antibody diluted in the blocking solution. The antibodies used were anti-DDX27 (Santa Cruz Biotechnology), anti-Fibrillarin (abcam), anti-mouse, and DAPI. Antibody concentrations are provided in Table 3. The following day cells were washed with 1xPBST 3 times (5 minutes each wash). Secondary antibody was added for 1 hour at RT diluted in 1xPBST. The cells were washed with 1xPBST three times for five minutes each. DAPI was added to the first wash (1:1000, D1306, Thermofisher Scientific). Slides were visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope.

Table 3: Antibodies and the associated dilutions used for in-situ hybridization and IF. All antibodies were diluted in 5% goat serum in 1xPBS.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DDX27</td>
<td>1:50</td>
<td>Anti-Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-Fibrillarin</td>
<td>1:50</td>
<td>Anti-Mouse</td>
<td>1:100</td>
</tr>
</tbody>
</table>

**Study of DEAD box (DDX) polypeptides**

To identify DDX encoding genes in humans, the HGNC database of human genes was used. There are 43 genes contained within the family (“DEAD-box helicases (DDX) Gene Family | HUGO Gene Nomenclature Committee,” n.d.). This information was employed to create a list that included the approved gene name, previous gene symbols,
and gene function. Gene function was obtained using Gene Cards: The human gene

**Primer Design for DDX genes**

To study the expression of DDX genes in skeletal muscle, RT-PCR primers were
designed for DDX genes involved in rRNA processing, translation, or ribosomal
biogenesis. Of the 43 DDX genes, 24 were involved in these processes. Therefore,
primers were designed for 24 DDX genes and ordered through Invitrogen Custom DNA
Oligos. To design RT-PCR primers, the mRNA sequence for mouse DDX genes was
identified in PubMed. The coding region was identified and primers were designed
outside this area in the 3’ or 5’ untranslated regions as many of the DDX genes share
sequence similarities in the coding regions. Using Primer 3 (“Primer3 Input (version
0.4.0),” n.d.), a primer was designed using a 200 – 250 base pairs target sequence. The
specificity of primers was checked in the mouse genome using BLAST (“Nucleotide
BLAST: Search nucleotide databases using a nucleotide query,” n.d.). A list of primer
sequences can be seen in Table 4.

<table>
<thead>
<tr>
<th>DDX Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX 1</td>
<td>Forward: 5’-CTGTTCCGAACCTTCTGACG-3’&lt;br&gt;Reverse: 5’-ATAGTTGTCAAGTTTATTTTTTCATGG-3’</td>
</tr>
<tr>
<td>EIF4A</td>
<td>Forward: 5’-TCAAGGGTTATGATGATTGC-3’&lt;br&gt;Reverse: 5’-CAATGCAGGCATGACAAGAG-3’</td>
</tr>
<tr>
<td>EIF4A2</td>
<td>Forward: 5’-AAGCGCGTGGACGTAATTAG-3’&lt;br&gt;Reverse: 5’-CTTTCTACAAAGTACCAGAAA-3’</td>
</tr>
<tr>
<td>DDX3X</td>
<td>Forward: 5’-TTTACTGAACCTGGGCTAAATCA-3’&lt;br&gt;Reverse: 5’-TGGCCACATTAGAAACTAATTCCA-3’</td>
</tr>
<tr>
<td>DDX3Y</td>
<td>Forward: 5’-GAACTGCTCATTATATCTGCAT-3’</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>DDX5</td>
<td>5’-AATGGTTAAACAAAATAGAATGTGCTG-3'</td>
</tr>
<tr>
<td>DDX6</td>
<td>5’-GAGTTAAGTGTCGAGGGTTG-3'</td>
</tr>
<tr>
<td>DDX10</td>
<td>5’-TTATCCCCACCATTGGA-3’</td>
</tr>
<tr>
<td>DDX18</td>
<td>5’-CACATCAGTAAGAAGCCAGC-3’</td>
</tr>
<tr>
<td>DDX21</td>
<td>5’-CCCTTACATATGTGCTC-3’</td>
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<tr>
<td>DDX24</td>
<td>5’-GTAAAGGCTTCCCTTGC-3’</td>
</tr>
<tr>
<td>DDX25</td>
<td>5’-GCAGCTATGGCGTCGTTACT-3’</td>
</tr>
<tr>
<td>DDX27</td>
<td>5’-TGAAAGATTTGAGTGACGCA-3’</td>
</tr>
<tr>
<td>DDX28</td>
<td>5’-GGACCTTTTGCTCTAAAA-3’</td>
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<tr>
<td>DDX31</td>
<td>5’-TGAGCTAATTGATTGACTTGTG-3’</td>
</tr>
<tr>
<td>DDX47</td>
<td>5’-TGAGACAACTTTTACAGTGTTGA-3’</td>
</tr>
<tr>
<td>EIF4A3</td>
<td>5’-ATCCGGATCTCAGGGACAT-3’</td>
</tr>
<tr>
<td>DDX49</td>
<td>5’-TGAGCTTTACAGGGACAT-3’</td>
</tr>
<tr>
<td>DDX50</td>
<td>5’-GGCCATAAAGGGAGTTTTGGA-3’</td>
</tr>
<tr>
<td>DDX51</td>
<td>5’-AAGTGCTTATACGTTAGCGTCTCC-3’</td>
</tr>
<tr>
<td>DDX52</td>
<td>5’-ATGACTGAGCGCAACTGGA-3’</td>
</tr>
<tr>
<td>DDX54</td>
<td>5’-AGAGAGCGGGTGTTAGGAGCG-3’</td>
</tr>
<tr>
<td>DDX55</td>
<td>5’-TGTAATTCCATCTTTTATAGACAGA-3’</td>
</tr>
</tbody>
</table>
C2C12 Cell Growth

C2C12 cells are an immortal line of mouse skeletal myoblasts (“ENCBS124ENC – ENCODE,” n.d.). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum, 100 units/mL penicillin and 100 µg/ml streptomycin. The cells were grown at 37°C in a humidified incubator with 5% CO2. The cells were grown and split into ten 10 cm culture dishes, with approximately 500,000 cells per dish. After day 0 collection, remaining six plates were changed to differentiation media. The differentiation media is made of DMEM supplemented with 2% donor equine serum and 1 µM Penicillin-Streptomycin-Glutamine. Dishes of cells were collected and frozen at day -2, day 0, day 2, day 5, and day 6.

RNA Extraction and cDNA Synthesis and PCR

RNA was extracted from the C2C12 cells using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. The RNA concentrations were quantified using Nanodrop. Equal concentrations of RNA from different samples were used to synthesize complementary DNA (1.87 µg/µL). The RNA was converted to complementary DNA (cDNA) using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA). The cDNA was created according to kit protocol using random hexamers. A polymerase chain reaction was set up for each DDX gene using cDNA template and the respective designed primer.
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed gene, was used as a control for normalization. (Stephens, Stephens, & Morrison, 2011) The samples were run on a 1% agarose gel to quantify the expression.

**IGHMBP2 Zebrafish**

*IGHMBP2* mutant fish were created using the CRISPR technology by targeting exon 10. This resulted in two different mutations in exon 10. Mutation 1 is a 30 base pair deletion beginning at base the -3 position of exon 10. Mutation 2 is a two base pair deletion at base 21 of exon 10. Heterozygous fish with either of these mutations are fully viable, fertile, and apparently unaffected. To study mutant embryos, one male and one female heterozygous fish were placed in mating cages overnight. Embryos were collected the next morning and evaluated over time. To evaluate the resulting phenotypes, physical appearance was monitored in addition to swimming behavior and touch evoked response. As phenotypes were characterized, DNA was extracted from the embryos and Sanger sequencing was performed to confirm their genotype. Sequences were analyzed using the Sequencher program. (*Sequencher*, n.d.)
RESULTS

*Immunofluorescence of Cultured Zebrafish Myofibers*

To identify both skeletal muscle and nucleolar defects in the *ddx27* mutant zebrafish, a number of different sarcomeric and nucleolar markers were used. Actinin, ryanodine receptor 1 (RYR1), and Lamin A antibodies were used to look at myofiber structure. The mutant myofibers showed disorganized sarcomeric structure in comparison to the wild-type myofibers (Figure 2). To investigate nucleolar defects, antibodies for UBF, Fibrillarin, and B23 were used. UBF labels the sites of RNA polymerase I transcription. Fibrillarin marks the dense fibrillar compartment of the nucleolus. B23 associates with nucleolar ribonucleoprotein structures. All nucleolar markers showed changes in organization when comparing the mutant fish to the wild-type fish. The fibrillin antibody showed merged, larger and more condensed structures in comparison to the WT control fish. The distribution of B23 was altered in the nucleoplasm. In the WT fish, B23 forms several small aggregates, while in the mutant fish B23 forms one large aggregate, colocalizing almost entirely with the nucleus. Ubf in the mutant fish was also drastically different from the wild-type fish. The organization changes from small punctate foci to larger condensed areas.
Figure 2: Immunofluorescence of Cultured Zebrafish Myofibers: Control and mutant myofibers were cultured and analyzed using a number of markers to visualize the sarcomeric and nucleolar structures.

Previous studies have shown that chromatin attaches to the nucleolus at several points within the cell. This localization of chromatin acts to regulate gene expression as the genes present in the attachment sites are often repressed. To determine the effects of nucleolar changes on chromatin state, 4 dpf zebrafish embryos were stained with Acetyl-
Histone H3 antibody and DAPI. DAPI is a marker for the cell nucleus, and acetyl-Histone H3 is a marker for transcriptionally active euchromatin within the nucleolus. Immunofluorescence analysis showed (Figure 3) an increase in the number of acetylated nuclei in the ddx27 mutant fish, suggesting an increase of activation of chromatin in mutants in comparison to the WT control.

Figure 3: Wild-type and ddx27 mutant fish stained with DAPI and Acetyl-H3 antibody: The mutant fish have an increased number of nuclei expressing acetylated H3 in comparison to wild-type.
**DDX27 deficiency results in an increase in muscle satellite cells**

In order to study myogenesis and the regenerative properties of *ddx27* mutant fish, IF was done using Pax7, MF20, and DAPI. Pax7 is a marker for satellite cells (precursors to skeletal muscle), and is important for muscle regeneration. MF20 stains myosin heavy chain and is a sarcomeric marker. DAPI stains the cell nuclei. The results of this experiment can be seen in Figure 4. The results show an increase in the number of Pax7 positive cells in the *ddx27* mutant fish.

**Figure 4: Whole Mount IF with Pax7, MF20 and DAPI in Wild-type and *ddx27* mutant fish. Pax7 is in green, MF20 is red, and DAPI is blue. There is an increase in the number of Pax7 positive cells in the mutant fish.**

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**Role of DDX27 in disease pathology in Myotonic dystrophy**

Control and patient myoblasts stained using combined fluorescent in situ hybridization can be seen in Figures 5 and 6. In Figure 5, the cells were stained with a
DDX27 antibody. Our results from this study show that the DDX27 protein in DM1 cells from patient A and patient C formed large aggregates in the center of the nucleolus, differing from the small and distributed aggregates observed in the control cells. The staining in DM1 patient B has a very different pattern of staining from both patients A, C, and the control cells. This patient also has the most severe form of the disease. In Figure 6, cells were stained with a Fibrillarin antibody. This is also a nucleolar protein known to associate with DDX proteins. The staining observed with this antibody corresponds to the DDX27 staining seen in Figure 5. These results lead us to believe that there may be a cell cycle or apoptotic defect present in these cells.
Figure 5: DDX27 forms abnormal protein aggregates in myoblasts of myotonic dystrophy patients: Combined fluorescent in situ hybridization (FISH) and immunofluorescence using FITC-labeled antisense oligonucleotide probe (green), DDX27 antibody (red) and DAPI (blue) show abnormal DDX27 protein aggregates in myoblasts from myotonic dystrophy patients.
Figure 6: Fibrillarin forms abnormal protein aggregates in myoblasts of myotonic dystrophy patients: Combined fluorescent in situ hybridization (FISH) and immunofluorescence using FITC-labeled antisense oligonucleotide probe (green), Fibrillarin antibody (red) and DAPI (blue) show abnormal Fibrillarin protein aggregates in myoblasts from myotonic dystrophy patients.
**DDX Expression in skeletal muscle cells**

To understand the biological functions of DDX genes in skeletal muscle development, expression of genes relevant to the protein translation process was analyzed in proliferating and differentiating muscle cells. The human genome contains 43 DDX genes and 24 of them are known to have a role in rRNA processing, translation, or ribosomal biogenesis. Table 5 outlines these DDX genes and their specific roles.

**Table 5: DDX RNA Helicases involved in ribosomal biogenesis or translation/initiation processes**

<table>
<thead>
<tr>
<th>Approved Symbol</th>
<th>Synonym</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDX1</strong></td>
<td>DBP-RB</td>
<td>• DNA duplex unwinding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Double-strand break repair</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Transcription, DNA templated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Translation initiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Spliceosomal complex assembly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• tRNA splicing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• tRNA processing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• mRNA processing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Response to virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Gene expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• RNA unwinding</td>
</tr>
<tr>
<td><strong>EIF4A1</strong></td>
<td>DDX2A, EIF-4A</td>
<td>• Nuclear-transcribed mRNA catabolic process; deadenylation-dependent decay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Nuclear-transcribed mRNA poly(A) tail shortening</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Translation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Translation initiation</td>
</tr>
<tr>
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<td>• Gene expression</td>
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<td>• RNA unwinding</td>
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<td>• Viral process</td>
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<td>• Cytokine-mediated signal pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Organ regeneration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cellular protein metabolic process</td>
</tr>
<tr>
<td><strong>EIF4A2</strong></td>
<td>DDX2B, EIF4A, BM-010</td>
<td>• Nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Nuclear-transcribed mRNA poly(A) tail shortening</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Translation initiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Gene expression</td>
</tr>
</tbody>
</table>

30
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein(s)</th>
<th>Functions and Processes</th>
</tr>
</thead>
</table>
| DDX3X | DBX, HLP2, DDX14 | - Immune system process  
- Transcription  
- **Translational initiation**  
- Apoptotic process  
- Chromosome segregation  
- Response to virus  
- RNA unwinding  
- Positive regulation of gene expression  
- Regulation of cell growth  
- DNA duplex unwinding  
- Regulation of interferon-beta production  
- Stress granule assembly  
- **Ribosome biogenesis**  
- **Mature ribosome assembly**  
- Regulation of ribosome biogenesis  
- Endopeptidase activity  
- Innate immune response  
- Cellular response to arsenic-containing substance  
- Cellular response to osmotic stress  
- Regulation of chemokine ligand 5 production  
- Regulation of G1/S transition of mitotic cell cycle |
| DDX3Y |  | - Chromosome segregation  
- Regulation of gene expression  
- RNA unwinding  
- **Translational initiation** |
| DDX5 | P68 | - Regulation of transcription from RNA polymerase II promoter  
- Regulation of alternative mRNA splicing via spliceosome  
- mRNA splicing  
- In utero embryonic development  
- Transcription  
- mRNA processing |
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Functional Categories</th>
</tr>
</thead>
</table>
| **DDX6** | **RCK** | - Cytoplasmic mRNA body assembly  
- Gene expression  
- Negative regulation of neuron differentiation  
- Nuclear-transcribed mRNA catabolic process; deadenylation-dependent decay  
- **Regulation of translation**  
- RNA unwinding  
- Stem cell maintenance  
- Viral RNA genome packaging  
- Exonucleolytics nuclear-transcribed mRNA catabolic process involved in deadenylation-dependent decay |
| **DDX10** | **HRH-J8** | - RNA unwinding  
- **Ribosome biogenesis (yeast)** |
| **DDX18** | **MrDb** | - RNA unwinding  
- rRNA processing (yeast; HAS1)  
- **Ribosome biogenesis (yeast; HAS1)**  
- **Ribosomal large subunit biogenesis (yeast; HAS1)**  
- **Ribosomal small subunit biogenesis (yeast; HAS1)** |
| **DDX21** | **RH-II/GU, GURDB** | - Osteoblast differentiation  
- Response to exogenous dsRNA  
- Response to virus  
- RNA unwinding  
- **rRNA processing**  
- Transcription from RNA polymerase II |
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Functions/Processes</th>
</tr>
</thead>
</table>
| DDX24 | promoter    | • RNA metabolic process  
|       |             | • RNA unwinding       
|       |             | • rRNA processing (Yeast; MAK5)  
|       |             | • Ribosome biogenesis (Yeast; MAK5)  |
| DDX25 | GRTH        | • mRNA export from nucleus  
|       |             | • Multicellular organismal development  
|       |             | • Regulation of translation  
|       |             | • RNA unwinding       
|       |             | • Spermatid development  |
| DDX27 | dJ686N3.1, DRS1 | • RNA unwinding       
|       |             | • Ribosome biogenesis (yeast; DRS1)  |
| DDX28 | MDDX28, FLJ11282 | • RNA unwinding       
|       |             | • Mitochondrial large ribosomal subunit assembly  
|       |             | • Ribosome biogenesis  |
| DDX31 | FLJ13633, FLJ23349, FLJ14578, PPP1R25 | • RNA unwinding       
|       |             | • Ribosome biogenesis  
|       |             | • Nucleotide binding       
|       |             | • Nucleic acid binding       
|       |             | • rRNA processing and ribosome biogenesis (yeast; DBP7)  |
| DDX47 | DKFZp564O176, FLJ30012, HQ0256, RRP3 | • rRNA processing       
|       |             | • mRNA processing       
|       |             | • Apoptotic process       
|       |             | • RNA splicing       
|       |             | • Extrinsic apoptotic signaling pathway via death domain receptors  
|       |             | • RNA unwinding       
|       |             | • rRNA processing and ribosome biogenesis (yeast; RRP3)  |
| EIF4A3 | DDX48, KIAA011, EIF4AIII | • Nuclear-transcribed mRNA catabolic process; nonsense-mediated decay  
|       |             | • Nuclear-transcribed mRNA poly(A)tail shortening  
|       |             | • mRNA splicing via spliceosome  
|       |             | • rRNA processing       
|       |             | • mRNA processing       
|       |             | • Regulation of translation       
|       |             | • Transport       
|       |             | • Gene expression       
|       |             | • RNA unwinding       
|       |             | • Cytokine-mediated signaling pathway       
<p>|       |             | • Embryonic cranial skeleton  |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX49</td>
<td>FLJ10432</td>
<td>DNA unwinding, rRNA processing and ribosome biogenesis (yeast; DBP8)</td>
</tr>
<tr>
<td>DDX50</td>
<td>GU2, MGC31</td>
<td>DNA unwinding, Nucleotide binding, Hydrolase activity, Ribosome biogenesis (review paper)</td>
</tr>
<tr>
<td>DDX51</td>
<td></td>
<td>rRNA processing, RNA unwinding, Ribosome biogenesis, rRNA processing and ribosome biogenesis (yeast; DBP6)</td>
</tr>
<tr>
<td>DDX52</td>
<td>ROK1</td>
<td>Metabolic processes, Nucleotide binding, Hydrolase activity, Helicase activity, rRNA processing and ribosome biogenesis (yeast; ROK1)</td>
</tr>
<tr>
<td>DDX54</td>
<td>MGC2835, APR-5, DP97</td>
<td>Transcription; DNA templated, RNA processing, RNA unwinding, Intracellular estrogen receptor signaling pathway, Negative regulation of nucleic acid-templated transcription, rRNA processing and ribosome biogenesis (yeast; ROK1)</td>
</tr>
<tr>
<td>DDX55</td>
<td>KIAA1595</td>
<td>RNA unwinding, rRNA processing and ribosome biogenesis (yeast; ROK1)</td>
</tr>
<tr>
<td>DDX56</td>
<td>NOH61</td>
<td>rRNA processing, RNA unwinding, Ribosome biogenesis, rRNA processing and ribosome biogenesis (yeast; ROK1)</td>
</tr>
</tbody>
</table>

The expression of each of these genes was studied during proliferating as well as differentiation phases of skeletal muscle cells. The expression of each of these genes was determined through reverse transcription-polymerase chain reaction (RT-PCR) analysis.
in C2C12 cells on day -2, 0, 2, 5, and 6. C2C23 cells are in proliferative stages during earlier stages (days -2 and 0). With the addition of differentiation media on day 0, the cells develop from myoblasts to differentiated myotubes. By collecting cells at different time points, expression of each DDX gene could be evaluated at different stages of development. These different stages of myoblast differentiation are shown in Figure 7.

**Figure 7:** C2C12 Cells at different developmental stages: Cells were collected at day -2, 0, 2, 5, and 6. The media was changed to differentiation media at day 0. Development of these cells can be seen from myoblasts (day -2) to multinucleated elongated myotubes.

Five major patterns of expression of the DDX genes were evident through RT-PCR amplification at each developmental stage. All expression was normalized with reference to GAPDH, a ubiquitously expressed protein, which served as a control. In group 1, high and consistent expression of DDX genes was seen at all stages of development. DDX21 and DDX24 showed this expression pattern. Group 2 genes showed lower to moderate expression that was consistent at all developmental stages.
Members in this group include DDX5, DDX10, and DDX28. Group 3 genes (DDX1, DDX6, DDX27) showed high expression during proliferation (days -2 and 0), with expression significantly decreasing during early differentiation on day 2, and increasing again during late differentiation on days 5 and 6. Group 4 genes showed a gradual increase in expression from proliferation to cell differentiation for DDX49 and DDX50. Finally, genes in Group 5, DDX3x, DDX25, and DDX31 genes showed no expression.

These RT-PCR results are shown in Figure 8.

**Figure 8:** Expression of DDX genes in C2C12 Cells at Different Stages of Differentiation: RT-PCR amplification of DDX genes involved with ribosomal biogenesis, rRNA, or translation and initiation processes in mammalian muscle cells.
IGHMBP2

Figure 9: *ighmBp2* Heterozygous Mutation 1: Genomic PCR and Sanger sequencing showed that these fish have a 30 base pair deletion in the *ighmBp2* gene. Figure 4 shows the sequencing result from a heterozygous fish.

Figure 10: *ighmBp2* Homozygous Mutation 1: The homozygous 30 base pair deletion in *ighmBp2* is shown here.

Figure 11: *ighmBp2* Heterozygous Mutation 2: Genomic PCR and Sanger sequencing showed that these fish have a 2 base pair deletion in the *ighmBp2* gene. Figure 4 shows the sequencing result from a heterozygous fish.

Figure 12: *ighmBp2* Homozygous Mutation 2: The homozygous 2 base pair deletion in *ighmBp2* is shown here.
**Phenotype of ighmBp2 fish**

IGHMBP2 heterozygous pairs were set up for mating and expected to result in 25% wild-type, 50% heterozygous, and 25% mutant embryos. Fish with both genetic backgrounds resulted in embryos that exhibited bent bodies, thin and pale colored embryos, and edema around the heart as seen in Figure 13 and Figure 14. Fish with obvious abnormalities also had difficulty swimming compared to the normal embryos. Sanger sequencing was confirmed on normal and abnormal looking embryos to have a genotype-phenotype and to confirm the genotype of mutant embryos as shown in Table 6.

*Figure 13: Phenotypes of ighmBp2 embryos observed on day 3*
Figure 14: Phenotypes of ighmBp2 embryos observed on day 5

Table 6: Predicted genotype versus actual genotype of embryos obtained from ighmBp2 Heterozygous matings. Fish were numbered 1 – 8, a phenotype was recorded for each fish and were then genotyped.

<table>
<thead>
<tr>
<th>Mutation 1 Predicted</th>
<th>Mutation 1 Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>Wild-type/Heterozygous</td>
</tr>
<tr>
<td>1, 2, 3, 4, 5</td>
<td>6, 7, 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutation 2 Predicted</th>
<th>Mutation 2 Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>Wild-type/Heterozygous</td>
</tr>
<tr>
<td>1, 2, 3, 4</td>
<td>5, 6, 7, 8</td>
</tr>
</tbody>
</table>

A consistent and accurate phenotype for mutant embryos versus wild-type or heterozygous embryos was not seen by Sanger Sequencing. As these fish were created by CRISPR technology, we predict that a second gene/genes may be affected by non-specific targeting. To address these issues, we have outcrossed IGHMBP2 heterozygous fish with wild-type fish. This may result in segregation of non-specific targeted genes in
the resulting generation. By genotyping the next generation we expect to identify
IGHMBP2 fish without any background mutations.
DISCUSSION

The purpose of this study was to better understand the molecular pathways that contribute to neuromuscular disorders. This project aimed specifically to understand the role of RNA helicases in neuromuscular development and disease pathology in muscular disease. Previous work done in this lab identified a zebrafish model with a mutation in the gene for RNA helicase *ddx27*, with the resulting phenotype similar to that of human patients with myotonic dystrophy. Previous studies suggest that mutations in *ddx27* lead to defects in ribosomal biogenesis, with more recent studies alluding to additional non-ribosomal functions of nucleolar genes. With this knowledge, an aim of this study was to further understand the role of *ddx27* in myogenesis and diseases. Additionally, this study hoped to identify other DDX genes that are expressed in mammalian muscle cells that regulate muscle protein translation by ribosomal biogenesis or translation initiation processes. Finally, we wanted to begin to understand the significance of IGHMBP2 in muscular development and disease pathology in zebrafish.

The immunofluorescence studies conducted on zebrafish myofiber culture allowed for visualization of both sarcomeric and nucleolar defects present in the *ddx27* mutant fish. The structural markers (actinin, RYR1, and Lamin A) showed sarcomeric disorganization in the mutant fish. Changes in nucleolar architecture were also present, and marked by UBF, Fibrillarin, and B23 antiboides. These markers were chosen in order to visualize the three morphologically distinct regions of the cell nucleolus: the fibrillar center, the dense fibrillar component, and the granular component. These three regions are believed to represent the sites of rRNA transcription, processing, and ribosomal
assembly. As mentioned previously, UBF labels the sites of RNA polymerase I transcription. Fibrillarin is a marker for the dense fibrillar component, which contains pre-rRNA. B23 marks the nucleolar matrix or the granular component of the nucleolus. These three markers represent distinct aspects of nucleolar activities from pre-rRNA transcription through late events of rRNA processing. (Cooper, 2000) Loss of ddx27 resulted in a change in the organization of Ubf from small punctate foci to larger more condensed areas. This suggests there may be a defect in active transcription sites in the nucleolus. Fibrillin, associated with early rRNA processing regions, was also disrupted and merged forming larger, more condensed structures. The distribution of B23 was drastically altered in the nucleoplasm of cells lacking ddx27. This suggests that a deficiency in ddx27 disrupts both early phases of nucleolus assembly and late steps in processing pathways (Németh et al., 2010).

The whole mount IF studies with acetyl-histone H3 allowed for visualization of transcriptionally active euchromatin within the nucleolus. The results of this study showed a significant increase in the number of nuclei staining positively for acetyl-histone H3 antibody in the ddx27 mutant fish. This shows the chromatin is in an activated state in the mutant fish, and further suggests that gene regulation may be altered. Over-activation of chromatin may be negatively impacting the mutant fish.

The implications of ddx27 deficiency in myogenesis were investigated. Whole mount IF with Pax7 and MF20 antibodies showed an increase in the number of Pax7 positive cells in ddx27 fish. Pax7 is a marker for satellite cells, while MF20 is a sarcomeric marker for myosin heavy chain. This result suggests that the regulation of
stem cells is perturbed in the mutant fish. Previous studies have shown that up regulation of Pax7 results in muscular atrophy. (He et al., 2013) Future studies using immunoprecipitation techniques may provide insight as to how ddx27 is involved in the regulation Pax7.

Through the in-situ hybridization and IF studies, it was apparent that there is a clear nucleolar defect in the DM1 patient cells compared to the control cells. While we don’t currently have an explanation for exactly what is causing this defect, it leads us to believe that ddx27 may play a role in cell cycle regulation or apoptosis. It is already known from studies previously conducted in this lab that ddx27 is a nucleolar protein involved with rRNA maturation and ribosomal biogenesis, that it is strongly expressed in muscle cells and that lack of this protein causes severe muscular defects in zebrafish. It is also known, as mentioned previously, that DEAD-box RNA helicases are involved in a wide variety of cellular functions including ribosome biogenesis, transcription, pre-mRNA splicing, microRNA processing, nonsense mediated decay and protein translation. (Linder & Jankowsky, 2011) In a study by Tsukamoto et. al., it was found that ddx27 expression in gastric cancer cells was associated venous invasion and liver metastasis, and ultimately poor prognosis. In order to have metastasis occur, colony formation of cells is required. This study found that knockdown of ddx27 inhibited colony formation of cells. Through the use of fluorescence-activated cell sorting, it was discovered that the suppression of colony formation occurred due to inhibition in the cell cycle independent of apoptosis. In the two gastric cancer cell lines studied, it was observed that lack of ddx27 caused either a G1 or G2 arrest in the cell cycle (Tsukamoto et al., 2015). Going
forward, it would be interesting to determine whether the levels of ddx27 protein in control and DM1 cells differs, and whether ddx27 might be involved in cell cycle regulation in myoblasts.

Through the expression studies of different DDX genes in mammalian muscle cells, it was evident that 18 of the 24 tested DDX genes showed moderate or high expression throughout progressive stages of differentiation. These DDX genes function similarly to ddx27 in that they all regulate protein translation by ribosomal biogenesis or translation initiation/elongation processes. All of these ddx genes could potentially play a role in neuromuscular development, and this preliminary expression data provides a good starting point for future research. It has been shown in a study by Laurent et al., 2012 that DDX5 is involved in myotonic dystrophy. This would be a particularly good gene to study. Future research of this gene could involve CRISPR knockout cells and zebrafish models to characterize the neuromuscular defects. Additionally, studies could be done to identify defects in protein translation due to issues with rRNA and ribosomal biogenesis.

Preliminary studies attempting to characterize a phenotype associated with a mutation in the RNA helicase IGHMBP2 were conducted. The embryos from many matings were analyzed and genotyped, but no one consistent phenotype was evident. Additionally, when predicting which embryos appeared to be mutant and which embryos appeared to be normal, our predictions were frequently incorrect as seen in Table 6. This leads us to believe that there may be another conflicting mutation that occurred when creating these lines of zebrafish. Moving forward, IGHMBP2 heterozygous fish will be outcrossed with wild-type fish. As previously mentioned, this may result in segregation
of non-specific targeted genes in the resulting generation. By genotyping the next
generation we expect to identify IGHMBP2 fish without any background mutations.

More than one million Americans suffer from neuromuscular disorders, and
approximately 40% of those individuals are under the age of 18 (“ALS & Neuromuscular
Center | UC Irvine Health | Orange County, CA,” n.d.). Many of these conditions
currently have no known treatments or therapies. By studying and understanding the
molecular pathways involved in these disorders, particularly RNA helicases and
associated RBPs, we can attempt to develop therapies or an approach to finding small
molecule therapies for neuromuscular diseases.
REFERENCES


Nucleotide BLAST: Search nucleotide databases using a nucleotide query. (n.d.).

Retrieved January 25, 2016, from
n&PAGE_TYPE=BlastSearch&BLAST_SPEC=

OMIM Entry - # 160900 - MYOTONIC DYSTROPHY 1; DM1. (n.d.). Retrieved
February 15, 2016, from http://www.omim.org/entry/160900

OMIM Entry - # 602668 - MYOTONIC DYSTROPHY 2; DM2. (n.d.). Retrieved
February 15, 2016, from
http://www.omim.org/entry/602668?search=myotonic%20dystrophy%202%20&highlight=2%20myotonic%20myotonia%20dystrophy


Primer3 Input (version 0.4.0). (n.d.). Retrieved January 25, 2016, from
http://bioinfo.ut.ee/primer3-0.4.0/

http://doi.org/10.1212/WNL.0000000000001220


http://doi.org/10.1186/1756-0500-4-410

http://doi.org/10.1096/fj.14-254680


http://doi.org/10.1016/j.nmd.2015.07.017

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EDUCATION

Boston University School of Medicine, Boston, MA
Master of Science in Medical Science, May 2016

The College of William & Mary, Williamsburg, VA
Bachelor of Science, May 2014
Major: Biology; pre-medical
Dean’s List Fall 2013

RESEARCH

College of William & Mary Forsyth Lab; Microbiology, Williamsburg, VA
• Student Researcher, September 2012 – May 2014
• Work under the mentorship of Dr. Mark Forsyth; Associate professor of biology
• Researching the potential stratification of Helicobacter pylori in the human stomach

Virginia Commonwealth University; Virginia Institute for Psychiatric and Behavioral Genetics, Richmond, VA
• Laboratory Assistant, June 2011, May 2012 – August 2012
• Worked for Dr. Brien Riley, Director of the Molecular Laboratory at VIPBG
• Assisted PhD student with project involving a gene correlating with alcohol dependence

Brigham & Women’s Hospital; Division of Genetics; Boston, MA
• Student Researcher, August 2015 – May 2016
• Working under the mentorship of Dr. Vandana Gupta, Assistant Professor of Medicine, Harvard Medical School
• Writing a thesis focused on understanding the role of a novel RNA binding protein in neuromuscular development and diseases

CO-CURRICULAR ACTIVITIES

Virginia Commonwealth University Medical Center, Richmond, VA
• Volunteer, June 2011 – August 2011
• Worked in the emergency department triage center
• Assisted Emergency Department staff, helped assist with the coordination of patient services to patients and families while admitted to the emergency department

Sailing Team, College of William & Mary, Williamsburg, VA
• *Team member*, August 2010 – May 2012
• Attended team practice, meetings, and assisted fundraising projects, and participated in team community service

Alpha Phi Omega Fraternity, College of William & Mary, Williamsburg, VA
• *Brother*, August 2012 – May 2014
• Service fraternity, Nu Rho Chapter
• Minimum 30 hours of service per semester

Campus Kitchens, Williamsburg Presbyterian Church, Williamsburg, VA
• *Volunteer*, August 2012 – May 2014
• Help prepare meals for low income subsidized housing communities in Williamsburg

Campus Escort, College of W&M, Williamsburg, VA
• *Volunteer*, August 2012 – May 2014
• The service provides nighttime transportation for students via golf cart or walking escort

Reach Out and Read Organization, Richmond VA
• *Volunteer*, 2007 – 2011
• ROR is a national non-profit organization of medical providers that promotes early literacy in pediatric exams by providing new books to children and advice to their parents
• Organized a book drive at a VCU basketball game in 2008
  o Those who donated a book received a half-price ticket to the event
• Wrote a testimonial as well as service project guides

**PUBLICATIONS & PRESENTATIONS**

*Authors contributed equally