Zebralfish Models of Congenital Myopathy

A dissertation presented

by

Laura Lindsay Smith

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

June 2015
Zebrafish Models of Congenital Myopathy

Abstract

The congenital myopathies are a diverse group of inherited neuromuscular disorders that manifest as skeletal muscle weakness at birth or in infancy, and are classically defined by the predominant morphological features observed on muscle biopsy. The goals of this dissertation were to better understand the pathophysiology behind these devastating diseases and to identify new therapeutic approaches through the use of faithful vertebrate models.

Due to their proliferative capacity, transparency, and well-characterized genome, zebrafish represent a robust vertebrate model system to study muscle development. In the first part of this work, we created and characterized a novel zebrafish model of centronuclear myopathy using antisense morpholinos targeting the bridging integrator 1 (bin1) gene. Bin1 morphant skeletal muscles revealed structural defects reported in human biopsies, and live calcium imaging offered new mechanistic insights linking abnormal triads to impairments in intracellular signaling.

Later studies focused on two forms of core myopathy, and utilized stable zebrafish models to guide development of targeted and effective therapies. We began by using TALE nucleases to generate germ line mutations in the zebrafish selenoprotein N (sepn1) gene, and in doing so created the first vertebrate to accurately model human SEPN1-related myopathy (SEPN1-RM). Sepn1 zebrafish mutants exhibited morphological abnormalities, reduced contractile strength, and skeletal muscle “cores” under electron microscopy. We then showed that the sepn1 phenotype could be ameliorated by pharmacological inhibition of a thiol oxidase localized at the sarcoplasmic reticulum. These data served as the first in vivo evidence to indicate that reactive oxygen species significantly contribute to SEPN1-RM, and may do so by impairing calcium re-uptake following muscle contraction. Finally, we performed a medium-
throughput chemical screen on the closely related \textit{relatively relaxed (rYr1b)} zebrafish, and identified JAK-STAT cytokine signaling as a druggable molecular pathway relevant to these pathologies.

In summary, these studies increase our knowledge of the affected systems in both centronuclear and core myopathies, and provide strong \textit{in vivo} support that these conditions arise from defects in skeletal muscle excitation-contraction coupling. This work also further establishes zebrafish-based small molecule screens as a powerful tool for lead compound identification and drug development in human genetic disease.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>GLOSSARY OF TERMS</td>
<td>xi</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xii</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION  
1  

1.1 CONGENITAL MYOPATHIES  
1.1.1 OVERVIEW  
1.1.2 CENTRONUCLEAR MYOPATHIES  
1.1.3 CORE MYOPATHIES  
1.1.4 NEMALINE MYOPATHIES  
1.1.5 CONGENITAL FIBER TYPE DISPROPORTION  

1.2 DEVELOPMENT OF TARGETED THERAPIES USING VERTEBRATE MODELS  
1.2.1 MAMMALIAN MODELS  
1.2.2 ZEBRAFISH MODELS  

1.3 MANIPULATION OF ZEBRAFISH GENE EXPRESSION  
1.3.1 MORPHOLINOS  
1.3.2 TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES  

1.4 SPECIFIC AIMS AND OVERVIEW OF DISSERTATION  

1.5 REFERENCES
CHAPTER 2:
ESTABLISHING THE ROLE OF bin1 IN EARLY ZEBRAFISH DEVELOPMENT
AND SKELETAL MUSCLE PATHOGENESIS

2.1 ABSTRACT
2.2 INTRODUCTION
2.3 MATERIALS AND METHODS
2.4 RESULTS
2.5 DISCUSSION
2.6 REFERENCES

CHAPTER 3:
THE sepn1 KNOCKOUT ZEBRAFISH:
A NOVEL GENETIC MODEL OF SEPN1-RELATED MYOPATHY

3.1 ABSTRACT
3.2 INTRODUCTION
3.3 MATERIALS AND METHODS
3.4 RESULTS
3.5 DISCUSSION
3.6 REFERENCES

CHAPTER 4:
SMALL MOLECULE SCREENING IN RYANODINE RECEPTOR MUTANT ZEBRAFISH
FOR THERAPEUTIC DEVELOPMENT IN CORE MYOPATHIES

4.1 ABSTRACT
4.2 INTRODUCTION
CHAPTER 5: CONCLUSIONS

5.1 OVERVIEW

5.2 ADVANCES IN UNDERSTANDING BIN1 FUNCTION IN SKELETAL MUSCLE

5.3 ADVANCES IN UNDERSTANDING PATHOPHYSIOLOGICAL MECHANISMS IN CORE MYOPATHIES

5.4 FUTURE DIRECTIONS

      5.4.1 BIOLOGICAL FUNCTIONS OF CAUSATIVE CONGENITAL MYOPATHY GENES

      5.4.2 DEVELOPING THERAPEUTICS FOR CONGENITAL MYOPATHIES

5.5 REFERENCES
LIST OF FIGURES

**Figure 1.1** – Known congenital myopathy genes and their encoded proteins

**Figure 1.2** – Characteristic histological findings in representative congenital myopathies

**Figure 1.3** – Zebrafish skeletal muscle development

**Figure 2.1** – BIN1 protein domains and tissue-specific isoforms

**Figure 2.2** – Zebrafish birefringence assay

**Figure 2.3** – Zebrafish *bin1b* shares strongest synteny with the human *BIN1* gene

**Figure 2.4** – *Bin1* gene expression in early zebrafish development

**Figure 2.5** – MO knockdown of *bin1* results in protein loss and mRNA mis-splicing

**Figure 2.6** – Bin1 deficiency leads to morphological abnormalities in zebrafish larvae

**Figure 2.7** – Knockdown of zebrafish *bin1* results in mild bradycardia

**Figure 2.8** – Abnormal motor functions in *bin1* morphants

**Figure 2.9** – *Bin1* zebrafish display similar histopathological defects as human CNM2

**Figure 2.10** – Neuromuscular junctions and myosepta are normal in *bin1* morphants

**Figure 2.11** – Triad markers are disrupted in Bin1 deficiency

**Figure 2.12** – Calcium signaling is impaired in *bin1* morphants

**Figure 2.13** – Overexpression of human *BIN1* mRNA in *bin1* MO-injected morphants

**Figure 2.14** – *Bin1* morphants are not rescued with *BIN1* transcripts containing exon 7

**Figure 2.15** – Neuronal exon 7 and nuclear centralization in *bin1* morphants

**Figure 3.1** – TALEN-mediated knockout of the zebrafish *sepn1* gene

**Figure 3.2** – Abnormal morphology and motor functions of *sepn1* mutants

**Figure 3.3** – Diminished spontaneous swimming in *sepn1* mutants

**Figure 3.4** – Skeletal muscle histology shows nuclear mislocalization in *sepn1* mutants

**Figure 3.5** – Integrity of myosepta and the sarcolemma are preserved in Sepn1 deficiency
**Figure 3.6** – Loss of Sepn1 causes ultrastructural defects in skeletal muscle

**Figure 3.7** – Contractile forces are reduced in sepn1 mutant zebrafish

**Figure 3.8** – Sepn1 deficiency results in increased oxidative stress and apoptotic markers

**Figure 3.9** – EN460 reduces sepn1 myofibrillar disorganization in a dose-dependent manner

**Figure 3.10** – ERO1 inhibition restores sepn1 birefringence and motor behaviors

**Figure 3.11** – Kaplan-Meier survival of sepn1 mutants are unaffected by ERO1 inhibition

**Figure 3.12** – EN460 may affect skeletal muscle relaxation in sepn1 mutants

**Figure 3.13** – EN460 treatment corrects sepn1 skeletal muscle ultrastructure

---

**Figure 4.1** – Chemical screening plate set-up

**Figure 4.2** – Numerical scoring of touch-evoked escape behaviors

**Figure 4.3** – Genotyping of ryr1b larvae

**Figure 4.4** – Schematic outline of two-tiered ryr1b chemical screen

**Figure 4.5** – Screened wells contain expected 3:1 ratio of unaffected to ryr1b larvae

**Figure 4.6** – Mobility and survival measurements of primary screen “hit” pools

**Figure 4.7** – Vitality scores of secondary screen “hit” compounds

**Figure 4.8** – Nifuroxazide acts on the ryr1b phenotype in a dose-dependent manner

**Figure 4.9** – Nifuroxazide increases contractile strength of ryr1b skeletal muscles

**Figure 4.10** – Nifuroxazide corrects ryr1b morphological features and swimming behaviors

**Figure 4.11** – Nifuroxazide does not significantly alter the life span of ryr1b mutants

**Figure 4.12** – Ketoprofen dose-response and proposed mechanistic link
LIST OF TABLES

**TABLE 4.1** – EMBRYONIC ZEBRAFISH SURVIVAL IN PRESTWICK2 LIBRARY CHEMICAL SCREEN

**TABLE 4.2** – CANDIDATE COMPOUNDS IDENTIFIED BY TWO-TIERED *ryr1b* CHEMICAL SCREEN
## Glossary of Terms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>BIN1</td>
<td>Bridging integrator 1</td>
</tr>
<tr>
<td>CCD</td>
<td>Central core disease</td>
</tr>
<tr>
<td>CFTD</td>
<td>Congenital fiber type disproportion</td>
</tr>
<tr>
<td>CM</td>
<td>Congenital myopathy</td>
</tr>
<tr>
<td>CNM</td>
<td>Centronuclear myopathy</td>
</tr>
<tr>
<td>DNM2</td>
<td>Dynamin 2</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERO1</td>
<td>Endoplasmic reticulum oxidoreductin 1</td>
</tr>
<tr>
<td>h/dpf</td>
<td>Hours/days post-fertilization</td>
</tr>
<tr>
<td>MmD</td>
<td>Multiminicore disease</td>
</tr>
<tr>
<td>MMD</td>
<td>Myotonic muscular dystrophy</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NM</td>
<td>Nemaline myopathy</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RYR1</td>
<td>Ryanodine receptor 1</td>
</tr>
<tr>
<td>SEPN1</td>
<td>Selenoprotein N</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>XLMTM</td>
<td>X-linked myotubular myopathy</td>
</tr>
</tbody>
</table>
DEDICATION

This dissertation is dedicated to my grandfather, Randlow Smith, who is the most genuine personification and greatest advocate of excellence in scientific education. Tales of his own academic achievements will forever be passed down in the Smith family alongside smiles and admiration, and rightfully so. Randlow graduated as valedictorian of Weatherford High School, received his acceptance letter from CalTech at age fifteen, and became one of the first Texans to graduate from CalTech as an undergraduate. He received two master’s degrees, including one earned while also raising four children. At ninety-five years old, he still works on unsolved dimensions of the Navier-Stokes equations. Despite these intellectual accomplishments, however, he himself has never been one to boast, and instead spends his time sharing simple wisdoms that lead to an honest and fulfilling life.

Granddad, I will always cherish our conversations over mealtimes and among the birch trees in Maine. Your advice regarding life in the classroom, in the laboratory, and at home is timeless, and it explains the vast amount of love and respect held for you in the hearts of so many. Thank you for being truly inspirational.
CHAPTER 1:

INTRODUCTION

Sections of this chapter are based on:

1.1 CONGENITAL MYOPATHIES

1.1.1 OVERVIEW

The congenital myopathies (CMs) are a heterogeneous group of inherited neuromuscular disorders that manifest as skeletal muscle weakness at birth or early in life, and are defined by the presence of specific morphological features on biopsy. The most common forms of CM can be roughly subdivided into four categories based on the predominant pathologic features observed under light and electron microscopy: (i) centronuclear myopathies; (ii) core myopathies; (iii) nemaline (or rod) myopathies; and (iv) myopathies with congenital fiber type disproportion. However, accurate diagnoses are often confounded due to broad variations in the clinical severity of each phenotype, and to substantial histological overlap between the different forms of these disorders. CMs can also result from mutations in more than one gene, with causative genes associated with multiple pathologies. Clinical features, such as the presentation of hypotonia during the newborn period, may be similar to features found in patients with congenital myasthenic syndromes, metabolic myopathies, spinal muscular atrophy, as well as muscular dystrophies. Thus, CMs are typically a diagnosis of exclusion and require detailed clinical data combined with electromyographic and histopathological findings to prioritize gene testing and establish a genetic basis.

More than twenty genes cause CM and their biological functions are widely studied in vitro and in vivo. Major pathophysiologic pathways responsible for weakness in CMs are hypothesized to result from either malformed contractile filaments, in the case of nemaline and other rod myopathies, or from disruptions in calcium homeostasis at the skeletal muscle triad, in the case of many centronuclear and core myopathies. However, it is becoming increasingly clear that each genetic condition likely involves multiple mechanisms, each of which provides potential therapeutic targets for development. In contrast to CMs, muscular dystrophies are characterized by skeletal muscle that retains the intrinsic ability to contract, but becomes progressively weaker due to myofiber death and eventually fails to compensate through regeneration.
Due to the vast genetic and clinical heterogeneity of CMs, molecular diagnosis is important, both for disease management and for the development of gene-specific therapeutic strategies. Today, investigators and clinicians are focused on understanding the biological basis underlying the CMs and on gene discovery, as the genetic cause in 30-40% of cases remains unknown.

Figure 1.1. Known congenital myopathy genes and their encoded proteins. Skeletal muscle disease genes discussed in the chapter text, the majority of which encode proteins of the myofilament or triad, are capitalized. Other important muscle proteins not presently known to be mutated in human CMs, but part of relevant complexes and responsible for other conditions, are selectively labeled in parentheses.
1.1.2 CENTRONUCLEAR MYOPATHIES

Centronuclear myopathies (CNMs) are classically defined by the abnormal centralization of nuclei in >25% of muscle fibers, although there can be considerable variability both in the number of myofibers with central nuclei and in the number of central nuclei within a single myofiber (Fig. 1.2 A) \(^8\). Several genetically distinct forms of CNM have been described based on age of onset, severity of symptoms, and mode of inheritance \(^9\), \(^10\). Clinically, these can be categorized as the severe X-linked recessive form with prenatal or neonatal onset, the autosomal recessive form with onset in infancy or childhood, and the autosomal dominant form, typically mild with late onset. Despite copious variability in the clinical features among these groups, emerging evidence suggests that defective excitation-contraction coupling at the level of the triad may be a unifying pathophysiological feature in CNM \(^11\).
Figure 1.2. Characteristic histological findings in representative congenital myopathies.

(A) H&E staining of muscle from a 1-month-old boy with X-linked myotubular myopathy. An MTM1 mutation is sufficient to reveal characteristic small myofibers with central nuclei. (B) NADH-tetrazolium reductase staining reveals multiple minicores, appearing as patchy lightly stained regions within myofibers, in the muscle of a 4-year-old boy with multiminicore disease caused by a mutation of SEPN1. (C) Gömöri trichrome stain of muscle from a 2 ½-year-old girl with nemaline myopathy due to a NEB mutation. Note dense subsarcolemmal accumulations of darkly-staining rod bodies in many fibers. (D) Fiber typing by histochemical staining for myosin ATPase at pH 4.6 reveals uniform smallness of the population of darkly staining slow twitch myofibers in a 1-year-old girl with congenital fiber type disproportion (genotype unknown).
X-linked centronuclear myopathy, also referred to as myotubular myopathy or X-linked myotubular myopathy (XLMTM), is caused by mutations in the myotubularin (MTM1) gene. Of the more than 500 known MTM1 mutations, most are believed to result in loss of protein expression, although there are a few recurring missense mutations which can cause either the classic severe phenotype or a milder presentation. XLMTM frequently presents in clinic with polyhydramnios and reduced fetal movements in utero, and in affected newborn boys as severe hypotonia, generalized weakness, and muscle wasting. Additional features include thin ribs, ophthalmoplegia, ptosis, pyloric stenosis, and contractures of the hips and knees. XLMTM carries a poor long-term prognosis, with death due to respiratory failure occurring within the first year of life in 20-25% of cases. Although a small proportion of boys may be less affected in the neonatal period and survive into childhood or even adulthood, most remain severely impaired and require permanent ventilation.

Mutations in the large GTPase dynamin 2 (DNM2) are the second most common cause of CNM and display dominant inheritance. Autosomal dominant CNM showing late adult onset with slowly progressive weakness, together with de novo forms of CNM with earlier onset, has a broader range of clinical presentation than the X-linked form. Generally, limb girdle, trunk, and neck muscles are involved, with degrees of ptosis and limitation of eye movements paralleling the age of onset. While most patients are ventilator independent, phases of respiratory decline may require non-invasive ventilation but rarely necessitate invasive support.

Autosomal recessive centronuclear myopathy is the rarest form of CNM, with causative mutations identified in the skeletal muscle ryanodine receptor (RYRI), titin (TTN), and bridging integrator 1 (BIN1) genes. The recessive form of the disease generally presents in infancy or early childhood with diffuse muscle weakness and respiratory distress. Facial diplegia, ptosis, and varying degrees of ophthalmoplegia are also common features. The clinical course of the disease is marked by slowly progressive weakness, development of scoliosis or kyphosis, as well as delays in motor milestones such as walking, running, and stair climbing.
1.1.3 Core Myopathies

Core myopathies are characterized by areas in the muscle fiber lacking oxidative and glycolytic enzymatic activity (Fig. 1.2 B). Central cores run along the length of the myofiber, whereas minicores are short zones of myofibrillar disorganization that are wider than they are long on longitudinal section. Based on the presence of these abnormal features, patients with core myopathies are traditionally subclassified as having either central core disease (CCD) or multiminicore disease (MmD) 30. The vast majority of CCD patients (>90%) have autosomal dominant or de novo dominant mutations in the RYR1 gene 31-34, while MmD core myopathy is most commonly caused by recessive mutations in the selenoprotein N gene (SEPN1) gene 35.

CCD was the first CM defined on the basis of specific morphological changes in skeletal muscle. In 1956, Magee and Shy described the first patient with single, well-circumscribed circular regions in the center of type I (slow twitch) fibers 36. The term “central core disease” was introduced soon afterwards to reflect the absence of oxidative enzymes, phosphorylase, and glycogen in the core area due to mitochondrial depletion 37. Whereas cores are best observed on sections stained for oxidative enzyme activity (e.g., succinate dehydrogenase [SDH], cytochrome-c-oxidase [COX], or nicotinamide adenine dinucleotide [NADH] dehydrogenase reacted sections), cores examined using electron microscopy contain densely packed and disorganized myofibrils, and have been divided into two types based on whether myofibrillar organization is maintained. Structured cores preserve basic sarcomeric architecture, although sarcomeres may be out of register with adjacent fibrils as well as with each other, whereas unstructured cores contain large areas of Z-line streaming 38. Clinically, CCD typically presents in infancy with hypotonia or in early childhood with delays in motor development. Although weakness preferentially affects proximal muscle, such as hip girdle and axial muscles, almost all CCD patients achieve the ability to walk independently. The primary exceptions are cases with debilitating hip dislocations or severe cases presenting with neonatal weakness, arthrogryposis, and respiratory failure 34.
Fifteen years after the initial description of CCD, Engel and colleagues reported a family with two affected siblings exhibiting multiple small cores within muscle fibers. Patients with the classic form of MmD generally present in infancy or childhood with pronounced hypotonia and proximal weakness, although select cases of prenatal or adult onset have been recognized. Axial muscle weakness, particularly affecting the neck and trunk flexors, is a prominent feature of MmD, and failure to acquire head control is an early clinical sign. Spinal rigidity and scoliosis are also common. The clinical course of MmD is static for the majority of patients. However, some experience cardiac involvement secondary to marked decline in respiratory function during adolescence or young adulthood.

MmD is diagnosed on muscle biopsy by the presence of multifocal, well-circumscribed areas in the muscle fiber with reduced oxidative staining and low myofibrillar ATPase activity. In contrast to central cores, “minicores” are typically unstructured, extend for only a short distance along the longitudinal axis of the myofiber, and may affect both type I (slow twitch) and type II (fast twitch) fibers. Minicores appear as regions of myofibrillar disruption lacking mitochondria in electron micrographs, with sarcomere degeneration and structural abnormalities of the triad.

1.1.4 NEMALINE MYOPATHIES

Clinically, nemaline myopathy (NM) phenotypes vary and are sub-classified into different groups according to age of onset as well as severity of motor and respiratory involvement: (i) severe congenital NM, (ii) intermediate congenital NM, (iii) typical congenital NM, (iv) childhood/juvenile-onset NM, (v) adult-onset NM, and (vi) other forms with atypical clinical features such as cardiomyopathy and ophthalmoplegia. Although these classifications have been well established and can be used to accurately predict prognosis, certain morphological and clinical features are common and shared between two or more groups. Nemaline rods, for example, are the pathological and diagnostic hallmark of NM, and by definition are shared among all genetic forms of this disorder.

Rods appear as red or purple structures against a blue-green myofibrillar background upon modified Gomori trichrome staining, and show a tendency to cluster under the sarcolemma and around
nuclei (Fig. 1.2 C). Rods are considered to derive from the lateral expansion of the Z-line, based on their structural continuity with Z-lines, electron density, and criss-cross pattern in electron micrographs. Rods also stain positively for antibodies to alpha-actinin isoforms 2 and 3, the two major components of the skeletal muscle Z-line. With few exceptions (e.g., patients with TPM3 mutations), rods are present in both type I and type II muscle fibers, although type I fiber predominance is a common feature of NM and fiber type disproportions tend to become more prominent with age. The proportion of myofibers containing rods varies considerably between cases, however, and the sizes and numbers of rods within a muscle specimen do not appear to correlate with disease severity. Nemaline rods are usually cytoplasmic. Intranuclear rods are occasionally a prominent feature, particularly in severe cases.

NMUs are considered diseases in which mutations disrupt the ability of the myofiber to generate adequate force during contraction. To date, mutations in ten different genes have been identified in a subset of NM patients: alpha-skeletal muscle actin (ACTA1); slow alpha-tropomyosin (TPM3); nebulin (NEB); slow troponin T (TNNT1); beta-tropomyosin (TPM2); muscle-specific cofilin (CFL2); leiomodin 3 (LMO3); kelch-like family members 40 (KLHL40) and 41 (KLHL41); and kelch repeat and BTB domain containing 13 (KBTBD13). Seven of these ten genes encode protein components of the muscle fiber thin filament, while the other three likely participate as regulators of the thin filament degradation/turnover apparatus.

1.1.5 CONGENITAL FIBER TYPE DISPROPORTION

Congenital fiber type disproportion (CFTD) is a histological diagnosis with multiple etiologies. Brooke and Engel first used the term in 1973 in a large morphology study of children’s biopsies to describe a group of 14 patients who all had clinical features of a CM and whose prevalent abnormality on muscle biopsy was a discrepancy in muscle fiber size (Fig. 1.2 D). It is now the consensus that the diagnosis of CFTD can be made when a mutation in a CM gene has been identified and type I fibers are consistently smaller than type II fibers by at least 35-40%. However, type I fiber hypotrophy is also observed in a variety of metabolic myopathies, in central nervous system malformations, and in the severe
neonatal form of myotonic muscular dystrophy . Some experts therefore include additional histologic features in the definition of CFTD, such as type I fiber predominance (>55% type I fibers) or a paucity (<5%) of type IIB fibers . While CFTD generally mimics the clinical course of other forms of CM that share the same genetic cause, early respiratory failure is frequent among CFTD patients and nocturnal hypoventilation should be monitored even in ambulant individuals .

Most cases of CFTD are associated with mutations in the \( TPM3 \) gene, encoding the type I fiber-specific protein slow alpha-tropomyosin . \( TPM3 \) mutations are hypothesized to alter the interaction between tropomyosin and actin , and some data suggest that clinical weakness may arise from misregulated actin–myosin interactions . \( RYR1 \) mutations are less commonly seen and only rarely have mutations in \( ACTA1 \), \( MYH7 \), \( SEPN1 \), \( TPM2 \), or an X-linked form been reported. Most recently, mutations of the \( LMNA \) gene, coding for lamin A/C, were identified in several Japanese patients with CFTD . Since \( LMNA \) defects are known to cause a variety of different muscular dystrophies and related cardiomyopathies, these patients may represent a subset of CFTD cases at risk for cardiac disease.

### 1.2 Development of Targeted Therapies Using Vertebrate Models

Treatment for the congenital myopathies has traditionally been limited to supportive, palliative care. Disease management commonly includes mechanical ventilation, feeding tubes, orthopedic interventions to prevent skeletal limb contractures, in addition to surgery to correct severe spinal deformities . However, with the identification of causative genes for many of these conditions, the prospect for development of targeted therapies becomes viable. Substantial advances in the creation of vertebrate animal models has fostered ideas for novel therapeutic strategies and set the stage for pre-clinical testing in the congenital myopathies.

#### 1.2.1 Mammalian Models

Mammals are often considered the premier model systems for biomedical research due to their
close genetic and physiological similarities to humans. For that reason, perhaps the most promising therapeutic advancement in the CMs to date has been that either gene or protein replacement can successfully ameliorate the disease process in mouse and dog models of XLMTM. Recombinant adeno-associated viruses (AAVs) are powerful tools to express transgenes in vivo, and several serotypes transduce skeletal muscle with very high efficiency. Local studies in Mtm1 knockout mice and in dogs carrying a naturally occurring MTM1 gene mutation have demonstrated the efficacy of AAV-mediated myotubularin delivery. Only a single intravascular injection is required to improve strength and force generation, as well as correct muscle pathology, in treated mouse and dog muscles. In dogs, treatment prolongs survival without any observed toxicity or an immune response. At the protein level, myotubularin replacement using a single chain fragment derived from the mouse monoclonal antibody 3E10 (3E10Fv) has also been published. Short-term exogenous myotubularin supplementation with a 3E10Fv-MTM1 fusion protein improves both muscle pathology and contractile function. Additionally, Mtm1 knockout mice have been treated via injections of a soluble activin-receptor type IIB fusion protein, which binds to TGF-beta family members and increases muscle fiber growth in vivo.

Alternative approaches have also been explored to rescue loss-of-function CM phenotypes. Reduction of Dnm2 expression in Mtm1 knockout mice, for example, is sufficient to rescue the early lethality and most pathological features associated with the X-linked disease. This finding also supports the hypothesis that MTM1 and DNM2 function in a common molecular pathway, with MTM1 acting as a negative regulator of DNM2 function in skeletal muscle. In NM, cardiac alpha-actin (the predominant actin isoform in fetal muscle) has been shown to successfully substitute for skeletal muscle alpha-actin and prevent the early postnatal death of Acta1 knockout mice. Overexpression of cardiac alpha-actin also ameliorates the phenotype and increases survival of transgenic mice with dominant ACTA1 p.D286G mutations. Finally, ex vivo treatment with N-acetylcysteine (NAC) significantly improves cell survival and decreases protein oxidation levels in cultured SEPN1-deficient myoblasts. As a reduced thiol donor, NAC is hypothesized to act by partially replacing the thiol activity of absent SEPN1 protein.
Alleviating oxidative and nitrosative stress in human SEPN1-related myopathy patients is currently being pursued as a therapeutic strategy to treat this debilitating disease.

1.2.2 ZEBRAFISH MODELS

Over the last four decades, the zebrafish (*Danio rerio*) has become increasingly valued as a vertebrate model system, particularly for understanding muscle development and disease. Zebrafish skeletal muscle shares numerous molecular and structural similarities with humans, and is one of its largest, most discernible organ systems (Fig. 1.3)\(^ {102} \). Compared to traditional mammalian models, exogenously fertilized zebrafish embryos develop rapidly and are optically clear, which allows real-time *in vivo* observations of development from the single-cell stage\(^ {58, 69, 103-110} \). In addition to their high genetic tractability and spawning productivity, the benefits of zebrafish research become even more explicit when modeling congenital diseases. Zebrafish embryos develop *ex utero* and their cardiovascular function is not essential during early embryogenesis. Zebrafish can therefore bypass most secondary defects that cause embryonic lethality in placental animals, and be used to investigate the primary cause of CMs at the molecular level\(^ {111} \).
**Figure 1.3. Zebrafish skeletal muscle development.** (A) Fully differentiated slow and fast muscle fibers span chevron-shaped somites by 48 hpf. Fibers anchor on either end at vertical myosepta, which are made of connective tissue and are equivalent to mammalian myotendinous junctions. The horizontal myoseptum develops at the apex of somites and is a partition that separates the zebrafish dorsal and ventral body masses. (B) Slow and fast muscle fibers are mixed in mammals, but are topographically separated in zebrafish. Zebrafish slow muscle derives from adaxial myoblasts, which originate adjacent to the notochord and migrate radially through the somites. Ultimately, these precursors form a single layer of superficial muscle cells and differentiate into mononucleated slow fibers. Fast muscle arises afterwards from a separate pool of myoblasts, which fuse to form multinucleated fibers and populate the majority of trunk musculature\(^{111,112}\). Image loosely adapted from a 2012 review by Yung-Yao Lin\(^{111}\).
Zebrafish models of MTM1 and RYR1 deficiency suggest that defects in either protein may promote skeletal muscle weakness in respective human centronuclear and core myopathies by disrupting the machinery necessary for excitation–contraction coupling\textsuperscript{106}. Targeted approaches with candidate compounds have also shown that NAC antioxidant can ameliorate muscle pathologies by reducing oxidative stress in the spontaneous zebrafish model of \textit{ryr1b} mutation\textsuperscript{113}. Zebras are also the only vertebrates suited for drug discovery and efficacy assessment in a high-throughput, whole-organism context. Phenotype-based screens performed in zebrafish have the advantages of not requiring validated molecular targets, and eliminating hits that have strong side effects, low uptake efficiency, and poor \textit{in vivo} activity. Large-scale zebrafish screens identified phosphodiesterase inhibitors as a viable treatment strategy for Duchenne muscular dystrophy\textsuperscript{114}.

\section*{1.3 Manipulation of Zebrafish Gene Expression}

Several zebrafish models of neuromuscular disease have been generated using “forward genetics” approaches, such as large-scale saturation screens involving chemical or retroviral mutagenesis\textsuperscript{115-119}. Recently, more and more zebrafish laboratories investigating the functions of causative genes are taking advantage of “reverse genetics” thanks to advancements in genetic manipulation technologies. Transient disruption of a gene’s normal function during early development can be achieved by microinjecting zebrafish embryos with exogenous DNA, mRNA, or antisense oligonucleotides called morpholinos\textsuperscript{120}. Stable genetic disruptions, however, are rapidly becoming the new gold standard, and often involve targeted transgenesis or sequence-specific endonucleases\textsuperscript{121}.

\subsection*{1.3.1 Morpholinos}

First developed as a way to inhibit the translation of RNA transcripts in living cells and organisms, morpholinos (MOs) have proven invaluable for the study of loss-of-function phenotypes during zebrafish embryogenesis\textsuperscript{122,123}. Generally, MOs are synthetic molecules consisting of 25 oligonucleotides connected by a chemically stable, neutrally charged phosphorodiamidate backbone\textsuperscript{120}.
Complementary base pairing targets MOs to the RNA of interest. Splice-blocking MOs bind to splice junctions and inhibit proper transcript processing, whereas translation-blocking MOs bind immediately adjacent to the ATG start site and prevent ribosome assembly. Both types of MOs reduce the expression of functional gene products.

1.3.2 TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES

Programmable nucleases now allow for precise genome editing through the introduction of DNA double-stranded breaks (DSBs) at specific loci. Although non-homologous end joining may accurately repair the break by directly rejoining the two ends of the DSB, repeated rounds of repair often result in small insertions or deletions at the break site. Such mutations introduced into the coding sequence of a gene can cause frameshifts that lead to mRNA degradation by nonsense-mediated decay, or the production of a nonfunctional, truncated protein. Alternatively, homology-directed repair machinery may use exogenous DNA templates to synthesize the DNA required for lesion repair. To date, three major classes of nucleases have successfully been applied in zebrafish: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease Cas9. ZFNs and TALENs are chimeric enzymes consisting of a DNA binding domain fused to a sequence-independent FokI nuclease, while CRISPR-Cas9 is targeted to specific DNA sequences by a short guide RNA that binds directly to the target DNA through Watson-Crick base pairing.

TALENs remain the most versatile custom restriction enzyme system in zebrafish with the lowest rates of off-target effects. First discovered in the plant pathogen Xanthomonas, TALENs are proteins that regulate host gene expression. The DNA-recognition domain of each TALEN consists of a repetitive motif approximately 30 amino acids in length. Two of these amino acids, referred to as repeat-variable di-residues, recognize different bases within the target DNA sequence. DNA-recognition domains are then linked to a FokI nuclease domain that must homodimerize to catalyze a DSB. TALENs are
therefore constructed as a pair of nuclease-guiding proteins that bind both sides of the intended break site. Such localized dimerization increases the cleavage specificity of this approach 121.

1.4 SPECIFIC AIMS AND OVERVIEW OF DISSERTATION

Spectacular progress has been made over the past twenty years in elucidating the genetic basis of neuromuscular disorders, using high-throughput next generation sequencing as well as traditional positional cloning to identify chromosomal linkage. However, only rarely have these advances in gene discovery led to development of safe and effective therapies. This conundrum is nowhere more apparent than in the congenital myopathies and muscular dystrophies.

My written dissertation work addresses this very significant translational disconnect in two major forms of congenital myopathy, and does so in a way that increases general understanding of the basic biology of these disorders as well as the mechanisms that lead to skeletal muscle weakness. Chapters 2 and 3 discuss the creation and characterization of zebrafish models of centronuclear and core myopathies, respectively, whereas Chapter 4 details use of a closely related model to identify targeted molecular therapies. These studies have been selected among others that I have published within the broader context of genetic disorders affecting skeletal muscle 133,134. This thesis has dual significance, in both improving clinical practice and care for patients with congenital myopathy, and further establishing zebrafish-based small molecule screens as a powerful tool for lead compound identification and drug development.

1.5 REFERENCES


CHAPTER 2:

ESTABLISHING THE ROLE OF bin1 IN EARLY ZEBRAFISH DEVELOPMENT

AND SKELETAL MUSCLE PATHOGENESIS

This chapter is based on:


2.1 ABSTRACT

Autosomal recessive centronuclear myopathy (CNM2) is a mildly progressive muscle disorder characterized by abnormally centralized nuclei on biopsy and caused by mutations in the bridging integrator 1 (BIN1) gene. Misregulated alternative splicing of BIN1 has also been reported in patients with myotonic muscular dystrophy, a disease that shares several clinical and histopathological features with CNM2. The encoded protein, BIN1, is important for membrane sensing and creating tubular membrane invaginations in vitro and in different cell types. However, to fully understand the biological roles of BIN1 in vivo and to answer critical questions concerning the muscle-specific function of BIN1 in vertebrates, robust small animal models are required. To address this current shortcoming in the field, we created and characterized a novel zebrafish model of CNM2 using antisense morpholinos. Immunofluorescence and histopathological analyses of Bin1-deficient zebrafish skeletal muscle revealed structural defects commonly reported in human CNM2 biopsies, and live imaging linked the presence of abnormal triads to impairments in intracellular calcium signaling. RNA-mediated rescue assays also demonstrated that knockdown of zebrafish bin1 can reliably be used to examine the in vivo pathogenicity of novel BIN1 mutations and alternatively spliced isoforms. Our results strongly suggest that the skeletal muscle-specific phosphoinositide-binding domain of BIN1 might be more critical for muscle maturation and maintenance than for early muscle development. Furthermore, faulty inclusion of a brain-specific BIN1 domain in skeletal muscle may be deleterious due to compromised protein-protein interactions. Overall, our data support that BIN1 plays an important role in membrane tubulation and may promote skeletal muscle weakness in inherited muscular disorders by disrupting the machinery necessary for excitation-contraction coupling. The reproducible phenotype of Bin1-deficient zebrafish, together with the generalized advantages of the teleost system, makes this model readily adaptable to test promising therapeutic strategies for CNM2 and related neuromuscular diseases in vivo.

2.2 INTRODUCTION

Centronuclear myopathies (CNMs) are a heterogeneous group of congenital disorders
characterized by muscle weakness and abnormal nuclear centralization in myofibers. Several genetic forms have now been described, and vary in terms of age of onset, severity of clinical symptoms, and mode of inheritance. Pathogenic CNM mutations are most commonly reported in the phosphoinositide phosphatase myotubularin (MTMI) and dynamin 2 (DNM2) genes, resulting in neonatal X-linked (XLMTM; MIM #310400) and autosomal dominant (CNM1; MIM #160150) forms of the disease, respectively. Variants in other genes including the ryanodine receptor calcium release channel (RYRI), titin (TTN) and myotubularin-related protein 14 (MTMR14) have also been identified in rare cases with centralized nuclei on muscle biopsy 3-6. Autosomal recessive CNM (CNM2; MIM #255200) is most often associated with homozygous partial loss-of-function mutations in the bridging integrator 1 (BIN1) gene, although heterozygous dominant BIN1 mutations have also been associated with a mild, adult-onset form of the disease 7,8. In comparison to MTM1- and DNM2-related CNM, pathological mechanisms underlying BIN1-related CNM remain particularly unclear, due to fewer available human biopsies as well as a lack of faithful and accessible animal models.

BIN1, also referred to as MYC box-dependent interacting protein 1, amphiphysin 2, or SH3P9, is a ubiquitously expressed scaffolding protein involved in the tubular invagination and remodeling of membranes 9-11. BIN1 is also an important regulator of the cytoskeleton, cell cycle progression, apoptosis, and DNA repair 12. The human BIN1 gene, located on chromosome 2q14, contains 20 exons and can be spliced into at least 10 differentially expressed isoforms (Fig. 2.1 A-D) 7,13. All known splice variants possess an N-terminal amphipathic helix, a Bin/Amphiphysin/RVS167 (BAR) domain able to sense and promote membrane curvature through dimerization, and a C-terminal Src homology 3 (SH3) domain with negative electrostatic potential important for protein-protein interactions (Fig. 2.1 E) 14-17. Variability arises in BIN1’s central region. The clathrin and AP2/α-adaptin (CLAP) binding domain encoded by exons 13-16, for example, is only found in brain isoforms, whereas the phosphoinositide (PI)-binding motif encoded by exon 11 is muscle-specific.
Figure 2.1. **BIN1 protein domains and tissue-specific isoforms.** (A-D) Predominant *BIN1* transcript isoforms present in brain, skeletal muscle, most other tissues, and in cancer. Alternatively spliced exons are indicated (^). Image adapted from a 2014 review by Prokic *et al* [12]. (E) Protein domains of BIN1. BIN1/Amphiphysin/Rvs167 domain (BAR); phosphoinositide-binding domain (PI); proline-serine rich domain (PS); clathrin/AP2/α-adaptin binding domain (CLAP); c-Myc-binding domain (MBD); Src homology 3 domain (SH3). (F) Gene organization of *BIN1* and the seven autosomal recessive CNM2 mutations reported in human patients to date. Mutation nomenclature is based on isoform 1 (NP_647593.1), with the exception of c.IVS10-1G>A, which is named based on isoform 8 (NP_004296.1) in accordance with its original report [18].
Six of the seven partial loss-of-function \textit{BIN1} mutations in CNM described to date are located in the ubiquitously expressed and evolutionarily conserved BAR and SH3 domains (Fig. 2.1 F). BAR missense mutations strongly decrease the membrane tubulating properties of BIN1 in cultured cells, while SH3 truncating mutations impair interactions with DNM2 \cite{7}. The first human \textit{BIN1} mutation affecting splicing of the muscle-specific PI-binding domain was identified only recently, and found to result in an unusually progressive form of the myopathy \cite{18}. Since the PI motif increases BIN1’s affinity for negatively charged lipids PI(4,5)P$_2$ and/or PI3P and PI5P \cite{19,20}, this mutation is hypothesized to disrupt BIN1 localization and/or conformation is skeletal muscles.

\textit{BIN1} expression increases dramatically during muscle cell growth and differentiation \cite{21,22}. In humans, the subcellular localization of BIN1 protein shifts during skeletal muscle development. BIN1 immunoreactivity appears as longitudinal striations along the muscle fiber in neonates and switches to a transverse orientation by three months of age \cite{23}. BIN1 co-localizes with transverse (T)-tubules throughout this reorganization, which are deep invaginations of the sarcolemma that, together with the sarcoplasmic reticulum, compose the triad and facilitate excitation-contraction coupling \cite{23,24}. Studies conducted \textit{in vivo} provide evidence to also implicate BIN1 in the biogenesis of these structures. Null mutations in the \textit{Drosophila melanogaster} orthologue of mammalian \textit{BIN1} result in viable, flightless flies with severely disorganized and reduced number of T-tubules \cite{25}. Similar disruptions in skeletal muscle, such as the mislocalization of T-tubules to Z-lines, have been observed in early BIN1 null mouse embryos and reproduced in isolated adult murine myofibers \cite{26,27}. Short hairpin RNA silencing of \textit{Bin1} gene expression in mouse skeletal muscle also leads to T-tubule structural abnormalities, disrupted expression patterns of nascent T-tubule markers, and defects in intracellular calcium release \cite{27}. Studies \textit{in vitro} and in cultured cells have attributed the localization patterns and membrane tubulating properties of BIN1 to the PI-domain specifically found in skeletal muscle isoforms \cite{7,19,28}. Nevertheless, the molecular basis for the muscle-specificity of CNM2 remains unresolved.

Robust animal models are necessary to fully elucidate the biological roles of BIN1 \textit{in vivo} and to answer critical questions concerning the muscle-specific function of BIN1 in vertebrates, similar to the
animal modeling already accomplished for the MTM1- and DNM2-related forms of CNM. Knockout mice generated by targeted mutagenesis of Mtm1 and Labrador retrievers with MTM1 mutations exhibit progressive motor deficits and triad defects. The zebrafish model for X-linked CNM generated by morpholino knockdown of the mtm1 gene displays similar developmental delays and ultrastructural abnormalities, including mislocalized nuclei and disorganized organelles. Tissue-specific excision of exon 4 in mouse Mtm1 suggests that the muscle phenotype observed in animal models of myotubularin deficiency is due to loss of Mtm1 function in skeletal muscle. Regarding autosomal dominant CNM, Dnm2 knockout mice die before embryonic day 10, but knock-in mice heterozygous for the most common pathogenic mutation, p.465W (c.1393C>T), present with a mild myopathic phenotype. Skeletal muscle weakness and disruptions of mitochondrial and T-tubule networks are also seen in a second murine model created by intramuscular AAV injections of DNM2-R465W into adult skeletal muscle tissue, as well as in zebrafish models overexpressing mutated human DNM2.

The Inherited Myopathy of Great Danes (IMGD) canine model newly characterized by Jocelyn Laporte and colleagues is the first animal model to reproduce CNM2 human pathology, since the perinatal lethality of BIN1 null mouse embryos precludes extensive studies in skeletal muscle. Although larger mammals such as mouse and dog are beneficial and necessary to study the effects of select lead therapeutic candidates, large-scale drug screening is not feasible in these models. There is thus significant benefit to developing small vertebrate models, especially for a disease still in the earliest stages of drug development. Zebrafish are greatly amendable to medium- and high-throughput chemical screens thanks to their ease of husbandry and breeding, rapid ex utero development, and high spawning productivity. Zebrafish embryos are also permeable to small molecules and their transparent chorions allow for simple developmental observations. For the study of skeletal muscle pathologies in particular, zebrafish offer important research advantages. Genes essential for muscle development and function, as well as the physical ultrastructure of the sarcomere, are highly conserved between mammals and zebrafish. Additionally, muscular disorganization and locomotive impairment can be quickly and non-invasively quantified in the zebrafish over the first few days post-fertilization using birefringence and touch-evoked
escape behavior assays.\(^3^9\)

Therefore, to develop a small vertebrate model of CNM2 suitable for use in most laboratories and for testing potential therapeutic strategies, we created and characterized the first zebrafish model of the disease using antisense oligonucleotides. Bin1-deficient zebrafish reliably reproduce the morphological abnormalities, impaired early motor functions, and histological defects in skeletal muscle observed in human CNM2 patients. Our \textit{bin1} knockdown morphants offer powerful insights into the biological functions of BIN1 \textit{in vivo} and its potential role in skeletal muscle pathogenesis.

### 2.3 Materials and Methods

#### Zebrafish lines and husbandry

Zebrafish (\textit{Danio rerio}) from the wild-type Oregon AB line were bred and maintained according to standard procedures in the Boston Children’s Hospital Aquatic Research Program facility.\(^4^0\) Embryos were collected by natural spawning, staged by hours (hpf) or days (dpf) post fertilization,\(^4^1\) and raised at 28.5°C in egg water. All animal work was performed with approval from the Boston Children’s Hospital Animal Care and Use Committee (14-05-2717R).

#### Morpholino knockdown and mRNA rescue

Two antisense morpholinos (MOs), one targeting the translational start site (ATG MO) and one targeting the exon 8-intron8 splice site (Ex8 MO), were designed to knockdown the zebrafish \textit{bin1} transcript (GeneTools LLC, Philomath, OR, USA). The morpholino sequences were \textit{bin1} ATG MO: 5’-TGACTCCTTTCCAACCTCTGCCAT-3’ and \textit{bin1} Ex8 MO: 5’-TCTCTTTATTATTGGCCTCAGTTTGCCAT-3’. A morpholino against human \(\beta\)-globin, which is not homologous to any sequence in the zebrafish genome by BLAST search, was used as a negative control for all injections (5’-CCTCTTACCTCAGTTACAATTTATA-3’). Morpholinos were dissolved in 1X Danieau buffer with 0.1% phenol red and 1–2 nL (1–10 ng) injected into the yolk of 1-cell stage wild-
type embryos.

For rescue experiments, full-length human BIN1 isoform 8 (NM_004305.3) and isoform 9 (NM_139350.2) cDNAs were cloned into pCSDest destination vectors (created by Nathan Lawson) using Gateway technology (Invitrogen, Carlsbad, CA, USA). Substitutions p.K35N (c.105G>T) and p.K575* (c.1723A>T) were incorporated into BIN1 isoform 9 cDNA using GENEART site-directed mutagenesis (Invitrogen). The four plasmids encoding enhanced green fluorescent protein (EGFP)-tagged splice variants of human BIN1 (-7-11, -7+11, +7-11, +7+11) were a gift from Nicolas Charlet-Berguerand. mRNA for all constructs was synthesized in vitro using mMessage mMachine SP6 kits (Ambion, Austin, TX, USA). mRNA (50–200 pg) was injected into embryos at the 1-cell stage independently or in combination with ATG MO, and subsequent phenotypic analyses performed at 4 dpf.

**Birefringence**

Muscle birefringence was analyzed as we have described previously in the *Journal of Visualized Experiments*, and is schematically diagrammed below (Fig. 2.2 A-B) \(^3^9\). Quantification data were calculated for five posterior somites (numbers 12-16) that exhibited flat orientation in both *bin1* morphants and wild-type controls. Mean pixel intensity was divided by the skeletal muscle area for each zebrafish birefringence image using Image J (National Institutes of Health, Bethesda, MD, USA), a value which was then normalized to wild-type controls.
Figure 2.2. Zebrafish birefringence assay. (A) Standard dissecting microscope fitted with top (i) and bottom (ii) polarized lenses. To assess muscle integrity in translucent zebrafish larvae, dechorionated, anesthetized embryos are placed directly on the bottom lens along the lateral axis of their bodies (iii). The top lens, with the embryo of interest in view, is then rotated until the axes of polarization of the two lenses are oriented at 90° from one another and the background is completely dark (iv). (B) Microscope view showing light to dark background transition. Image adapted from Smith et al (2013) 39.
**Western blotting**

Zebrafish embryos at 3 dpf were homogenized in buffer containing Tris-Cl (20 mM, pH 7.6), NaCl (50 mM), EDTA (1 mM), NP-40 (0.1%) and complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA). Following centrifugation at 11000g at 4°C for 15 min, protein concentration in supernatants was determined by BCA protein assay (Pierce, Rockford, IL, USA). Proteins were separated by electrophoresis on 4–12% gradient Tris–glycine gels (Invitrogen) and transferred onto polyvinylidene difluoride membrane (Invitrogen). Membranes were blocked in PBS containing 5% casein and 0.1% Tween-20, then incubated with either rabbit polyclonal anti-bridging integrator 1 (1:250, SAB1408547, Sigma, St. Louis, MO, USA) or mouse monoclonal anti-β-actin (1:1000, A5441, Sigma) primary antibodies. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (1:2500, 170-6515) or anti-mouse (1:5000, 170-6516) IgG secondary antibodies (BioRad, Hercules, CA, USA). Proteins were detected using the SuperSignal chemiluminescent substrate kit (Pierce).

**Touch-evoked escape response assay**

Mechanosensory stimuli were delivered to 3 dpf embryos by touching the yolk sac or tail with an insect pin as described previously. Embryonic motor behaviors were recorded using a SPOT RT3 digital camera system (SPOT Imaging Solutions, Diagnostic Instruments Inc., Sterling Heights, MI, USA) mounted on a Nikon SMZ1500 stereomicroscope (Nikon Instruments Inc., Melville, NY, USA). Video frame capture (30 Hz) was performed using Image J (NIH).

**RT-PCR**

Total RNA was prepared from zebrafish embryos using RNeasy fibrous tissue mini kits (Qiagen, Valencia, CA, USA). cDNAs were synthesized from 1-2 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers. To quantify extent of bin1 knockdown in 3 dpf embryos, quantitative real-time RT-PCR amplification of cDNAs was performed with a Taqman assay for bin1.
exon10-exon11 (Applied Biosystems, Austin, TX, USA) on a 7300 Real Time PCR System (Applied Biosystems). Gapdh served as the control to normalize bin1 expression using the $2^{-\Delta\Delta\text{Ct}}$ method. To detect relative bin1 expression levels in early zebrafish development, RT-PCR was performed on cDNAs from <1 to 7 dpf embryos on a Tetrad 2 thermocycler (BioRad). Primer sequences to zebrafish genes bin1 or ef1α were as follows: bin1 forward: 5′-TGTCTGGCGAATGTATGACC-3′; bin1 reverse: 5′-TATCACTCAGATTCTGGTTCAGTTTG-3′; ef1α forward: 5′-TCACCCTGGGAGTGAAACAGC-3′; and ef1α reverse: 5′-ACTTGCAGGCGATGTGAGCAG-3′.

**Myofiber cultures and immunofluorescence**

Mixed cell cultures from 3 dpf embryos were obtained as described previously. Fixed cells were blocked in PBS containing 10% goat serum and 0.3% Triton X, incubated in primary antibody overnight at 4°C, washed in PBS, incubated in secondary antibody for 1 hour at room temperature (RT), washed in PBS, then mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Primary antibodies used were rabbit polyclonal anti-bridging integrator 1 (1:50, SAB1408547, Sigma), and mouse monoclonal anti-sarcomeric alpha-actinin (1:100, A7732, Sigma), anti-dihydropyridine receptor 1 alpha (1:100, ab58552, Abcam, Cambridge, MA, USA), anti-triadin (1:100, T3569, Sigma). After washing in PBS several times, samples were incubated with anti-mouse fluorescein FITC (1:100, 115-095-003) or anti-rabbit rhodamine TRITC (1:100, 111-025-144) secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). Imaging was performed using an UltraVIEW VoX spinning disk confocal microscope (Perkin Elmer, Wellesley, MA, USA).

Indirect immunofluorescence staining was performed on frozen sections from 3 dpf zebrafish embryos as described previously. Mouse monoclonal anti-dystrophin primary antibody (1:500, D8043, Sigma) was used and revealed by FITC anti-mouse secondary antibody at 1:100 dilution (Jackson Immunoresearch). Nuclei were stained with DAPI and imaged using a Nikon eclipse 90i microscope. Whole-mount immunofluorescence with phalloidin was performed on 3 dpf embryos as described previously. Briefly, embryos were fixed in 4% PFA overnight at 4°C, then washed as follows: 2 x 10
min in PBS, 2 x 10 min in PBS-T (0.1% Tween-20), 1 x 60 min in PBS-TR (2% Triton X), and 2 x 5 min in PBS-T. Embryos were blocked in PBS-T containing 5% goat serum for 1 hour at RT, and incubated with Alexa Fluor® 488 phallolidin (1:20, A12379, Invitrogen) overnight at 4°C. Embryos were washed 4 x 15 min in PBS-T before being mounted in 70% glycerol and visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope. A similar procedure was used for mouse monoclonal anti-slow myosin heavy chain (1:100, MF20, Developmental Studies Hybridoma Bank, University of Iowa, USA) primary followed by anti-mouse fluorescein FITC (1:100, 115-095-003, Jackson Immunoresearch) secondary antibodies.

**Calcium imaging**

Zebrafish embryos were injected at the 1-cell stage with a plasmid encoding the GCaMP3-EGFP calcium reporter under control of the alpha-actin promoter independently or in combination with ATG MO, resulting in wild-type or bin1 morphant embryos that mosaically expressed GCaMP3 within skeletal muscle. Before imaging at 3 dpf, tricaine was removed and the embryos allowed to recover in Evans solution (134 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, 10 mM HEPES) for 10 min and treated with 200 mM N-benzyl-p-toluene sulphonamide to inhibit contraction for 5 min. Hundred micromolar KCl was added to initiate muscle contraction. GCaMP3-EGFP-expressing cells were imaged at 30 Hz using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope. Perkin Elmer Volocity 6.3 confocal software was used to measure relative fluorescence intensity changes of induced Ca²⁺ transients.

**Histopathology**

For hematoxylin/eosin sections, 3-4 dpf zebrafish embryos were anesthetized and fixed overnight at 4°C in 4% PFA in PBS, washed in PBS, dehydrated in alcohols and xylenes, and embedded in paraffin. Microtome sections were cut at 5 μm using a Leica 2255 cryostat (Leica Microsystems Inc., Wetzlar, Germany) and H&E was done per standard protocol. For electron microscopy, 4 dpf zebrafish embryos
were fixed in formaldehyde-glutaraldehyde-picric acid in cacodylate buffer overnight at 4°C, followed by osmication and uranyl acetate staining. Subsequently, embryos were dehydrated in a series of ethanol washes and embedded in TAAB Epon (Marivac Ltd., Halifax, Nova Scotia, Canada). Sections (95 nm) were cut with a Leica UltraCut microtome, picked up on 100-µm Formvar-coated copper grids, and stained with 0.2% lead citrate. Sections were viewed and imaged under a Tecnai BioTwin Spirit electron microscope (Philips, Amsterdam, Netherlands) at the Harvard Medical School Electron Microscopy Core.

**Whole mount in situ hybridization**

Riboprobes were constructed from the 3’UTR of *bin1* using adult zebrafish RNA. Total RNA was extracted from adult zebrafish muscle tissue using RNeasy mini kits (Qiagen). cDNAs were synthesized using the Superscript RT-PCR system (Invitrogen) and PCR-amplified with product ends carrying T7 or SP6 RNA polymerase recognition sequences. Sense or antisense digoxigenin-labeled riboprobes were synthesized by *in vitro* transcription using dig-labeling kits (Roche Applied Sciences). Whole mount in situ hybridization was performed as described 45. Images were taken using a Nikon SMZ1500 stereoscope equipped with a SPOT RT3 digital camera system.

**Acetylcholine receptor labeling**

Embryos (3 dpf) were fixed in 4% paraformaldehyde for 2 hours at RT and dehydrated in 100% methanol overnight at -20°C. Embryos were rehydrated and permeabilized in 1X TBS-T (1% Triton X) for 2 hours at RT. Blocking was performed with 5% goat serum in TBS-T for 1 hour at RT followed by incubation with fluorescent conjugated α-bungarotoxin (5 mg/mL) for 30 min at RT (Invitrogen). Embryos were washed several times in TBS-T and mounted in 70% glycerol and visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope.

**Statistical analysis**

Data were statistically analyzed by parametric Student t-test (two-tailed) and were considered
significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)]. All data analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). All quantitative data are shown as the mean ± standard deviation.

**2.4 RESULTS**

**Morpholino knockdown of zebrafish bin1**

The Ensembl genome browser identifies two zebrafish genes as orthologues to human BIN1: bridging integrator 1a (*bin1a*; ENSDARG00000042114) and bridging integrator 1b (*bin1b*; ENSDARG00000058820). Of these two, only the 1878-base pair zebrafish *bin1b* located on chromosome 6 shares strong synteny with the human gene, suggesting it to be the truest orthologue of human BIN1 (Fig. 2.3). Zebrafish *bin1b* will hereinafter be referred to simply as *bin1*.

![Syntenic organization of human BIN1 compared with zebrafish bin1b (ENSDARG00000058820) and bin1a (ENSDARG00000042114). Blue arrows indicate common human and zebrafish orthologues within the 1.5 megabase pair region shown for each chromosome.](image)

**Figure 2.3. Zebrafish bin1b shares strongest synteny with the human BIN1 gene.** Syntenic organization of human BIN1 compared with zebrafish bin1b (ENSDARG00000058820) and bin1a (ENSDARG00000042114). Blue arrows indicate common human and zebrafish orthologues within the 1.5 megabase pair region shown for each chromosome.
Zebrafish \textit{bin}1 encodes a 364 amino acid protein that is highly conserved (67\% identity, 84\% similarity) with the longest human BIN1 isoform (Fig. 2.4 B i-ii). RT-PCR and Western blots confirmed that \textit{bin}1 is actively expressed in the developing fish (Fig. 2.4 C-D) and whole-mount \textit{in situ} hybridizations localized this expression to skeletal and cardiac muscle (Fig. 2.4 A). Expression of \textit{bin}1 in zebrafish striated muscle is consistent with \textit{in situ} data generated previously using high-throughput analyses and made available on the Zebrafish Model Organism Database (http://zfin.org) \textsuperscript{46}.
Figure 2.4. Bin1 gene expression in early zebrafish development. (A) Whole-mount in situ hybridization in wild-type embryos at 6 hpf, 18 hpf, 1 dpf, and 2 dpf confirm that bin1 is expressed in zebrafish cardiac and skeletal muscle. Heart (H); somites (SK); pectoral fin (PF). (B) Protein structure of (i) zebrafish Bin1 and of selected human BIN1 isoforms: (ii) brain-enriched, longest isoform of human BIN1; (iii) ubiquitous BIN1 isoform 9 which lacks the PI-binding domain encoded by exon 11; (iv) muscle-specific BIN1 isoform 8 containing the PI-binding domain. The two human CNM2 mutations used for subsequent rescue experiments are indicated. Domain abbreviations are defined in Fig. 2.1. (C) RT-PCR of wild-type zebrafish cDNA shows that bin1 gene expression peaks at ~1-2 dpf during the first week of development. Non-template control (NT). (D) Total Bin1 protein levels decrease and reflect decreased amounts of bin1 transcript between 18 hpf and 7 dpf. β-Actin was used as the loading control.
To ablate the function of *bin1* in zebrafish, two independent MOs were designed to the translational start site (ATG MO) and to the splice donor site of exon 8 (Ex8 MO) (Fig. 2.5 A&C). The ATG MO was predicted to block *bin1* translation and the Ex8 MO was expected to introduce premature stop codons to the transcript by retaining the adjacent intron. Both MOs were independently injected into 1-cell stage embryos. A standard MO directed against human β-globin, a sequence of nucleotides not found in the zebrafish genome, was used in all experiments to control for injection-related non-specific effects in wild-type embryos.

Efficacy of the ATG MO to interfere with translation of zebrafish *bin1* was verified by Western blot. Reduced levels of endogenous zebrafish Bin1 protein were observed in 3 dpf *bin1* morphants (Fig. 2.5 B). Efficacy of the Ex8 MO to alter *bin1* mRNA processing and stability was first examined by RT-PCR using primers targeted to flanking exons (Fig. 2.5 E). DNA sequencing showed retention of the 105-base pair intron between exons 8 and 9 in *bin1* morphants at 3 dpf and contains at least two putative stop codons likely to result in a truncated protein. Further analysis by Western blot and quantitative real-time RT-PCR confirmed that injection of the Ex8 MO at high concentrations (10 ng) results in a lower molecular weight, presumably non-functional, form of Bin1 protein and that expression of the full-length *bin1* transcript decreases with increasing concentrations of Ex8 MO (Fig. 2.5 D&F).
Figure 2.5. MO knockdown of bin1 results in protein loss and mRNA mis-splicing. (A-B)

Translation-blocking MO (ATG MO) results in reduced levels of Bin1 protein as shown by Western blot. (C) Splice-blocking MO (Ex8 MO) causes an intron containing putative stop codons to be retained. (D) Quantitative real-time RT-PCR (primers targeting exon 10 boundary) demonstrates that full-length bin1 expression decreases with increasing concentrations of Ex8 MO. (E) RT-PCR confirms presence of lengthened bin1 transcript in Ex8 MO-injected bin1 morphants compared to wild-type controls. (F) Western blot shows that Ex8 MO results in a truncated form of Bin1 protein at higher concentrations. All MO analyses described here were performed with 3 dpf zebrafish larvae.
**Loss of zebrafish Bin1 results in abnormal morphology and impaired locomotor activity**

Zebrafish embryos undergo rapid skeletal muscle development. Multinucleated myofibers are present by 1 dpf and muscle cells fully differentiate by 2 dpf. Morphological abnormalities in *bin1* MO-injected embryos arise within this same time frame. Both MOs yielded indistinguishable phenotypes throughout early zebrafish development. As early as 1 dpf, *bin1* morphants display a dorsal curvature that is absent from wild-type controls (Fig. 2.6 A). This curvature is reminiscent of the kyphosis/scoliosis often seen in CNM2 patients\(^\text{47,48}\), and has been observed in other zebrafish models of congenital myopathy, including the closely related X-linked form of CNM\(^\text{31}\). By 3-4 dpf, *bin1* morphants develop a more pronounced kyphosis and exhibit bent and/or foreshortened tails, together resulting in an overall S-shaped appearance (Fig. 2.6 B-C).
Figure 2.6. Bin1-deficiency leads to morphological abnormalities in zebrafish larvae. (A) Manually dechorionated wild-type control embryos straighten quickly, whereas bin1 morphants begin to exhibit dorsal curvature as early as 1 dpf (arrowheads). (B-C) The bin1 morphant phenotype is highly reproducible throughout early development using two independent MOs targeting different regions of the zebrafish bin1 gene. Wild-type controls show normal morphology by 4 dpf, whereas bin1 morphants injected with translation-blocking ATG MO or splice-blocking Ex8 MO are S-shaped in appearance with pronounced kyphosis (asterisks) and bent tails (arrows).
Mild bradycardia was also noted in *bin1* MO-injected larvae (Fig. 2.7 C), and is likely due to disruptions in actin-myosin contractile apparatuses within striated muscles (Fig. 2.7 A-B). This finding is consistent with earlier reports of diminished calcium transients and impaired ventricular contractility in Bin1-deficient zebrafish hearts \(^{49}\), as well as with cardiac arrhythmias in human CNM2 patients \(^{50}\).

**Figure 2.7. Knockdown of zebrafish *bin1* results in mild bradycardia.** (A) Loss of Bin1 leads to reduced sarcomeric assemblies in zebrafish heart. Electron micrographs of wild-type zebrafish hearts at 4 dpf show well-organized sarcomeres (left). Bin1-deficient morphant hearts exhibit fewer actin-myosin contractile structures and sarcomeres that are present have scattered thin/thick filaments and lack Z-lines (right). (B) Whole-mount immunofluorescence of wild-type and *bin1* morphant hearts with MF20 antibody that recognizes both atrial and ventricular myosin. (C) Average heart rate of *bin1* morphants is reduced compared to wild-type controls at 4 dpf (WT: 206.0 ± 6.9 beats per minute (bpm), \(n = 20\); *bin1* MO: 148.8 ± 8.6 bpm, \(n = 20\); Student’s t-test, \(P < 0.01\)). Scale bars: 500 nm.
Consistent with defects in morphology, *bin1* morphants display deficits in early motor functions. The first recognizable muscle-dependent motor activity in zebrafish is spontaneous embryo coiling, detectable between 17 and 26 hpf. On average, wild-type embryos coil 10.6 ± 0.9 times per 15 seconds whereas *bin1* morphants coil only 7.7 ± 0.8 times within the same time interval (Fig. 2.8 A). Motor functions in early zebrafish development can also be evaluated by measuring the rate of chorion hatching. By 60 hpf, 95.9 ± 0.7 % of wild-type zebrafish embryos hatch naturally from their protective chorions, as opposed to only 46.0 ± 1.7 % of *bin1* morphants. This atypical delay suggests a mild early muscle weakness in the *bin1* morphant phenotype (Fig. 2.8 B).

Zebrafish embryos swim in response to touch by 26 hpf, and the frequency of muscle contractions during swimming increases to a value comparable to that of adult zebrafish shortly thereafter. By 3 dpf, *bin1* morphants display markedly impaired locomotion. Wild-type larvae respond to tactile stimuli by swimming quickly out of the field of view, whereas *bin1* morphants exhibit fluttering motions that either propel the larvae in circles or for only a few short lengths (WT: 6.30 ± 0.30 cm/0.1 s; *bin1* MO: 0.30 ± 0.05 cm/0.1 s) (Fig. 2.8 C-M). The highly diminished motor behaviors of *bin1* morphants are indicative of reduced muscle function and overall skeletal muscle weakness.
Figure 2.8. Abnormal motor functions in *bin1* morphants. (A) Quantification of spontaneous embryo coiling at 22 hpf. Control wild-type fish coil 10.6 ± 0.9 times per 15 seconds, while *bin1* morphants coil 7.7 ± 0.8 times in the same period. (B) Quantification of chorion hatching. At 60 hpf, 95.9 ± 0.7 % of wild-type controls are hatched from their protective chorions compared to only 46.0 ± 1.7 % of *bin1* morphants. (C) Average swimming distance of 3 dpf larvae in response to touch (WT: 6.30 ± 0.30 cm/0.1 s; *bin1* MO: 0.30 ± 0.05 cm/0.1 s). (D-G) Mechanosensory stimulation induces wild-type controls to swim away rapidly and fully exit the field of view within 100 ms. (H-M) Touch-evoked *bin1* morphants do not swim normally. Twittering motions propel morphant larvae in small circles within the field of view. All motor functions were quantified using 20 embryos from three independent injections (n = 60). Significance was determined by Student’s t-test, P < 0.01.
Bin1 deficiency results in disorganized myofibers and molecular abnormalities similar to CNM2

The skeletal muscle architecture in Bin1-deficient morphants was examined to see if their abnormal morphologies and diminished locomotive behaviors result from structural defects in this tissue. Hematoxylin/eosin staining of skeletal muscle in 4 dpf larvae revealed that the sarcomeric organization of myofibers is regularly disrupted around myonuclei in bin1 MO-injected embryos. Moreover, bin1 morphant myonuclei are mislocalized, rounded, and grouped. Morphant myonuclei are also frequently larger and filled with darkly-staining nucleoli compared to the smaller wild-type myonuclei, which are typically singular, elongated, and located towards the fiber periphery (Fig. 2.9 A-F).

Figure 2.9. Bin1 zebrafish display similar histopathological defects as human CNM2. (A-F) H&E stained longitudinal myofibers from 4 dpf bin1 morphant larvae and wild-type controls. Myonuclei from
(Figure 2.9, continued) *bin1* morphants are prominently mislocalized, rounded, and grouped (brackets). Morphant myonuclei also appear large and contain discrete, darkly-staining nucleoli (arrows).

Conversely, wild-type controls have singular, elongated myonuclei located at the fiber periphery. (G-H) Z-stacked images of wild-type and *bin1* morphant skeletal muscle captured using confocal laser scanning microscopy following whole-mount immunostaining with fluorophore-conjugated phalloidin. *Bin1* morphant myofibers appear criss-crossed and mildly disorganized at 3 dpf compared to wild-type controls. (I-J) Myofiber disorganization in *bin1* morphants becomes more apparent with individual image planes (no Z-stack). (K-N) Transmission electron micrographs of 4 dpf zebrafish larvae reveal that the fraction of abnormal triads in wild-type skeletal muscle is significantly lower in *bin1* morphants (arrowheads; see also Fig. 2.13 C&E). (P) Unusual membranous structures are commonly observed in the perinuclear regions of *bin1* morphants (arrow) but are absent in wild-type controls. (Q-R) Higher magnifications of a representative membranous structure. At least three embryos from three independent injections were processed and examined for all histochemical and electron micrograph analyses. Scale bars: (A-J) 10 µm, (K-R) 500 nm.
Whole-mount direct immunostaining of 3 dpf larvae with phalloidin, a high-affinity probe for filamentous actin, showed that myofibrillar organization is disrupted in bin1 morphants compared to wild-type controls. Major trunk skeletal muscle fibers overlap and often cross over one another in bin1 morphants, whereas wild-type myofibers generally appear parallel (Fig. 2.9 G-J). Such gross observations may at least partially explain the curved bodies of bin1 morphants, and are in accordance with the extensive myofibrillar disarray reported in CNM2 patients \(^{50}\). Additional immunofluorescence studies confirmed that the number and appearance of neuromuscular junctions, as well as dystrophin expression at the myosepta, in Bin1-deficient zebrafish skeletal muscle are normal (Fig. 2.10). Together, these data suggest that the bin1 morphant phenotype is a primary defect within myofibers, and does not arise from abnormalities at the sarcolemma and/or extracellular matrix.

Figure 2.10. Neuromuscular junctions and myosepta are normal in bin1 morphants. (A-D)

Acetylcholine receptor staining with α-bungarotoxin in 3 dpf embryos showed no significant differences in number or distribution of neuromuscular junctions between bin1 morphants and wild-type controls (left: fluorescence inverted; right: normal fluorescence). (E-F) Indirect immunostaining for dystrophin protein in 3 dpf embryos suggests that Bin1 deficiency in zebrafish does not affect the structure or proteins of the sarcolemma and/or extracellular matrix. Scale bars: 10 µm.
Transmission electron microscopy was performed at 4 dpf to visualize the effect of bin1 knockdown on underlying skeletal muscle ultrastructure. The architectural integrity of Bin1-deficient myofibers is generally preserved. Bin1-deficient fish showed normal organization of the contractile apparatus with no necrotic fibers or apoptotic nuclei, consistent with a non-degenerative myopathy. The most remarkable pathological defect of bin1 morphant muscle was the presence of malformed and highly disorganized triad structures. The majority of triads were abnormal in bin1 morphants, with distortions in size and/or shape, in comparison to wild-type controls (Fig. 2.9 K-N; quantified in Fig. 2.13 E). Terminal cisternae of the sarcoplasmic reticulum (SR), which are localized adjacent to T-tubules, appeared to be collapsed in most bin1 morphant myofibers, while no significant abnormalities were detected in SR longitudinal vesicles. Other ultrastructural features unique to bin1 morphant skeletal muscle and absent from wild-type controls were large whorled membranous structures of ambiguous origin localized to perinuclear regions (Fig. 2.9 O-R). Similar structures and T-tubule deformities have been reported in human CNM biopsies and in myotubularin-deficient zebrafish. Overall, these data support the hypothesis that BIN1 is important for membrane remodeling and maintaining the structural integrity of skeletal muscle, and that bin1 morphant zebrafish are a suitable model for human CNM2 on both the morphological and molecular levels.

**Bin1 deficiency disrupts localization of triad markers and calcium signaling in zebrafish skeletal muscle**

BIN1 is localized to T-tubules in human skeletal muscle and is hypothesized to play a role in the formation and/or maintenance of these structures. To test if the triad defects observed in bin1 morphants are a direct consequence of Bin1 deficiency at T-tubules, localization of Bin1 in zebrafish skeletal muscle was examined in cultured zebrafish myofibers. Indirect immunofluorescence revealed that zebrafish Bin1 protein is expressed in a distinctive striated pattern in wild-type embryos that overlaps with that of the dihydropyridine receptor (Dhpr), an established T-tubule marker (Fig. 2.11 A, top). The presence of Dhpr protein at T-tubule membranes was reduced in bin1 morphants (Fig. 2.11 A, bottom). Knockdown of bin1...
also perturbed expression patterns of other triad markers, including triadin (Trdn), an SR transmembrane protein (Fig. 2.11 B). However, it did not appear to affect non-triad muscle proteins, such as the Z-line marker alpha-actinin (Actn) (Fig. 2.11 C). These data agree with studies of BIN1 in human skeletal muscle\textsuperscript{20}, and importantly suggest that BIN1 is involved in maintaining the structural organization of triads \textit{in vivo}.
Figure 2.11. Triad markers are disrupted in Bin1 deficiency. Double label immunofluorescence on 3 dpf isolated myofibers. (A) Bin1 is localized in a striated pattern similar to the dihydropyridine receptor (Dhpr), a known T-tubule marker, in wild-type zebrafish (top). Loss of Bin1 disrupts normal Dhpr localization (bottom). (B) Expression patterns of alternative triad markers, such as triadin (Trdn), are perturbed in Bin1 deficiency. (C) Localizations of non-triad muscle proteins, such as alpha-actinin (Actn), appear unaffected. Images are representative of 20 myofibers analyzed from each of three independent experiments. Scale bars: 20 µm.
The triad is the membrane structure controlling excitation-contraction coupling (ECC) in skeletal muscle by regulating the release of calcium from the SR to the cytosol. To directly examine whether disorganized triads in bin1 morphants may lead to impaired ECC and defects in calcium homeostasis in vivo, calcium transients were imaged in 3 dpf zebrafish embryos mosaically expressing the alpha-actin-driven GCaMP3 Ca\(^{2+}\) reporter (Fig. 2.12 A)\(^{42,53}\). Skeletal muscle contraction was evoked by the application of potassium chloride, which depolarizes cells and activates voltage-gated calcium channels. Peak calcium release in fluorescing myofibers was found to be significantly reduced in bin1 morphants compared to wild-type controls (WT: 3.8 ± 0.4 RFU; bin1 MO: 1.8 ± 0.2 RFU) (Fig. 2.12 D). Fluorescence propagation through skeletal muscle over a fixed time interval was also qualitatively assessed in the examined embryos, and was noticeably diminished in bin1 morphants (Fig. 2.12 B-C). These findings provide strong in vivo evidence to suggest that defective ECC and abnormal calcium signaling in BIN1 deficiency, resulting from structural alterations at triads, may be a primary cause of CNM2. The Bin1-deficient zebrafish is the first animal model to demonstrate this important link in skeletal muscle.
Fig. 2.12. Calcium signaling is impaired in *bin1* morphants. (A) Representative example of a 3 dpf wild-type embryo mosaically expressing the alpha-actin-driven GCaMP3-EGFP Ca\(^{2+}\) reporter in skeletal muscle. (B-C) Calcium propagation through the trunk region is diminished in *bin1* morphants compared to wild-type controls following KCl treatment. Individual frames shown depict the time at which EGFP fluorescence is most widespread throughout the analyzed region of skeletal muscle. (D) Peak Ca\(^{2+}\) release is decreased between *bin1* morphant and wild-type myofibers expressing GCaMP3-EGFP (WT: 3.8 ± 0.4 RFU, *n* = 12; *bin1* MO: 1.8 ± 0.2 RFU, *n* = 12). Significance was determined by Student’s t-test, *P* < 0.01. Relative fluorescence units (RFU). Scale bars: (A) 200 µm, (B-C) 25 µm.

**The phosphoinositide-binding domain is not crucial for Bin1 function during early zebrafish development**

RNA-mediated rescue studies were performed to confirm that the muscular phenotype observed in *bin1* morphants directly results from Bin1 deficiency. Human *BIN1* encodes at least 10 different alternatively spliced transcripts and those specifically expressed in skeletal muscle are distinguished by the presence of a phosphoinositide (PI)-binding domain encoded by exon 11 (Fig. 2.1 B). The polybasic residue sequence of exon 11 (RKKSKLFSRLRRKKN) is required for BIN1-induced membrane tubulation when exogenously expressed in cultured cells \(^7,19\). However, until recently, all human *BIN1* mutations had been reported in ubiquitously expressed exons \(^18\). To investigate the functional significance of exon 11 in vivo, we exogenously overexpressed two distinct human BIN1 isoforms in *bin1* morphant
embryos (Fig. 2.4 B iii-iv), and evaluated the ability of each to rescue the skeletal muscle phenotype of the fish in terms of morphology, birefringence assay, and electron microscopy at 4 dpf. Intriguingly, overexpression of either isoform 8 (with exon 11) or isoform 9 (without exon 11) rescues the severe skeletal muscle morphology of bin1 morphants, and both do so to a similar degree (Fig. 2.13 A, B&D). Ubiquitous overexpression of either BIN1 isoform also significantly improves birefringence, a non-invasive measure of skeletal muscle organization. As the pronounced dorsal and tail curvature of bin1 morphants did not allow for accurate birefringence quantification of the whole fish, birefringence was quantified in five posterior somites consistently displaying flat orientation across all captured images. Bin1 MO-injected embryos displayed 53.8 ± 3.7% of the maximal birefringence observed in wild-type controls, whereas co-injection of wild-type human BIN1 mRNA and bin1 MO increased the mean pixel intensity of the selected somites to 78.4 ± 7.9% (isoform 8) or 75.8 ± 6.9% (isoform 9) (Fig. 2.13 E). Skeletal muscle ultrastructure was largely corrected in RNA-rescued bin1 morphants. The fraction of abnormal triads in bin1 MO-injected embryos decreased from 74.4 ± 2.3% to 26.2 ± 5.0% when rescued with isoform 8 mRNA, and to 31.0 ± 4.3% when rescued with isoform 9 mRNA (Fig. 2.13 C&E). This result strengthens the hypothesis that BIN1 plays a role in T-tubule biogenesis, and may do so independently of the PI-binding domain. Our rescue data demonstrate the specificity of MO-mediated bin1 knockdown and suggest that the BIN1 PI-binding domain may be more critical for skeletal muscle maturation and maintenance rather than for early development.
Figure 2.13. Overexpression of human BIN1 mRNA in bin1 MO-injected morphants. (A) Wild-type control larvae (4 dpf) are unaffected by overexpression of BIN1 or control mRNA (top panel: live larvae in normal light; bottom panel: same larvae in polarized light). (B) Morphology and organized musculature is fully or partially restored to 4 dpf bin1 morphants upon overexpression of human wild-type BIN1 mRNA. Isoform 8 (Iso8) and isoform 9 (Iso9) BIN1 transcripts show similar rescue as quantified in D and E. In contrast, human BIN1 mRNAs containing pathogenic CNM2 mutations K35N or K575* completely fail to rescue the bin1 morphant phenotype. (C) Electron microscopy shows normal structure of skeletal muscle triads in bin1 morphant fish rescued with either BIN1 Iso8 or Iso9 mRNA but disorganized triads in zebrafish embryos injected only with ATG MO. (D) Quantification of morphological changes in 4 dpf wild-type and bin1 morphants observed with mRNA overexpression. Representative images of living phenotypes specified in the color legend are starred in B. (E) Quantification of birefringence and fraction of disorganized triads in 4 dpf wild-type controls and bin1 morphants observed with mRNA overexpression. Birefringence data were calculated by dividing the mean intensity of five posterior
(Figure 2.13, continued) somites by the selected area of the fish (Image J), and normalizing the percentage values to wild-type controls (WT: 100 ± 10.2%; bin1 MO: 53.8 ± 3.7%; Iso8 RNA: 78.4 ± 7.9%; Iso9 RNA: 75.8 ± 6.9%; K35N RNA: 55.7 ± 6.4%; K575* RNA: 49.2 ± 11.0%; Student’s t-test, P < 0.01). Fifteen images were quantified per experimental group, five from each of three independent rescue studies (n = 15). Triad fractions were calculated as the percentage ratio of abnormal triads to the total number of triads counted (WT: 12.5 ± 5.2%, n = 96; bin1 MO: 74.4 ± 2.3%, n = 129; Iso8 RNA: 26.2 ± 5.0%, n = 151; Iso9 RNA: 31.1 ± 4.3%, n = 161). Significance was determined by Student’s t-test, P < 0.01.

**Human BIN1 mutations and splicing alterations are pathogenic in Bin1-deficient zebrafish**

*In vivo* RNA-mediated overexpression experiments were also used to test the ability of mutant human BIN1 to rescue the morphological abnormalities observed in bin1 morphants. Two pathogenic BIN1 mutations affecting the most evolutionarily conserved BIN1 protein domains were independently introduced into the wild-type human BIN1 gene by site-directed mutagenesis and the mutant mRNA transcribed *in vitro* (Fig. 2.4 B iv). The p.K35N (c.105G>T) missense change alters the charge of the BIN1 BAR domain N-terminus, and is predicted to compromise BIN1 interactions with negatively charged membranes and its capacity to generate membrane curvature. The p.K575* (c.1723A>T) nonsense mutation removes the last alpha helix and two beta strands of the SH3 domain. This change alters the three-dimensional structure of the SH3 region and disrupts important BIN1 protein-protein interactions, such as those with DNM2, a large GTPase that promotes membrane fission by constricting vesicle necks and is defective in autosomal dominant CNM. Overexpression of either mutant BIN1 was not able to rescue bin1 morphant morphology or birefringence (Fig. 2.13 B, D&E). These data show that our bin1 morphant fish is a reliable tool for characterizing the pathogenicity of BIN1 mutations *in vivo*, and could be used to quickly analyze novel genetic alterations in BIN1 as they are identified.

One such analysis was conducted via collaboration with Dr. Nicolas Charlet-Berguerand (Institut Génétique Biologie Moléculaire Cellulaire, France) after his group identified exon 7-containing BIN1.
transcripts in the skeletal muscles of human patients with myotonic muscular dystrophy (MMD). Exon 7 is normally only found in the BAR domain of neuronal BIN1 isoforms, where it strongly mediates interactions with DNM2 and improves efficiency of endocytosis. An RNA-mediated rescue approach was used to examine the potential pathogenicity of different BIN1 splicing variants in skeletal muscle. Overexpression of EGFP-tagged BIN1 mRNAs lacking exon 7 was consistently found to rescue 3 dpf abnormal morphant morphologies, including their prominent dorsal curvature and bent tails (Fig. 2.14 A&C). Nuclear centralization, a shared histopathological feature of both CNM and MMD, was also corrected (Fig. 2.15 A-C). Rescued skeletal muscles contained higher ratios of peripheral to central nuclei (Fig. 2.15 G). In contrast, EGFP-tagged BIN1 mRNA containing exon 7 failed to rescue either morphological or histological features (Fig. 2.14 A&C; Fig. 2.15 D-F, G). The presence of muscle-specific exon 11, similar to findings described in the above section, did not affect these results. EGFP expression was strongly detected through 2 dpf and faintly at 3 dpf (Fig. 2.14 B).
Figure 2.14. Bin1 morphants are not rescued with BIN1 transcripts containing exon 7. (A) Wild-type and bin1 larvae (3 dpf) are unaffected by overexpression of control mRNA. Abnormal morphology of bin1 morphants is fully restored upon overexpression of human EGFP-tagged BIN1 mRNAs lacking exon 7, with or without exon 11 (left). Human EGFP-tagged BIN1 mRNAs containing exon 7, in contrast, do not rescue bin1 morphology (right). These RNA-injected morphant embryos presented with dorsal curvature, bent tails, and mild bradycardia accompanied by cardiac edema. (B) Strong detection of
(Figure 2.14, continued) EGFP expression at 1-2 dpf. Wild-type embryos injected with EGFP-tagged $\textit{BIN1}$ mRNA only are shown here, and appeared normal in all experimental groups. (C) Quantification of morphological changes in 3 dpf wild-type and $\textit{bin1}$ morphants observed upon EGFP-tagged mRNA overexpression. All rescue studies were performed in triplicate, with injection replicates obtained using independent clutches on different days. The size of experimental groups within each replicate ranged from 20-100 embryos.
Figure 2.15. Neuronal exon 7 and nuclear centralization in bin1 morphants. Transverse sections of H&E stained myofibers from 3 dpf embryos. (A, D) Nuclei in skeletal muscles of wild-type control larvae are traditionally found at the myofiber periphery, while bin1 myonuclei are often mislocalized to the myofiber center. (B-C) Skeletal muscles rescued with human EGFP-tagged BIN1 mRNAs lacking exon 7, with or without exon 11, exhibit reduced proportions of central nuclei and are statistically
(Figure 2.15, continued) indistinguishable from wild-type controls. Peripheral nuclei are indicated with black arrowheads. (E-F) However, larvae injected with human EGFP-tagged BIN1 mRNAs containing exon 7, with or without exon 11, are not histologically rescued and display similar proportions of central nuclei as bin1 morphants. Centralized nuclei (CN) are shown using black arrows. (G) Quantification of centralized versus peripheral nuclei in 3 dpf wild-type control and bin1 morphant skeletal muscles observed with mRNA overexpression (WT: 4.3 ± 3.8% CN, n = 213; bin1 MO: 38.2 ± 11.1% CN, n = 259; -7-11 RNA: 6.8 ± 4.2% CN, n = 484; -7+11 RNA CN: 3.9 ± 4.0%, n = 335; +7-11 RNA: 39.7 ± 9.7% CN, n = 416; +7+11 RNA: 45.2 ± 11.7% CN, n = 192). Significance was determined by Student’s t-test, P < 0.01. Fibers were counted using H&E stained sections from 2-6 larvae from each experimental group, depending on the quality of the stain.

2.5 DISCUSSION

**Bin1-deficient zebrafish as a small vertebrate model for CNM2**

Although small animal models have been carefully characterized for the MTM1- and DNM2-related forms of CNM, the perinatal lethality of BIN1 null mice has hindered progress on this front to date. In our studies, we used a MO-mediated knockdown approach to examine the role of Bin1 in early zebrafish development and to develop a faithful small vertebrate model of autosomal recessive centronuclear myopathy (CNM2). Our data demonstrate that Bin1 localizes to T-tubules in zebrafish skeletal muscle, similar to published observations in mouse, canine, and human tissue. The loss of Bin1 in zebrafish disrupts this characteristic expression pattern and leads to similar morphological abnormalities and skeletal muscle defects as those reported in CNM2 patients. Furthermore, because Bin1-deficient zebrafish have a reproducible and clear phenotypic readout, this model is particularly suited for drug discovery applications. Zebrafish models have already been used successfully to demonstrate efficacy and toxicity of potential chemical therapeutics for human disease.
PI-binding domain of BIN1 may be more critical for late skeletal muscle maintenance rather than for early development

The molecular basis behind the muscle-specificity of CNM2 remains unclear. Of the 10 known alternatively spliced BIN1 isoforms, a PI-binding domain encoded by exon 11 is present only in isoforms expressed in skeletal muscle. In vitro and cell culture studies have shown that the exon 11 encoded PI-binding motif is required for the membrane tubulating properties of BIN1 in cultured muscle cells. Its unique muscle-specific expression may be of particular relevance to CNM as defects in the remodeling of PI-rich membranes are common to all of the major genetic forms, yet almost all CNM2 missense mutations described to date have been found in ubiquitously expressed exons and result in mild to moderate disease progression. Only recently has the first human splice mutation involving the skipping of the muscle-specific exon 11 been documented in two siblings with CNM2.

The BIN1 PI-binding domain is not required to rescue the bin1 phenotype in zebrafish skeletal muscle. Exogenous ubiquitous expression of human BIN1 transcript variants with and without the canonical PI-binding domain both drastically improved bin1 morphant morphology, as well as muscle birefringence and ultrastructure, and did so to a similar degree. These data reinforce the notion that BIN1 PI-binding ability may play a greater role in muscle later maturation and maintenance rather than in early muscle formation, and agree with the progressive integration of exon 11 in BIN1 mRNA during skeletal muscle cell differentiation and development. These observations also explain why exon 11 acceptor splice site mutations in dogs and humans present as a highly progressive phenotype later in life but are unaffected at birth and during early childhood. Additionally, the PI phosphatase activity of MTM1 is not required to completely rescue the X-linked CNM phenotype of myotubularin-deficient mice. Our rescue studies in zebrafish, coupled with recently published work in the CNM2 dog model, build on earlier findings that MTM1 is essential for skeletal muscle maintenance rather than for myogenesis by extending this functional role to another protein implicated in human CNM.
BIN1 functions in skeletal muscle and pathomechanisms of CNM

Immunohistochemical and ultrastructural analyses of Bin1-deficient zebrafish skeletal muscle revealed highly abnormal T-tubule morphology and mislocalization of triad proteins, supporting the hypothesis that BIN1 is essential for the formation and organization of these structures. Overall, Bin1 protein levels were greatly reduced in bin1 MO-injected zebrafish embryos, although not completely abolished. Residual Bin1 protein in bin1 morphant muscle fibers appeared to be mildly accumulated around myonuclei. Similar findings were observed on biopsies from patients with BIN1 and MTM1 mutations, and are in accordance with the presence of a nuclear localization sequence in BIN1. Studies performed in cultured cells demonstrate BIN1 to have both nuclear and cytoplasmic subcellular localizations under wild-type conditions, but point towards the primary role of BIN1 in the cytoplasm. However, it is feasible that BIN1 may be concentrated in the nucleus under certain functional or disease states. Our data are consistent with the hypothesis that CNM2 may not be due to an absence of BIN1 protein, but rather to a decreased association of BIN1 with triads.

Triad abnormalities are consistent with the known biochemical function of BIN1 and other members of the BAR protein family, to sense and induce membrane curvature through BAR domain dimerization and the insertion of an amphipathic helix into membrane bilayers. Previous in vivo experiments have demonstrated that mutations in the Drosophila orthologue of mammalian BIN1 result in viable, flightless flies with disorganized triads. Murine Tibialis anterior injected with a U7 small nuclear RNA construct containing an antisense sequence to promote alternative splicing of Bin1 also display T-tubule abnormalities and skeletal muscle weakness. However, the Bin1-deficient zebrafish is the first animal model of CNM2 to provide a direct connection between malformed triads and disruptions in excitation-contraction coupling. Calcium transients are reduced in bin1 morphant embryos compared to wild-type controls in terms of both peak calcium release and visualized signal propagation. Structural modifications to triads leading to disruptions in the transport of ion channels and subsequent intracellular calcium signaling may explain the skeletal muscle weakness and atrophy observed in CNM2.

Membrane remodeling defects very similar to those discovered in Bin1-deficient zebrafish have
been reported in other published CNM animal models and in human patients. Disorganized triads and membranous whorls seen in bin1 morphant embryos are also found in myotubularin-deficient dog, mouse, and zebrafish models, as well as in patients with MTM1 mutations involving protein loss23,29-31,61. In agreement with our results for the dihydropyridine receptor and triadin, abnormal triad markers have also been observed in MTM1- and DNM2-related CNM 23,62. Together, these commonalities suggest that MTM1, DNM2, and BIN1 may act in the same molecular pathway regulating membrane remodeling in skeletal muscle, and indicate a shared pathological mechanism between the different genetic forms of CNM.

2.6 REFERENCES


CHAPTER 3:

THE sepnl KNOCKOUT ZEBRAFISH:

A NOVEL GENETIC MODEL OF SEPN1-RELATED MYOPATHY
3.1 ABSTRACT

SEPN1-related myopathies (SEPN1-RM) are a heterogeneous group of early onset muscular disorders characterized by hypotonia, respiratory insufficiency, and spinal rigidity. SEPN1-RM is caused by mutations in the human gene encoding selenoprotein N (SEPN1). However, SEPN1 function and the mechanisms behind these pathologies remain poorly defined. To understand the role of SEPN1 in skeletal muscle development, TALE nucleases were used to generate germ line mutations in the zebrafish sepn1 gene. Homozygous sepn1 knockout embryos exhibit morphological abnormalities and impaired early motor functions, as evidenced by their diminished spontaneous movements and slow touch-evoked swimming. Moreover, electron micrographs of sepn1 skeletal muscles reveal amorphous cores lacking intact contractile apparatuses that surround enlarged vesicles of the sarcoplasmic reticulum, as well as mitochondria with swollen inner membranes inside the wide gaps between myofibers. These ultrastructural defects, combined with extensive myofibrillar disarray, likely contribute to the reduced contractile forces produced by sepn1 mutants compared to unaffected controls. Importantly, several aspects of the severe sepn1 phenotype can be ameliorated by pharmacological inhibition of ERO1, a thiol oxidase of the sarcoplasmic reticulum. Our studies provide the first in vivo evidence to suggest that reactive oxygen species play a significant role in SEPN1-deficient vertebrate organisms, and may do so by reducing activity of the SERCA calcium re-uptake pump. Functional interactions between SEPN1 and SERCA potentially connect perturbations in redox homeostasis to the skeletal muscle weakness observed in SEPN1-RM via defects in calcium signaling.

3.2 INTRODUCTION

Selenium is trace element required in minute dietary quantities for proper organismal growth, and is covalently attached to proteins in the form of a selenocysteine residue. Selenocysteine, the twenty-first amino acid of the genetic code, is co-translationally inserted into growing peptide chains of selenoproteins in response to a reprogrammed opal stop codon (UGA) within the open reading frame. For the 25 selenoproteins identified in higher eukaryotes, distinction between the UGA of selenocysteine
and that of the traditional termination codon involves complex translation machinery. This discrimination also requires a selenocysteine insertion sequence, which is a cis-acting stem-loop RNA structure located in the 3’ untranslated region of selenoprotein messenger RNA.5,4

Selenocysteine differs from cysteine by only a single atom, yet is substantially more reactive in biological reactions due to the strong acidity of its selenol group.5-7 It is therefore not surprising that most selenoproteins of known function are catalytically active in cellular redox processes and play an essential role in embryogenesis.8-10 Dietary deprivation of selenium has been associated with multiple pathologies, such as viral infection, thyroid dysfunction, and Keshan cardiomyopathy.11-13 Epidemiological studies are also underway to investigate whether polymorphisms in selenoprotein genes might modulate different forms of cancer, cardiovascular disease, and pregnancy pre-eclampsia.14-17

Autosomal recessive mutations in the selenoprotein N gene (SEPNI, MIM #606210) cause a subset of congenital muscle disorders collectively known as the SEPNI-related myopathies (SEPNI-RMs).18 These include rigid spine muscular dystrophy, classic multiminicore disease, desmin-related myopathy with Mallory body-like inclusions, and congenital fiber type disproportion.20 SEPNI-RMs are characterized by a homogeneous, early-onset clinical phenotype, including predominant atrophy and affection of axial muscle groups, with relative sparing of proximal muscles.23 These conditions typically manifest within the first two years of life and present as poor head control.24 Nonetheless, almost all patients achieve motor milestones and independent ambulation at a normal age. During later infancy and childhood, symptoms arise due to the pattern of muscle involvement. Most commonly, these include spinal stiffness due to extensor muscle contractures, progressive scoliosis, and respiratory insufficiency.24 Histopathology among the SEPNI-RMs, however, can show significant variability. For example, myofibrillar disorganization may range from a few altered Z-lines to large amorphous accumulations of Z-line proteins (i.e., Mallory body-like inclusions). Most biopsies also display some degree of hypotrophy, fiber size variation, type I fiber predominance, and/or mild dystrophic signs, such as connective tissue or regenerating fibers. Another characteristic feature is the presence of numerous
small focal regions of sarcomeric disorganization and mitochondrial depletion throughout the muscle fiber known as “minicores” 25-27.

SEPN1 is a glycoprotein localized in the endoplasmic reticulum whose expression peaks early in vertebrate development, from mid-gestation to immediately before birth, and decreases during the transition from fetal to adult tissues 28-30. Although the exact function of SEPN1 in muscle remains unclear, substantial insights have emerged in recent years and are of distinct clinical interest given the muscle-specificity of SEPN1-RM. A role for SEPN1 in calcium ion homeostasis, for example, is suggested by its structural similarity to calcium-binding proteins 19, in addition to its close spatial and functional relationships with calcium-handling proteins. The redox states of the skeletal muscle ryanodine receptor (RyR1) calcium release channel and the Ca$^{2+}$-ATPase (SERCA) calcium re-uptake pump are both regulated by SEPN1 31,32. SEPN1, like many other selenoproteins, is also believed to function in cellular protection against oxidative stress 33. SEPN1-deficient patient myotubes exhibit higher basal oxidative activity than controls, as well as excessive oxidation of actin and myosin heavy chain contractile proteins. Furthermore, patient-derived fibroblasts are significantly more susceptible to hydrogen peroxide-induced damage 34,35. Successful mitigation of this elevated sensitivity by pretreatment with antioxidant indicates that SEPN1 may be involved in buffering reactive oxygen species and/or repairing malfunctional proteins 27,36.

In vivo vertebrate models continue to be tremendously valuable resources for understanding the molecular pathways and pathogenesis of congenital myopathies and muscular dystrophies. For SEPN1-RM, two independent morpholino-based studies have shown that knockdown of the sepn1 gene in zebrafish embryos reproduces most of the phenotypic features observed in human patients, such as skeletal muscle weakness and defects in the organization and attachment of myofibrils 31,37. Sepn1-deficient zebrafish morphants were also used to show that SEPN1 is required for full activity of the RyR1 31. Translation of SEPN1-RM from human to mammalian models has proven less reliable. SEPN1 null mice are largely indistinguishable from their wild-type littermates under normal conditions, and although Sepn1 is abundantly expressed in fetal muscle precursors, loss of murine SEPN1 does not alter
somitogenesis or the expression of myogenic factors\textsuperscript{30}. Only after being subjected to severe physical or oxidative stress do \(\text{Se}pn1^{-/-}\) mice present with atrophy and kyphosis in trunk muscles\textsuperscript{32,38}. Disruptions in satellite cell function have also been reported in \(\text{Se}pn1^{-/-}\) mice, but only in adults 4 to 10 months of age or in younger mice following cardiotoxin injury\textsuperscript{39}.

To better understand the role of SEPN1 in skeletal muscle development within a stable model system suitable for future small molecule screens, germ line mutations were generated in the zebrafish \(sepn1\) gene using TALENs. This chapter describes the characterization of the \(sepn1\) knockout zebrafish, and begins to explore how pharmacological approaches can be used to elucidate the pathomechanisms behind \(SEPN1\)-RM. Our studies on SEPN1 were largely inspired by the Lee and Penny Anderson Family Foundation and members of the Groethe family.

### 3.3 Materials and Methods

**Zebrafish lines and husbandry**

Zebrafish (\textit{Danio rerio}) were bred and maintained under standard conditions\textsuperscript{40}, as detailed in Chapter 2. Wild-type embryos used for TALEN injections were obtained from the Oregon AB line. Embryos were collected by natural spawning, staged by hours (hpf) or days (dpf) post fertilization\textsuperscript{41}, and raised at 28.5°C in egg water. All animal work was performed with approval from the Boston Children’s Hospital Animal Care and Use Committee (14-05-2717R).

**TALEN-mediated knockout of the zebrafish \(sepn1\) gene**

Exon sequences of the zebrafish \(sepn1\) gene (ENSDARG00000033616; NM\_001004294.4) obtained from the Zv9 Zebrafish Genome Assembly were scanned for potential TALEN target sites using the TAL Effector Nucleotide Targeter 2.0 program at https://tale-nt.cac.cornell.edu. Design parameters used for site selection were 1) spacer length (14–18 base pairs), 2) TALE repeat array length (15–21 RVDs), and 3) applying all additional options that restrict target choice. The uniqueness of potential
TALEN target sequences was determined using the TAL Effector Nucleotide Targeter 2.0 program’s Target Finder and Paired Target Finder, as well as a BLAST analysis, ensuring that highly similar left and right binding sites in close proximity to each other did not exist at other regions within the zebrafish genome. The University of Utah Mutation Generation and Detection Core then constructed the TALENs using a modified Golden Gate assembly system. RVD repeat array sequences were cloned into pCS2TAL3-DDD (Addgene, #48637) and pCS2TAL3-RRR (Addgene, #48636) expression vectors in order to generate left and right TALENs, respectively. Following linearization of the TALEN plasmid templates with NotI enzyme, 5’-capped mRNA was generated by in vitro transcription and purified using mMESSAGE mMACHINE SP6 and MEGAclear kits (Ambion). Equal amounts of left and right TALEN mRNAs (50 pg) were co-injected into the cytoplasm of one hundred 1-cell stage wild-type zebrafish embryos.

At 3 dpf, genomic DNA was extracted from 15 larvae via treatment with alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA) for 2 hours at 95°C. PCR was then performed using primers that span the target site of interest: sepn1 forward 5’-TTGCACCATTTCTCTGTGGA-3’ and sepn1 reverse 5’-AAAAAGACAGAGAGGGGGA-3’. Genomic PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), cloned into a TOPO® TA vector (Life Technologies, Carlsbad, CA, USA), and transformed into chemically competent Top10 E. coli bacterial cells (Life Technologies). Sequencing was performed by the Boston Children’s Hospital Molecular Genetics Core Facility using M13 forward and reverse primers. The somatic DNA mutation rate was calculated as the number of mutant sequences divided by the total number of sequences that covered the target region multiplied by 100. All remaining injected embryos were raised to the adult stage as founders and outcrossed with wild-type fish to produce F1 progeny. To screen for germline transmission events at the sepn1 locus, genomic DNA was isolated from F1 fin clips at 6-8 weeks of age, amplified by PCR, and Sanger sequenced. Sexually mature heterozygous adults were mated to obtain clutches containing ~25% homozygous sepn1 embryos.
mRNA rescue

For rescue experiments, full-length human SEPN1 cDNA (Dharmacon, Pittsburgh, PA, USA, #100062356) was cloned into a pCSDest destination vector (Addgene, #22423) using Gateway technology (Invitrogen). mRNA was synthesized in vitro using the mMESSAGE mMACHINE SP6 kit (Ambion). mRNA (50-200 pg) was injected into embryos obtained from heterozygous sepn1 mating pairs at the 1-cell stage, and subsequent phenotypic analyses performed at 3 dpf.

RT-PCR

Total RNA was prepared from zebrafish embryos using RNeasy fibrous tissue mini kits (Qiagen). cDNAs were synthesized from 1 to 2 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers. To detect relative sepn1 transcript levels in control and knockout embryos at 5 dpf, RT-PCR was performed on cDNAs in a Tetrad 2 thermocycler (BioRad). Primer sequences to zebrafish genes sepn1 or ef1α were as follows: sepn1 forward: 5′-TTGCACCATTTCTCTGTGGA-3′; sepn1 reverse: 5′-AAAAAGACAGAGAGGGGGGA-3′; ef1α forward: 5′-TCACCCTGGGAGTGAAACAGC-3′; and ef1α reverse: 5′-ACTTGCAGGCGATGTGAGCAG-3′.

Western blotting

Western blotting was performed on groups of 50-100 wild-type or mutant zebrafish larvae at 5 dpf, as described in Chapter 2. The primary antibody against SEPN1 was custom made by Invitrogen by injecting rabbits with the peptide KEGLRRGLPLLQP, which corresponds to the last 13 amino acids of the human protein. Crude serum from immunized rabbits (7637) was diluted 1:500, and mouse monoclonal anti-β-actin (1:1000, A5441, Sigma) was used as a loading control. HRP-conjugated anti-rabbit (1:2500, 170-6515) and anti-mouse (1:5000, 170-6516) IgG (BioRad) were used as secondary antibodies.
Activity monitoring

Zebrafish behavioral parameters were defined using the Daniovision activity monitoring system (Noldus, Leesburg, VA, USA). In 6-well plates, wild-type and mutant larvae (4 dpf) were placed into the machine with the light box off. After a 10 min acclimation to the dark, larvae were stimulated by light exposure for 20 min. This cycle was repeated three times during the course of a single trial. Swimming behaviors were recorded over the entire 1.5-hour period with an infrared light source. Four independent trials were performed with larvae from four different clutches, examining a total of 12 wild-type and 12 sepn1 larvae. Behavioral parameters recorded include frequency, mean velocity, total distance, and cumulative duration of movement, and reported values reflect the average of all larvae in an experimental group.

Histopathology

For electron microscopy, 6 dpf zebrafish embryos were fixed in formaldehyde-glutaraldehyde-picric acid in cacodylate buffer overnight at 4°C, followed by osmication and uranyl acetate staining. Subsequently, embryos were dehydrated in a series of ethanol washes and embedded in TAAB Epon. Semi-thin sections (0.5 µm) were then cut with a Leica UltraCut microtome and transferred to a drop of water on a glass slide. After drying on a hot plate, sections were stained with 1% toluidine blue and 1% sodium borate in dH2O for viewing under a Nikon E600 light microscope. Next, sections (95 nm) cut with the same microtome were picked up on 100-µm Formvar-coated copper grids and stained with 0.2% lead citrate. Sections were viewed and imaged under a Philips Tecnai BioTwin Spirit electron microscope at the Harvard Medical School Electron Microscopy Core.

Immunofluorescence

Whole-mount staining on wild-type and sepn1 embryos (30 hpf) for activated caspase 3 was performed as described previously with no modifications43. The primary and secondary antibodies used in these studies were rabbit anti-activated caspase 3 (1:500, BDB559565, Fisher Scientific, Waltham,
MA, USA) and Alexa Fluor ® 488 donkey anti-rabbit (1:500, A-21206, Life Technologies), respectively. Whole-mount staining for filamentous actin was performed as described in Chapter 2 with Alexa Fluor ® 488 phalloidin (1:20, A12379, Invitrogen) at 4 dpf. Embryos were mounted in 70% glycerol and visualized using a Perkin Elmer UltraVIEW VoX spinning disk confocal microscope.

Indirect immunofluorescence staining was carried out on frozen sections from 3 dpf zebrafish embryos as described previously 44. Mouse monoclonal anti-dystrophin (1:500, D8043, Sigma) and anti-β-dystroglycan (1:200, NCL-β-DG, Leica Novocastra) primary antibodies were each revealed by Alexa Fluor ® 594 goat anti-mouse secondary antibody (1:100, A-11005, Life Technologies). Stained sections were imaged using a Nikon eclipse 90i microscope.

Evans blue dye staining was performed as described previously 45. Briefly, the dye (Sigma) was injected at 0.1 mg/mL into the pre-cardiac sinus of anesthetized embryos at 3 dpf. Embryos were examined and imaged 4-6 hours later.

**ROS and enzymatic quantification**

To quantify ROS, groups of 30 wild-type or mutant zebrafish larvae (5 dpf) were euthanized in tricaine and homogenized in 100 µl buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM phenylmethylanesulphonyl fluoride, pH 7.4). Following centrifugation at 4°C (10000g, 15 min), 20 µl of supernatant, 100 µl PBS, and 8.3 µl 2',7'-dichlorofluorescin diacetate (DCFDA) stock (10 mg/mL, Sigma) was added to each well. Samples were incubated for 30 min at 37°C, and then fluorescence intensities were read using a Synergy-2 microplate reader (BioTeK, Winooski, VT, USA) with the excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Samples were run in triplicate and values were normalized protein concentrations. Enzymatic assays (Cayman Chemical, Ann Arbor, MI, USA) were used to measure the activities of glutathione reductase and thioredoxin reductase in protein homogenates of groups of 30 wild-type or mutant zebrafish larvae (5 dpf) as per manufacturer’s instructions.
Pharmacological treatment

Clutches obtained from heterozygous sepn1 mating pairs were collected at birth and treated with either dimethyl sulfoxide (DMSO) alone (0.1%) or EN460 in DMSO (0.25-2.5 µg/mL) at 1 dpf. Solutions were changed daily. Sepn1 embryos and unaffected clutchmates were identified by phenotype at 3 dpf. The effect of EN460 on sepn1 mutant morphology, myofibrillar organization, and contractile strength was studied between 3-5 dpf.

Force assays

Experimental set-up. All physiological studies were performed in fish bicarbonate buffer (117.2 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25.2 mM NaHCO₃, 11.1 mM glucose)⁴⁶. Each larva was anesthetized in buffer containing 0.02% tricaine and decapitated. The head was stored and later used for genotyping. The larval body was transferred to a small experimental chamber filled with buffer maintained at 25°C and equilibrated with 95% O₂, 5% CO₂. While submerged in buffer, the proximal end of the preparation was attached to a wire extending from an isometric force transducer (Aurora Scientific, Ontario, Canada, Model 403A) and the distal end to a wire extending perpendicularly from the lever arm of a high-speed position motor (Aurora Scientific, Model 308B). The proximal portion of the preparation was secured at the gastrointestinal opening using an overhand loop of 10-0 monofilament nylon suture, which slid over both the preparation and wire. The distal end of the preparation was secured in a similar manner with the suture tie located several myotomes anterior from the tip of the tail. The distance between suture attachments averaged 1.3 ± 0.1 mm when preparations were at their optimal length.

Measurement of peak force. The preparation was viewed at 450X and sarcomere length adjusted to approximately 2.05-2.15 µm, which is equal to, or slightly shorter than, the 2.15 µm optimal sarcomere length reported for these larval preparations⁴⁶. After a 10-15 min equilibration period, the preparation was tetanized using 9 biphasic, 200 µs square-wave pulses at 300 Hz. The pulse train was delivered via platinum electrodes that flanked the preparation. Output from the motor and transducer were sampled at
1000 Hz using a PC and a multipurpose data acquisition device (National Instruments, Austin, TX, USA, Model PCI-6229). Custom programs written in LabVIEW (National Instruments) controlled the preparation stimulator (Aurora Scientific, Model 701A) and coordinated data acquisition, storage, and analysis. Stimulation current was systematically increased until force plateaued. To minimize any fatigue effects, contractions were separated by a minimum of 60 seconds. Once maximal current was obtained, the length of the preparation was systematically adjusted until force was maximal (i.e., until the preparation was at optimal length). The preparation was then stimulated with a single pulse to elicit a twitch. Twitches were obtained at both positive and negative polarities and the trial with the greatest force was used in analysis.

Caffeine contractions. Twitch force was assessed first in normal buffer and then in buffer containing 1 mM caffeine. Preparations were given 2 min to equilibrate in the caffeine-containing buffer prior to stimulation.

Preparation of cross-sectional area. After all contractile measurements had been completed, the monofilament tie securing the proximal end of the preparation was loosened and the preparation carefully rotated so that images of its widest and narrowest axes could be captured at 40X. Image J (NIH) was used to convert pixels into distance using an internal calibration present on each image. Cross-sectional area was modeled as an ellipse.

Normalization of force responses. Preparations were mounted at a consistent anatomical location, as described above, but even the most skilled investigator may deviate slightly from this location due to the small size of the preparations. Preparations may also vary in size due to biological factors, such as their genotype. To allow for valid comparisons between preparations, force was normalized to the largest anatomical cross-section of the preparation (i.e., the cross-sectional area of the proximal portion of the preparation as described above). This approach does not take into account the complex fiber architecture of fish muscle or the fact that the anatomical cross-section contains non-contractile elements. Due to this lack of information, specific force could not be calculated. Corrected cross-sectional area values are instead referred to as normalized force.
Statistical analysis

GraphPad Prism 7 software (GraphPad Software Inc.) was used to graph all quantitative data and perform statistical analyses. *P* values for pairwise comparisons were determined using a two-tailed Student’s t-test. *P* values for Kaplan-Meier survival curves were calculated using a log-rank test. Multiple comparison tests were calculated using one-way ANOVAs, followed by Tukey’s honestly significant difference (HSD) post hoc tests (alpha = 0.05). Studies were considered significant when *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***). All quantitative data are shown as the mean ± standard deviation.

3.4 Results

Generation of the *sepn1* knockout zebrafish using TALENs

Morpholino (MO)-based loss-of-function studies have offered substantial insights regarding the role of *sepn1* in early zebrafish development. However, the transiency and variability of MO injections precludes using knockdown approaches to faithfully model human genetic disorders, or to screen therapeutic small molecules in a medium- to high-throughput context. Therefore, to stably knockout expression of *sepn1* in zebrafish, exon sequences of the single zebrafish orthologue of human *SEPN1* (*sepn1*; ENSDARG00000033616; NM_001004294.4) were obtained from the Zv9 Zebrafish Genome Assembly and scanned for potential TALEN target sites. The TAL Effector Nucleotide Targeter 2.0 program (https://tale-nt.cac.cornell.edu) identified one potential site in exon 2 of the zebrafish *sepn1* gene, and the corresponding TALEN pair was constructed using a modified Golden Gate assembly method. One hundred wild-type zebrafish embryos were then co-injected with 50-100 pg of left and right TALEN mRNAs at the 1-cell stage. TOPO-cloned PCR products amplified from the genomic DNA of 12 injected embryos revealed the somatic mutation rate of *sepn1* alleles to be 19.0% (4 of 21). All mutated alleles detected were deletions, ranging from 3-7 base pairs in length.

The remaining injected embryos were raised to adulthood as founders. At 3 months of age, 10 mosaic adult founders were out-crossed to wild-type fish in order to examine germline transmission of
mutant sepn1. Among the 10-20 F1 progeny genotyped from each mating, 47.5 ± 7.8 % were found to be heterozygous carriers of a mutated sepn1 allele. The predominant mutation was a two base pair deletion in exon 2 (c.191_192delAG) (Fig. 3.1 A-B). This deletion was predicted to encode a truncated Sepn1 protein with only the first 63 of Sepn1’s 557 total amino acids, in addition to 19 mutated residues, preceding a premature stop codon. This heterozygous sepn1+/− mutant line was grown to sexual maturity and in-crossed to yield homozygous sepn1−/− knockout embryos for further experiments. Homozygous knockouts will henceforth be referred to as sepn1 mutants.

**Figure 3.1. TALEN-mediated knockout of the zebrafish sepn1 gene.** (A) Schematic representation of the genomic structure of the zebrafish sepn1 gene, with its 12 coding exons and untranslated regions depicted as solid and open boxes, respectively. The location of the TALEN target site in exon 2 is indicated in red. Above, the TALEN target sequence is shown in bold, with left and right TALEN
(Figure 3.1, continued) monomer binding sites labeled on either side. (B) Sequence analysis of genomic DNA isolated from wild-type, heterozygous, and homozygous sepn1 mutant embryos at 5 dpf. The sepn1 gene contains a 2 base pair deletion in exon 2 (c.191_192delAG). (C) RT–PCR of sepn1 cDNA indicates a similar amount of sepn1 transcripts in control and mutant embryos at 5 dpf (top). Homozygous sepn1 mutants show a complete absence of Sepn1 protein in comparison to wild-type swimming clutchmates at 5 dpf, as demonstrated by Western blot (bottom). Ef1α and β-Actin were used as controls, respectively. (D) Overexpression of full-length wild-type human SEPN1 mRNA results in a significant decrease of sepn1 mutant embryos in injected clutches at 3 dpf. In non-injected controls, 18.4 ± 2.0 % of all embryos exhibited the sepn1 phenotype, compared to only 7.5 ± 1.0 % in rescued clutches. Results were obtained from six independent clutches and three separate sets of injections. Significance was determined by Student’s t-test, $P = 3.0 \times 10^{-7}$.

**Sepn1 mutants exhibit prominent skeletal muscle and locomotive defects**

TALEN-mediated sepn1 knockout zebrafish exhibit a variety of early morphological abnormalities. External features include small heads and eyes, as well as a distinct dorsal curvature throughout the trunk and tail region (Fig. 3.2 A-B). Internally, sepn1 mutant skeletal muscles display dark patches under polarized light. This non-invasive birefringence readout reliably indicates myofibrillar degeneration and disorganization within the somites. Muscle degeneration appears to be restricted to skeletal muscles, however, as there is no strong evidence of cardiac dysfunction in sepn1 larvae. Mutant heart rates are statistically indistinguishable from wild-type controls through 5 dpf (data not shown). Regarding life span, the majority of all sepn1 homozygotes die by 5 dpf for reasons most likely related to severe skeletal muscle dysfunction, although few can live as long as 7 dpf. Heterozygous larvae, in contrast, are fully viable and seemingly unaffected. Heterozygotes are indistinguishable from their homozygous wild-type clutchmates in terms of morphology, birefringence, and swimming behaviors.

Zebrafish swim in response to tactile stimulation with a frequency of muscle contractions similar to that of an adult zebrafish within the first two days of development. At 3 dpf, sepn1 mutants exhibit
significantly slower swimming than wild-type controls. Swimming phenotypes were first examined using a touch-evoked escape behavior assay and captured by video microscopy. Young wild-type larvae normally respond to mechanosensory stimuli by swimming in a straight line and rapidly exiting the field of view. In contrast, sepn1 larvae twitch and/or swim only a few lengths, but rarely disappear out of the visualized area (WT/HT: 6.2 ± 0.9 cm/0.1 s; sepn1: 0.6 ± 0.3 cm/0.1 s) (Fig. 3.2 C-D). Motor behaviors of 4 dpf wild-type and sepn1 larvae were then quantified using the Noldus Daniovision. Four movement parameters, including frequency, velocity, distance, and cumulative duration, were recorded via an infrared light source for each individual larva over a defined 1.5-hour trial period (Fig. 3.3 A). For each trial, larvae were first placed in the dark and then exposed to light in order to stimulate movement. As expected, sepn1 larvae moved significantly less and at slower speeds than did wild-type controls (Fig. 3.3 B-E). Impaired motor functions are suggestive of an overall skeletal muscle weakness in sepn1 mutants.
Figure 3.2. Abnormal morphology and motor functions of sepn1 mutants. (A) Lateral views of wild-type/heterozygous control and sepn1 mutants during the first week of development using brightfield (left) and polarized light (right) microscopy. Mutants survive 5-7 dpf and exhibit a variety of morphological abnormalities, including small heads and eyes, in addition to dorsal curvature throughout the back and tail. Their “patchy” pattern of birefringence under polarized light suggests extensive myofibrillar disarray and degeneration. Wild-type and heterozygous controls appear bright, indicating normal sarcomeric alignment and organization. (B) Aerial view of several sepn1 mutants at 4-5 dpf. The mutant phenotype is highly reproducible and consistent among the ~25% of knockout embryos obtained from crossing heterozygous sepn1 parents. (C) Average swimming distance of 3 dpf larvae in response to touch (WT/HT: 6.2 ± 0.9 cm/0.1 s; sepn1: 0.6 ± 0.3 cm/0.1 s; P = 7.0 x 10^{-20}). Motor functions were quantified using 15 embryos per group from three independent clutches. Significance was determined by a Student’s
(Figure 3.2, continued) t-test. (D) Mechanosensory stimulation induces wild-type/heterozygous controls to swim away rapidly and fully exit the field of view, whereas sepn1 mutants swim, but slowly and for only short distances within the field of view. Birefringence and touch-evoked escape behavior assays were performed exactly as described in Chapter 2.
Figure 3.3. Diminished spontaneous swimming in sepn1 mutants. (A) Wild-type and sepn1 zebrafish imaged in 6-well plates at 4 dpf using Noldus Daniovision, a high-resolution system that allows for automated analysis of larval locations and orientations. Each well represents a single larva, with the red signal mapping movement. Four independent trials were performed with larvae from four different clutches, two of which are shown. Movement was tracked for 1.5 hours using the light-dark cycle specified. (B-E) Swimming behaviors were quantified in terms of number, mean velocity, total distance, and cumulative duration of movements for 12 wild-type and 12 sepn1 larvae. All larvae were genotype-confirmed. Significance was determined by a Student’s t-test.
Knockout of zebrafish sepn1 is specific and results in the absence of target protein

The severe sepn1 phenotype prompted experimental evaluation of target gene expression at both the RNA and protein levels. Reverse transcription polymerase chain reaction (RT-PCR), used as a fast qualitative measurement of sepn1 transcripts, showed similar band intensities in both mutants and wild-type controls at 5 dpf, indicating that sepn1 mRNA levels are relatively constant between groups (Fig. 3.1 C, top). Quantitative real-time RT-PCR using fluorescently tagged Taqman probes later confirmed any slight differences in sepn1 gene expression as statistically insignificant (data not shown). Expression of Sepn1 protein, however, was virtually undetectable in knockout zebrafish at 5 dpf, whereas Western blot analysis revealed a protein product of expected size in wild-type clutchmates (Fig. 3.1 C, bottom).

Finally, to validate that the sepn1 skeletal muscle phenotype is a true consequence of Sepn1 deficiency, rescue studies were performed. Embryos obtained from crossing two heterozygous adults were injected with full-length wild-type human SEPN1 mRNA at the 1-cell stage. The mutant phenotype was assigned to a larva if it exhibited a small head and eyes, pronounced dorsal curvature, reduced birefringence, and/or diminished touch-evoked swimming at 3 dpf, while designation of a wild-type phenotype was made in the absence of all of these features. In non-injected clutches, 18.4 ± 2.0 % of embryos were consistently mutant, comparable to the 25% that would be expected according to traditional Mendelian ratios (Fig. 3.1 D). In contrast, embryos injected with SEPN1 mRNA showed a significant reduction in the percentage of mutants to only 7.5 ± 1.0 %. Rescue of the mutant phenotype upon overexpression of SEPN1 is strong evidence for specificity of the gene knockout. Moreover, the ability to rescue mutant zebrafish with the human gene demonstrates that SEPN1 is genetically and functionally conserved between species.

Sepn1 deficiency in zebrafish causes histological and ultrastructural abnormalities

To better understand the pathological progression of Sepn1 deficiency, toluidine-blue stained longitudinal and transverse sections of wild-type and sepn1 skeletal muscles were examined at 6 dpf. All knockout larvae exhibit gross alterations in myotome architecture. Notably, somites in sepn1 zebrafish
lack well-defined boundaries and are rounded, as compared to the chevron shape of wild-type somites (Fig. 3.4 A). Mutant muscles also contain interlacing and/or detached muscle fibers, in contrast to wild-type fibers that extend the entire length of the somite. In terms of nuclear organization, sepn1 myonuclei larvae are rounded, grouped, and often mislocalized to the center of myofibers (Fig. 3.4 B). These strongly differ from the elongated, singular, and peripheral nuclei typical of wild-type muscles.
Figure 3.4. Skeletal muscle histology shows nuclear mislocalization in sepn1 mutants. (A)

Longitudinal sections of wild-type and sepn1 skeletal muscles stained with toluidine blue at 6 dpf show sepn1 myonuclei are grouped and round in shape, compared to the singular, elongated myonuclei found in
(Figure 3.4, continued) wild-type controls (orange arrows). Additionally, mutant myosepta do not stain as darkly as their wild-type equivalents (green arrowheads). (B) Mislocalization and centralization of sepn1 mutant myonuclei is most apparent in transverse sections. Normal, wild-type myonuclei are typically found at the fiber periphery (white arrowheads). Histological sectioning and staining was performed as described in Chapter 2. Scale bars: (A) 10 µm, (B) 4 µm.

The staining discrepancies described between wild-type and sepn1 somite boundaries were particularly interesting, since several proteins mutated in different forms of congenital muscular dystrophy are localized to these regions (e.g., laminin α2, α-dystroglycan). To investigate whether the absence of Sepn1 affects protein stability at the peripheral ends of myofibers, immunofluorescence studies were performed on wild-type and sepn1 mutant larvae at 3 dpf (Fig. 3.5 A-D). No differences in expression levels or localization of membrane proteins, including dystrophin and β-dystroglycan, were detected between groups. Evans blue dye was also used as in vivo confirmation that Sepn1 deletion does not disrupt integrity of the sarcolemma (Fig. 3.5 E-F). This intravital stain labels cells with compromised plasma membranes. No uptake of dye was observed in either wild-type or sepn1 mutant somites following injection of the dye into the pre-cardiac sinus of 3 dpf embryos.
Figure 3.5. Integrity of myosepta and the sarcolemma are preserved in Sepn1 deficiency.

Localizations of dystrophin-glycoprotein complex components are preserved in sepn1 mutants. Lateral views of 3 dpf larvae labeled with antibodies directed against (A-B) dystrophin and (C-D) β-dystroglycan. (E-F) Myofiber permeability was evaluated in vivo by the injection of Evans blue dye in the pre-cardiac sinus of wild-type and sepn1 mutant embryos at 3 dpf. No significant uptake of dye was observed in muscle cells of either group, suggesting that the sarcolemma remains relatively intact in Sepn1 deficiency.

Scale bars: 10 µm.
The most striking differences between fully differentiated wild-type and sepn1 skeletal muscles were detected at the ultrastructural level. Transmission electron micrographs at 6 dpf revealed enlarged vesicles of the sarcoplasmic reticulum (SR) in sepn1 mutants, in addition to irregularly wide gaps between myofibers (Fig. 3.6 A-D). These gaps almost always contained malformed mitochondria with dilated inner membranes. Cores, defined as areas of mitochondrial depletion in mammalian skeletal muscles, are a prominent histological feature of SEPN1-RM. In zebrafish, the term “core” has been used to describe amorphous areas in muscle fibers that lack intact contractile apparatuses. Bundles of actin and myosin were regularly missing around the SR and from significant portions of whole myofibers in sepn1 mutants (Fig. 3.6 E-F). Since these areas also lack mitochondria and other organelles, it is likely that they are the zebrafish equivalent of human cores and do not exhibit mitochondrial activity.
Figure 3.6. Loss of Sepn1 causes ultrastructural defects in skeletal muscle. Transmission electron micrographs of zebrafish skeletal muscle at 6 dpf. (A-B) Longitudinal sections show enlarged
(Figure 3.6, continued) vesicles of the SR in sepn1 mutants compared to wild-type controls (orange arrows). (C-D) Transverse sections of skeletal muscle distinguish the well-organized sarcolemmal boundaries of wild-type myofibers from the irregular, wide gaps containing malformed mitochondria found between adjacent sepn1 myofibers (yellow arrows). (E-F) Importantly, sepn1 skeletal muscle contains large, amorphous cores lacking intact contractile apparatuses, a prominent histological feature in human SEPN1-RM. Smaller core regions surround the SR (green arrows). Electron microscopy was performed as described in Chapter 2. Scale bars: (A-B) 500 nm, (C-D) 2 µm, (E-F) 200 nm.

**Reduced contractile strength of sepn1 mutants is a primary defect of skeletal muscle**

Muscular lesions observed in sepn1 zebrafish, such as cores and disruptions in sarcomeric organization, suggested that contractile forces are weaker in mutant than in wild-type skeletal muscles. Weaker contractions by trunk and tail muscles would also explain the diminished locomotive behaviors of sepn1 mutants. Therefore, to quantify contractile strength in embryonic zebrafish, peak twitch and tetanic forces of individual preparations were measured following electrical stimulation. Force was then normalized to the largest anatomical cross-section of the preparation in order to allow for valid comparisons between embryos. As anticipated, peak twitch as well as peak tetanic force per cross sectional area were significantly decreased in sepn1 mutants compared to wild-type controls (Fig. 3.7 A-C, F). These data trends were consistent across all ages tested (data not shown). Furthermore, because larvae were decapitated prior to analysis, potential contributions of the central nervous system towards the sepn1 phenotype and defects at the neuromuscular junction could both be eliminated. Thus, sepn1 mutants with prominent skeletal muscle weakness arise from a primary defect within this tissue.
Figure 3.7. Contractile forces are reduced in sepn1 mutant zebrafish. (A) Sepn1 larvae stimulated with a single supramaximal (500 µs) square wave pulse display weaker twitch force per cross-sectional area (CSA) than wild-type swimming clutchmates from 3-5 dpf (WT: 50.4 ± 14.3 kPa, n = 6; sepn1: 26.8 ± 5.3 kPa, n = 6; P = 0.003). (B) Sepn1 mutants also show diminished tetanic force per CSA, following stimulation with 9 biphasic, 200 µs square-wave pulses at 300 Hz (WT: 59.0 ± 15.0 kPa, n = 6; sepn1: 31.1 ± 7.1 kPa, n = 6; P = 0.005). (C) Representative twitch peaks from wild-type and sepn1 mutant larvae. (D-E) Twitch peaks from wild-type and sepn1 mutant larvae with (gray) and without (black) caffeine stimulation. Twitch force increases due to caffeine were comparable between the two experimental groups, suggesting that calcium release is not impaired in Sepn1 deficiency. (F) Quantification of A and B (WT, n = 6; sepn1, n = 5-6). (G) Quantification of D and E (WT, n = 2; sepn1, n = 3). Abbreviations: milliNewtons (mN); kilopascals (kPa).
**Sepn1-deficient zebrafish show increased markers of oxidative stress and apoptosis**

Myoblasts and fibroblasts obtained from human SEPN1-RM patients exhibit increased levels of reactive oxygen species (ROS), suggesting a role for SEPN1 in maintaining the redox status of skeletal muscles. To evaluate oxidative stress resulting from Sepn1 deficiency *in vivo*, DCFDA fluorescence-based plate assays were used to quantify levels of intracellular reactive oxygen species in whole zebrafish embryos at 5 dpf. Similar to studies conducted *in vitro*, fluorescence emission in *sepn1* mutants was significantly increased to 2.70-fold the levels measured in wild-type controls (Fig. 3.8 A). Activities of enzymes involved in the detoxification of ROS, including glutathione reductase and thioredoxin reductase, were also reduced in *sepn1* mutants (Fig. 3.8 B-C). Finally, because ROS contribute to the induction of apoptosis under both physiological and pathological conditions, whole-mount staining with activated caspase-3 antibody was performed. *Sepl* mutant skeletal muscles showed significantly increased levels of this reliable apoptotic marker relative to wild-type clutchmates at 30 hpf (Fig. 3.8 D-H). Together, these data indicate that the absence of Sepn1 is associated with measurably increased oxidative stress and cell death in zebrafish tissues.
Figure 3.8. Sepn1 deficiency results in increased oxidative stress and apoptotic markers. (A) ROS measured in pools of 30 wild-type and sepn1 larvae at 5 dpf, using DCFDA as a ROS indicator. Average fluorescence intensities were normalized to wild-type controls (WT/HT: 1.00 ± 0.06 RFU; sepn1: 2.70 ± 0.15 RFU; $P = 5.0 \times 10^{-5}$). (B-C) Important redox enzymes, including thioredoxin reductase (TrxR) and
(Figure 3.8, continued) glutathione reductase (GR), are dysregulated and show reduced enzymatic activities in sepn1 mutants (GR – WT/HT: 52.4 ± 4.6 nmol/min/mL; sepn1: 17.4 ± 0.9 nmol/min/mL; P = 0.0002) (TrxR – WT/HT: 0.047 ± 0.016 µmol/min/mL; sepn1: 0.011 ± 0.009 µmol/min/mL; P = 0.02).

(D-G) Activated caspase 3 staining in representative wild-type and sepn1 embryos at 30 hpf. All stained larvae were genotype-confirmed. (H) Quantification of caspase 3 staining demonstrates that more apoptotic nuclei are present in sepn1 embryos compared to wild-type controls. Regions of interest (ROIs) with identical areas were drawn around the trunk somites of 10 embryos per experimental group using Image J (yellow box in E). Average fluorescence intensities were then normalized to wild-type controls in relative fluorescence units (RFU) (WT/HT: 1.00 ± 0.34 RFU; sepn1: 2.86 ± 0.53 RFU; P = 0.007). Significance was determined by a Student’s t-test.

**SEPN1-RM pathology may result from oxidative dysregulation of calcium re-uptake channels**

Calcium homeostasis is a crucial function in skeletal muscle and relies closely on the redox status of calcium-handling proteins. Among SR calcium-handling proteins, SEPN1 has been shown to physically and functionally interact with both RyR1 and SERCA. To first investigate whether Sepn1 deficiency impairs RyR1-mediated calcium release, electrophysiological studies were conducted in the presence of caffeine, an established RyR1 agonist. Interestingly, force increases due to caffeine were comparable between the two experimental groups. These data contradicted the hypothesis that Sepn1-deficient zebrafish would exhibit increased sensitivity to caffeine due to a higher ratio of defective to normal RyR1 channels, and suggested that Sepn1 might have an alternative function in vivo (Fig. 3.7 D-E, G).

The slow-twitch muscle isoform of SERCA, SERCA2, is a redox partner of SEPN1. SEPN1 enhances SERCA2 activity by reducing luminal cysteines that are normally oxidized by a thiol oxidase known as endoplasmic reticulum oxidoreductin 1 (ERO1). ERO1 is an enzyme conserved among eukaryotes and uses flavin adenine dinucleotide (FAD) as an electron acceptor to promote the formation of catalytic intramolecular disulfide bonds, which are then transferred to protein substrates via protein...
disulfide isomerase. As reduced FAD (FADH₂) is re-oxidized upon reaction with molecular oxygen (O₂), ERO1 activity results in a stoichiometric production of hydrogen peroxide (H₂O₂)⁵⁴⁻⁵⁷. Cells lacking SEPN1 are hypersensitive to ERO1 overexpression and exhibit defects in SERCA2-mediated calcium re-uptake due to the loss of SEPN1’s redox activity.⁵² To test whether mild ERO1 inhibition might improve the sepn1 mutant phenotype in vivo, sepn1 mutant embryos were treated with EN460, a commercially available ERO1 inhibitor, from 1 to 4 dpf. At 4 dpf, whole-mount phalloidin staining was used to measure efficacy of the compound in a dose-dependent manner. Skeletal muscles of sepn1 larvae treated with 2.5 µg/mL EN460 exhibited significantly fewer disorganized somites and detached myofibers than sepn1 larvae treated with 0.25 µg/mL EN460 or DMSO only (Fig. 3.9 A-B). At the 2.5 µg/mL dose, birefringence and spontaneous swimming behaviors of EN460-treated sepn1 mutants were also restored to wild-type levels (Fig. 3.10 A-B), although mutant life spans remained unaffected by treatment (Fig. 3.11).
Figure 3.9. EN460 reduces sepn1 myofibrillar disorganization in a dose-dependent manner. (A)

Whole-mount phalloidin staining of filamentous actin in 4 dpf zebrafish. The ratio of normal to abnormal somites is significantly increased in sepn1 mutants treated with 2.5 µg/mL EN460, compared to mutants treated with DMSO or a lower dose of the same compound (0.25 µg/mL). Somites containing detached myofibers (arrows) were considered abnormal. Higher concentrations of EN460 were also tested (up to 10 µg/mL), but were found to be lethal to all embryos. This lethality likely resulted from decimating ERO1’s key role in the unfolded protein response, an adaptation of the ER/SR to cope with the accumulation of misfolded proteins. (B) Quantification of myofibrillar organization imaged in A. Fourteen somites in individual larvae were assessed at 4 dpf, and quantified in terms of abnormal somites divided by total somites multiplied by 100 (WT-DMSO/0.25 µg/mL EN460/2.5 µg/mL EN460: 100.0 ± 0.0 %, n = 3;
(Figure 3.9, continued) *sepn1*-DMSO: 2.4 ± 2.4 %, *n* = 3; *sepn1*-0.25 µg/mL EN460: 32.1 ± 3.6 %, *n* = 3; *sepn1*-2.5 µg/mL EN460: 92.9 ± 4.5 %, *n* = 5). Significance was determined by a one-way ANOVA (*F* = 108.1, *P* < 0.0001), followed by pairwise Tukey’s HSD post hoc tests.

Figure 3.10. ERO1 inhibition restores *sepn1* birefringence and motor behaviors. (A) The reduced and “patchy” birefringence characteristic of *sepn1* zebrafish skeletal muscles is restored to bright wild-type levels by 4 dpf after beginning treatment with EN460 at 1 dpf. (B) Swimming behaviors quantified by the Noldus Daniovision, such as distance traveled during a single trial period, is also improved in EN460-treated *sepn1* mutants at 4 dpf (WT-DMSO: 1985.9 ± 101.8 mm, *n* = 4; WT-EN460: 2058 ± 143.9 mm, *n* = 6; *sepn1*-DMSO: 765.7 ± 43.0 mm, *n* = 8; *sepn1*-EN460: 1960.6 ± 16.0, *n* = 6). All larvae were genotype-confirmed. Significance was determined by a one-way ANOVA (*F* = 46.21, *P* < 0.0001), followed by pairwise Tukey’s HSD post hoc tests.
Figure 3.11. Kaplan-Meier survival of \textit{sepn1} mutants are unaffected by ERO1 inhibition.

Homozygous \textit{sepn1} zebrafish survive to 5-7 dpf with or without 2.5 \textmu{}g/mL EN460 treatment. A log-rank test verified that no significant differences existed between the two experimental groups ($P > 0.5$).

The thiol redox state of ER proteins, such as SERCA2, are crucial for calcium homeostasis. Operating on the hypothesis that SEPN1 deficiency leads to ERO1-mediated hyperoxidation of SERCA2, we next examined whether ERO1 inhibition affects skeletal muscle contraction and relaxation \textit{in vivo}. Electrophysiological studies were repeated in 4 dpf wild-type and \textit{sepn1} mutant larvae after 3 days of EN460 treatment. Notably, mild ERO1 inhibition influences how \textit{sepn1} zebrafish relax from tetanus. Whereas \textit{sepn1} mutant muscles do not typically reach a full resting state following tetanus, EN460 treatment corrects this abnormality without altering other aspects of muscle force, such as peak tetanic force or rate of relaxation (Fig. 3.12 A-C).
Figure 3.12. *In vivo* force assays suggest EN460 may affect skeletal muscle relaxation in *sepn1* mutants. (A) Tetanic force measurements from individual zebrafish preparations at 4 dpf following electrical stimulation and normalized for cross-sectional area. *Sepl* mutants consistently display reduced rates of tetanic force decline compared to wild-type controls. Mutant skeletal muscles also do not appear to return to a full resting state as quickly. (B) EN460 treatment does not significantly increase the overall rate of tetanic force decline in *sepn1* mutants (WT-DMSO: $-113.8 \pm 13.7 \text{ mN} \cdot \text{µm}^2/\text{s}$, $n = 3$; WT-EN460: $-127.6 \pm 5.3 \text{ mN} \cdot \text{µm}^2/\text{s}$, $n = 5$; *sepn1*-DMSO: $-15.0 \pm 1.6 \text{ mN} \cdot \text{µm}^2/\text{s}$, $n = 2$; *sepn1*-EN460: $-30.1 \pm 5.9 \text{ mN} \cdot \text{µm}^2/\text{s}$, $n = 4$; $F = 66.33$, $P < 0.0001$). (C) However, EN460-treated *sepn1* skeletal muscles do return to a resting state comparable to wild-type by 0.20 seconds post-stimulation. This recovery is not
(Figure 3.12, continued) observed in sepn1 mutants treated with DMSO (WT-DMSO: 1.54 ± 0.80 kPa, n = 3; WT-EN460: 2.23 ± 1.25 kPa, n = 5; sepn1-DMSO: 10.34 ± 2.92 kPa, n = 2; sepn1-EN460: 3.04 ± 0.36 kPa, n = 4; F = 7.10, P = 0.008). Significance for B and C was determined by a one-way ANOVA, followed by pairwise Tukey’s HSD post hoc tests.

It has been suggested that skeletal muscle cores form as a secondary cellular response to isolate regions of defective calcium regulation from regions of normal calcium homeostasis. To evaluate the effect of EN460 treatment on cores, electron microscopy was performed on transverse sections of sepn1 skeletal muscles 5 days following administration of the compound (Fig. 3.13 A-F). Cores in 6 dpf sepn1 zebrafish treated with EN460 were either completely absent (similar to wild-type), or did not exceed 50 nm in diameter, while cores in untreated larvae averaged between 50-500 nm in diameter. The ERO1 inhibitor also substantially improved mitochondrial morphologies, consistent with the idea that ERO1 increases SR stress levels by burdening the cell with potentially toxic ROS. Together, these data serve as the first in vivo evidence to suggest that ERO1 inhibition may effectively treat the skeletal muscle pathology seen in SEPN1 deficiency, and support calcium re-uptake as a potential pathomechanism of SEPN1-RM.
**Figure 3.13. EN460 treatment corrects sepn1 skeletal muscle ultrastructure.** Transverse transmission electron micrographs of zebrafish skeletal muscle at 6 dpf. (A-B) Wild-type controls show healthy bundles of actin and myosin in areas immediately adjacent to the SR (yellow arrow), as well as normal mitochondria (orange arrow). (C-D) In contrast, intact contractile apparatuses are often absent around the SR in sepn1 mutants (yellow arrow). These cores are typically 50-500 nm in diameter at this stage in sepn1 development. Sepn1 muscles also contain abnormal mitochondria with swollen inner membranes, reminiscent of mitochondrial permeability transition that has previously been reported in muscular dystrophies (orange arrow) 59. (E-F) Although EN460 does not correct all aspects of sepn1 ultrastructure, such as large disorganized spaces between myofibers, ERO1 inhibition restores mitochondrial morphologies (orange arrow). EN460 also reduces the number and diameter of amorphous cores in mutant skeletal muscles (yellow arrow). Scale bars: 500 nm.
3.5 DISCUSSION

**Sepn1 knockout zebrafish are the first reliable vertebrate model of human SEPN1-RM**

Four early-onset skeletal muscle disorders, collectively known as the SEPN1-related myopathies (SEPN1-RMs), arise from loss-of-function mutations in the human SEPN1 gene. Pathological SEPN1 mutations consist of frameshift micro-deletions or insertions, splice site alterations, or single nucleotide missense changes centered around the selenol catalytic site. All of these genetic defects lead to a drastic reduction of SEPN1 protein levels, and present similarly in clinic. SEPN1-RM phenotypes involve generalized hypotonia with predominant impairment of axial muscles, spinal rigidity and scoliosis, as well as life-threatening respiratory difficulties. The mechanisms behind these pathologies, however, remain poorly understood as the exact functions of SEPN1 are still being defined. One hypothesis is that SEPN1 acts as a reductase in the ER lumen and redox-regulates the SERCA2 calcium pump, thereby enhancing ER calcium re-uptake, although much of the supportive evidence has been conducted in vitro. Studying SEPN1 function has proven more difficult in vivo due to a present lack of reliable vertebrate models. The Sepn1−/− mouse is the best example, since it does not exhibit an overt muscle phenotype under normal conditions. SEPN1-deficient mice only develop select features of the human disease, such as skeletal muscle cores, after being subjected to considerable physical or oxidative stress. MO-based zebrafish models of SEPN1-RM have also been created. Zebrafish Sepn1 protein shares 65% amino acid identity and 76% sequence similarity with the human orthologue, with the entire 31 amino acid block containing the catalytic selenocysteine conserved between humans and zebrafish. While these knockdowns phenocopy most hallmarks of SEPN1-RM, the transiency and variability of MOs preclude this model from being used to investigate suitable therapeutic approaches in a high-throughput context.

The sepn1 knockout zebrafish serves as the first true vertebrate model of SEPN1-RM, by accurately reproducing both the genetic and clinical aspects of the human disorder. TALEN-generated sepn1 mutants homozygous for a two base pair deletion in exon 2 of the zebrafish gene survive 5-7 dpf and have Sepn1 protein levels undetectable by Western blot. Importantly, sepn1 knockout embryos
exhibit morphological abnormalities and impaired early motor functions, evidenced by slow touch-evoked swimming and reduced spontaneous movements quantified in an automated activity monitor. Myofibrillar disorganization and the presence of detached muscle fibers by 3 dpf was confirmed both by non-invasive birefringence assay under polarized light, as well as by whole-mount phalloidin staining for filamentous actin. With regard to ultrastructure, electron micrographs of sepn1 mutant skeletal muscle at 6 dpf show amorphous cores lacking intact contractile apparatuses that surround enlarged vesicles of the SR. Additionally, malformed mitochondria, potentially undergoing mitochondrial permeability transition (MPT), are found in wide gaps between myofibers. These frequently contain swollen inner membranes and inflated matrices, reflective of the heavy oxidative stress placed on muscle cells in SEPN1-deficient conditions. Furthermore, induction of MPT can lead to cell death via apoptosis or necrosis depending on the particular biological setting. Sepn1 mutant skeletal muscle demonstrates significantly increased levels of apoptosis relative to wild-type controls. Such pronounced skeletal muscle defects likely contribute to the reduced twitch and tetanic contractile forces generated by sepn1 mutants following electrical stimulation.

**Pathomechanism of SEPN1-RM may arise from disruptions in calcium re-uptake**

SEPN1’s role in the regulation of calcium signaling within skeletal muscle was brought to light when SEPN1 was found to co-precipitate the ryanodine receptor calcium release channel and serve as a modifier of its activity *in vivo* 37. More recently, studies have expanded the list of proteins that functionally interact with and are redox-regulated by SEPN1 to include the calcium import SERCA2 pump 32. In skeletal muscle, SERCA pumps are majorly responsible for calcium re-uptake into the SR during excitation contraction coupling. Deficits in SERCA activity have been shown to result in dystrophic muscle, cellular necrosis through calpaine activation, as well as mitochondrial swelling 59,61-63. The observation that Sepn1-deficient zebrafish mirror many of these same traits, including “patchy” birefringence due to detached and disorganized myofibers, higher numbers of apoptotic myonuclei, and abnormal mitochondria, suggests that SEPN1 and SERCA2 are physiologically linked.
Endoplasmic reticulum oxidoreductin (ERO1) is the main protein disulfide oxidase that channels electrons from protein disulfide isomerase to molecular oxygen, the terminal electron acceptor, and generates hydrogen peroxide. It has therefore been proposed that excessive ROS produced by ERO1 oxidize luminal thiols of the SERCA2 pump \(^{32}\). In SEPN1 deficiency, the lack of SEPN1 reductase activity traps these thiols in a hyperoxidized state and inhibits SERCA2, disrupting calcium homeostasis. This tie between SEPN1 and ERO1 provided rationale for testing an ERO1 inhibitor \textit{in vivo}. As anticipated, attenuation of ERO1 activity in \textit{sepn1} knockout zebrafish protects against some of their most severe phenotypic features. \textit{Sepn1} motor behaviors and birefringence are restored to wild-type levels, in addition to improvements in their skeletal muscle ultrastructure, such as corrected mitochondrial morphologies and fewer large cores. Most importantly, mild ERO1 inhibition with EN460 allows \textit{sepn1} skeletal muscles to fully relax following tetanus, whereas untreated mutants often remain in a semi-contracted state. Together, these data are consistent with the hypothesis that SEPN1 has a crucial role in maintaining calcium homeostasis \textit{in vivo}, and may assist via redox-regulation in SERCA-mediated calcium re-uptake. ERO1 inhibition is certainly a therapeutic strategy for \textit{SEPN1}-RM to be explored in future studies.

3.6 References


CHAPTER 4:

SMALL MOLECULE SCREENING IN RYANODINE RECEPTOR MUTANT ZEBRAFISH
FOR THERAPEUTIC DEVELOPMENT IN CORE MYOPATHIES
4.1 Abstract

Ryanodine receptor 1 (RYR1) mutations are a common cause of congenital myopathies with both dominant and recessive inheritance in humans. Mutations in RYR1 have been described in patients with central core disease, congenital fiber type disproportion, as well as with multiminicore, centronuclear, core-rod, and nemaline myopathies. RYR1 mutations are also associated with malignant hyperthermia susceptibility and with leaky calcium release channels in certain forms of muscular dystrophy. Although RYR1 mutations are the underlying cause of a number of genetic diseases, no specific and effective therapies are currently available. We therefore performed a two-tiered chemical screen aimed at identifying lead FDA-approved compounds that can rescue the function of skeletal muscle in the relatively relaxed zebrafish model of ryr1b mutation. In our primary screen, pools of four chemicals from the Prestwick2 chemical library (280 pools, 1120 chemicals) were scored for their ability to improve survival and postnatal motility of ryr1b homozygous mutant fry. Individual chemicals from hit pools were further evaluated in a secondary screen, and 12 candidate compounds were identified that could ameliorate the relatively relaxed muscle phenotype. One chemical, nifuroxazide, which is known to inhibit JAK-STAT cytokine signaling, restores the locomotive activities and corrects mild morphological abnormalities observed in ryr1b mutants. The identification of chemicals, especially JAK-STAT inhibitors and anti-oxidizing agents previously described to treat mouse models of muscular dystrophy, validates our in vivo screening protocol and offers valuable mechanistic insights for the development of targeted therapies in RYR1-related disorders.

4.2 Introduction

Human cells express three distinct isoforms of the ryanodine receptor, each encoded by a different gene: RyR1, RyR2, and RyR3. RyR1 and RyR2 are predominantly expressed in skeletal and cardiac muscles, respectively, whereas RyR3 has widespread expression during development but is present at relatively low levels. The skeletal muscle RYR1 gene encodes a large, homotetrameric transmembrane ion channel that serves as one of the major intracellular calcium channels in this tissue.
First identified in the 1980s and named for its ability to bind the plant alkaloid ryanodine, RyR1 resides on the terminal sarcoplasmic reticulum (SR) and exists in close proximity with the T-tubule. Its functional role is to mediate excitation-contraction coupling, by releasing calcium from the SR into the cytosol in response to motor neuron stimulation at the neuromuscular junction.

Causative mutations in RYR1 are associated with a wide range of pathologies. Inherited as a dominant trait, RYR1 mutations often give rise to malignant hyperthermia (MH) and/or central core disease (CCD). MH is a pharmacogenetic condition manifesting as muscle rigidity and a dramatic rise in body temperature upon exposure to certain anesthetics or environmental conditions, whereas CCD is characterized by infantile hypotonia and weakness affecting proximal muscles. Pathologically, the cardinal diagnostic feature of patients with CCD is the presence of “central cores” running along the long axis of myofibers that are devoid of mitochondria and deficient in oxidative enzymes, phosphorylase activity, and glycogen. Only occasionally will autosomal recessive RYR1 mutations yield CCD pathology. More frequently, recessive mutations will lead to alternative findings on muscle biopsy, including characteristic features of multiminicore disease (MmD), centronuclear and core-rod myopathies, congenital fiber type disproportion, and muscular dystrophy.

The molecular mechanisms underlying RYR1-related disorders are not yet precisely defined, although trends regarding some genotype-phenotype correlations are emerging. Vis-à-vis the functional effects of dominant RYR1 mutations, MH is believed to arise from “hyperactive” RyR1 channels that show increased sensitivity to RyR1 agonists in vitro. Caffeine and halothane both activate RyR1 channels carrying MH mutations at lower concentrations than those required to stimulate Ca\(^{2+}\) release in normal channels. RyR1 dysfunction in CCD is debated between two models: increased cytosolic calcium levels and depletion of SR calcium stores (“leaky channel” hypothesis) and disruptions in excitation-contraction coupling (“uncoupling” hypothesis). In vitro studies have demonstrated reduced levels of stimulated calcium release consistent with both hypotheses. The functional consequences of recessive RYR1 mutations, however, have only begun to be elucidated, though poorly functioning and reduced numbers of RyR1 channels are a common finding. Protein levels of RyR1 and the physically
coupled dihydropyridine receptor (DHPR) are both reduced in autosomal recessive RYRI patient muscles. Localization of the DHPR alpha subunit is also perturbed in cultured myotubes treated with RYRI-targeting siRNAs. These two alterations result in impaired excitation-contraction coupling. Recessive RYRI mutations have also been tied to changes in cellular secretion of interleukin. This cytokine’s involvement in a variety of biological events across many tissues and cell types highlights the importance of identifying new molecular pathways that might be involved in RYRI disease pathogenesis.

Several vertebrate models have been created to help define the histopathological course of various RYRI-related disorders. Heterozygous mice carrying the p.R163C and p.Y522S mutations undergo full MH episodes when exposed to volatile anesthetics or heat. Their skeletal muscles display caffeine- and heat-induced contractures in vitro, as well as increased calcium release from leaky RyR1 channels under conditions of oxidative stress. Another established murine model is the heterozygous line carrying the p.I4895T uncoupling mutation at the C-terminus of RYRI. These mice exhibit a slowly progressive myopathy and age-dependent formation of cores and nemaline rods in skeletal muscles. There are currently no working mouse models of recessive RYRI-related myopathies, since homozygous Ryr1−/− mice die perinatally with skeletal muscles unresponsive to electrical stimulation. Fortuitously, a spontaneous zebrafish mutant that closely mimics human MmD has opened new doors for the study of these diseases in vivo.

The relatively relaxed (ryr1b) zebrafish mutant is homozygous for a recessive nonsense mutation in the ryr1b gene, which results in reduced levels of Ryr1 protein. Whereas wild-type zebrafish larvae swim away in response to tactile stimulation, ryr1b mutants swim slowly due to weak muscle contractions despite normal output from the central nervous system. Ryr1b mutants exhibit small amorphous cores in myofibers, similar to both human MmD patients and our sepn1 zebrafish model described in the previous chapter. Furthermore, Ca2+ transients in ryr1b mutants are also drastically reduced, consistent with the prevailing hypothesis that RYRI-related disorders are chiefly due to impairments in excitation-contraction coupling.
Following initial characterization of the *ryr1b* zebrafish, comparative microarray expression analysis was performed on RNA isolated from mutants and wild-type clutchmates in order to identify novel pathogenic pathways associated with the loss of Ryr1 function. Several of the mis-expressed transcripts were involved in redox and cellular homeostasis. This finding was particularly noteworthy since the redox regulation of RyR1’s cytosolic thiol groups is known to affect the channel’s gating properties, its opening in response to ions and caffeine, as well as its ability to bind calstabin and calmodulin (two proteins that help dictate RyR1-mediated Ca\(^{2+}\) release in skeletal muscle). A candidate compound approach confirmed a role for oxidative stress in these disorders when the antioxidant N-acetylcysteine was shown to ameliorate select aspects of the *ryr1b* phenotype, including major histopathological abnormalities. In an attempt to better understand *RYR1*-related pathophysiology and elucidate new molecular pathways that might contribute to disease phenotypes, we performed a chemical screen aimed at identifying lead compounds that can rescue the function of skeletal muscle in the *relatively relaxed* zebrafish.

### 4.3 Materials and Methods

**Zebrafish lines and husbandry**

Zebrafish (*Danio rerio*) were bred and maintained under standard conditions, as detailed in Chapter 2. The *relatively relaxed* (abbreviated *ryr1b*) line was acquired from John Kuwada at the University of Michigan after its full characterization as a zebrafish model of MmD. The skeletal muscle phenotype in homozygous *ryr1b* mutants is transmitted in a recessive manner such that 25% of the offspring from mating heterozygous *ryr1b*\(^{+/}\) adults show diminished touch-evoked swimming by 5 dpf. Embryos were staged by hours (hpf) or days (dpf) post fertilization at 28.5°C. All animal work was performed with approval from the Boston Children’s Hospital Animal Care and Use Committee (14-05-2717R).
Chemical library

The Prestwick2 chemical library was supplied by the Institute of Chemistry and Cell Biology Screening Facility at Harvard Medical School and used as the source of small molecules for chemical screening experiments. This library contains 1,120 small molecule composed of 90% marketed drugs and 10% bioactive alkaloids or related substances. Compounds in the Prestwick2 collection were selected for their high chemical and pharmacological diversity, as well as their established bioavailability and safety in humans 37.

Chemical screening

Primary screen. For the primary screen, twenty embryos (1 dpf) resulting from a mating of heterozygous ryr1b zebrafish were manually sorted into 48-well plates containing 250 µL embryo 1X E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂·2H₂O, 0.33 mM MgSO₄·7H₂O) and four pooled chemicals from the Prestwick2 chemical library. Each chemical pool was assayed in duplicate using embryos from two independent clutches, for a total of 24 distinct pools tested per plate (Fig. 4.1). The chemicals were dissolved to a final concentration of 2.0 µg/mL and 0.4% DMSO, which is similar to concentrations other groups have used in zebrafish studies 37,38. As a negative control, twenty embryos from each clutch were cultured without chemicals in vehicle only (0.4% DMSO) (Fig. 4.1). Larvae obtained from wild-type (Oregon AB) matings served as positive controls in separate plates. All plates containing embryos were incubated at 28.5°C and maintained as initially treated for 5 days. Dead embryos were removed from wells when observed during the first 4 days of the study. On day 5, dead or “motion-dead” larvae were scored alongside living larvae.
Figure 4.1. Chemical screening plate set-up. Representative diagram of a 48-well plate used in the ryr1b primary screen. No two rows of the plate were filled with embryos from the same clutch, and each row contained a negative control well treated with vehicle only (0.4% DMSO). Experimental replicates were placed vertically from one another. An example treatment of the first two rows is illustrated, with negative controls in the first column shown in gray and replicate wells treated with the same chemical pool shown using different colors.

At 5 dpf, pools were scored for their ability to improve the survival and touch-evoked escape behaviors of ryr1b homozygous mutants using the following numeric scoring system: 3 = 4-6 cm, fast (wild-type); 2 = 2-4 cm (moderate); 1 = < 2 cm, slow (ryr1b); 0 = no movement (dead) (Figure 4.2 A). Survival, swimming, and combined endpoints of the primary screen were then examined for their ability to distinguish positive from negative controls. Since the survival component of the primary screen proved to be most statistically robust ($Z'$-factor$_{survival} = 1 - [(3 * (\sigma_p + \sigma_n)) / |\mu_p-\mu_n|] = 0.60$), chemical pools with
significantly higher ryrb survival rates than DMSO-treated controls were considered hits \( (P < 0.005) \). Taking into account both experimental replicates \( (n = 40) \), hit pools contained at least 37 alive larvae on day 5 (i.e., 70-100% of the larvae assumed to be ryrb mutants survived the duration of the study), and were selected for analysis in a secondary screen.

**Figure 4.2. Numerical scoring of touch-evoked escape behaviors.** (A) Four days following treatment with DMSO or drug, all larvae present in each screened well were counted and evaluated in terms of mobility using a touch-evoked escape behavior assay. Behaviors were scored using the following numeric scoring system: 3 = 4-6 cm, fast (wild-type); 2 = 2-4 cm (moderate); 1 = < 2 cm, slow (ryrb); 0 = no movement (dead). A representative response of each score is shown. (B) Average swimming distance of 5 dpf larvae in response to touch (WT/HT: 6.2 ± 0.8 cm/0.1 s; ryrb: 0.5 ± 0.3 cm/0.1 s). Motor functions were quantified using 15 embryos per group from three independent clutches. Significance was determined by a Student’s t-test, \( P = 1.2 \times 10^{-25} \).
**Secondary screen.** In the secondary screen, hit pools were separated and tested as 68 individual chemicals. Screening was performed precisely as described for the primary screen with few modifications. Embryos were raised in wells containing 750 µL 1X E3 medium, as opposed to 250 µL, although the final concentration of each compound was maintained at 2.0 µg/mL. Additionally, because full establishment of our heterozygous ryr1b line resulted in improved robustness of a combined survival and mobility assay, ryr1b larvae in this tier of the screen were evaluated in terms of an overall “vitality” score (Z’-factor = 0.37). Vitality scores, weighting survival and swimming ratios appropriately, were calculated as (# Dead * 0) + (# ryr1b * 1) + (# Moderate * 2) + (# Wild-type * 3). Theoretical maximum and minimum scores were 60 and 0, respectively, with an expected score of 50 in DMSO-treated controls. Secondary screen hits (P < 0.05), considered “candidate” compounds, are currently being examined in dose-response and long-term studies.

**Genotyping of ryr1b mutants**

The relatively relaxed zebrafish was initially identified due to a spontaneous autosomal recessive mutation within the ryr1b gene\(^30\). Specifically, ryr1b mutants carry a 4046 base pair (bp) DNA insertion in the intron between exons 48 and 49 that includes an additional 32 bp sequence found in mutant cDNA (Figure 4.3)\(^30\). Genotypes of individual embryos were examined using three-primer genomic PCR, with an expected wild-type band of 1,668 bp and a mutant band of 653 bp. Primer sequences were as follows: 
*ryr1b* #1 (forward): 5′-GTGGGTTCCTGTGCGATATGAGCTTCA-3′; *ryr1b* #2 (reverse): 5′-AACAGTGCGACATTAGGTGAGCAGAGG-3′; and *ryr1b* #3 (reverse): 5′-CTTTAAATAAGCTCTGAATGCGATGGTTGA-3′.
Figure 4.3. Genotyping of ryr1b larvae. (A) The ryr1b mutant allele carries a 4046 bp DNA insertion in the intron between exons 48 and 49 that includes an additional 32 bp sequence found in mutant cDNA. Schematic image adapted from Hirata et al. Genotypes of individual embryos were examined using three-primer genomic PCR. Primer binding sites are indicated. (B) Representative agarose gel used for PCR-based genotyping of individual wild-type, heterozygous, and ryr1b mutant larvae.

Western blotting

Western blotting was performed on groups of 50-100 wild-type or mutant zebrafish larvae at 5 dpf, as described in Chapter 2. Mouse monoclonal anti-phospho-STAT3 (1:1000, D128-3, MBL International, Woburn, MA, USA) and mouse monoclonal anti-β-actin (1:1000, A5441, Sigma) were used as primary antibodies, and were visualized with HRP-conjugated anti-rabbit (1:2500, 170-6515) and anti-mouse (1:5000, 170-6516) IgG (BioRad). Of three candidates, a reliable antibody for the detection of total Stat3 in zebrafish was not found. Phospho-Stat3 band intensities were normalized to control bands and quantified in Image J (NIH).
Real-time RT-PCR

Total RNA was prepared from zebrafish embryos using RNeasy fibrous tissue mini kits (Qiagen). cDNAs were synthesized from 1-2 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers. To assess relative stat3 expression levels in 5 dpf embryos, quantitative real-time RT-PCR amplification of cDNAs was performed with a Taqman assay for stat3 exon15-exon16 (Applied Biosystems) on a 7300 Real Time PCR System (Applied Biosystems). Gapdh served as the control to normalize stat3 expression using the $2^{-\Delta\Delta Ct}$ method.

Activity monitoring

Zebrafish behavioral parameters were defined using the Noldus Daniovision activity monitoring system. In 12-well plates, wild-type and mutant larvae (5 or 20 dpf) were placed into the machine with the light box off. After a 10 min acclimation to the dark, larvae were stimulated by light exposure for 50 min. This cycle was repeated two times during the course of a single trial. Swimming behaviors were recorded over the entire 2.0-hour period with an infrared light source. Three independent trials were performed with larvae from three different clutches, examining a total of 14-20 wild-type and ryr1b larvae from both DMSO- and nifuroxazide-treated groups. Behavioral parameters recorded include frequency, mean velocity, total distance, and cumulative duration of movement, and reflect the average of all larvae in an experimental group.

Calcium imaging in murine cell culture

Primary myoblasts from C57BL/6N mice were isolated and differentiated as described previously. Calcium imaging was performed 5 days after differentiation in myotubes loaded with 3 µM Fluo-4-AM (Life Technologies). Myotubes were imaged in imaging buffer (125 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 6 mM glucose, 2 mM Ca$^{2+}$, 25 mM Hepes/Tris, pH 7.4) at 490-500 nm with a Stanford Photonics 10 bit digital intensified CCD using a DG4 multi-wavelength light source. Fluorescent emission at 510 was acquired and analyzed using QED Imaging software (QED Software, Pittsburgh, PA,
USA) from regions of interest within each myotube at 30 frames per second. Sensitivity to $K^+$-depolarization and caffeine-activation were determined by a 5 second perfusion with 5-6 volumes of KCl (10 mM to 60 mM) or caffeine (3 mM to 40 mM).

**Statistical analysis**

GraphPad Prism 7 software (GraphPad Software Inc.) was used to graph all quantitative data and perform statistical analyses. $P$ values for pairwise comparisons were determined using a two-tailed Student’s t-test. $P$ values for Kaplan-Meier survival curves were calculated using a log-rank test, and for dose response curves in murine myotubes using an extra sum-of-squares F test. Multiple comparison tests were calculated using one-way ANOVAs, followed by Tukey’s honestly significant difference (HSD) post hoc tests (alpha = 0.05). Studies were considered significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

4.4. **RESULTS**

**Primary screening of ryr1b zebrafish**

Homozygous ryr1b mutants do not have morphological or birefringence abnormalities during the first week of development, and are typically only distinguishable from wild-type and heterozygous clutchmates by touch-evoked swimming behaviors (Fig. 4.2 B). However, mutants obtained from newly established ryr1b$^{+/ -}$ lines also exhibit increased mortality and die before 5 dpf, prioritizing survival as the most robust endpoint for chemical screening. Therefore, for our primary screen (schematically outlined in Fig. 4.4), 280 chemical pools from a total of 1,120 chemicals in the Prestwick2 chemical library were tested for their ability to improve the survival rate of homozygous ryr1b mutants. Of these chemical pools, 41 (14.6%) resulted in the death of all embryos, whereas the remaining 239 yielded surviving larvae after four full days of treatment (Table 4.1). Surviving wells were expected, and subsequently confirmed, to contain approximately 25% homozygous ryr1b mutants (Fig. 4.5). Assuming this ratio, the
17 chemical pools found to significantly improve ryr1b mutant survival compared to DMSO-treated controls were considered hits ($P < 0.005$) and selected for analysis in a secondary screen (Fig. 4.6).

**Figure 4.4. Schematic outline of two-tiered ryr1b chemical screen.** Primary and secondary ryr1b chemical screening procedures with the Prestwick2 chemical library. Images of homozygous ryr1b mutants at 5 dpf under brightfield (top) and polarized light (bottom) are also included, illustrating that mutants exhibit wild-type birefringence levels and must therefore be assayed for a skeletal muscle phenotype using an alternative assay, touch-evoked escape behavior (TEEB).
Table 4.1. Embryonic zebrafish survival in Prestwick2 library chemical screen.

<table>
<thead>
<tr>
<th>Survival in Primary Screen</th>
<th>Total</th>
<th>Viable</th>
<th>Lethal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Pools</td>
<td>280</td>
<td>239</td>
<td>41</td>
</tr>
<tr>
<td>Chemicals</td>
<td>1,120</td>
<td>956</td>
<td>164</td>
</tr>
<tr>
<td>Percentage</td>
<td>100.0</td>
<td>85.4</td>
<td>14.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Survival in Secondary Screen</th>
<th>Total</th>
<th>Viable</th>
<th>Lethal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemicals</td>
<td>68</td>
<td>58</td>
<td>10</td>
</tr>
<tr>
<td>Percentage</td>
<td>100.0</td>
<td>85.3</td>
<td>14.7</td>
</tr>
</tbody>
</table>
**Figure 4.5.** Screened wells contain expected 3:1 ratio of unaffected to *ryr1b* larvae. Genotypes and phenotypes compared in three independent DMSO-treated controls verify approximate 3:1 ratio of wild-type/heterozygous larvae to *ryr1b* mutant larvae. Each group represents the contents of two wells, considered experimental replicates (*n* = 40). Note: Genotyping of one larva in “Control 1” was not conclusive (*).
Figure 4.6. Mobility and survival measurements of primary screen “hit” pools. All treatments were performed in duplicate, with each replicate consisting of 20 larvae from heterozygous ryr1b matings \( n = 40 \). Larvae obtained from wild-type AB matings served as positive controls. Significance was determined by pairwise comparisons between experimental and control pools using a Student’s t-test. “LS” and “VG” designates the person who performed each trial, as two individuals conducted the ryr1b primary screen over the course of two years.
Secondary screening of *ryr1b* zebrafish

The second tier of our chemical screen was performed two years following the primary screen, with a fully established *ryr1b*+/− line. Healthier clutches tended to result in better survival of homozygous *ryr1b* mutants. Median mutant life spans more accurately adhered to the 7-15 day estimate previously published 30. Therefore, in our secondary screen, hit chemical pools were separated into individual compounds and each of 68 total compounds was examined for its ability to positively influence both *ryr1b* survival and motor function (referred to in combination as “vitality”). Weighing survival and swimming appropriately, twelve compounds were found to improve *ryr1b* mutant vitality compared to DMSO-treated controls (Table 4.2; Fig. 4.7). While these twelve compounds varied with regard to annotated function, several showed considerable overlap. These included two anti-inflammatory agents (sulfasalazine and ketoprofen), two monoamine oxidase inhibitors (pargyline hydrochloride and tranylcypromine hydrochloride), and two ion channel modulators (metolazone and nimodipine).
Table 4.2. Candidate compounds identified by two-tiered ryr1b chemical screen.

<table>
<thead>
<tr>
<th>Vitality Score</th>
<th>Chemical Name</th>
<th>Formula</th>
<th>MW</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>58.0</td>
<td>Pargyline hydrochloride *</td>
<td>C₁₁H₁₂ClN</td>
<td>195.69</td>
<td>Irreversible monoamine oxidase (MAO) inhibitor</td>
</tr>
<tr>
<td>57.0</td>
<td>Sulfasalazine</td>
<td>C₁₈H₁₄N₄O₅S</td>
<td>398.39</td>
<td>NF-KB inhibitor; anti-inflammatory</td>
</tr>
<tr>
<td>55.5</td>
<td>Metolazone **</td>
<td>C₁₆H₁₅ClN₃O₃S</td>
<td>365.83</td>
<td>Sodium-chloride channel inhibitor</td>
</tr>
<tr>
<td>55.0</td>
<td>Zimelidine dihydrochloride monohydrate **</td>
<td>C₁₆H₂₁BrCl₂N₂O</td>
<td>408.16</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>54.0</td>
<td>Miconazole ***</td>
<td>C₁₈H₁₄Cl₄N₂O</td>
<td>416.13</td>
<td>Anti-fungal agent</td>
</tr>
<tr>
<td>54.0</td>
<td>Ticlopidine hydrochloride ***</td>
<td>C₁₄H₁₅Cl₂NS</td>
<td>300.25</td>
<td>Inhibitor of platelet aggregation</td>
</tr>
<tr>
<td>53.5</td>
<td>Iohexol</td>
<td>C₁₉H₂₆I₃N₃O₇</td>
<td>821.14</td>
<td>Low-osmolality contrast agent</td>
</tr>
<tr>
<td>52.5</td>
<td>Benoxinate hydrochloride ***</td>
<td>C₁₇H₂₉ClN₂O₃</td>
<td>344.88</td>
<td>Surface anaesthetic</td>
</tr>
<tr>
<td>52.0</td>
<td>Ketoprofen</td>
<td>C₁₀H₁₄O₂</td>
<td>254.28</td>
<td>Cyclooxygenase inhibitor; anti-inflammatory</td>
</tr>
<tr>
<td>52.0</td>
<td>Nifuroxazide</td>
<td>C₁₂H₉N₃O₅S</td>
<td>275.22</td>
<td>JAK/STAT signaling inhibitor</td>
</tr>
<tr>
<td>52.0</td>
<td>Nimodipine</td>
<td>C₂₁H₂₈N₂O₇</td>
<td>418.44</td>
<td>Dihydropyridine calcium channel blocker</td>
</tr>
<tr>
<td>52.0</td>
<td>Tranylcypromine hydrochloride *</td>
<td>C₂₀H₁₂ClN</td>
<td>169.65</td>
<td>Irreversible MAO inhibitor</td>
</tr>
</tbody>
</table>

*Asterisks indicate compounds originating from the same chemical pool. Molecular weight (MW).*
Figure 4.7. Vitality scores of secondary screen “hit” compounds. Each scored compound represents an average of two replicates, consisting of 20 larvae from heterozygous ryr1b matings (n = 40). Larvae obtained from wild-type AB matings served as positive controls. Experimental treatments that significantly improved ryr1b mutant survival and mobility (combined as “vitality”) compared to DMSO-treated controls were considered candidate “hit” compounds warranting further study. Significance was determined by pairwise comparisons between control and experimental pools using a Student’s t-test. Vitality score = (# Dead * 0) + (# ryr1b * 1) + (# Moderate * 2) + (# Wild-type * 3). Standard deviations shown for positive and negative controls were calculated based on results from 5 and 21 independent experiments, respectively.
Dose-response identifies nifuroxazide as a modulator of the ryr1b phenotype

Our primary and secondary screens only tested chemical pools and individual compounds at a single concentration (2.0 µg/mL). Although beneficial in a high-throughput context, this approach can result in false positives and false negatives. All twelve candidate compounds are now being investigated to identify those that exhibit a dose-dependent, positive effect on the ryr1b mutant phenotype.

Nifuroxazide (NIF), a potent inhibitor of the JAK-STAT signaling pathway, was one of the first candidates found to improve ryr1b mobility in a dose-dependent manner (Fig. 4.8 A). For analysis, pools of 20 larvae obtained from heterozygous ryr1b matings were treated in duplicate with DMSO or with one of three different concentrations of NIF (1.0-100.0 µg/mL) at 1 dpf and scored for touch-evoked escape behaviors at 5 dpf. All scored larvae were then genotyped using three-primer genomic PCR. An intermediate dose of 10 µg/mL resulted in confirmed ryr1b larvae that swam similar to wild-type controls. Next, hit expansion was used to verify JAK-STAT as a potential molecular pathway involved in ryr1b mutant pathology. Whereas NIF acts by reducing tyrosine phosphorylation of JAK2 kinase and blocking downstream phosphorylation of the transcription factor STAT3, 5,15-diphenylporphyrin (5,15-DPP) is a cell permeable porphyrin that specifically inhibits interleukin 6-induced STAT3 activation by preventing its dimerization. As anticipated, 5,15-DPP also showed a dose-response relationship with ryr1b mutants and exhibited optimal activity at 100.0 µg/mL (Fig. 4.8 B). A higher effective dose for 5,15-DPP was consistent with the IC50 measurements reported for each compound 40, 41.

In skeletal muscle, activation of the JAK-STAT pathway is correlated with myogenic differentiation, and inhibition of JAK-STAT signaling in dystrophic mice has been shown to rescue defects in muscle regeneration 42-44. To determine whether zebrafish stat3 is differentially expressed in the wild-type and Ryrl-deficient background, quantitative real-time RT-PCR was performed using fluorescently tagged Taqman probes. Interestingly, stat3 expression is significantly increased in ryr1b mutants in comparison with unaffected controls at 5 dpf (Fig. 4.8 C). Western blots subsequently showed that zebrafish with homozygous ryr1b mutations are also more sensitive to pharmacological inhibition of Stat3. Levels of phosphorylated Stat3 protein experienced a larger decrease in ryr1b mutants than in
unaffected controls following NIF treatment (10 µg/mL) (Fig. 4.8 D).

**Figure 4.8. Nifuroxazide acts on the ryr1b phenotype in a dose-dependent manner.** (A) Dose-response for nifuroxazide (NIF). In two independent experiments, two pools of 20 larvae from heterozygous ryr1b matings were treated with DMSO or with one of three different concentrations of NIF (1.0-100.0 µg/mL) at 1 dpf and evaluated in terms of touch-evoked escape behaviors at 5 dpf ($n = 40$). Following phenotypic scoring, all living embryos were genotyped using three-primer genomic PCR. Swimming scores for larvae genotype-confirmed as ryr1b homozygous mutants are shown as a percentage of the total number of living larvae in each dose category: 1 = poor swimming; 2 or 3 = moderate or wild-type swimming. NIF improved mobility of ryr1b mutants at a dose of 10 µg/mL.
(Figure 4.8, continued) (B) Dose-response for 5,15-DPP, a known inhibitor of STAT3. 5,15-DPP improved mobility of select ryr1b mutants at a dose of 100 µg/mL, but also showed evidence of toxicity since treated pools consistently contained less than 25% genotyped ryr1b larvae. (C) Zebrafish stat3 expression is significantly increased in ryr1b mutants compared to unaffected larvae from the same clutch at 5 dpf ($P < 0.005$). (D) Western blot at 5 dpf showing levels of phosphorylated Stat3 in zebrafish suggests that ryr1b mutations may increase Stat3 sensitivity to pharmacological inhibition. Phospho-Stat3 band intensities were normalized to β-Actin control bands and quantified in Image J (NIH).

**Nifuroxazide improves contractile forces in Ryr1-deficient larvae**

Ryr1b mutants display weaker muscle contractions than wild-type clutchmates as early as 2 dpf. To investigate whether NIF affects contractile strength in zebrafish, ryr1b mutants and unaffected controls were treated with the compound for several days. At 5 dpf, electrophysiological studies were performed with the help of Dr. Jeffrey Widrick. Whereas ryr1b mutants exhibited reduced tetanic force per cross-sectional area compared to unaffected controls, tetanic forces in NIF-treated mutants were restored to wild-type levels (Fig. 4.9 A). These data, together with published observations that Ca$^{2+}$ transients are smaller in Ryr1-deficient zebrafish and mouse muscles relative to wild-type, prompted the hypothesis that nifuroxazide may act, either directly or indirectly, on defective excitation-contraction coupling in ryr1b mutants.

Depolarization of the muscle membrane causes a transient increase in cytoplasmic Ca$^{2+}$, a result of excitation-contraction coupling that leads to actin-myosin sliding and skeletal muscle contraction. To examine the effect of NIF on Ca$^{2+}$ transients in vertebrate muscle, wild-type murine myotubes were cultured in the presence or absence of compound for 5 days. Depolarization-induced calcium release was then studied in response to increasing doses of caffeine. As expected, only very modest increases in calcium release were observed in the wild-type background (Fig. 4.9 B). While these data are supportive of an effect of the compound on RyR1-mediated calcium release, more experiments are necessary and must be designed to look at other aspects of calcium homeostasis in RyR1 deficiency, such as SR calcium
load, resting free calcium concentration, and/or passive calcium entry. This particular experiment was not possible in RYR1 null muscle cells since these do not respond to any direct agonist of this channel (e.g., caffeine, potassium).

Figure 4.9. Nifuroxazide increases contractile strength of ryr1b skeletal muscles. (A) DMSO-treated ryr1b mutants exhibit reduced tetanic force per cross-sectional area (CSA) compared to DMSO-treated wild-type larvae at 5 dpf. Tetanic forces in mutants treated with NIF, however, are restored to wild-type levels at 5 dpf. Tetanic forces were measured following stimulation with 9 biphasic, 200 µs square-wave pulses at 300 Hz (WT-DMSO: 45.6 ± 4.2 kPa, n = 5; WT-NIF: 52.8 ± 4.1 kPa, n = 5; ryr1b-DMSO: 11.4 ± 1.3 kPa, n = 7; ryr1b-NIF: 25.4 ± 4.0 kPa, n = 7). Significance was determined by a one-way ANOVA ($F = 29.61, P < 0.0001$), followed by pairwise Tukey’s HSD post hoc tests. (B) Dose response relationship between caffeine concentration and depolarization-induced calcium release in wild-type mouse myotubes. Prior to testing, myotubes were differentiated in either DMSO or NIF (20 µM) for 5 consecutive days (DMSO EC$_{50}$: 8.6 mM; NIF EC$_{50}$: 7.9 mM). Non-linear regression data of curves were not statistically different, as determined by an extra sum-of-squares F test ($P = 1.0$).
**Long-term Stat3 inhibition as a potential treatment for homozygous ryr1b zebrafish**

Wild-type zebrafish larvae display straight bodies throughout the first 20 days of life, with angles that do not deviate significantly from 180 degrees. Although homozygous ryr1b mutants do not exhibit early morphological defects, ryr1b trunks are frequently bent by 20 dpf with angles reduced to 140 degrees. This bending is likely a consequence of skeletal muscle weakness, and may be analogous to neck and trunk flexor weakness observed in human patients with MmD (Fig. 4.10 A). To determine if NIF could correct this abnormality, larvae were treated with the compound from 1 to 20 dpf. After 20 days, body angles were measured as the angle of intersection between two lines, one drawn between the eyes of each larva and one drawn along the trunk midline. Body angles of NIF-treated ryr1b mutants were significantly increased and statistically indistinguishable from unaffected controls (Fig. 4.10 B). These improvements in morphology reflect long-term strengthening of ryr1b skeletal muscles as a result of exposure to NIF.

Next, to determine if long-term NIF treatment affects ryr1b motor functions, spontaneous swimming behaviors of 20 dpf larvae were quantified using the Noldus Daniovision. As expected, untreated ryr1b mutants moved significantly less than control clutchmates. In contrast, NIF treatment resulted in a large and robust increase on the distance travelled by ryr1b zebrafish, restoring their movements to the levels of NIF-treated controls (Fig. 4.10 C-D). Together with our dose-response data, these findings indicate that NIF improves the motor phenotype and endurance of ryr1b mutants.
Figure 4.10. Nifuroxazide corrects ryr1b morphological features and swimming behaviors. (A) Body angles of ryr1b mutants after 20 days of NIF treatment (10.0 µg/mL) are corrected and statistically indistinguishable from wild-type controls. (B) Quantification of body angle measurements. Angles were measured by first drawing/extending straight lines between the eyes of the larva as well as along the midline of the trunk (shown by dotted red lines). The angle of intersection was determined using Adobe Photoshop CS3 software (WT-DMSO: 179.1 ± 0.9 °, n = 6; WT-NIF: 176.8 ± 1.1 °, n = 6; ryr1b-DMSO: 142.6 ± 6.2 °, n = 11; ryr1b-NIF: 174.4 ± 2.0 °, n = 4). Significance was determined by a one-way ANOVA (F = 13.60, P < 0.0001), followed by pairwise Tukey’s HSD post hoc tests. (C) Swimming behaviors of 20 dpf larvae quantified in terms of distance traveled during a 2.0-hour period using the
(Figure 4.10, continued) Noldus Daniovision, a high-resolution system that allows for automated analysis of larval locations and orientations. Three independent trials were performed with larvae from three different clutches, examining a total of 14-20 wild-type and \( ryr1b \) larvae from both DMSO- and NIF-treated groups. Genotypes of individual embryos were confirmed by genomic PCR following the study. Significance was determined by a one-way ANOVA \( (F = 10.03, P = 0.0009) \), followed by pairwise Tukey’s HSD post hoc tests. (D) Monitored activities of DMSO- and NIF-treated wild-type and \( ryr1b \) zebrafish at 20 dpf using the Noldus Daniovision’s infrared light source. One representative 20-minute time interval within the recording period is shown.
In addition to their muscular defects, *ryr1b* mutants die prematurely. To examine whether long-term treatment with NIF positively impacts the life span of *ryr1b* mutants, affected larvae (selected at 5 dpf by touch-evoked escape behavior assay) were treated with compound from 1 to 50 dpf. Despite drug-mediated improvements in anatomy and swimming, Kaplan-Meier survival curves and median survival did not significantly differ between NIF- and DMSO-treated *ryr1b* larvae, although select *ryr1b* mutants treated with NIF did survive the complete duration of the study (Fig. 4.11).

![Graph showing survival curves for NIF and DMSO treated *ryr1b* mutants](image)

**Figure 4.11. Nifuroxazide does not significantly alter the life span of *ryr1b* mutants.** Kaplan-Meier survival curves of *ryr1b* mutants treated with either DMSO or NIF (10 µg/mL) from 1 to 50 dpf. Mutants were sorted from unaffected controls at 5 dpf using a touch-evoked escape behavior assay. Median survival of DMSO- versus NIF-treated mutants was 15.0 and 13.0 days, respectively. No statistically significant differences were detected according to a log-rank test ($P = 0.5$).
Ketoprofen candidate further supports a role for JAK-STAT signaling in Ryrl deficiency

A second candidate compound, ketoprofen, is a nonsteroidal anti-inflammatory drug that reversibly inhibits cyclooxygenase-1 and -2 and reduces the production of pro-inflammatory prostaglandin precursors (Fig. 4.12 A, top). A literature-based search revealed interleukin 6 (IL-6) to be one of the most prominently down-regulated precursors 46, and one that acts in the same molecular pathway as nifuroxazide (Fig. 4.12, bottom). Preliminary dose-response experiments have confirmed a relationship between ketoprofen and improvements in ryrlb mobility (Fig. 4.12 B). Although further studies are necessary, it is plausible that increased IL-6 production in Ryrl deficiency leads to increases in JAK-STAT signaling downstream, and to changes in skeletal muscle gene expression that are not altered in healthy muscle (Fig. 4.12 C) 17.
Figure 4.12. **Ketoprofen dose-response and proposed mechanistic link.** (A) Chemical structures of ketoprofen and nifuroxazide. (B) Dose-response graph for ketoprofen (KETO). Two pools of 20 larvae from heterozygous \textit{ryr1b} matings were treated with DMSO or four different concentrations of KETO (1.0-100.0 µg/mL) at 1 dpf and evaluated in terms of touch-evoked escape behaviors at 5 dpf \((n = 40)\). Following phenotypic scoring, all living embryos were genotyped using three-primer genomic PCR. Swimming scores for larvae genotype-confirmed as \textit{ryr1b} homozygous mutants are shown as a percentage of the total number of living larvae in each dose category: 1 = poor swimming, 2 or 3 = moderate or wild-type swimming. (C) Potential mechanistic link between candidate compounds via the IL-6/JAK2/STAT3 signaling pathway. Phosphorylated STATs form dimers, translocate to nuclei, and from there are associated with various transcriptional activities.\(^{47}\).
4.5 DISCUSSION

Chemical screening in the ryr1b zebrafish

Current research on congenital myopathies and muscular dystrophies is focused on using traditional methods to develop gene- and protein-based treatments. These methods, while having great potential, are fraught with methodological and logistical problems that are general to the approach (i.e., delivery, immune rejection, carcinogenic potential, cost, etc.). Our ryr1b chemical screen represents a paradigm shift by taking a more pragmatic approach to drug development, by testing bioactive compounds in a validated vertebrate model and then using the identity of positive “hits” to learn more about the molecular pathways associated with core myopathies. The use of zebrafish to screen small molecule libraries was developed by our collaborative neighbors in the Boston Children’s Hospital Division of Genetics and Genomics, and is innovative in its application to the ryr1b phenotype.

In contrast to target-based strategies, chemical screens in zebrafish are guided by a desired phenotype and allow for drug discovery without knowledge of specific molecular targets and mechanisms. Embryonic and larval zebrafish are also permeable to small molecules and can be assayed in large numbers. Since zebrafish with homozygous recessive mutations in the ryr1b gene have been characterized as excellent models of core myopathy, we screened the Prestwick2 library for chemicals that could correct the severely impaired swimming behaviors of ryr1b mutants. Our two-tiered screening strategy of first pooling chemicals and then screening individual compounds resulted in the identification of twelve candidate compounds that decreased the number of phenotypically affected ryr1b larvae. Dose-response experiments are now in progress to identify those candidates that clearly demonstrate a causal relationship with improvements in the ryr1b phenotype.

JAK-STAT signaling and ROS in RyR1 deficiency

Genetic- and pharmacological-based approaches have recently shown that increases in JAK-STAT signaling impair muscle regeneration by suppressing myogenic activities and satellite cell

148
functions\textsuperscript{42-44}. Follow-up studies conducted \textit{in vitro} and in mice demonstrated that inhibition of either JAK kinase (using inhibitor AG490) or STAT3 (using inhibitor 5,15-DPP) promotes satellite cell expansion and rescues defects in muscle generation in both aging and dystrophic mouse models\textsuperscript{43,44}. Intriguingly, nifuroxazide, a nitrofuran inhibitor of STAT3 transcription factor signaling, was one candidate compound found to rescue the muscle weakness of \textit{ryr1b} larvae in a dose-dependent manner. Genotyping of the \textit{ryr1b} gene in 5 dpf larvae treated with nifuroxazide (10 or 100 µg/mL) or with 5,15-DPP (100 µg/mL) beginning at 1 dpf revealed that \textit{ryr1b} mutants were among larvae that exhibited wild-type touch-evoked escape behaviors. These data suggesting that STAT3 inhibition might prevent the onset of muscular abnormalities in zebrafish with \textit{ryr1b} mutation, together with reports linking JAK-STAT signaling to myogenesis, prompted further studies of nifuroxazide in our model system. Stat3 transcripts were significantly increased in \textit{ryr1b} mutants relative to unaffected controls, while levels of Stat3 protein were similar between groups. However, phosphorylated Stat3 protein levels in \textit{ryr1b} mutants treated with nifuroxazide at the lowest effective dose were largely reduced compared to \textit{ryr1b} mutants treated with vehicle only. Such a decrease was not observed in between DMSO- and drug-treated unaffected control larvae. These observations indicate an increased sensitivity of Ryr1-deficient zebrafish to the inhibitor, and strongly suggest that JAK-STAT signaling is relevant to induction of the \textit{ryr1b} phenotype. Although nifuroxazide did not dramatically impact \textit{ryr1b} survival, chemical treatment definitively improved contractile strength, body morphology, and locomotive activities of mutants.

NADPH oxidase-generated reactive oxygen species (ROS) have been implicated in STAT3 activation\textsuperscript{58}. Since Ryr1-deficient zebrafish demonstrate excessive production of ROS\textsuperscript{31} as well as increased \textit{stat3} expression, it is quite plausible that there is a causal relationship between the two. Independent experiments showing that \textit{ryr1b} swimming abnormalities can be treated with either N-acetylcysteine antioxidant\textsuperscript{31} or JAK-STAT inhibitors serve as further support of this model. Though more studies will be necessary for verification, it is also important to note that other candidates identified in our screen may also act within the same signaling pathway. Ketoprofen, a nonsteroidal anti-inflammatory drug that reversibly inhibits cyclooxygenase-1 and -2, inhibits the production of IL-6 \textit{in vitro} and shows a
dose-response relationship with *ryr1b* mutants. Similarly, sulfasalazine is another anti-inflammatory candidate shown to inhibit IL-6 release in skeletal muscle. IL-6 is a pleiotropic cytokine that exerts both pro-inflammatory and anti-inflammatory effects depending on the cellular context, and has been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis. IL-6 preferentially activates STAT3. Activation of STAT3 also induces IL-6 mRNA production and increases secretion of IL-6 through the direct activation of IL-6 promoters. Based on these reports, we hypothesize that ketoprofen (and potentially sulfasalazine) might act by decreasing JAK-STAT signaling in Ryr1-deficient zebrafish and restoring a feedback loop that becomes dysregulated under oxidizing conditions within their muscle cells.

Apart from candidates modulating JAK-STAT signaling, a fourth compound identified in our chemical screen has previously been reported to have beneficial effects on vertebrate models of muscle disease. Notably, the monoamine oxidase inhibitor pargyline hydrochloride significantly decreases myofiber apoptosis and increases muscle strength in mouse models of Duchenne and Ullrich congenital muscular dystrophies. This study also found that ROS produced in mitochondria oxidize myofibrillar proteins important for contractile function in dystrophic muscle, such as tropomyosin, and pargyline hydrochloride corrects this defect at the molecular level.

The four candidates discussed in this chapter validate our screening strategy as a means to find pathways that might influence abnormalities within skeletal muscle, and also open up promising avenues for future studies with the *ryr1b* and *sepn1* zebrafish. We look forward to exploring their mechanisms of action in greater depth, and to testing each of the eight remaining compounds on these two vertebrate models of core myopathy.

### 4.6 REFERENCES


CHAPTER 5:

CONCLUSIONS
5.1 OVERVIEW

This dissertation sought to advance current understanding of the pathological mechanisms that lead to skeletal muscle weakness in centronuclear and core forms of congenital myopathy. With this objective in mind, we first created a transient zebrafish model of BIN1 deficiency and demonstrated that knockdown of the \textit{bin1} gene results in disrupted calcium signaling \textit{in vivo}. Next, we generated a TALEN-mediated zebrafish model of \textit{SEPN1}-RM and used a targeted pharmacological approach to show that loss of SEPN1-SERCA interactions may be principally responsible for the \textit{SEPN1}-RM skeletal muscle phenotype. Lastly, we performed a medium-throughput chemical screen on an established zebrafish model of \textit{ryr1b} mutation and identified JAK-STAT signaling as a molecular pathway clinically relevant to \textit{RYRI}-related disorders.

5.2 ADVANCES IN UNDERSTANDING BIN1 FUNCTION IN SKELETAL MUSCLE

Since the initial discovery that homozygous partial loss-of-function mutations in the human \textit{BIN1} gene result in autosomal recessive CNM (CNM2), the majority of follow-up studies aimed at understanding the true functions of BIN1 have been conducted \textit{in vitro}. This shortcoming is partially due to a lack of available human biopsies (since \textit{BIN1} mutations have been reported in fewer than 20 families), but is largely due to a lack of robust vertebrate models. Cardiac complications cause perinatal lethality in BIN1 null mouse embryos \textsuperscript{1}, and although the Inherited Myopathy of Great Danes canine model reproduces many aspects of CNM2 pathology \textsuperscript{2}, large mammals are precluded from experiments requiring numerous subjects (e.g., chemical screens) and from many laboratories due to the expenses and logistics involved in their care.

Our work introduces the first small vertebrate model of BIN1 deficiency that replicates characteristic clinical features of the human condition. \textit{Bin1} zebrafish morphants generated using antisense oligonucleotides display dorsal curvature reminiscent of human kyphosis/scoliosis, mild bradycardia, and impairments in early motor behaviors (i.e., spontaneous coiling, chorion hatching, touch-evoked swimming). Such evidence for skeletal muscle weakness is further substantiated by structural...
defects in this tissue, including diminished birefringence and myofibrillar disorganization. Importantly, *bin1* morphant muscles exhibit the major histopathological hallmarks of CNM2, including centralized myonuclei on H&E stain and large membranous structures on electron microscopy.

Alterations in T-tubule structure and biogenesis have previously been reported in association with *BIN1* mutations\(^3\).\(^4\). Our studies used cultured zebrafish myofibers to show that BIN1 is localized at T-tubules *in vivo*, and electron micrographs to demonstrate that triads are malformed in BIN1-deficient vertebrates. To then probe whether structural disruptions at the skeletal muscle triad compromise proper excitation-contraction coupling, we imaged calcium transients in *bin1* morphants mosaically expressing an alpha-actin-driven calcium reporter. As anticipated, calcium release and fluorescence propagation through skeletal muscle was significantly diminished in *bin1* morphants relative to wild-type controls. Our data complement earlier observations of abnormal intracellular calcium release in isolated murine muscle fibers following *Bin1* shRNA-mediated knockdown\(^5\). These experiments are the first to tie the loss of BIN1 to defects in excitation-contraction coupling within a whole organism.

Creation of the Bin1-deficient zebrafish also allowed novel *in vivo* investigations into the specific roles of two *BIN1* exons discussed in the literature. Mis-splicing of the muscle-specific exon 11, which encodes BIN1’s phosphoinositide-binding domain, has been reported in different forms of myotonic muscular dystrophy\(^6\). This particular splicing defect was also recently identified in dizygotic twins affected with a rapidly progressive form of centronuclear myopathy, but only with parallel inclusion of a brain-specific exon\(^2\). Exon 7 is typically only found in the BAR domain of neuronal BIN1 isoforms, and modulates protein-protein interactions with DNM2\(^6\).\(^8\). Interestingly, our RNA-mediated overexpression experiments showed that BIN1 isoforms with and without exon 11 are both able to rescue the skeletal muscle phenotype of *bin1* morphants but cannot do so in the presence of exon 7. These data indicate that muscle-specific exon 11 is more important for muscle development and maintenance of triads rather than for initial formation, and correspond to reports of human patients unaffected at birth and during early childhood\(^2\). They also begin to assess the impact of the faulty inclusion of exon 7 in skeletal muscle, and support a role for this exon in nuclear centralization. Collaborative studies are now in progress with the
Charlet-Berguerand laboratory to test our working hypothesis that exon 7-mediated defects are caused by mislocalized interactions between BIN1 and DNM2.

5.3 Advances in Understanding Pathophysiological Mechanisms in Core Myopathies

Rapid changes in intracellular calcium concentration are essential for many signaling events in eukaryotic cells ⁹. The sarcoplasmic reticulum (SR) is a highly specialized organelle in striated muscles for Ca²⁺ storage and regulation of cytoplasmic Ca²⁺ levels, equivalent to the endoplasmic reticulum (ER) in most other mammalian cells. In muscle cells, the SR forms a junction with internalization of the plasma membrane, known as transverse (T)-tubules, at every sarcomere. These types of junctions spread regularly along the length of the muscle fiber allow for controlled and uniform calcium release from intracellular stores when the muscle is stimulated, a series of events collectively as excitation-contraction coupling ¹⁰.

Mitochondria occupy ~20% of the cell volume in skeletal muscle fibers, and are strategically positioned within muscle cells throughout development to supply the energy required for muscle movement ¹¹. Mitochondrial ATP production, for example, locally supports the activity of the SERCA Ca²⁺ ATPase and its refilling of SR calcium stores ¹²-¹⁴. One mole of ATP is required for every two moles of Ca²⁺ pumped into the lumen of the SR ¹⁵. There is also substantial evidence for localized calcium transfer between these two organelles, calcium that is primarily used for the stimulation of oxidative metabolism. In vivo recordings in skeletal muscle show that increases in ATP and calcium concentrations within mitochondria mirror the rise in cytosolic calcium concentrations resulting from sarcolemmal depolarizations ¹⁶,¹⁷. However, close coordination between mitochondria and SR also results in the production of reactive oxygen species (ROS). ROS generated by mitochondria through the electron transport chain and/or by the SR/ER via NADPH oxidase enzymes play a major regulatory role in skeletal muscle ¹⁴,¹⁸-²¹. Our studies with Sepn1- and Ryr1-deficient zebrafish offer novel mechanistic interpretations regarding how defects in these two proteins might physiologically link oxidative stress to skeletal muscle weakness.
The sepn1 knockout zebrafish is the first genetic model to accurately reproduce human SEPN1-RM in a vertebrate system. Sepn1 mutants exhibit morphological abnormalities, including smaller bodies (as determined by cross-sectional areas) and dorsal curvature reminiscent of patient kyphosis/secoliosis, as well as reduced contractile strength and skeletal muscle cores under electron microscopy. Mutants also show increased intracellular oxidant activity relative to unaffected controls, but on par with levels previously measured in ryr1b mutant zebrafish. We therefore considered the idea that, since SEPN1 and RyR1 are physically associated in vivo and SEPN1 is required for full RyR1 activity, SEPN1-mediated redox regulation of the RyR1 channel may negatively impact calcium release in SEPN1 deficiency. However, subsequent studies of sepn1 mutant contractile forces suggested that their cellular defects might influence muscle relaxation (calcium re-uptake) more than muscle contraction (calcium release). Sepn1-deficient skeletal muscles do not completely relax following tetanic stimulation.

In parallel with our characterization of the sepn1 zebrafish, Marino and colleagues successfully unmasked the hidden muscle phenotype of the Sepn1 knockout mouse model via AAV-mediated overexpression of endoplasmic reticulum oxidoreductin 1 (ERO1). Increases in ERO1, a thiol oxidase localized at the ER/SR, result in the hyperoxidation of SERCA’s luminal cysteines and attenuate pump activity. Thioredoxin-related protein ERp57 has also been shown to promote disulfide bond formation between two cysteines on the luminal L7-8 loop of SERCA, and to decrease SERCA-mediated calcium re-uptake in the presence of high SR Ca\(^{2+}\) concentrations. The redox functions of SEPN1 are believed to protect ER/SR-associated proteins involved in calcium transport from ROS. This targeted function would explain defects in intracellular calcium signaling observed in SEPN1 deficiency. To date, perhaps the largest contribution of the sepn1 zebrafish model is the observation that ex vivo treatment with an ERO1 inhibitor (EN460) can correct its severe skeletal muscle phenotype. ERO1 inhibition ameliorates sepn1 defects in myofibrillar organization, motor functions, and muscle relaxation. It is also important to note the ultrastructural improvements seen in EN460-treated sepn1 larvae, primarily because the appearance of skeletal muscle cores and abnormal mitochondrial morphologies (only present in untreated sepn1 controls) are thought to reflect changes in physiological parameters, such as calcium...
homeostasis and ROS production. These data strongly implicate ROS and their dysregulation of necessary ER/SR calcium transport mechanisms as the pathological basis of SEPNI-RM.

Candidate compounds identified in our chemical screen of the relatively relaxed (ryr1b) zebrafish extends this model of ROS-mediated disruptions in calcium signaling from SEPNI-RM to other subgroups of core myopathy. Pargyline hydrochloride, for example, is a monoamine oxidase inhibitor shown to reduce mitochondria-generated ROS in vivo and to improve the contractile strength of dystrophic mouse muscles. However, although RyR1 channels possesses many cysteine residues that are susceptible to redox modifications, it is unlikely that ROS acting directly on RyR1 are solely responsible for the myopathic phenotype seen in RYR1 null backgrounds. This speculation is consistent with prior observations that ex vivo antioxidant treatment only improves select aspects of the ryr1b zebrafish phenotype, such as histopathology and certain swimming parameters, but not the contractile properties of skeletal muscle.

Three other candidate compounds have annotated functions that suggest elevated levels of ROS might indirectly cause features of the ryr1b phenotype through dysregulation of JAK-STAT cytokine signaling. Ketoprofen and sulfasalazine are two anti-inflammatory agents known to decrease intracellular levels of interleukin 6 (IL-6), a pro-inflammatory cytokine that is increased four-fold in myotubes from patients with CCD relative to those with MH or to unaffected controls. IL-6 is also the primary cytokine to preferentially initiate the JAK-STAT signaling cascade, and uses this pathway to mediate changes in cytosolic calcium concentrations within specific cell types. Nifuroxazide, a JAK2 inhibitor, demonstrates a dose-response relationship with improvements in ryr1b mobility, while also correcting contractile strength and mild morphological abnormalities typical of older larvae. Furthermore, nifuroxazide treatment of ryr1b mutants was shown to reduce levels of phosphorylated Stat3, a downstream target of Jak2. A direct inhibitor of STAT3 was also confirmed to increase ryr1b mobility in a dose-dependent manner. Taken together with reports that ROS can activate STAT3, it is feasible that ROS (generated by disruptions in calcium signaling due to loss of RyR1 channels) might upregulate JAK-
STAT signaling and disrupt feedback mechanisms necessary for the prevention of inflammation or the transcription of genes differentially expressed in normal skeletal muscles.

5.4 Future Directions

5.4.1 Biological Functions of Causative Congenital Myopathy Genes

The zebrafish models described in this work, as well as our bin1 knockout model currently in the pipeline, will be invaluable tools for future studies aimed at understanding the in vivo functions of congenital myopathy genes. First and foremost, our group is deeply interested in helping to define the molecular basis behind the muscle-specificity in many of these diseases. Almost all autosomal recessive CNM mutations described to date are in ubiquitously expressed exons, yet abnormalities in the brain, heart, and organs other than skeletal muscle are relatively rare. SEPN1 is also a ubiquitously expressed glycoprotein whose defects primarily manifest in skeletal muscle. We anticipate crossing adult bin1 and sepn1 heterozygous zebrafish with transgenic lines overexpressing BIN1 or SEPN1 (respectively) under the control of either ubiquitous or tissue-specific promoters in order to identify the expression patterns required for rescue. Similar overexpression approaches, whether transient or genetic, could also be employed to probe the therapeutic benefits of SERCA overexpression in Sepn1-deficient zebrafish tissues, similar to what has already been shown in a dystrophic background. Finally, while a handful of missense and nonsense mutations affecting the selenocysteine residue of SEPN1 have been identified in patients, the majority of SEPN1 mutations are found within its non-catalytic protein domains. Transient RNA overexpression could be used to explore the importance of SEPN1’s catalytic activity in the context of disease pathogenesis, by attempting to rescue the sepn1 knockout phenotype with human wild-type or mutant SEPN1 mRNAs.
5.4.2 Developing Therapeutics for Congenital Myopathies

Many laboratories are pursuing different therapeutic strategies for the treatment of congenital myopathy. Gene replacement therapies based on localized or systemic administration of AAV vectors have successfully been used to treat other monogenic diseases, such as metabolic disorders and retinal degeneration, and are now showing considerable promise in the context of X-linked myotubular myopathy (XLMTM). Recently, Jocelyn Laporte and colleagues also reported that decreasing Dnm2 expression in mouse skeletal muscles could be utilized to revert XLMTM disease progression. Since interactions between BIN1 and DNM2 are critical for healthy skeletal muscle and because BIN1, DNM2, and MTM1 likely act in the same molecular pathway, it is also reasonable to suspect that the down-regulation of Dnm2 might also treat mice with CNM2. The Laporte laboratory is now characterizing constitutive (CMV) and muscle-specific (HSA) knockout mouse lines generated by targeted homologous recombination in embryonic stem cells for exon 11 (encoding the phosphoinositide-binding domain) and exon 20 (the last exon) of BIN1. Whereas both CMV and HSA knockouts of exon 20 were found to be perinatally lethal, CMV deletion of exon 11 in mice results in a very mild muscle phenotype with nuclear mislocalization. Our two groups our currently working as part of a constructive collaboration to test DNM2 downregulation as a potential therapy to treat vertebrate models of CNM2. Our experiments will involve knockdown of the zebrafish dnm2 gene using different morpholinos in both transient and stable bin1 models.

Next steps for the sepn1 knockout line also center on therapeutic development. Sepn1 zebrafish, for instance, are ideally suited for a medium- or high-throughput chemical screen. Our prior experience screening the ryr1b zebrafish, in addition to the fact that sepn1 mutants exhibit a muscle phenotype easily detectable by birefringence assay, will allow us to optimize this protocol and complete screening in a shorter time frame. However, before we begin to examine new lead drugs and targeted options for therapy, our plan is to test additional ERO1 inhibitors (apart from EN460) and anti-oxidizing agents in the sepn1 model. The mechanism of action behind EN460 and other promising compounds will also need to be further scrutinized. For example, it will be necessary to investigate whether these drugs reduce ROS
and rates of apoptosis *in vivo*, as this correlation is currently just speculative. It would also be prudent to analyze whether EN460 and related compounds improve *sepn1* recovery following cardiotoxin injury, since impaired satellite cell functions and defects in muscle regeneration have been reported in SEPN1-deficient mice.\(^{38}\)

Upcoming experiments with the *ryr1b* zebrafish will involve validating the twelve candidate compounds identified in our chemical screen. Three candidates potentially modulate components of the JAK-STAT signaling pathway, and JAK-STAT upregulation is believed to suppress myogenic transcription factors and muscle repair.\(^{39-41}\) Although this connection is encouraging, it is based almost entirely on the annotated functions of these compounds. We must therefore explore whether the phenotypic improvements observed in *ryr1b* zebrafish treated with nifuroxazide (and related drugs) truly result from JAK-STAT inhibition. This could be achieved by assessing whether *ryr1b* mutants injected with JAK- or STAT-targeted morpholinos phenocopy those treated with drug. The active site of each drug could also be altered in order to prevent substrate binding. After confirming a role for JAK-STAT in *RYRI*-related disorders, we might then investigate whether the numbers or functions of satellite cells are decreased in *ryr1b* zebrafish, and if so, could one or more of our drug candidates restore these defects. In parallel to our experiments at the bench, all drugs identified to positively and specifically influence the *ryr1b* phenotype will be brought to the attention of suitable potential industry partners, or academic facilities with medicinal chemistry expertise, for further lead compound development. Our colleague, Dr. Louis Kunkel, is currently pursuing this plan of action with one of his laboratory’s dystrophin-deficient mutants.

In its entirety, this dissertation work provides important insights into the biochemical activities of congenital myopathy genes and investigates the potential of small molecules to compensate for the loss of these functions in a disease state. Our studies support a tight connection between calcium homeostasis and cellular redox conditions in skeletal muscle, and tie disruptions in proper excitation-contraction coupling to disease pathogenesis in both centronuclear and core myopathies.
5.5 REFERENCES


