Characterization of a Zebrafish Model of X-linked Centronuclear Myopathy for Therapeutic Drug Development

by

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Molecular Genetics
University of Toronto

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Abstract

X-linked centronuclear myopathy (XLCNM) is a congenital skeletal muscle disorder caused by mutations in MTM1. Despite being associated with a high rate of neonatal mortality there are currently no effective therapies for this disorder. MTM1 is a phosphoinositide 3-phosphatase that antagonizes class II and III phosphatidylinositol 3-kinases (PI3K). Loss of Pik3c2b, a class II PI3K, rescues lethality in Mtm1 knockout mice. Importantly, Pik3c2b knockout mice are viable and have no apparent phenotype. Therefore, pharmacological inhibitors of PIK3C2B have the potential to be highly effective in the treatment of XLCNM. I have characterized an mtm1 mutant using the zebrafish, Danio rerio, and found that PIK3C2B inhibition improves the mtm1 mutant phenotype. In parallel, a phenotypic drug screen identified valproic acid as a suppressor of an mtm1 mutant phenotype. The work presented herein demonstrates that PIK3C2B inhibitors and drugs like valproic acid may represent putative therapeutics for translation into MTM1 patients.
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Chapter 1
Introduction

1 X-linked Centronuclear Myopathy

X-linked centronuclear myopathy (XLCNM), or myotubular myopathy, is a childhood skeletal muscle disorder caused by loss of function mutations in \(MTM1\). It is a rare disorder that is estimated to occur in 1 / 50,000 male births. XLCNM is characterized by clinical signs of a congenital myopathy and the presence of centrally positioned nuclei on muscle biopsy\(^1\). Some clinical features of the severe or classic form of XLCNM include marked muscle weakness at birth, generalized hypotonia, respiratory failure, and high rates of neonatal mortality\(^1\). Other distinguishing presentations include ophthalmoparesis (weak eye movement), elongated fingers and facial features, and body length in the 90\(^{th}\) percentile or higher\(^1,2\). The severe clinical presentation is also associated with significant disabilities such as lifelong ventilator and wheelchair dependence. Patients with milder forms of the disorder are often ambulant, showing only mild muscle weakness or hypotonia and may or may not require transient ventilator support\(^2\).

Despite a strong phenotypic classification, the natural history of XLCNM has yet to be well-defined making it difficult to predict patient outcomes and to define a therapeutic window. To address these issues, a prospective cross-sectional clinical study was recently completed to characterize the disease course and natural history of XLCNM\(^3\). While there have been substantial improvements in supportive therapy and our molecular understanding of disease pathogenesis, there are currently no effective treatments for this disorder.

XLCNM is part of a genetically heterogeneous group of disorders known as centronuclear myopathies (CNM). Among all forms of CNM, the X-linked form is the most common and is associated with the most severe clinical presentations. Three other classical CNM genes include dynamin-2 (\(DNM2\))\(^4\), amphiphysin 2 (\(BIN1\))\(^5\), and ryanodine receptor 1 (\(RYR1\))\(^1\), and are the next most commonly encountered and are generally associated with milder forms of CNM. CNM has also been associated with mutations in titin (\(TTN\))\(^6\), striated muscle preferentially expressed protein kinase (\(SPEG\))\(^7\), myotubularin related protein 14 (\(MTMR14\))\(^1\) and coiled-coil domain-
containing protein 78 (CCDC78) thanks to advances in whole-exome, -genome and RNA sequencing.

Skeletal muscle requires several specialized substructures to produce contractile force, including the neuromuscular junction (NMJ), the excitation-contraction (EC)-coupling machinery, and the sarcomere. There is evidence for NMJ defects in XLCNM based on abnormal stimulation single fiber EMG in patients and NMJ defects in MTM1 models. XLCNM is also associated with defects in the EC-coupling machinery. EC-coupling begins with excitation at the NMJ causing a membrane depolarization that is propagated down membrane invaginations known as Transverse (T)-tubules. The T-tubule is abutted on both sides by terminal sarcoplasmic reticulum (SR), forming the triad. The triad is a key structure in EC-coupling that translates the membrane depolarization at the T-tubule into mechanical release of Ca\(^{2+}\) from SR stores. It is the rise in intracellular Ca\(^{2+}\) that results in muscle contraction via shortening of the sarcomeres, completing the EC-coupling process. One of the hallmark pathologies found in XLCNM is disorganization of the triad, which in addition to NMJ defects, is thought to explain the profound muscle weakness seen in patients. The sarcomeric structure in XLCNM appears normal.

2 Function, Structure, Localization and Protein-Protein Interactions of MTM1

MTM1 protein, traditionally referred to as myotubularin, was originally described as a protein tyrosine phosphatase (PTP) based on sequence homology. It has since been shown that it acts as a lipid phosphatase that dephosphorylates membrane phosphoinositides (PIPs) at the 3’ position of the inositol headgroup. Specifically, MTM1 can convert phosphatidylinositol 3-phosphate [PtdIns(3)P] into phosphatidylinositol (PtdIns) and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P\(_2\)] into phosphatidylinositol 5-phosphate [PtdIns(5)P]. In C2C12 myoblasts and myotubes, MTM1 is found in a dense cytoplasmic network and can localize at the plasma membrane as shown by immunofluorescence. There is no MTM1 detected in the nucleus. Another report combined immunogold labelling with electron microscopy provided evidence that MTM1 acts at the sarcoplasmic reticulum while another used immunofluorescence and fluorescently tagged proteins to show MTM1 acts in the sorting and recycling endosomes where it dephosphorylates PtdIns(3)P.
Figure 1. The structure of phosphatidylinositol (PtdIns). PtdIns can be phosphorylated at the 3’, 4’, and 5’ hydroxyl groups of the myo-inositol ring, serving as the precursor to seven phosphoinositides (PIPs). PIPs are the mono-, bi-, or triphosphorylated forms of PtdIns. The fatty acyl chains and glycerol backbone are hydrophobic while the phosphate linker and head group are hydrophilic. The head groups of PIPs are negatively charged due addition of phosphate groups. (This figure was adapted from Figure 1 of Falasca and Maffucci, 2012).

MTM1 has several conserved functional domains. The PH-GRAM domain is thought to bind PIPs. There is evidence that the PH-GRAM domain of MTM1 binds PtdIns(5)P which allosterically activates its 3-phosphatase activity. A more recent report investigating the role of PH-GRAM domain in MTMR6 suggests that phosphoinositide binding of the PH-GRAM domain is low affinity and insufficient on its own to localize MTMR6 to its physiologic compartment in the cell. Specifically, MTMR6 requires both the PH-GRAM domain and the coiled coil (CC) domain to bind both to bind the lipid PtdIns(3,5)P2 and the protein KCa3.1 in order to perform its function. Thus, it is possible that in the context of the whole protein the PH-GRAM domain of MTM1 serves other functions than to bind PtdIns(5)P. Indeed, purified C. elegans MTM1 can bind to PtdIns(4,5)P2 in liposome sedimentation and flotation assays but not to other phospholipid species including PtdIns(3)P, PtdIns(4)P, PtdIns(3,4)P2, PtdIns(3,4,5)P3, phosphatidylserine, or phosphatidylcholine. They further showed that genetic manipulation of the kinases and phosphatases that alter PtdIns(4,5)P2 levels heavily influences the localization of MTM1 in C. elegans gonadal sheath cells. Importantly, overexpression of human MTM1 was able to rescue a PtdIns(4,5)P2-dependent phenotype associated with loss-of-function mtm-1 mutation in the worm, supporting the idea that MTM1 affinity towards PtdIns(4,5)P2 is conserved across species.

The Rac1-induced localization domain (RID) was aptly named by Laporte and colleagues who found that it mediates localization of MTM1 to membrane ruffles induced by overexpression of constitutively activated Rac1. They suggested that MTM1 may be recruited to the plasma membrane upon activation of Rac1 or a phosphatidylinositol 3-kinase (PI3K). Of note, this is a
highly conserved domain and is a hotspot for missense variants in human patients (see Figure 2). Another important domain is the PTP domain which contains the highly conserved catalytic active site motif, CSDGWDR. The PTP domain is responsible for phosphoinositide 3-phosphatase activity of MTM1 and also represents a hotspot region for missense variants in patients (see Figure 2).

MTM1 has been shown to physically interact with many proteins. In skeletal muscle, myotubularin-related protein 12 (MTMR12)\textsuperscript{20,21}, BIN1\textsuperscript{22}, and desmin\textsuperscript{23} have all been identified as interactors of MTM1 by co-immunoprecipitation and co-localization. MTMR12 is a catalytically inactive member of the myotubularin family of phosphatases. It was shown to increase the stability of MTM1 in the cell, and morpholino knockdown of \textit{mtmr12} in zebrafish was shown to result in a severe muscle phenotype similar to the \textit{mtm1} morphant phenotype\textsuperscript{21}. Furthermore, MTM1 homo-oligomerizes in the presence of substrate and its allosteric activator, PtdIns(5)P\textsuperscript{13}. \textit{In vitro} assays performed with purified human proteins also showed that adding phosphatase-dead MTM1\textsuperscript{C375S} to MTM1 increased its PtdIns(3)P phosphatase activity\textsuperscript{13}. Given these findings, it is possible that MTMR12 hetero-oligomerizes with MTM1 to promote its catalytic activity and stability. The interaction of MTM1 with BIN1 is intriguing because \textit{BIN1} is another CNM gene. It is one known genetic cause of autosomal recessive CNM. BIN1 has a PIP binding motif and a membrane curvature-generating BAR domain and is thought to be involved with T-tubule generation and maintenance, given its ability to tubulate membranes\textsuperscript{22}. The interaction of MTM1 and BIN1 was shown to promote more extensive tubulation of membranes than by BIN1 alone\textsuperscript{22}. MTM1 interacts with desmin, a cytoplasmic intermediate filament, via several surface residues\textsuperscript{23}. Mutation of these residues abolishes interaction with desmin and leads to disruption of mitochondrial dynamics and motility\textsuperscript{23}. Interestingly, mutation of these residues does not abolish the phosphatase activity of MTM1, suggesting that MTM1 has phosphatase-independent functions in the cell\textsuperscript{23}. However, no missense mutations have been identified in patients in the residues predicted to abolish an interaction with desmin, suggesting that a loss in desmin interaction alone does not give rise to XLCNM. This does not necessarily mean that the loss of phosphatase-independent functions of MTM1, such as regulating mitochondrial dynamics, are unimportant in the pathogenesis of XLCNM. But until these missense mutations are modeled in mice or found in human patients, it will remain difficult to interpret the mechanistic role that loss of desmin interaction alone plays in XLCNM.
MTM1 has also been shown to interact with sorting nexin-17 (SNX17), tripartite motif family-like 2 (TRIML2), and tubulin alpha-3C/D chain (TUBA3C)\textsuperscript{24} through a large-scale proteomics method. Briefly, HEK293 cells expressing an HA-tagged MTM1 “bait” were lysed and subjected to affinity purification followed by mass spectrometry to detect “prey” protein interactors\textsuperscript{24}. Most recently, purified forms of PI4K2α and Sec6 were shown to interact with MTM1 by immunoprecipitation\textsuperscript{16}. PI4K2α is a phosphatidylinositol 4-kinase that catalyzes the phosphorylation of PtdIns to PtdIns(4)P, while Sec6 is a component of the exocyst complex involved in fusion of exocytic vesicles to the plasma membrane\textsuperscript{16}. These interactions support a role for MTM1 in a phosphoinositide conversion mechanism in HeLa cells, from PtdIns(3)P to PtdIns(4)P, that provides recycling endosomes their identity and allows them to dock successfully to the plasma membrane\textsuperscript{16}.

3 Genetics of XLCNM

\textit{MTM1} is located on the X chromosome (Xq28.1) and is an X-linked recessive disorder. Penetrance is considered 100% in males with a pathogenic variant. Disease-associated mutations are found along the entire gene. Over 300 mutations have been reported in peer-reviewed literature\textsuperscript{25}. There are currently 529 DNA variants that have been submitted to the Leiden Open-source Variation Database (LOVD)\textsuperscript{26}. The majority of variants found in this database are substitutions (69.8%; includes missense and nonsense mutations), followed by deletions (20.6%), duplications (6.8%), insertions (1.3%), insertion/deletions (1.3%), as well as one allele containing 2 variants (0.2%). The majority of mutations are found within exons (75.0%), followed by introns (21.8%), and mutations spanning several exon/intron boundaries (3.2%).

To help visualize genotype-phenotype correlations, I prepared a schematic of the \textit{MTM1} gene indicating important functional domains and the location of 134 point mutations and 25 deletions along with their clinical severity (Figure 2). The domain layout is based on previously published work by Hnia and colleagues\textsuperscript{23}. Of note, the mutations shown in this figure represent only the deletions and nonsynonymous substitutions found within exons. All mutations were from male patients, so milder phenotypes associated with mosaicism in females are not represented in clinical severity. Lastly, I only used mutations submitted from peer-reviewed articles. From this schematic we see that mutations that introduce a frameshift, be they deletions or nonsense mutations, are predominantly associated with severe phenotypes. Missense mutations within the
catalytic phosphatase site are highly conserved from yeast to humans and typically correlate with a severe clinical presentation. On the other hand, milder forms tend to correlate with missense mutations that fall outside this domain such as in the RID domain. These correlations are consistent with previous reports\textsuperscript{12,25}. 
Figure 2. Landscape of mutations and their clinical severity in XLCNM. This schematic shows the amino acid sequence of the human MTM1 gene where alternating exons are shown in black and blue, beginning with exon 2 (the first coding exon). The locations of MTM1 functional domains are shown above this sequence and various patient mutations are provided below. Clinical severity associated with each mutation is indicated for severe (red), moderate (orange), and mild (green) forms of XLCNM. Missense mutations are indicated by circles, nonsense mutations are indicated by triangles, and deletions are indicated by bars/rectangles. Only exonic point mutations and deletions that were identified in males are shown. In cases where clinical severity of a point mutation was variable, e.g. associated with mild and severe XLCNM, each reported instance of that mutation is shown. Therefore, the number of point mutations at a given residue is not indicative of the relative frequency of its mutation in this schematic because other frequently mutated residues may only be shown once.

4 Model Organisms of XLCNM

Various model organisms harbouring mutations in MTM1 orthologs have been described, from yeast to dogs. Of note, the CSDGWDR phosphatase active site motif is conserved across species. The Saccharomyces cerevisiae ortholog of MTM1 is called ymr1, encoding a myotubularin-like phosphoinositide 3-phosphatase with 32% amino acid sequence identity to the human protein. Parrish and colleagues showed the ymr1 knockout mutant itself is viable but is synthetically lethal in a triple mutant background with two synaptojanin-like polyphosphatidylinositol phosphatases, sjl2 and sjl3, owing to toxic accumulation of PtdIns(3)P. They carried out a genetic screen to identify genes that, when overexpressed, could suppress lethality of the triple mutant. As expected, they showed that either ymr1, sjl2, or sjl3 overexpression rescued lethality by reducing PtdIns(3)P levels. They also found multiple genes that disrupt a Rho1-mediated MAPK pathway involved in maintenance of cell wall integrity and actin organization that could suppress synthetic lethality without reduction of PtdIns(3)P levels. Additionally, two genes involved in vesicle trafficking, ypt1 and vam6, suppressed triple mutant lethality independently of PtdIns(3)P reduction. These findings demonstrate that it is possible to suppress loss-of-function mutations in MTM1 without lowering PtdIns(3)P levels. Hence, this work suggests that a therapy for XLCNM might not necessarily have to correct the toxic accumulation of PtdIns(3)P in order to be effective. Of course, a major caveat here is that the yeast amino acid sequence is only 32% homologous to the human sequence.

Moving up the complexity scale are the soil-dwelling nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster. The C. elegans mtm-1 gene encodes a protein with 45% amino acid sequence homology to human MTM1. The fruit fly’s mtm encodes a
phosphoinositide 3-phosphatase with 56% amino acid sequence identity to MTM1. In *C. elegans*, it has been shown that both *mtm-1* and *piki-1*, the class II PI3K ortholog in worms, regulate PtdIns(3)P levels on phagosomes and both single mutants have a strong cell corpse clearance (Ced) phenotype\(^2\). The defects in cell corpse clearance are rescued in the double mutant\(^2\). On the other hand, mutants of the class III PI3K-encoding *vps-34* had a mild Ced phenotype and *vps-34; mtm-1* doubles showed only a modest improvement of the severe *mtm-1* Ced phenotype\(^2\). In *Drosophila*, loss of *mtm* has been associated with defects in cortical remodeling, endocytic trafficking, and integrin signalling, all of which are rescued by loss of *Pi3K68D*, the class II PI3K ortholog in flies\(^29,30\). Meanwhile, loss of the class III PI3K *Vps34* only suppressed endolysosomal defects but not other defects associated with loss of *mtm*\(^29\). Similarly, it was shown that depletion of MTM1 in HeLa cell lines results in PtdIns(3)P accumulation and promotes pro-apoptotic signalling via decreased Akt phosphorylation, an effect that was suppressed by silencing *PIK3C2B* but not *VPS34*\(^31\). Taken together, these data support the idea that distinct pools of PtdIns(3)P are generated by class II and class III PI3Ks and assign major and minor roles for *PIK3C2B* and *VPS34*, respectively, in suppressing *MTM1* mutant phenotypes.

There are currently three murine models that closely mirror the skeletal muscle pathology found in the human disorder. This is unsurprising given the high degree of amino acid sequence identity between mouse and human proteins (92%). The first mouse model, described in 2002, is an exon 4 knock-out (KO) generated by Cre-Lox recombination which introduces a premature termination codon in *Mtm1*\(^32\). This KO allele results in the complete loss of MTM1 protein expression. The KO mice display many histological features in skeletal muscle found in the human form including the hallmark feature of centralized nuclei, decreased fiber size, and type I fiber predominance\(^32,33\). The skeletal muscle of these mice also has elevated PtdIns(3)P levels and various defects in the triads\(^33,34\). Of note, the KO mice have reduced muscle strength by various measures and have greatly reduced survival (median lifespan = 35 days) compared to WT littermates\(^32,33\). Of the three mouse models, this model has been the most extensively characterized in the literature, including by our lab. This model has provided important insights into a drug-targetable pathway for XLCNM therapy development. Of note, our lab has shown that knockout of *Pik3c2b*, a class II PI3K, rescues lethality of *Mtm1* mice\(^33\). Importantly, mice
with whole body knockout of Pik3c2b are phenotypically normal. Therefore, pharmacological inhibitors of PIK3C2B have the potential to be highly effective in the treatment of XLCNM.

A second model was generated in 2011 to model milder forms of XLCNM. It was generated by knock-in (KI) of a missense mutation in exon 4 to model a p.R69C change that is consistently found in mild cases of XLCNM (Figure 2). The KI mice also show elevated levels of PtdIns(3)P, centralized nuclei, muscle weakness, and decreased myofiber size (each measured at different time points over 1-5 months). The KI mouse has reduced survival (median lifespan = 66 weeks) compared to WT, but live far longer than the KO mouse. Unexpectedly, the missense mutation was found to result in the complete loss of MTM1 protein expression, with a KO mouse used as a positive control. The mutation was shown to cause aberrant splicing of the mRNA where exon 4 was excluded resulting in an out-of-frame transcript. Both protein and mRNA expression analyses were performed on quadriceps muscle, and it is possible that there is differential splicing and normal MTM1 expression in other muscle groups, explaining the milder phenotype. This model, along with the KO model, has been used to demonstrate that loss of Mtm1 causes structural abnormalities in the neuromuscular junction.

In early 2013, a group described a third mouse model that they generated by inserting a gene trap containing a 3’ splice acceptor site, selection cassette and polyadenylation signal in intron 1 upstream of the ATG site for Mtm1. The gene trapped allele results in loss of protein expression, elevated PtdIns(3)P levels, centralized nuclei, smaller muscle fibers, and muscle weakness. Using this model, Fetalvero and colleagues showed that loss of Mtm1 was associated with impaired autophagy, accumulation of polyubiquitinated proteins, and hyperactivation of mTORC1. They point out that this is inconsistent with elevated PtdIns(3)P which would be expected to promote autophagy through the recruitment of PIK3C3/VPS34 effectors such as Atg18/WIPI and DFCP1 to nascent autophagosomes. I think that this data suggests that MTM1 does not act on PtdIns(3)P generated by PIK3C3 in skeletal muscle. Indeed, this is supported by the absence of a positive genetic interaction between Pik3c3 and Mtm1 in either double KO (unpublished data from our lab) or Pik3c3\(^{3/4}\); Mtm1\(^{-/-}\) mice. Moreover, we found that Pik3c2b\(^{3/-}\); Mtm1\(^{-/-}\) mice are normal. Taken together, these data support the idea that MTM1 regulates distinct sub-pools of PtdIns(3)P in skeletal muscle as opposed to non-specifically dephosphorylating PtdIns(3)P wherever it is found in the cell.
The zebrafish *Danio rerio* has also been used to model XLCNM. The zebrafish protein shares 73% identity to human MTM1 by amino acid sequence. Our lab previously characterized an *mtm1* morphant phenotype in zebrafish and shown that it develops several features that are consistent with XLCNM. The *mtm1* morphant was generated by using translation-blocking or “ATG” morpholinos. These are proprietary antisense oligonucleotides (Gene Tools, LLC) that bind to mRNA to block protein production. Our lab found that morphants have impaired motility, abnormal skeletal muscle morphology, triad defects, and larval lethality. Importantly, several of these findings were reproduced independently by another group. Despite the observation that the *mtm1* knockdown model nicely recapitulated some of the phenotypes specific to XLCNM, a more recent study re-examined 24 morpholino knockdown phenotypes previously described in the literature and found that only five of those phenotypes were observed in their corresponding genetic mutants. Based on this observation, an *mtm1* mutant zebrafish would likely serve as a more reliable model of XLCNM than a morpholino-based model. This led us to generate a mutant *mtm1* zebrafish using zinc finger nucleases. The characterization of this mutant and its use in hypothesis-based and unbiased chemical screens forms the basis of my graduate work.

5 Pathogenic Mechanisms in XLCNM

Various pathogenic mechanisms underlying XLCNM have been proposed based on work carried out with these models. Structural abnormalities in both the neuromuscular junction and the triads have been shown to contribute to muscle weakness. While these represent structural or physiologic mechanisms underlying the disease, the molecular mechanisms by which these defects arise are not well understood. They may be directly related to the accumulation of PtdIns(3)P that results from loss of MTM1. Indeed, we recently showed that a class II PI3K, *Pik3c2b*, is a strong suppressor of *Mtm1* in mice, where double KO mice have normalized PtdIns(3)P levels and normal triad structure. Presumably, the NMJ in these mice were normal as well given that the double KO mice have equivalent grip strength and treadmill endurance to wildtype mice. Two additional lines of evidence suggest possible links between PtdIns(3)P and the triad. First, there is evidence that PtdIns(3)P is found at the sarcoplasmic reticulum (SR) in skeletal muscle and that MTM1 overexpression can change the physical shape of the SR. Second, MTM1 and BIN1 physically interact and display a synergistic role in promoting membrane tubulation, so it is possible that PtdIns(3)P regulation is involved in T-tubule
biogenesis and maintenance. Given that the SR and T-tubules make up the triad, I hypothesize that PtdIns(3)P and its protein effectors are involved in the formation and maintenance of triad structure and function. This leaves some major questions about the pathogenic mechanism due to PtdIns(3)P accumulation. What are the protein effectors of PtdIns(3)P that make its accumulation toxic? How many effectors are there? Is accumulation of PtdIns(3)P in a specific subcellular compartment, e.g., the sarcoplasmic reticulum, the primary cause of disease? Answers to these questions will shed important insight not only on XLCNM pathogenesis but also on other disorders caused by errors in phosphoinositide metabolism.

Interestingly, it was shown by Amoasii and colleagues that expressing phosphatase-dead MTM1 in Mtm1 KO mice improved muscle performance and histopathological signs of disease. The authors concluded that MTM1 has several important roles that are separate from its lipid phosphatase function. For example, a phosphatase-independent function of MTM1 the regulation of desmin and mitochondrial dynamics. It is important that we revisit these conclusions considering our finding that the genetic ablation of Pik3c2b fully rescues Mtm1 KO mice. Their findings suggest that loss of Pik3c2b would not rescue Mtm1 fully simply by lowering PtdIns(3)P, given that MTM1 has important phosphatase-independent functions. Meanwhile our findings strongly suggests that the loss of lipid phosphatase activity of MTM1 and accumulation of PtdIns(3)P is the primary cause of the Mtm1 KO phenotype. Indeed, it is very unlikely that PIK3C2B has both kinase-dependent and -independent functions that happen to suppress both the phosphatase-dependent and -independent functions of MTM1. It is more parsimonious to conclude that loss of PIK3C2B kinase activity rescues the loss of MTM1 phosphatase activity alone. For these two findings to be consistent would require an alternative conclusion for the partial rescue via overexpression of phosphatase-dead MTM1. One plausible explanation is that the phosphatase-dead MTM1C375S binds PtdIns(3)P in the cell, masking its accumulation and preventing binding of effector proteins. Indeed, the same group showed a year later that overexpression of MTM1C375S was able to bind to PtdIns(3)P without catalysis of the phosphate group. This masking effect, together with restoration of phosphatase-independent functions of MTM1, could together explain the rescue they saw. Importantly, this mechanism is consistent with suppression of Mtm1 null phenotypes by loss of Pik3c2b.

Another important insight into the pathogenic mechanism of XLCNM comes from identification of Dnm2 as a genetic suppressor of Mtm1 in mice. Based on the fact that DNM2 mutations are
associated with autosomal dominant form of CNM and DNM2 levels are elevated in Mtm1 KO mice, Cowling and colleagues hypothesized that these genes act in the same pathway and that reduction of DNM2 could suppress Mtm1\textsuperscript{40}. Complete loss of Dnm2 is embryonically lethal in mice, whereas heterozygous Dnm2\textsuperscript{+/−} mice, which express roughly half the normal DNM2 protein level, show no detectable phenotype\textsuperscript{40}. Cowling and colleagues showed that Dnm2\textsuperscript{+/−};Mtm1\textsuperscript{-/y} mice had a normal lifespan, muscle function, and triad ultrastructure\textsuperscript{40}. Around the same time it was demonstrated that DNM2 mutations found in autosomal dominant CNM are hypermorphic or gain-of-function\textsuperscript{41}. DNM2 encodes dynamin-2, a large GTPase that catalyzes the membrane fission step in clathrin-mediated endocytosis (CME)\textsuperscript{41}. Hypermorphic forms of dynamin-2 excessively fission membranes, resulting in T-tubule fragmentation and triad disorganization in skeletal muscle\textsuperscript{41}. Thus, in an Mtm1 mutant background, where we find elevated levels of DNM2, it is possible that the phenotype results from hyperactivity of DNM2. It is important to note that this mechanism of suppression likely does not involve a reduction of PtdIns(3)P, although this has not been tested. Is it possible, then, that DNM2 is a direct effector of PtdIns(3)P? DNM2 has a pleckstrin homology (PH) domain that is reported to bind to PtdIns(4,5)P\textsubscript{2} with low affinity but may bind other PIPs, and perhaps can bind PtdIns(3)P in vivo in the context of locally high concentrations of the lipid. This is highly speculative, but what is clear from this data that MTM1 and DNM2 act in a common pathway involved in the maintenance of specialized structures in skeletal muscle such as the T-tubule.

Importantly, it was shown that the Mtm1 KO mouse phenotype could be rescued after the onset of overt symptoms by genetically ablating Pik3c2b or Dnm2 with tamoxifen-inducible Cre lines\textsuperscript{33,40}. This indicates that muscle pathology in XLCNM is likely reversible and amenable to therapeutic intervention.

## 6 Therapy Development for XLCNM

There are currently no effective therapies available to treat XLCNM. The simplest solution, but certainly not the easiest, would be to stably re-introduce the MTM1 protein or gene in the muscle. Indeed, protein and gene replacement therapy approaches are currently the focus of most research efforts in the XLCNM field. Given that XLCNM is a monogenic disorder caused by mutation in a relatively small gene (MTM1 coding sequence is 3.5 kb including UTRs, 1.8 kb excluding UTRs), it is a great candidate for gene replacement therapy (GRT). The principle
behind GRT is to stably reintroduce a wild-type gene to tissues affected by endogenous mutation in that gene via viral vector or other means such as local injection. In a landmark study published in 2014, Childers and colleagues showed that GRT was highly efficient and successful in rescuing both murine and canine models of XLCNM. Specifically, they showed that a single dose of a muscle-trophic AAV2/8 vector carrying full-length wildtype MTM1 cDNA (cloned from either mouse or dog) under control of a muscle-specific desmin promoter was sufficient to provide significant, long-lasting rescue of the disease phenotype in both mice and dogs.

Gene therapy has tremendous potential to become a curative therapy for XLCNM. Proponents of this approach could argue that it would make other therapeutics obsolete once it is available to patients. A cure, after all, is the best possible therapy for a disease. However, there is still a strong rationale for identifying other therapeutic drugs. First, there could be a role for a therapeutic drug as either a primary or adjunct therapy prior to or while receiving GRT. It is even possible that a primary drug therapy could significantly improve patient quality of life to a degree that GRT is unnecessary. Secondly, it remains unknown if GRT will be as effective in humans as it has been in mice and dogs. Even if it will be effective, it could take several years of clinical trials before GRT reaches patients. Lastly, if and when GRT becomes available, its cost will likely be exorbitantly high as the drug maker seeks to recover its cost of development. This is true for most therapies developed for rare disorders. Indeed, for seven different rare disorders evaluated from 2008-2012, the annual treatment costs were all >$100,000 per patient (Canadian dollars), with two drugs costing >$1,000,000 annually per patient. High treatment costs like these can easily exceed public funding allocations, highlighting a very realistic problem for XLCNM patients seeking GRT in the future. Importantly, the cost analysis mentioned above includes drugs that need to be repeatedly administered, allowing drug companies to recover costs over multiple treatments. The question then becomes, “how will drug companies monetize single-dose curative therapies for rare diseases?” We might idealistically predict a cost similar to the one year cost of treatment for other rare diseases, e.g., $100,000. But we might more reasonably expect a drug company to maximize profit by setting the cost of a GRT therapy at the limit of public and private donor funding capability. Given these reasons, it is of utmost importance to pursue alternative, cost-effective treatment strategies for XLCNM.

A highly cost-effective means of treating rare diseases is through re-purposing drugs that are already on the market. Many of today’s approved drugs are affordable because their patents have
expired or they are used to treat conditions that are prevalent in the population. As such, our lab has taken a drug re-purposing approach to find potential therapies for XLCNM. Remarkably, the first attempt at taking this approach has been very promising. Our lab has previously shown that FDA-approved anticholinesterase inhibitors such as pyridostigmine can improve patient outcomes as well as phenotypes in MTMI disease models. In one case study, a boy with a genetically confirmed case of XLCNM (c.695A>G in MTMI corresponding to p.His232Arg change) was wheelchair dependent by age 13 and only able to walk a few steps with support\textsuperscript{10}. A year later, the patient was no longer able to bear weight, but saw a significant improvement when given pyridostigmine, regaining his ability to stand and to swim long distances without stopping\textsuperscript{10}. This was the first case of a positive drug treatment for XLCNM and raised the possibility that pyridostigmine treatment could be beneficial to all XLCNM patients. Further support for pyridostigmine as a potential therapy was shown by subsequent studies in our lab, carried out using both the KO and KI mice. It was shown that Mtm1 mutants have abnormalities in their neuromuscular junctions and their muscles fatigue more quickly\textsuperscript{11}. The mutant mice showed a significant improvement in grip strength and treadmill endurance with pyridostigmine treatment\textsuperscript{11}.

More recently, our lab has explored other potential drug targetable pathways as therapeutic avenues for XLCNM. Because MTM1 is a PtdIns(3)P phosphatase and that accumulation of PtdIns(3)P in skeletal muscle is a molecular hallmark of the disease, our lab hypothesized that an effective approach to the treatment of XLCNM could involve inhibiting the kinase(s) that generate PtdIns(3)P to restore PtdIns(3)P to normal levels. We tested this genetically by ablating Pik3c2b in Mtm1 KO mice and found this completely rescued the mutant phenotype\textsuperscript{33}. We have also seen that PIK3C2B appears to be a strong genetic suppressor of MTM1 mutations across species. Unfortunately, there are no safe and specific inhibitors available for PIK3C2B. The only PI3K inhibitors that are currently in clinical trials, to my knowledge, are specific to the class I kinases. Thus, although class II PI3K inhibition shows great promise as a therapy for XLCNM in the laboratory, it is outside the scope of our lab to develop a class II specific inhibitor as a therapeutic. However, I have tested the efficacy of available PIK3C2B inhibitors in the zebrafish mutant to determine whether this is a viable therapeutic strategy. An alternative approach that our lab is taking is to identify FDA-approved drugs that have inhibitory activity against PIK3C2B through \textit{in silico} and \textit{in vitro} based screens.
Another therapy development approach involves novel target identification through unbiased screens. As mentioned earlier, a yeast genetic screen for suppressors of ymr1; sjl2; sjl3 triple mutant lethality identified several genes that rescue the phenotype but do not reduce PtdIns(3)P accumulation. This was the first suggestion that a drug-targetable pathway that does not lower PtdIns(3)P levels could be a therapeutic strategy for XLCNM. Our lab demonstrated that this is possible with pyridostigmine, which improves muscle function through a physiologic mechanism in spite of elevated PtdIns(3)P levels. Additionally, Dnm2 was identified as a strong suppressor of Mtm1 in mice, indicating that multiple pathways are capable of suppressing MTM1. Thus, it is possible that additional pathways exist that have yet to be identified. To identify these pathways, one could perform chemical-genetic screens using an organism that has muscle, for example, *C. elegans*, *Drosophila*, or zebrafish. Given my primary interest in repurposing drugs as treatment for XLCNM, I have performed a chemical-genetic screen using an FDA-approved drug library and the zebrafish mtm1 mutant.

7 Function and Chemical Inhibitors of PIK3C2B

PIK3C2B is a member of the phosphatidylinositol 3-kinase (PI3K) family. It is one of three members belonging to the class II PI3Ks. Relatively little is known about the function of class II PI3Ks when compared to the well-defined roles of class I PI3Ks (e.g., PIK3CA/p110a) in canonical PI3K-Akt-mTOR signalling and the involvement of the sole class III PI3K, PIK3C3/VPS34, in regulating autophagy. It is known that PIK3C2B is capable of generating phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P$_2$] from phosphatidylinositol 4-phosphate [PtdIns(4)P] and PtdIns(3)P from PtdIns in *vitro*, although there is a strong preference for the latter reaction in *vivo*.

Despite being less well-characterized, PIK3C2B is rapidly emerging as a potential drug target for several diseases. Our lab has found evidence that PIK3C2B is an attractive therapeutic target for XLCNM. But PIK3C2B has also been shown to be overexpressed at the protein and mRNA level in several subtypes of cancers including acute myeloid leukemia, glioblastoma multiforme, medulloblastoma, neuroblastoma, and small cell lung cancer. Moreover, inhibiting PIK3C2B by siRNA reduced the proliferative capacity of many cancer cell lines and primary cancer cultures. PIK3C2B was also recently shown to regulate migration and invasiveness of prostate cancer cells. In addition to having a role in certain cancers, PIK3C2B has been linked to
insulin-resistant type 2 diabetes. Mice harbouring a kinase-dead version of PIK3C2B (produced by knock-in of a D1212A mutation disrupting the ATP-coordinating DFG motif) showed enhanced insulin sensitivity and glucose tolerance, and were protected from high-fat diet induced steatosis\textsuperscript{46}. This suggests that PIK3C2B inhibition could represent a new means to increase insulin sensitivity and treat diabetes.

PIK3C2B inhibition may have therapeutic value against XLCNM, certain cancer subtypes, and type 2 diabetes. This provides a strong rationale to design potent and specific PIK3C2B inhibitors. There are currently two groups that have described relatively specific inhibitors to class II PI3Ks. Freitag and colleagues developed a series of chemical modifications to the structure of a class I PI3K-specific inhibitor parent compound, XL147, and tested the derivatives against a panel of the 8 mammalian PI3Ks covering all three classes \textsuperscript{47}. They identified 3 compounds with greater inhibitory specificity towards PIK3C2B versus the remaining seven PI3K isoforms. However, the most potent inhibitor among them, compound 30, only achieved an IC\textsubscript{50} value of 2.71 µM for PIK3C2B\textsuperscript{47}. Boller and colleagues describe two molecules, PI701 and PI702, as having IC\textsubscript{50} values of 528 nM and 632 nM for PIK3C2B, respectively, while IC\textsubscript{50} values for class I PI3Ks were >10 µM\textsuperscript{44}.

These molecules have all the desired features of class II PI3K-specific inhibitors and would be excellent as research tools to determine the effect of small molecule PIK3C2B inhibition has on MTM1 phenotypes. Unfortunately, they were not commercially available during the completion of this project. But there are many other so-called \textit{pan}-PI3K inhibitors that inhibit all three classes of PI3K to some degree including wortmannin, LY294002, and PI-103 (Table 1). Of note, PI-103 has greater inhibitory activity towards PIK3C2B versus the class III kinase. To dissect the role of PIK3C2B using these small molecule inhibitors, I tested in parallel the effect of class I- and class III-specific inhibitors such as GDC0941 and VPS34-IN1, respectively (Table 1).

8 Summary

Based on the information gathered, the specific aims of my Master’s Thesis were: 1) to characterize the \textit{mtm1} mutant phenotype of zebrafish to facilitate small molecule screening, 2) to address the therapeutic potential of PIK3C2B inhibitors for XLCNM and 3) to identify novel potential therapies for this disorder through an unbiased drug screen.
Towards the first aim, another member of our lab generated a mutant \textit{mtm1} zebrafish using zinc finger nucleases. This mutant has an 8 bp deletion in exon 5 that introduces a premature termination codon which is predicted to encode an unstable truncated protein. This mutant allele will be referred to as Δ8 or used in the form of \textit{mtm1}^{Δ8/Δ8}. Importantly, the phosphatase active site conserved in all catalytically active members of the myotubularin family is encoded by exon 11 and is expected to be lost. Furthermore, nonsense and frameshift mutations in exon 5 of \textit{MTM1} have been identified in XLCNM (see Figure 2). This mutant allele produces a milder phenotype than observed by morpholino knockdown; however, the characterization of our \textit{mtm1} mutant shows phenotypic overlap in impaired motility and early lethality, and these phenotypes segregate with homozygosity for the 8 bp mutation in \textit{mtm1}. The data presented hereafter will highlight these and other features of the \textit{mtm1} mutant.

My second aim is based on the reasonable expectation that \textit{mtm1} and \textit{pik3c2b} have conserved roles in zebrafish given that loss-of-function mutations in class II PI3K orthologs found in \textit{C. elegans}, \textit{Drosophila}, and mice are able to suppress adverse phenotypes in \textit{MTM1} mutants. Additionally, drug screening can be performed using zebrafish disease models in a more time- and cost-effective manner than in mice without sacrificing the potential translational impact of the findings\textsuperscript{48,49}. Thus, I have tested whether \textit{pik3c2b} acts as a suppressor of \textit{mtm1} in zebrafish through morpholino knockdown of \textit{pik3c2b} and pharmacologic inhibition of PIK3C2B in the \textit{mtm1}^{Δ8/Δ8} background. A parallel approach was taken by other members of our lab to test one available PIK3C2B inhibitor directly in mice, but these data will not be presented here.

Finally, the third aim of my project was to perform an unbiased screen with \textit{mtm1} mutants to identify FDA-approved drugs that correct an \textit{mtm1}-specific phenotype. To my knowledge, this screen is the first of its kind using a model of XLCNM with the potential to have translational impact. The drugs identified in this screen that correct an \textit{mtm1} mutant phenotype will be validated further using the zebrafish and \textit{Mtm1} KO mouse models and may represent putative therapeutics for translation into patients with myotubular myopathy.
Chapter 2
Characterization of the \textit{mtm1} Mutant Phenotype in Zebrafish

I have characterized salient features of the \textit{mtm1}^{Δ8/Δ8} mutant phenotype including transcript expression, gross morphology, survival, motor behaviour, and muscle pathology of mutants, e.g., triad morphology, nuclei positioning, and PtdIns(3)P levels.

9 Transcript and Protein Levels in \textit{mtm1} Mutants

At the transcriptional level, RT-PCR revealed maternal deposition of \textit{mtm1} transcript, as it is detectable prior to the first wave of zygotic genome activation, which begins at the 64-cell stage\textsuperscript{50} (Figure 3A). The \textit{mtm1} mutant lacks an early embryonic phenotype (the earliest phenotype begins at 3 dpf; see below) which might be explained by compensation from wildtype maternal transcript. This may justify generation of a maternal-zygotic \textit{mtm1} mutant as maternal gene products are often important in early embryonic development\textsuperscript{51}. To test whether the premature termination codon flags \textit{mtm1} transcript for nonsense-mediated decay, I performed a comparative qPCR with three independent clutches of 7 dpf larvae (\(n = 35\) each). The beta actin gene, \textit{actb1}, was used as an endogenous control. There was no fold difference in the levels of \textit{mtm1} transcript between WT siblings and \textit{mtm1} mutants (Figure 3B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{A) RT-PCR of WT embryos with two different probes shows \textit{mtm1} transcript is maternally loaded as it is detectable prior to activation of the zygotic genome at the 64-cell stage. B) At 7dpf, quantitative real-time PCR shows no significant fold change between \textit{mtm1} mutants to WT siblings in \textit{mtm1} transcript levels relative to \(\beta\)-actin (\textit{actb1}) endogenous control. Results are shown for three independent biological replicates (Rep 1-3). Each sample comprised 35 larvae.}
\end{figure}
Anecdotally, our lab has noticed this is a common feature of zebrafish genes that contain premature termination codons. This may suggest that an alternative means of surveillance is taking place to protect from deleterious effects of a truncated protein, such as nonsense-associated alternative splicing or nonsense-mediated translational decay. However, the former scenario is unlikely given that I have performed RT-PCR spanning multiple exon-exon boundaries with \textit{mtm1} mutant cDNA and found there were no detectable alternative transcripts. Therefore, it remains possible that either an unstable, non-toxic truncated protein is made or nonsense-mediated translational decay prevents truncated protein formation.

To try to answer this question, I attempted to detect the effect of the mutation on MTM1 protein level by Western blot. However, there are no commercially available antibodies specific to the zebrafish MTM1. Our lab had three antibodies specific to mouse MTM1 in the lab and I found that the immunogens used to generate them were 68\%, 72\%, and 75\% identical by amino acid sequence to the same region in zebrafish MTM1. I thought it might be worthwhile to try them in case the structural epitopes were conserved in zebrafish. However, the antibodies bound non-specifically in zebrafish lysates and it could not be determined whether MTM1 was being detected.

10 Survival of \textit{mtm1} Mutants

I tracked the survival of whole clutches of embryos every day from 5 – 12 dpf. When fed live paramecia starting at 5 dpf, \textit{mtm1} mutants have a mean and median survival of 7 and 9 dpf, respectively, whereas WT siblings can live up to 2 years (Figure 4A). A Chi-square test of independence shows the proportion of mutants in a given clutch does not deviate significantly from the expected Mendelian ratio of 0.25 for a recessive trait (Figure 4B). This suggests that embryonic lethality is not a specific feature of \textit{mtm1} mutants and I could assume \textit{mtm1} mutants survive as well as their siblings until 5 dpf when I began tracking survival.
**Figure 4.** *mtm1* is essential for zebrafish development. Specifically, *mtm1* is required for larval survival. **A)** *mtm1* larvae have median and maximum survival of 7 and 9 days, respectively, while most WT siblings survive into adulthood ($n = 150$ per group; $P < 0.001$, Mantel-Cox test). **B)** The proportion of mutants in a given clutch does not deviate significantly from 0.25 ($P > 0.05$ for each clutch; Chi-square test of independence). Note: Content in this figure has been published in the *Journal of Clinical Investigation*: Sabha et al., 2016.

11 Non-Muscle Phenotypes of *mtm1* Mutants

Several additional aspects of the *mtm1* mutant phenotype were characterized and found to segregate with *mtm1* mutation. Mutants develop an enlarged, globular, and fatty liver (Figure 5). The phenotype becomes apparent in a few larvae around 5 dpf, though the majority exhibit the phenotype at 6 dpf. The phenotype is fully penetrant, with all mutants developing the phenotype by 7 dpf. Treatment with 0.2 mM phenylthiourea (PTU) starting at 1 dpf prevents pigment formation and makes the liver phenotype even more apparent as it becomes yellowish (Figure 5B). In addition to the liver phenotype, the gastrointestinal tract appears to be very simplified or underdeveloped in *mtm1* mutants. Interestingly, a similar liver and gastrointestinal phenotype is apparent in *cdipt* mutants. *cdipt* encodes an enzyme that catalyzes the addition of the myo-inositol headgroup onto a CDP-diacylglycerol backbone, generating PtdIns. Thus, *cdipt* is responsible for generating the precursor to all PIPs. The fact that both *mtm1* and *cdipt* mutations cause liver and intestinal defects suggests that PIP metabolism must be tightly regulated in these tissues in the larval stages of zebrafish development.
Figure 5. mtm1 mutant larvae develop a severe liver phenotype. A) At 7 dpf, mtm1 mutants have a distinctly discoloured liver (outlined in red). B) The liver is enlarged and amorphous (arrowhead). Note that these larvae were treated with 0.2 mM PTU from 1 dpf to depigment the larvae and better visualize the liver. This has the effect of making the liver appear yellowish in mtm1 mutants. C) The liver of mtm1 mutants weakly stains for Oil Red O, indicating the abnormal presence of neutral lipids in the liver. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

I also noticed that mtm1 mutants appeared smaller on average than their WT siblings. I confirmed this observation by quantifying their heights, lengths, and weights and found that mtm1 mutants were significantly smaller in all categories (Figure 6). These findings are consistent with both liver and gastrointestinal defects given that zebrafish are lecithotrophic for the first 5 days of life, meaning that their only source of nutrition is via the yolk. It suggests that mutants are defective in their processing or the uptake of the yolk which results in a smaller size. Heights and lengths were measured with built-in tools in cellSens software (Olympus). Weight was measured using a fine balance by pooling 35 larvae in a pre-weighed 1.5 mL tube, removing excess water with a capillary pipette, and re-weighing the tube containing the larvae. While the differences between wildtype and mutant fish with these phenotypes are statistically significant, the variability between individuals precludes using this phenotype as a robust readout for drug screens. Additionally, taking these measurements has very low throughput.
**Figure 6.** *mtm1* mutant larvae are significantly smaller than their WT siblings. **A, B)** From 3 to 5 dpf, the myotome length of *mtm1* larvae only increases by 3.34% ± 0.52% while the length of WT siblings increases by 8.80% ± 0.47% (***P* < 0.001). Similarly, the dorsoventral height of the *mtm1* myotome only increases by 3.24% ± 0.97% while that of WT siblings increases by 8.82% ± 0.87% (***P* < 0.001). *n* = 20 each; Student’s *t* test, 2-tailed. Error bars indicate SEM. **C)** At 7 dpf, *mtm1* larvae weigh significantly less than their WT siblings (**P* = 0.0011). Each data point represents *n* = 35 larvae weighed as a group. Pair-wise comparisons of the groups, indicated by lines, were made between WT siblings and *mtm1* mutants from the same parents to control for any influence of genetic heterogeneity on weight; paired *t* test, 2-tailed.

The *mtm1* mutant also has dysmorphic pectoral fins (Figure 7). Similar to the liver phenotype, a difference becomes apparent in some larvae at 5 dpf but most mutants develop a visible phenotype at 6 dpf. It is also fully penetrant by 7 dpf, though there is some variability in the appearance of the dysmorphic fins (Figure 7A). Interestingly, it was reported that morpholino knockdown of *pi4ka*, a type III phosphatidylinositol 4-kinase (PI4K) that generates PtdIns(4)P from PtdIns, significantly impairs pectoral fin development by 3 dpf53. At the same time, it was shown that PI3K inhibition by exposure to 10 µM LY294002 from 50% epiboly stage impaired pectoral fin development by 3 dpf53. Taken together, these data again suggest that PIP metabolism must be tightly regulated in the pectoral fin during larval stages of zebrafish development. Interestingly, MTM1 was recently and elegantly implicated in a PtdIns(3)P to PtdIns(4)P conversion mechanism in HeLa cells16 which makes it plausible that *mtm1* may interact with *pi4ka* in the pectoral fin, despite being a different PI4K with different substrate preference. However, it appears that *mtm1* regulates pectoral fin morphology later in development (beginning at 5 dpf) and so it may or may not interact with the PI4K or PI3K pathways in this tissue.
Figure 7. A) By 7 dpf, mtm1 larvae have outwardly kinked pectoral fins (white arrowheads). Less frequently they may appear stubby due to degeneration (turquoise arrowheads) or simply frayed (orange arrowheads). Note: all images were taken from the dorsal side of the larvae. Scale bars: 100 µm. B) At 7dpf, formalin-fixed, paraffin-embedded cross-sections stained with hematoxylin and eosin (H&E) show that the mtm1 mutant pectoral fin is highly abnormal histologically. Scale bars: 20 µm. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

The liver, small body size, and pectoral fin phenotypes were the first non-muscle phenotypes that I observed. One issue with these phenotypes is that they only become apparent at 5 dpf and they are not fully penetrant by 5 dpf. This precluded the collection of large numbers of mutants that would facilitate other forms of characterization at earlier stages because the genotype of putative mutants had to be confirmed by PCR. One possibility was to consider pursuing a transgenic method to easily identify the mtm1 homozygotes such as introducing a chromosomal fluorescent marker (or any other dominant marker) physically linked to the wild-type mtm1 allele in trans to the mtm1∆8 allele. In this way, mtm1+/+ and mtm1+/∆8 would be fluorescent and distinct from non-fluorescent, mtm1Δ8/Δ8 mutants. I attempted to find a marker that mapped close to the mtm1 locus; however, the locations for the vast majority of transgenic insertions in zebrafish have not been mapped, which meant I had to take another approach.

Another suggestion was to determine which phenotypes segregate with the mutant allele given the incomplete penetrance of visible phenotypes in the mutants. I addressed this by genotyping whole clutches and correlating phenotypes with their genotype. This is how I found that the abnormal liver and pectoral fin phenotypes always segregated with mtm1 mutation. But during
this process I observed an overt phenotype that segregated completely in the mutants that had previously gone unnoticed: the larval fin folds of *mtm1* mutants progressively degenerate starting at 3 dpf (Figure 8). There is a spectrum of severity in fin degeneration between clutches, i.e., different parents (Figure 8B), and between individual mutants (Figure 8C). This variability is possibly due to the presence of different genetic modifiers in the background, or perhaps reflects differences in the amount of maternally loaded WT protein and transcript. In any case, I developed a 3-point ordinal scale to rank the severity of fin degeneration that includes mild, moderate, and severe categories. This would allow me to perform statistical analysis using nonparametric tests where all “mild” mutants were counted as 1, “moderate” as 2, and “severe” as 3 to generate an average rank score. In cases where “WT-like” larvae were included, they were given a rank score of 0.

Figure 8. A) Bright-field of WT siblings compared with *mtm1* larvae at 4 dpf illustrating complete loss of the anterior and posterior ventral fin folds (white arrowhead indicates urogenital opening; scale bar: 200 µm). This represents a “severe” mutant phenotype. B) The severity of *mtm1* mutants is variable between different clutches, i.e., different parents. C) Bright-field images tracking the fin morphology of individual WT and *mtm1* larvae from 3 dpf to 5 dpf (scale bar = 200 µm). As shown, *mtm1* fin folds progressively degenerate and the extent of
Degeneration is variable between individuals. The severity of fin degeneration is given an ordinal score from "mild" to "severe" depending on the degree of fin loss. A mild phenotype is scored when the avff and pvff are incompletely lost on either side of the presumptive cloaca (white arrowhead) while the cf and dff remain intact. Moderate is defined as the complete loss of the avff and partial loss of the pvff while the cf and dff remain intact. Severe mutants have lost the avff, pvff, and significant portions of the cf and dff. Abbreviations: avff=anterior ventral fin fold; pvff=posterior ventral fin fold; dff=dorsal fin fold; cf=caudal fin. Note: Content in this figure has been published in the *Journal of Clinical Investigation*: Sabha et al., 2016.

This overt phenotype was surprising given that it was a phenotype that wasn't previously reported in F₃ ENU mutagenesis screens for fin mutants. It became clearer after reviewing the selection methods used in those screens. The F₃ embryos were categorized into four broad phenotypic classes of mutants and the entire class of mutants with general abnormalities, i.e. degeneration, retardation, or necrosis, were discarded. Moreover, mutants with fin necrosis were discarded if they failed to develop a swim bladder. Given that many mtm1Δ⁸/Δ⁸ mutants fail to inflate their swim bladders (albeit for unknown reasons), it is possible that any mtm1 mutants with fin necrosis would have been categorized in the general abnormality class. Another possibility is that any mutations in mtm1 produced through ENU mutagenesis led to mild reduction of function which produce a more subtle, later onset phenotype. For fin-specific mutants, F₃ embryos were screened at 48 and 60 hpf, before the onset of the mtm1 mutant fin phenotype.

Given that this phenotype is the earliest observable phenotype, it lent itself to performing an RNA rescue experiment to confirm the specificity of the phenotype to the mtm1 mutation. Briefly, WT mtm1-GFP mRNA was prepared at approximately 66.7 ng/µl in sterile water with phenol red and a total of 200 pg mRNA was injected into 1-cell stage embryos. At 1 dpf, larvae were dechorionated and sorted into GFP(+) and GFP(−) groups. At 4 dpf, larvae were anaesthetized with tricaine and severity of fin degeneration scored on the 3-point ordinal scale. I found that the majority of mtm1 mutants injected with WT mtm1-GFP RNA had WT-like or very mild fin phenotypes (Figure 9). This confirmed that the fin degeneration is caused by hypomorphic or loss of function mutation in mtm1.
Figure 9. Expression of mtm1+::EGFP RNA prevents or ameliorates fin degeneration in mtm1 mutants at 4 dpf. A) Proportions of mtm1 larvae with a fin fold phenotype. The majority of mutants that were not injected (n=21) had moderate to severe fin degeneration where the avff is lost and significant portions of the pvff, cf, and df are lost. In contrast, the majority of mtm1 mutants injected with mtm1::EGFP RNA had WT-like or mild fin phenotypes where the fin folds remain intact. B) Representative bright-field images of genotyped mtm1 mutants from each group showing their phenotype (white arrowhead indicates the cloaca; scale bar = 200 μm). Note that mild mutants that were not injected often had partial loss of the avff. Abbreviations: avff=anterior ventral fin fold; pvff=posterior ventral fin fold; dff=dorsal fin fold; cf=caudal fin. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

Fin degeneration was not previously described in mtm1 morpholino knockdown experiments, as the authors were interested in muscle-specific phenotypes. I was interested in whether mtm1 knockdown would produce a similar phenotype as seen in the mutant. I repeated the mtm1 knockdown by injecting the yolks of wild-type embryos at the 1-cell stage with 1.5 nL of 0.6 mM (~7.5 ng) of a splice acceptor morpholino targeting exon 3 (5'-CAATGTTCCGTGTGTGTGACAGG-3'). This morpholino is predicted to introduce an early stop codon through exclusion of exon 3 and produce a truncated, non-functional protein. Notably, morpholino knockdown of mtm1 phenocopies the mtm1 mutant (Figure 10). A morpholino designed to a random sequence (5'-CCTCTACACCTCAGTTACATTTATA-3') with no homology to other genes in the zebrafish genome was used as a control (Gene Tools, LLC).
Figure 10. Morpholino knockdown of mtm1 phenocopies the fin fold degeneration seen in mtm mutants. A) Proportions of WT larvae with a fin fold phenotype at 4 dpf after injection with 7.5 ng of either control (n=40) or mtm1 exon 3-splice acceptor (Ex3-SA; n=100) morpholino. B) DIC images focused near the presumptive cloaca (white arrow) at 3 dpf show that mtm1 mutants and mtm1 morphants have similar morphology at the beginning of ventral fin fold degeneration. Both mutant and morphant fins have visible clusters (white arrowheads) and depressions (black arrowheads). Note: DIC images were taken at the same magnification. Abbreviations: avff=anterior ventral fin fold; pvff=posterior ventral fin fold. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

I was next interested in whether the severity of the fin phenotype was regulated by external force, such as mechanical stress on the fin. To create mechanical stress, larvae were placed in 40 mL system water with the indicated chemical in a 50 mL conical tube on a rotating shaker from 1-4 dpf. When mtm1 mutants were grown under mechanical stress conditions, it significantly exacerbated the fin degeneration (Figure 11). In contrast, immobilizing the larvae by inhibiting skeletal muscle myosin II with N-benzyl-p-toluene sulphonamide (BTS) lessens the severity of degeneration (Figure 11).
Figure 11. The mtm1 fin fold phenotype is influenced by mechanical stress on the fin. Immobilizing mtm1 larvae with 20 µM N-benzyl-p-toluene sulphonamide (BTS) starting from 1 dpf tends to decrease the severity of the fin fold phenotype at 4 dpf ($P = 0.1122$) compared to mtm1 grown in 0.2% DMSO alone. In fact, there were some mtm1 mutants with a WT-like phenotype that were identified by PCR genotyping. In contrast, fin degeneration was significantly more severe when mtm1 larvae were grown in mechanical stress conditions (***$P < 0.001$). From left to right: $n = 50, 47, 41$; Kruskal-Wallis rank test followed by Dunn’s post-test.

12 Muscle Phenotypes of mtm1 Mutants

Given the conserved role of mtm1 in skeletal muscle function, I wanted to assess whether mtm1 mutants have motor defects. All motor behavioural analysis was performed using Zebrabox software (ViewPoint, France). This motion-tracking software detects the movement of dark objects, i.e., larvae against a white background. The software also allows you to set speed thresholds to focus on subjects that are moving predominantly within a given range. However, in all of my experiments using the Zebrabox I did not use thresholds and instead captured and analyzed all movements of larvae.

In my initial attempts to characterize the natural or spontaneous motor behaviour of zebrafish larvae, I found that there was considerable well-to-well and plate-to-plate variability. Well-to-well variability was most likely due to different states of arousal of individual larvae. This could be overcome by tracking the movements of larvae for longer intervals, e.g., >1 hour. Plate-to-plate variability was encountered when tracking the spontaneous activity of fish at different times of the day. Hence, I did my best to track spontaneous activity within at a set time frame (3-6 PM). I found in a 1 hour trial the spontaneous swimming behaviour is impaired in mtm1 mutants (Figure 12). This finding was reproducible on separate days in different plate formats.
Figure 12. The *mtm1* mutant shows reduced spontaneous activity over a 1 hour motion tracking period. **A)** The *mtm1* mutants spent 16.95 min ± 2.06 min swimming versus 26.43 min ± 2.03 min spent by WT siblings (**P = 0.0015**). **B)** Likewise, mutants covered significantly less distance over 1 hour, traveling only 82.84 cm ± 11.36 cm whereas WT siblings travelled an average of 150.2 cm ± 12.5 cm (**P < 0.001**). Each data point indicates an individual larva. *n* = 40 per group; Student’s *t* test, 2-tailed. Error bars indicate SEM.

While the differences were statistically significant, I wanted to minimize the time required and reduce the plate-to-plate variability. I hypothesized that if larvae were coaxed to move at the same time with the same stimuli, then a less variable and more robust difference between the WT and mutant response would be detectable. I addressed the variability by using optovin to photochemically induce the larvae to move when presented with light stimuli\(^5^6\). Photoactivation by optovin or its analogs is mediated by an ion channel, TRPA1b, expressed in sensory neurons in the fish\(^5^6\). Activation of sensory neurons presumably activates skeletal muscle through a reflex arc. After working with optovin and two analogs, I found optovin analog-6b8 had the most favourable properties as it works with acute treatment. Briefly, larvae are incubated in chemical at 28.5°C for 5 minutes. Care is taken to protect the plate from light. Then larvae are exposed to timed pulses of white light and their movement is captured using the Zebrabox tracking system.

Using this system, I found that the *mtm1* mutants also show impaired motor behaviour in their response to photoactivation by optovin-6b8 at 7 dpf (Figure 13). I also observed significant motor defects in larvae as early as 5 dpf. Importantly, using this optogenetic tool can detect motility defects in just 30 seconds compared to >1 hour required when tracking spontaneous activity. This greatly increased the throughput of my subsequent experiments using motility as an endpoint assay.
Figure 13. The mtm1 mutant has significantly impaired motor function compared to WT siblings. A) In a 30-second optovin-induced photoactivation period performed at 7 dpf, mutants only traveled an average of 69.06 mm ± 3.94 mm, about half (55%) the distance of 125.0 mm ± 2.83 mm covered by their siblings (***P < 0.001). n = 48 each; Student’s t test, 2-tailed. Error bars indicate SEM. B) Photoactivation is induced with a pulse of white light and 10 μM optovin analog 6b8, a photoactive TRPA1b agonist thought to elicit movement by depolarizing sensory neurons in the fish which initiates a reflex arc to the skeletal muscle. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

I characterized the muscle of mtm1 mutants further through several additional experiments. Birefringence is a technique that is based on the refraction pattern of polarized light. In zebrafish models of muscular dystrophy like the dmd mutant, muscle fibers detach from myosepta and the birefringence appears dark or patchy and less bright in some myotomes. To measure birefringence, I mounted tricaine-anaesthetized 7 dpf larvae in 3% methylcellulose on glass slides and imaged them under polarized light on a dissecting microscope. The muscle integrity and/or sarcomere organization of mtm1 mutants is normal as shown by a normal birefringence pattern (Figure 14).

Figure 14. At 7 dpf, the mtm1 mutant has a normal birefringence pattern indistinguishable from WT siblings, indicative of normal sarcomere organization. Representative images for each are shown.
Despite the normal macroscopic appearance of muscle, I wanted to examine the ultrastructure of the muscle by electron microscopy given that triad defects are a pathologic feature of X-linked centronuclear myopathy (XLCNM). To do this I anaesthetized 5 dpf larvae in tricaine and fixed them in Karnovsky’s fixative overnight at 4°C. Then I sent the samples to the Advanced Bioimaging Center (Mount Sinai, Toronto, Canada) where larvae were processed. Briefly, they were rinsed in buffer, post-fixed in 1% osmium tetroxide in buffer, dehydrated, and embedded in Quetol-Spur resin. Then 90 nm sections thick were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with an FEI Tecnai 20 transmission electron microscope. Electron micrographs revealed abnormalities in mtm1 muscle at the ultrastructural level that are consistent with those found in XLCNM. These features included disorganized triads, longitudinal-tubules, and fragmented T-tubules (Figure 15). These ultrastructural findings are consistent with the observed motor behaviour defects.

Figure 15. At 5 dpf, electron micrographs show aberrant skeletal muscle ultrastructure in mtm1 larvae (scale bars: 500 nm; inset: 100 nm). WT larvae have normal triad structure where T-tubules (inset; black arrow) are apposed by terminal cisternae/SR (inset; white arrows), whereas mtm1 muscle has L-tubules (black arrowhead), triads lacking SR (white arrowhead), and fragmented T-tubules (inset; black arrows). Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

I also examined the skeletal muscle for central nuclei, the histopathologic hallmark of XLCNM. Briefly, larvae were anaesthetized in cold tricaine and fixed in 10% neutral buffered formalin overnight at 4°C. The next day, formalin was replaced with 70% ethanol and sent to be paraffin-embedded at the pathology lab at SickKids. Finally, cross-sections were stained with hematoxylin and eosin. Larvae needed to be oriented for cross-section to see the position of nuclei within myofibers. At the histological level, I found that skeletal muscle from both WT siblings and mtm1 mutants contain centrally located nuclei at 7 dpf (Figure 16). If the mtm1
mutants lived longer than 7 dpf, we could compare them to wildtype at a later stage in development where we expect the nuclei of wildtype muscle to be found peripherally. Indeed, in the Bio-Atlas database curated by Pennsylvania State University, cross-sections from wildtype larvae show exclusively peripheral nuclei after 21 dpf. Thus, perhaps central nuclei would be a feature of a skeletal muscle-specific \textit{mtm1} knockout zebrafish that lives beyond 7 dpf.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{At 7 dpf, formalin-fixed, paraffin-embedded cross-sections stained with hematoxylin and eosin (H&E) show that both \textit{mtm1} mutants and WT siblings have central nuclei. Scale bars: 20 μM; green arrows indicate a central nucleus.}
\end{figure}

Lastly, I attempted to detect changes in PtdIns(3)P levels in the \textit{mtm1} mutant. First, I took an immunofluorescence approach using an anti-PtdIns(3)P antibody in isolated myofibers as previously performed. Briefly, myofibers were isolated by collagenase II digestion of either 5 or 6 dpf larvae, plated on poly-L-lysine coated glass coverslips, and fixed with 4% paraformaldehyde. Next they were blocked and then treated with primary and secondary antibodies. Mouse anti-PtdIns(3)P antibody (Echelon Biosciences) was used at 1:250 dilution. In the end, I found there were considerable problems with this approach. First, the signal did not localize to intracellular vesicles as I would expect for PtdIns(3)P (Figure 17A). Second, there was a lot of background fluorescence that was not present in samples treated with secondary
antibody only, suggesting that there was primary antibody that had precipitated or was binding non-specifically. Due to these issues, I took an alternative strategy to assess changes in this phosphoinositide. I tried injecting DNA constructs containing a specific PtdIns(3)P reporter, 4xFYVE<sup>Hrs</sup>-GFP, into one-cell stage embryos. Injection of 20 pg of the construct reliably produced mosaic animals with GFP(+) myofibers. At 1 dpf, embryos were placed in 0.2 mM phenylthiourea to prevent the formation of pigment. At 2 dpf, I selected GFP(+) mosaic embryos that showed expression in their muscles for analysis by confocal microscopy. Taking this approach, I found PtdIns(3)P-positive vesicles in myofibers as one would expect (Figure 17B). Qualitatively, I noticed that some mtm1 mutant fibers have larger GFP(+) vesicles while others have very small GFP(+) vesicles, consistent with disregulation of PtdIns(3)P metabolism. However, a larger number of animals and myofibers will need to be assessed and quantified before any definitive conclusions can be drawn.

![Figure 17. A) Isolated myofibers from 7 dpf larvae stained with an anti-PtdIns(3)P antibody. The signal produced was very punctate and did not localize to intracellular vesicles as expected. Myofibers were counterstained with DAPI to visualize nuclei. B) Live myofibers in 2 dpf embryos expressing 4xFYVE<sup>Hrs</sup>-GFP show the presence of intracellular vesicles. Images are not representative of all fibers captured but are included to show that this method could be a reliable method for assessing changes in PtdIns(3)P metabolism.](image-url)
way to investigate PtdIns(3)P dynamics in live embryos. Images on the left represent a single section from a z-stack. Images on the right are 3D volume views of the same fiber where they are pseudocoloured blue and yellow to help visualize depth of the fiber.
Chapter 3
Investigating the Interaction of *mtm1* and *pik3c2b* in Zebrafish

Given the conserved role of PIK3C2B as a suppressor of MTM1 phenotypes across species, PIK3C2B inhibition may represent a viable therapeutic strategy for XLCNM. I tested whether *pik3c2b* is a suppressor of *mtm1* in zebrafish by morpholino knockdown and CRISPR/Cas9-mediated mutation of *pik3c2b*. The effect of *pik3c2b* perturbation has on *mtm1* mutants were assessed using phenotypic readouts described in Chapter 2.

13 Genetic Manipulation of *pik3c2b* in *mtm1* Mutants

The effects of *pik3c2b* morpholino knockdown were first characterized in wildtype fish before testing the effect in *mtm1* homozygotes. I designed a splice donor morpholino targeting exon 24 (Ex24-SD; 5'-AGCTCTTTTCAGTGCTCCTACCGTTT-3') of *pik3c2b*. This morpholino is designed to disrupt RNA processing by promoting aberrant mis-splicing or complete exclusion of the targeted exon. In particular, the purpose of targeting exon 24 is to perturb the kinase domain via mis-splicing or exclusion of the PI3K catalytic kinase domain. The Ex24-SD morpholino did not cause complete exclusion of exon 24 which would have resulted in a band ~170 bp smaller (Figure 18A). Rather, sequencing of the mis-spliced product from Ex24 morpholino-injected animals revealed that 43 bp of exon 24 were excluded from the transcript which causes a frameshift and loss of the kinase domain (Figure 18B).
I then demonstrated that this morpholino disrupts *pik3c2b* RNA processing in a time- and dose-dependent manner by RT-PCR of morpholino-injected embryos (Figure 19A). Through this process I noticed that morphants injected with 4 ng Ex24-SD MO are phenotypically normal while still disrupting RNA processing. Hence, I injected *mtm1* mutants with 3 nL of 0.16 mM (~4 ng) of Ex24-SD morpholino into the yolk at the 1-cell stage and found that knockdown of *pik3c2b* significantly improves fin severity in *mtm1* mutants at 4 dpf compared to mutants injected with control morpholino (Figure 19B).
Figure 19. Knockdown of pik3c2b by morpholino (MO) injection is time- and dose-dependent and ameliorates the mtm1 mutant fin phenotype. A) RT-PCR of appropriate regions of pik3c2b shows that when WT embryos are injected with 4 ng of Ex24-SD morpholino there are two bands visible where the slightly larger WT transcript only begins to reappear at 3 dpf. Injection with 4 ng Ex24-SD results in qualitatively decreased abundance of transcript until 3 dpf when WT transcript is detectable. B) Knockdown of pik3c2b significantly lessens the severity of fin fold degeneration in 4 dpf mtm1 mutants (*P<0.0403). Control MO, n=46; pik3c2b MO, n=38; Kruskal-Wallis test, followed by Mann-Whitney U test. Note: Content in this figure has been published in the Journal of Clinical Investigation: Sabha et al., 2016.

For CRISPR/Cas9-targeted mutation of pik3c2b, I designed guide RNAs (gRNA) based on the principles described by Jao and colleagues that targets the ATP-coordinating DFG motif of PIK3C2B which is found in a highly conserved region of the PI3K kinase domain encoded by exon 24(Figure 20A). In mice, mutating the aspartic acid residue of the DFG motif to an alanine abolishes lipid kinase activity. One gRNA efficiently mediated Cas9 cutting at the site as shown by restriction fragment length polymorphism (RFLP) analysis of F1 adults (Figure 20B). I designed my RFLP protocol with a restriction enzyme that cuts at two sites (XcmI) so that any residual undigested wildtype band would not be confused with a digestion-resistant mutant allele. I sequenced XcmI resistant bands to categorize mutations. Using this method, I successfully screened through F0 and F1 lines to isolate eight mutations of interest (Table 2).
Figure 20. A) The PI3K domain of pik3c2b and its orthologs is highly conserved evolutionarily. I targeted the ATP-coordinating DFG motif (shown in orange) that is highly conserved and was compatible with CRISPR/gRNA design criteria. B) RFLP analysis of F₁ adult fin clips. As shown, XcmI digestion cuts wildtype amplicons into three fragments (a 70 bp band is very faint). In fish where the CRISPR/Cas9 mutated the XcmI site at the gRNA site, the 70 bp combines with the 270 bp band to produce a fourth 340 bp band (indicated by white asterisks). The XcmI resistant 340 bp band was used for sequencing to categorize the pik3c2b mutants.

F₁ mutant heterozygotes were outcrossed to WT and then F₂ carriers were outcrossed to mtm1 lines. I focused on the 6 bp substitution allele (hereafter referred to as a6) given that it results in in-frame missense changes that disrupt the DFG motif. Specifically, substitution changes the “IDFG” amino acid sequence in wildtype to “KFLG”. This mutation would be expected to abolish kinase activity. The effect of this mutation on protein level was not determined. If protein stability was unaffected, this allele could be expected to mimic the effect of pharmacologic inhibition of PIK3C2B. For this reason, I generated F₃ pik3c2b<sup>a6/a6</sup>; mtm1<sup>+/Δ8</sup> adults. To characterize the fin phenotype of pik3c2b<sup>a6/a6</sup>; mtm1<sup>+/Δ8</sup> double mutants, I crossed pik3c2b<sup>a6/a6</sup>; mtm1<sup>+/Δ8</sup> adults to pik3c2b<sup>+/a6</sup>; mtm1<sup>+/Δ8</sup> adults and at 4 dpf identified the mtm1 mutants by their fin phenotype. Had the double mutants showed no phenotype, my alternative approach would have been to genotype all WT-like larvae for the mtm1 mutation. I scored each on the 3-point ordinal scale and subsequently genotyped each for the pik3c2b a6 allele. This allowed me to have an internal control where half of the mtm1 mutants were heterozygous for the
pik3c2b mutation. Taking this approach, I found that there was no significant difference between the two groups (Figure 21). However, the double mutants have more mild (25% vs. 18.8%), fewer moderate (53.1% vs. 57.9%) and fewer severe (21.8% vs. 23.1%) phenotypes. This trend was observed in four independent clutches, i.e., in each clutch there were more mild mtm1 phenotypes in a pik3c2b homozygous mutant versus heterozygous background. I conclude that this represents a weak genetic interaction in the fin that is difficult to detect without a very large sample size.

![Figure 21](image)

**Figure 21.** Double mutants analysis shows that mutation of the DFG motif in pik3c2b does not significantly affect the severity of fin fold degeneration in 4 dpf mtm1 mutants ($P=0.1817$). pik3c2b$^{+/\alpha6}$; mtm1$^{\Delta8/\Delta8}$, $n=138$; pik3c2b$^{\alpha6/\alpha6}$; mtm1$^{\Delta8/\Delta8}$, $n=128$; Kruskal-Wallis test, followed by Mann-Whitney U test.

### 14 Pharmacologic Inhibition of PIK3C2B in mtm1 Mutants

Until recently, highly specific inhibitors of PIK3C2B had not been described in the literature. Dr. Stefan Laufer's group took a medicinal chemistry approach to modify the pan class I PI3K inhibitor, XL147, and characterized several analogs with high specificity towards PIK3C2B$^{47}$. Some of these were then shown to test negative for inhibitory activity against 60 additional kinases, underscoring the high specificity of these chemicals for PIK3C2B$^{47}$. However, at the time of these experiments we were unable to acquire any of these chemicals.

Instead, to pharmacologically inhibit PIK3C2B, I used three isoform non-specific, commercially available inhibitors of PIK3C2B: LY294002, wortmannin, and PI-103. To isolate PIK3C2B as the variable, I also tested a class I specific inhibitor, GDC-0941, and a class III specific inhibitor, VPS34-IN1. The inhibitory profile of each chemical towards each PI3K is provided in (Table 1). Chemical stocks were prepared in DMSO and added to 0.1% or 0.2% of final volume to egg
water to prepare working concentrations. Note that methylene blue was not added to the egg water. Groups of approximately 50 embryos (1 dpf) were distributed into sterile 10-cm tissue culture dishes (Corning) containing 40 mL of egg water plus chemical. Every day, 50% of the egg water was discarded and replaced with freshly prepared egg water plus chemical. Culture dishes were wrapped in aluminum foil and incubated at 28.5°C. Using this method, I showed that the chemicals that inhibit PIK3C2B improve several *mtm1* phenotypes. PIK3C2B inhibition improves fin severity, ameliorates motor function, and increases lifespan of *mtm1* mutants (Figure 22).

Of note, there appears to be a small effect of inhibiting the class III PI3K with VPS34-IN1 in improving the motor function of *mtm1* larvae. Importantly, given that there is only evidence of a weak genetic interaction between *mtm1* and *pik3c2b* in the larval fin fold, the large effect size seen after treatment with these chemical inhibitors most likely represents synergistic effects of inhibiting multiple PI3Ks. To rule out this possibility, one could quantify these phenotypes in single, double, and triple mutants of *pik3c2b, vps34*, and *pik3ca* in an *mtm1* mutant background.

**Figure 22.** Treatment with pan-PI3K inhibitors improves the *mtm1* zebrafish phenotype. A) Severity of fin fold phenotype in 4 dpf *mtm1* mutants is significantly improved after treatment with 5 µM LY294002, 50 nM wortmannin, or 250 nM PI-103 (**P < 0.001), but not by inhibition of class III PI3K with 500 nM VPS34-IN1 (P = 0.4955) or class I PI3Ks with 500 nM GDC-0941 (P = 0.3494). From left to right: n = 92, 110, 108, 97, 92, 94; Kruskal-Wallis test, followed by Dunn’s post-test. B) At 7 dpf, the mean distance traveled in a 30-second optovin-
induced photoactivation period by mtm1 larvae treated with DMSO alone is 57% that of WT siblings (***$P < 0.001$). Class I inhibition with GDC-0941 does not improve mtm1 motor function (45% WT, ***$P < 0.001$). In contrast, mtm1 motor function is improved relative to cognate-treated WT siblings by treatment with LY294002 (74% WT, **$P = 0.0059$), wortmannin (70% WT, **$P = 0.0012$), and, surprisingly, VPS34-IN1 (73% WT, *$P = 0.0155$). Though these groups remained significantly different from WT, this improvement suggests an mtm1-specific chemical effect for this phenotype. Notably, mutants treated with PI-103 are statistically indistinguishable from PI-103–treated WT siblings (80% WT) and move significantly better than DMSO-treated mtm1 (*$P < 0.0171$). 

C) Kaplan-Meier survival curve showing that treatment with LY294002, wortmannin, and PI-103 improves median survival from 8 to 9 days and maximum survival from 9 to 11 to 12 days in comparison with DMSO-treated mtm1 larvae ($P < 0.001$ compared with mtm1 DMSO, Mantel-Cox or Gehan-Breslow-Wilcoxon tests). Inset legend top to bottom: $n = 132, 43, 46, 47, 44$. Note: Content in this figure has been published in the *Journal of Clinical Investigation*: Sabha et al., 2016.33

Importantly, multiple clutches were required to obtain a mutant sample size large enough for chemical screens because the distribution in phenotype severity of mtm1 mutants is variable between different clutches (Figure 8). For this reason, it was ensured that each treatment group received equal proportions of embryos from a given clutch. The number of embryos used from a single clutch was no fewer than 16 for each group, usually ranging between 30-40. For example, clutches of at least 96 embryos from a heterozygote in-cross were required for testing 6 different chemicals, where each treatment group would receive 16 embryos at random. Of the 16, 4 embryos were expected to be mutants with a similar phenotypic distribution. Continuing with this example, to achieve a mutant sample size of 40, 16 embryos would be taken at random from 10 clutches of 96 embryos and pooled together in 6 different groups ($n = 160$ total/group). This method ensured that the phenotypic distribution of each group was consistent prior to chemical treatment and changes in population distribution due to chemical effect could be detected.

In addition to these inhibitors, we received a compound from AstraZeneca that was found to have specific inhibitory activity towards PIK3C2B versus other PI3Ks. I performed a dose response curve against mtm1 fin degeneration and found that it had no effect on the fin phenotype (Figure 23). One possibility is that the compound precipitated. Indeed, in a separate set of experiments with this chemical I found that it precipitated from solution when added to aqueous solution. However, precipitation occurred when I was preparing a higher concentration than in my previous experiments. Another possible interpretation for this result is that pharmacologic PIK3C2B inhibition alone is insufficient to improve the fin phenotype. Indeed, the effect size of PIK3C2B knockdown, while significant, was quite small (Figure 19) and there
was a statistically undetectable difference in \textit{mtm1}; \textit{pik3c2b} double mutants. Given that the commercially available PI3K inhibitors that inhibit multiple PI3K isoforms significantly improved the phenotype with a larger effect, we might conclude that inhibition of PIK3C2B along with other PI3Ks has the greatest effect in improving the severity of \textit{mtm1} fin degeneration.

\textbf{Figure 23.} Putative PIK3C2B-specific chemical inhibitor does not improve \textit{mtm1} fin degeneration. \textbf{A)} There was no significant difference in fin severity distribution after chemical treatment. \textbf{B)} As a quality control, there was no significant difference found in the distribution of siblings and mutants between plates. Kruskal-Wallis test, followed by Dunn’s post-test.

Our lab has also collaborated with a lab that performed an \textit{in silico} screen of FDA-approved drugs to predict molecules that could bind the catalytic pocket of the PIK3C2B kinase domain. I have performed dose response testing of several of the top hits against \textit{mtm1} fin degeneration and found none that significantly ameliorate the fin phenotype (Figure 24). Two possible interpretations include that either the \textit{in silico} prediction failed to identify inhibitors of PIK3C2B or that there are PIK3C2B-independent effects of the drugs that concomitantly worsen the \textit{mtm1} phenotype, masking any improvement from PIK3C2B inhibition. Other possibilities include poor aqueous solubility of the drugs, poor uptake of drugs into the larval tissues, or rapid metabolism of the drugs. If these drugs do in fact inhibit PIK3C2B as predicted, the data may simply reflect the small genetic interaction between \textit{pik3c2b} and \textit{mtm1} in the fin fold and reaffirm that this phenotype is not a reliable readout to test potential inhibitors of PIK3C2B.
Figure 24. Drugs predicted to bind the PIK3C2B kinase domain do not improve *mtm1* fin degeneration. There was no significant difference in fin severity distribution after treatment with A) tetrahydrobiopterin (THB), B) clindamycin, C) finasteride, or D) pravastatin. There was no significant difference found in the distribution of siblings and mutants between plates. Kruskal-Wallis test, followed by Dunn’s post-test.
Chapter 4
Phenotypic Drug Screen of mtm1 Mutant Zebrafish to Identify Therapeutic Leads for X-linked Centronuclear Myopathy

In mice, two genetic suppressors of Mtm1 have been identified in Pik3c2b and Dnm2. Other pathways have also been implicated in MTM1 mutant animal models, and additional pathways may yet to be discovered. Thus, my goal was to identify a novel drug that can modulate an mtm1-specific phenotype in zebrafish by screening a library of FDA-approved drugs. This will hopefully lead to novel cost-effective treatment strategies for X-linked centronuclear myopathy (XLCNM).

15 Phenotypic Drug Screen of mtm1 Mutants

To identify novel drug targets for myotubular myopathy, I screened a library of 1280 FDA-approved drugs (MicroSource US Drug Collection) for drugs that could suppress mtm1-mediated fin fold degeneration. The library was selected to facilitate potential future translation of promising hits to patients, given that the drugs already have FDA-approval and have been used safely in humans. My rationale to use fin degeneration as a phenotypic readout included:

1) Fin degeneration is specific to loss of mtm1
2) Fin degeneration is readily observable in a dissecting microscope and easily screenable
3) Fin degeneration is fully penetrant by 4 dpf
4) The spectrum in phenotypic severity could allow for the identification of both enhancers and suppressors of fin degeneration
5) A large scale screen for drug modifiers of mtm1 using a vertebrate model has not been performed previously

In the primary screen, I screened each drug at 10 µM against 16 embryos derived from a heterozygous parent incross (Figure 25). Briefly, 150 µL of egg water was added to wells of four separate 96-well plates. Four embryos were added to each well (1536 embryos) at 1 dpf while still in their chorions. Care was taken to pipette embryos to the bottom of a Pasteur pipette tip
and deposit embryos into the well by surface tension to minimize changes to well volume. I prepared a 40 µM working concentration of the drug library in a separate 96-well plate by adding 1 µL of the 10 mM drugs to 249 µL of egg water. Next, I added 50 µL of the 40 µM working stock to the 150 µL water containing embryos for a final concentration of 10 µM drug. Plates were parafilmed, wrapped in aluminum foil and incubated at 28.5°C until 4 dpf to assay fin degeneration. The screen was performed at 4 dpf when there is a relatively even distribution of mild and moderate phenotypes and smaller proportion of severe degeneration.

**Figure 25.** Screen methodology. Screening involved a primary chemical suppressor screen with *mtm1* mutants from a heterozygous carrier in-cross. At 1 dpf (Day 1), four embryos were placed in a single well of a 96-well plate and repeated in four separate plates. Next, FDA-approved drugs were added to the wells containing the embryos to a final concentration of 10 µM. At Day 4, 16 embryos/drug were assessed for fin degeneration. A secondary screen was then performed with 24 embryos to eliminate false positives and identify real targets. For drugs that retested, tertiary screens with 250 embryos were performed to determine optimal concentration and begin to elucidate mechanism(s) of action.

Out of 1280 drugs, 89 drugs were lethal to all embryos; of the remaining 1191, fin severity scores were recorded for individual *mtm1* mutants identified. In total, 40 drugs were identified where 0/16 plated embryos showed degeneration, corresponding to a hit rate of 3.36% (Figure 26A). In total, 256 DMSO control wells were screened of which 6 wells contained 0/16 mutant phenotypes, corresponding to a false positive rate of 2.34% (Figure 26A). This suggested that
~12 hits were real. I also ensured that mutants were randomly distributed into wells by comparing the proportion of mutants across all DMSO and experimental wells (Figure 26B).

Figure 26. A) The majority of wells contained 3-4 mutants per 16 embryos that were plated. DMSO wells (n = 256) had an average of $3.625 \pm 0.113$ mutants per 16 embryos and experimental wells (n = 1191) had $3.377 \pm 0.049$. As shown, the number of experimental wells with 0 mutants was greater than in DMSO wells, suggesting some of these represented true phenotypic suppression. B) The average proportion of $mmtm1$ mutants randomly distributed into was $0.227 \pm 0.007$ for DMSO wells and $0.211 \pm 0.003$ for experimental wells which was not significantly different ($P = 0.1947$; Kolmogorov-Smirnov non-parametric test to compare cumulative distributions). Values indicate mean ± SEM.

I retested each of the 40 drugs at 10 µM against 24 embryos and identified six drugs where only very mild or near WT-like $mmtm1$ phenotypes were observed. I genotyped all 24 larvae from these wells and for 4/6 drugs I found that in addition to the very mild mutants identified, there were $mmtm1$ mutants with a WT phenotype that had not been identified. This suggested that these drugs could completely suppress fin degeneration in some mutants at this concentration. Note that I did not genotype WT-like larvae from DMSO controls given that I have previously determined that the fin degeneration phenotype is fully penetrant in $mmtm1$ mutants by 4 dpf.

Next, I performed dose response curves with the six drugs against large clutches of embryos ($n = 250$). I found that three were false positives (trinitroglycerin, tannic acid, flunixin meglumine) as they did not appreciably suppress the phenotype (Figure 27). However, tannic acid and flunixin both made the phenotype significantly milder at 10 µM compared to controls, perhaps explaining why they were identified as suppressors in the primary and secondary screens. The three remaining drugs were cycloheximide, haloperidol, and sodium valproate/valproic acid (VPA). All three significantly suppressed the fin degenerative phenotype in a dose-responsive manner (Figure 28). However, both haloperidol and cycloheximide treatment produced significant adverse phenotypes; importantly, larvae treated with these drugs
were underdeveloped by ~1 day at the 4 dpf assay time point, raising the possibility that the
larvae had not reached a developmental stage where fin would begin to degenerate appreciably.
Therefore, I chose not to further characterize their mechanism of action. In contrast, VPA
treatment up to 250 µM was not associated with any toxicity and completely suppresses fin
degeneration in the majority of mtm1 mutants.

Figure 27. Three drugs identified in the primary and secondary screens turned out to be false
positives as they do not appreciably improve mtm1 fin degeneration. While there was a
significant improvement in fin severity distribution after treatment with either A) 10 µM tannic
acid (**P = 0.0087) or B) 10 µM flunixin (**P = 0.0029), higher concentrations of either drug
were lethal to all treated embryos. There was no improvement in the mtm1 phenotype after
treatment with C) trinitroglycerin. There was no significant difference found in the distribution
of siblings and mutants between plates. Kruskal-Wallis test, followed by Dunn’s post-test.
Figure 28. Three drugs identified in the primary and secondary screens improved *mtm1* fin degeneration in a dose-responsive manner. A) With valproic acid (VPA), there was no significant improvement in fin severity distribution of mutants despite a clear trend towards milder mutants; however, a significant difference found in the distribution of siblings and mutants at 100 µM VPA (**P < 0.001) reveals there are *mtm1* mutants with WT-like phenotypes that could not be identified as mutants. B) Cycloheximide treatment significantly improved *mtm1* fin degeneration at both 1 µM and 10 µM (**P < 0.001). Similar to VPA, the significant change in WT/*mtm1* distribution at 10 µM (**P < 0.001) suggests the presence of phenotypically rescued *mtm1* mutants. C) Haloperidol treatment did not significantly improve *mtm1* fin degeneration despite a clear trend towards milder mutants; however, a significant change in WT/*mtm1* distribution at 10 µM (**P < 0.001) suggests the presence of phenotypically rescued *mtm1* mutants. Kruskal-Wallis test, followed by Dunn’s post-test.

In addition to those drugs that completely suppress the fin degeneration phenotype, I was interested in whether certain classes or categories of drug were associated with either milder or more severe fin degeneration. To begin exploring this idea, I compared the severity of all mutants identified in DMSO wells with those identified in experimental drug wells (Figure 29).
did this in two ways, first by comparing the severity distributions using the 3-point ordinal scale as before (this is the most appropriate method given that it is non-parametric data). As shown, there is no significant difference in the severity between these wells ($P=0.6527$), suggesting that mutants were evenly distributed at random between all wells. Second, I treated each score as continuous data in order to calculate the mean severity score in each group. This method also showed no significant difference in the severity between these wells ($P=0.2946$).

**Figure 29.** A) Severity distribution comparison of *mtm1* mutant fin degeneration shows no screen-wide difference ($P = 0.02946$; Mann-Whitney non-parametric rank test) between DMSO ($n = 928$) and experimental wells ($n = 4014$). B) The mean severity score of DMSO wells was $1.496 \pm 0.0178$ while that of experimental wells was $1.518 \pm 0.0092$ which was not significantly different ($P = 0.2946$; Student’s t-test, 2-tailed).

Next, I assigned 1165 drugs to one of 73 drug categories based on their reported function in the MicroSource data file or literature (Table 3). I subcategorized drugs with similar function that were also structurally highly similar. For each drug category I assembled the severity distributions on the 3-point ordinal scale and also calculated mean severity scores. When I compared the severity distributions for experimental drug wells with the severity distribution for DMSO wells, I found that one drug category is significantly associated with a more severe phenotype (Figure 30A) while no drug categories were significantly associated with milder phenotypes (Figure 30B). Similarly, when I compared the mean severity scores for experimental drug wells with the mean severity scores for DMSO wells, I found that two drug categories are significantly associated with a more severe phenotype (Figure 30C) while two drug categories are significantly associated with milder phenotypes (Figure 30D).
Figure 30. A,B) Severity distribution comparison for experimental drug wells and DMSO wells \((n = 4014)\) shows that glucocorticoid/mineralocorticoid steroids (category #1; \(n = 183\)) make fin degeneration more severe \((**P < 0.003)\) while no drug categories significantly improve the phenotypic distribution. Kruskal-Wallis test, followed by Dunn’s post-test. C) Similarly, the mean severity score for glucocorticoid steroids is significantly more severe compared to DMSO wells \((***P = 0.0008)\). Additionally, the mean severity score for leukotriene antagonists/H1
antihistamines (category #19; \( n = 12 \)) was also associated with a more severe fin phenotype (\( ***P = 0.0004 \)). A 1-way ANOVA followed by Dunnett’s post-test was performed to compare all wells to the control mean but not with each other. **D** The mean severity score for antipsychotics that are haloperidol analogs (category #15; \( n = 2 \)) and antipsoriatics including cycloheximide (category #70; \( n = 2 \)) were both significantly milder compared to DMSO wells (\( *P = 0.0109 \) and \( **P = 0.0076 \), respectively). 1-way ANOVA, followed by Dunnett’s post-test.

Given that glucocorticoid/mineralocorticoid steroid hormones tend to worsen the phenotype of \textit{mtm1} mutant fins, it may be worthwhile to investigate this effect in the mouse KO model of \textit{Mtm1} to inform us on the safe use of these drugs in patients with XLCNM. Re-testing the leukotrienes/H1 antihistamines (category #19) may provide insight into how MTM1 functions and regulates zebrafish fin fold development. Likewise, further testing of the antipsoriatics (category #70) and antipsychotics (category #15) that ameliorate the fin phenotype may shed light on the mechanisms underlying fin degeneration in \textit{mtm1} mutants.

### 16 Determining the Mechanism of Action of Valproic Acid

VPA is an anticonvulsant that is routinely and safely used in paediatric populations. Its anticonvulsant activity is attributed to inhibition of voltage-gated sodium channels or NMDA ionotrophic glutamate receptors\(^{59,60}\). Given that many other voltage-gated sodium channel inhibitors (e.g., phenytoin and lamotrigine) and ionotrophic glutamate receptor inhibitors (e.g., riluzole and topiramate) present in the library did not suppress fin degeneration, it seemed unlikely that VPA was acting via this mechanism and I chose to focus on alternative mechanisms.

VPA is also a known histone deacetylase (HDAC) inhibitor\(^ {61}\). To test whether VPA suppresses fin degeneration by this mechanism, I tested other known HDAC inhibitors against the \textit{mtm1} phenotype including trichostatin A (TSA), 4-phenylbutyrate (PBA), and suberanilohydroxamic acid (SAHA). In addition to VPA, I found that both TSA and PBA suppressed fin degeneration in a dose-dependent manner while SAHA had no effect on the phenotype (Figure 31 and Figure 32). Interestingly, PBA was in the drug library that was screened originally at 10 µM; however, as shown it only begins to suppress fin degeneration at 250 µM, explaining why it was not identified in the primary screen. Given that VPA is structurally different from both TSA and PBA, this strongly suggests that their shared ability to suppress fin degeneration is attributable to their shared property as HDAC inhibitors. As an additional piece of validation, I showed that
250 µM VPA could suppress the fin degeneration caused by *mtm1* Ex3-SA morpholino knockdown (Figure 33).

**Figure 31.** Structurally dissimilar HDAC inhibitors suppress *mtm1* fin degeneration. A) At higher valproic acid (VPA) concentrations there is a significant improvement in *mtm1* fin degeneration (**P = 0.0056; ***P < 0.001). Starting at 100 µM VPA, there is evidence that *mtm1* mutants are completely rescued. Notably, at 500 µM VPA it appears that all *mtm1* mutants have WT-like fins. B) Treatment with 4-phenylbutyrate (PBA) shows there is a significant amelioration in *mtm1* fin degeneration (*P = 0.0372; ***P < 0.001). Notably, at 500 µM PBA it appears that all *mtm1* mutants have WT-like fins. C) Treatment with trichostatin A (TSA) shows
there is a significant amelioration in \textit{mtm1} fin degeneration (\(*\!*\!\!* P < 0.001\)). At 250 nM TSA there are many phenotypically rescued \textit{mtm1} mutants while affected \textit{mtm1} mutants have very mild fin degeneration. \textbf{D)} Treatment with suberanilohydroxamic acid (SAHA) has no effect on this \textit{mtm1} phenotype up to 20 \(\mu\)M. However, higher concentrations have not been tested. Kruskal-Wallis test, followed by Dunn’s post-test.

\textbf{Figure 32.} Treatment with \textbf{A}) 100 \(\mu\)M VPA, \textbf{B}) 500 \(\mu\)M PBA or \textbf{C}) 250 nM TSA prevents fin degeneration in \textit{mtm1} mutants.
Figure 33. A) Treatment with 250 µM VPA can prevent fin degeneration in mtml morphants. B) There is a significant degeneration in mtml morphants (***P < 0.001) and significant improvement in mtml fin degeneration with exposure to 250 µM VPA (***P < 0.001).

To determine the mechanism by which HDAC inhibition suppresses mtml fin degeneration, I reviewed the literature for other mutants that develop fin degeneration. I found that three integrin pathway mutants (itga3b, fermt1, and ilk) have similar fin morphology to the mtml mutant and their fins begin to degenerate around 2-3 dpf and are mostly lost by 6 dpf\(^62–64\). These findings highlight the importance of integrin signalling in larval fin fold development. It is well established that integrin signalling is dependent on proper endocytic transport and that integrins are trafficked through the endosomal compartment\(^65\). In fact, a direct role for mtml in regulating β1 integrin traffic has previously been shown in both Drosophila and human skeletal muscle\(^30\).

Thus, it is plausible that loss of mtml results in aberrant PtdIns(3)P-dependent endosomal trafficking in the larval fin fold resulting in mislocalization of integrin pathway proteins away from their normal signalling compartments. Based on this model, I hypothesize that HDAC inhibitors like VPA are working by upregulating some combination of these integrin genes. By increasing their protein abundance in the cell, it could overcome a protein deficit at their normal signalling compartments. To test this idea, I performed a qPCR of itga3b, fermt1, and ilk in 4 dpf WT siblings and mtml larvae either left untreated or treated with 250 µM VPA. I found that across three biological replicates, itga3b is more highly expressed in WT siblings and mtml mutants treated with VPA compared to their untreated counterparts, supporting the hypothesized mechanism of suppression by VPA (Figure 34). For the future, a combination of genetic epistasis experiments and live imaging to illustrate how this integrin, ITGA3B, is trafficked under different conditions will be needed to convincingly confirm this mechanism of action.
Figure 34. At 4 dpf, quantitative real-time PCR shows that both WT siblings and mtm1 mutants treated with 250 µM VPA have dramatically elevated levels of itga3b transcript compared to their DMSO treated counterparts. Results are shown for three independent biological replicates (A-C). Each sample comprised 5 larvae exposed from 1 dpf to 4 dpf with the indicated condition.

Lastly, given the role of MTM1 in regulating PtdIns(3)P and endosomal trafficking\(^{16}\), I sought to test whether mtm1 mutants would be hypersensitive to perturbations in membrane traffic. I tested brefeldin A, a molecule that collapses the Golgi, preventing both secretory (Golgi-to-plasma membrane) and retrograde (Golgi-to-ER) traffic and found that it significantly worsens fin degeneration in mtm1 mutants (Figure 35). This is consistent with a role for MTM1 in regulating integrin delivery to the membrane.
Figure 35. Brefeldin A worsens mtm1 fin phenotype. A) Treatment of mtm1 mutants with 7.13 µM brefeldin A starting at 3 dpf significantly worsened the fin severity distribution of mutants at 4 dpf (**$P = 0.0037$). B) As a quality control, there was no significant difference found in the distribution of siblings and mutants between plates (ns: $P = 0.5985$). Mann-Whitney U test.
Chapter 5
Discussion

The purpose of my research project was to support the development of therapies for X-linked centronuclear myopathy (XLCNM). There are currently no treatments available for this a devastating childhood muscle disorder. Gene and protein replacement therapies hold the promise to cure the disorder; however, they are still years away from being used safely in humans. Not to mention that once they become available they may not be accessible to or affordable for all patients. Therefore, there is still a necessity to develop alternative therapies that are both effective and affordable.

To accomplish this, I characterized an mtm1 mutant zebrafish to serve as model of XLCNM. The purpose of developing a new model was to facilitate the testing and identification of potential drug therapies for this devastating inherited disorder. Zebrafish have many advantageous properties when it comes to drug screening, including their large brood sizes and ex utero development. Importantly, zebrafish larvae can be easily and cost-effectively exposed to many drugs by addition of compound to their water.

In line with the overarching goal of my project, I showed that the mtm1 mutant has several phenotypes that involve both the skeletal muscle and other tissues such as the liver and larval fin folds. This characterization of the mutants has advanced the field in several ways. First, I have characterized a new model that can serve as a research tool for us and other researchers to use to answer questions related to the function of MTM1 in various tissues. For example, the triad abnormalities could allow us to investigate mechanisms underlying triad biogenesis and maintenance in skeletal muscle. Additionally, studying the liver phenotype in this model may shed light into the pathomechanism(s) involved in patients with XLCNM that develop liver complications. Second, my observations have provided insight into the cause versus effect relationship between central nuclei and muscle weakness seen in XLCNM. Wildtype larvae have central nuclei in their muscle up to 7 dpf and still exhibit normal motor behaviour, whereas mtm1 mutants have disorganized triads and accompanying motor defects at 7 dpf. This observation supports the current belief in the field that the centralized nuclei are not causative of muscle weakness and rather represent downstream changes that occur in muscle in the absence of MTM1.
As part of my characterization of the mtm1 mutant, I optimized methods to visualize in vivo PtdIns(3)P dynamics in the skeletal muscle of zebrafish larvae. This will help us address some of the critical unanswered questions regarding MTM1 and its regulation of PtdIns(3)P. Specifically, by co-expressing markers of PtdIns(3)P and a given subcellular compartment we may determine whether or not PtdIns(3)P accumulates in a specific subcellular compartment, e.g., the early endosome or the sarcoplasmic reticulum. It may also allow us to visualize the effect of relevant genetic manipulations have on PtdIns(3)P in an mtm1 mutant background which will inform us about the function of MTM1. For example, we could track PtdIns(3)P after knocking down BIN1 or overexpression DNM2 in both wildtype and mtm1 mutant larvae, which would provide us with a better picture of how these centronuclear myopathy genes interact in vivo.

Next, I tested whether mtm1 and pik3c2b interact in the zebrafish larval fin fold by genetic manipulations and examined the effect of pharmacologic inhibition of PIK3C2B on several phenotypes. I expected to see a strong genetic interaction between pik3c2b and mtm1 in the fish, based on the finding that ablation of Pik3c2b in Mtm1 KO mice completely suppresses their adverse phenotypes as well as many other reports in other models where the two genes interact. However, I only found evidence of a weak genetic interaction in the larval fin fold. I chose the larval fin fold because it was the earliest phenotype to arise in the mtm1 mutant (appearing at 3 dpf) and thus was amenable to morpholino knockdown of pik3c2b. Additionally, I showed that pan-PI3K inhibitors that inhibit PIK3C2B suppressed fin degeneration in mtm1 mutants while specific class I and class III PI3K inhibitors had no effect on their own. These data suggest that pik3c2b and at least one other PI3K interact together with mtm1 in the larval fin fold. I also showed that the pan-PI3K inhibitors had a positive effect on the motor behaviour and survival of mtm1 mutants. Meanwhile, a parallel approach was taken by our lab to test one available PIK3C2B inhibitors, wortmannin, directly in mice. Wortmannin treatment significantly increased the muscle strength and survival of Mtm1 KO mice compared to controls. Altogether these data supported the hypothesis that mtm1 and pik3c2b interact and that PIK3C2B inhibitors may have therapeutic value in XLCNM.

Future work should focus on characterizing the effect of pik3c2b mutation on the other observable mtm1 phenotypes such as the defects in liver, pectoral fin morphology, and motor behaviour. Indeed, should we want to test the effects of novel inhibitors of PIK3C2B in the mtm1 mutant, it would be prudent to first validate additional phenotypes in the mtm1; pik3c2b double
mutant. We can now test the interaction of \textit{mtm1} with \textit{pik3c2b} for these phenotypes in the \textit{mtm1; pik3c2b} double mutants.

Finally, the third aim of my project was to perform an unbiased screen with \textit{mtm1} mutants to identify FDA-approved drugs that correct an \textit{mtm1}-specific phenotype. To my knowledge, this screen is the first of its kind using a model of XLCNM with the potential to have translational impact. My preliminary characterization of valproic acid (VPA) revealed that suppression of \textit{mtm1} mutant fin degeneration was likely attributed to histone deacetylase (HDAC) inhibition. This suggests that in the zebrafish larval fin fold loss of MTM1 leads to either 1) transcriptional reprogramming, specifically repression, of specific genes, 2) reduced localization of proteins in subcellular compartments or 3) some combination of these scenarios. In the first scenario, HDAC inhibition could be reprogramming cells by promoting open, transcriptionally active chromatin states. Alternatively, HDAC inhibition may suppress fin degeneration by upregulating specific genes (e.g. \textit{itga3b}) to promote increased protein expression in compartments where the protein level is deficient. The possibility that defects in integrin recycling may underlie the pectoral fin and fin fold phenotypes in \textit{mtm1} mutants presents us with the opportunity to learn more about how MTM1 functions in intracellular trafficking pathways \textit{in vivo}.

The drug screen also identified several drug families with the ability to modulate the \textit{mtm1} fin phenotype. It may be informative to characterize the mechanisms responsible for their effects by taking a similar approach as I did with valproic acid. Continuing studies to elucidate the mechanism of suppression by VPA and mechanism(s) for phenotypic modulation by other drug families should inform us on novel drug targetable pathways for XLCNM.

Ultimately, the drugs identified in this screen that correct the \textit{mtm1} mutant fin phenotype will be validated further using the zebrafish and \textit{Mtm1} KO mouse models. These drugs may represent putative therapeutics for translation into patients with myotubular myopathy and hopefully one day will fulfill an unmet need in this rare disease population.
Chapter 6
Future Directions

There are many remaining questions surrounding X-linked centronuclear myopathy (XLCNM) and the function of MTM1. The most urgent problem is that there are still no therapies available to treat this disorder. For this reason, I screened for drugs that suppress an $mtm1$ mutant phenotype and identified valproic acid (VPA), an anticonvulsant with HDAC inhibitory activity.

It would be prudent to understand the mechanism underlying the fin degeneration phenotype in $mtm1$ mutants and how VPA rescues this phenotype. There is preliminary evidence that suggests the fin phenotype is a result of defective integrin trafficking. I hypothesize that VPA overcomes this defect by epigenetic modifications which result in upregulation of integrins, specifically $itga3b$, in the fin.

It will also be important to characterize the effect of this drug on the $Mtm1$ KO mice. The mice represent an excellent pre-clinical model of the human disorder and will allow us to determine whether or not VPA has therapeutic potential for XLCNM.

Finally, despite our increased understanding of pathomechanisms involved in XLCNM, we still don’t have a real picture of how MTM1 functions in skeletal muscle. MTM1 is a PtdIns(3)P phosphatase, but what is the function of PtdIns(3)P in skeletal muscle and how does PtdIns(3)P accumulation result in a deleterious phenotype? There has yet to be a systematic investigation to answer these questions. I propose to perform a comparative proteomic analysis of subcellular compartments obtained from the skeletal muscle of wildtype and $Mtm1$ KO mice. This will provide us with a global map of how proteins traffic through various compartments within muscle and how these processes are perturbed when MTM1 is lost and PtdIns(3)P accumulates.

17 Specific Aim 1: Determine the cause of $mtm1$ fin degeneration and mechanism underlying suppression by valproic acid

 Trafficking defects are a consistent feature in models of XLCNM. For example, there is evidence of abnormal $\beta1$ integrin localization in XLCNM patient muscle and Drosophila $mtm$ mutants and evidence of transferrin receptor and $\beta1$ integrin recycling defects in HeLa cells depleted of
MTM1. I hypothesize that the fin degeneration seen in the mtm1 zebrafish mutant is a direct result of a defect in integrin trafficking (given the strong phenocopy in integrin pathway mutants) and represents a conserved mechanism involved in the pathogenesis of XLCNM.

Specifically, I hypothesize that mtm1 acts upstream of itga3b promoting the normal traffic of ITGA3B to the plasma membrane in the zebrafish larval fin fold. To test this, I will perform genetic epistasis experiments. For example, I expect that overexpression of itga3b RNA would rescue the mtm1 fin phenotype whereas mtm1 RNA overexpression would have no effect on fin degeneration in itga3b mutants. I expect that a sub-optimal level of itga3b morpholino, i.e., one that produces no phenotype in a wildtype background, would exacerbate the mtm1 phenotype. I could also test the effect of VPA under these conditions where I would expect that itga3b knockdown would prevent suppression by VPA.

I will also perform complementary live imaging to test this hypothesis. Using fluorescently labeled ITGA3B (e.g., GFP), I expect to see an abnormal expression pattern in mtm1 mutant fins compared to wildtype. To complement these studies, I will co-express markers of endosomal compartments to compare the subcellular distributions of ITGA3B. Again, I expect that VPA treatment would show a significant increase in the labeled ITGA3B at the cell membrane by virtue of increasing the abundance of endogenous ITGA3B in the cell.

Interestingly, the hypothesized mechanism by which VPA suppresses fin degeneration is consistent with the finding that SNX17 is a protein interactor of MTM1. In HeLa cells, loss of SNX17 results in a diversion of integrins, including β1 and α3, from recycling compartments towards lysosomes, resulting in their degradation and decreased expression at the plasma membrane. Given that MTM1 has been implicated in a phosphoinositide conversion mechanism from PtdIns(3)P-positive sorting endosomes to PtdIns(4)P-positive recycling endosomes, it is plausible that MTM1 coordinates with SNX17 during this conversion process to mediate integrin cargo traffic to the recycling endosomal compartment. Based on this, it would be worthwhile to test whether SNX17-dependent sorting of integrins is involved in mtm1-mediated fin degeneration. This could be tested in our zebrafish model by morpholino (MO) knockdown of snx17. One possibility is that it results in a fin degenerative phenotype similar to integrin pathway mutants or mtm1 mutant. I expect that if SNX17 is necessary in zebrafish for
sorting integrins away from lysosomes towards the plasma membrane then *snx17* knockdown will exacerbate the fin degenerative phenotype in *mtm1* mutants.

18 Specific Aim 2: Determine the effect of valproic acid on *Mtm1* KO mice

I will treat *Mtm1* KO mice beginning at 21 days of age using methods previously established in our lab (appropriate drug vs. placebo groups, blinding, and sample size). Mice will be treated until animal death (median lifespan of *Mtm1* KO = 35 days). In addition to tracking survival, I will also determine the effect of VPA on other phenotypes such as grip strength and body weight.

If there appears to be a significant benefit with VPA treatment, I will characterize the effect on muscle by histopathology (e.g., central nuclei, myofiber size, fiber type proportions) and electron microscopy (triad and sarcomeric ultrastructure) at various timepoints.

It is difficult to accurately predict what effect VPA might have in a mammalian model of XLCNM; however, it is worthwhile given that VPA is an FDA-approved drug with the ability to suppress an *mtm1* mutant phenotype. Furthermore, our established experimental protocol will be sufficiently powered to determine if there is any improvement. I expect that VPA will have a positive effect on the *Mtm1* KO mice based on the hypothesized mechanism of overcoming intracellular trafficking defects. There is also emerging evidence that HDAC-4 is dramatically upregulated in muscle from XLCNM patients corresponding to significant transcriptional changes in genes such as *RYR1*\(^{67}\). Bachmann and colleagues suggest that these changes are secondary or downstream causes of MTM1 mutation but nevertheless contribute to the profound muscle weakness seen in patients\(^{67}\). Altogether, this suggests that HDAC inhibitors that inhibit HDAC-4, like VPA, could have some therapeutic value to XLCNM.

Ultimately, I must consider what happens if VPA does not have an effect on *Mtm1* KO mice. If this is the case then I could test other HDAC inhibitors such as 4-phenylbutyrate which is also FDA-approved and suppressed the zebrafish *mtm1* fin phenotype. I could also evaluate other drugs identified in the drug screen that were associated with a milder fin phenotype.
Specific Aim 3: Determine the function of MTM1 and PtdIns(3)P in skeletal muscle

What is the function of MTM1-regulated PtdIns(3)P in skeletal muscle and why does the accumulation of PtdIns(3)P cause a severe muscle phenotype? One method to address these important questions would be to perform comparative proteomics on subcellular fractions collected from muscle tissue of Mtm1 mutant and wildtype mice. If we could isolate pure fractions of the early endosome, late endosome and recycling endosome and compare their protein content based on genotype we may gain a more complete understanding of how the loss of MTM1 affects protein trafficking within the cell. It would allow us to analyze how MTM1 controls the global trafficking of membrane-bound and intravesicular proteins in the cell. I would expect this analysis would reveal many proteins that are mislocalized in the mutant or have differential expression levels between endosomal compartments.

To carry out this aim I would first focus on developing and validating a method. I would modify previously developed methods used to purify subcellular fractions and build organellar maps that don’t rely on metabolic labelling, e.g., SILAC\textsuperscript{68,69}. Briefly, subcellular fractionation would begin with mechanical breakdown and detergent-free lysis of the tissue to preserve the membrane integrity of endosomal compartments. This would be followed by density gradient ultracentrifugation to separate subcellular fractions. With a given endosomal fraction, we could either perform subsequent centrifugation or use antibody-based purification to purify the distinct endosomal compartments. The purity of the fractionation protocol could be validated by Western blot, e.g., a Rab5(+) compartment is enriched for EEA1 and does not contain Lamp1 while the opposite pattern is observed in the Rab7(+) compartment. Once sufficient purity is achieved, then the various subcellular fractions would be analyzed by comparative mass-spectrometry. Fractions would be subjected to tryptic protein digest and labelling with tandem mass tags, followed by mass spectrometry and analysis as previously described\textsuperscript{68,69}.

With proteins of interest, I could further validate changes in subcellular localization by immunohistochemistry or immunofluorescence on sections from Mtm1 KO mice. To determine their importance in pathogenesis, I could further validate the effect of their genetic manipulation in both wildtype and mtm1 mutant fish.
Developing this method would be challenging but possible through collaborations with SPARC BioCentre at Sickkids and well-established proteomics labs at University of Toronto. Importantly, it would provide novel insight into how MTM1 regulates protein trafficking or translocation events in the cell, specifically through the endosomal compartments.
References


59. Farber, N. B., Jiang, X., Heinkel, C. & Nemmers, B. Antiepileptic drugs and agents that


Appendix: Supplementary Tables

**Table 1.** *In vitro* IC$_{50}$ values (μM) for PI3K inhibitors against phosphoinositide kinases. Data compiled from multiple sources. Note: Content in this table has been published in the *Journal of Clinical Investigation*: Sabha et al., 2016$^{33}$.

<table>
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*Single values represent IC$_{50}$ of p110α catalytic subunit. Otherwise, the range of IC$_{50}$ values for all four isoforms is shown.

$\dagger$Exact values were not reported.
Table 2. Mutant alleles generated by CRISPR/Cas9 targeting exon 24 of *pik3c2b*. The ATP-coordinating “DFG” motif of the kinase domain is highlighted in yellow. In the partial sequence of exon 24 shown, the PAM sequence of the gRNA is highlighted in red while the remainder of the gRNA sequence is highlighted in gray. Red letters represent nucleotide or amino acid differences from wildtype.

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<td>HMFHIIMTLSGTHRCEATIN</td>
<td>CACATGTTCA---CATGCAATTGGCCACATGTTTGGCAACATTAAACG</td>
</tr>
<tr>
<td>36 bp deletion</td>
<td>H--------------AQMFGNIK</td>
<td>CAC-------------CATGCAATTGGCCACATGTTTGGCAACATTAAACG</td>
</tr>
<tr>
<td>48 bp deletion</td>
<td>HMFH-------------K</td>
<td>CAC-------------CATGCAATTGGCCACATGTTTGGCAACATTAAACG</td>
</tr>
<tr>
<td>5 bp deletion</td>
<td>HMFHI---WQVPGARTDVQH*</td>
<td>CACATGTTCA---CATGCAATTGGCCACATGTTTGGCAACATTAAACG</td>
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<td>6 bp deletion</td>
<td>HMFH---DFGKFLGHAQMFGNIK</td>
<td>CACATGTTCA---CATGCAATTGGCCACATGTTTGGCAACATTAAACG</td>
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</table>
Table 3. Categorization of drugs in the MicroSource US Drug Collection by known function/bioactivity/mechanism of action. The number of drugs in each category is given by $N$ and was used to compare screen-wide mean severity scores. There had to be at least three drugs in order to form a category. $N_{\text{dist}}$ represents how many $mtm1$ mutants were sampled in that drug category when calculating the severity distribution. Data compiled from multiple sources but primarily from the supplier data file.

<table>
<thead>
<tr>
<th>Category #</th>
<th>Bioactivity</th>
<th>$N$</th>
<th>$N_{\text{dist}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Steroid hormones: Glucocorticoids/mineralocorticoids</td>
<td>52</td>
<td>183</td>
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<tr>
<td>2</td>
<td>Steroid hormones: Progesterones/estrogens/testosterones</td>
<td>34</td>
<td>101</td>
</tr>
<tr>
<td>3</td>
<td>Non-steroidal anti-inflammatory drug (NSAID)</td>
<td>48</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>NSAID; fenamate derivatives</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Analgesics</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>Anaesthetics/muscle relaxants</td>
<td>46</td>
<td>146</td>
</tr>
<tr>
<td>7</td>
<td>Serotonin agonist including SSRIs/SNRIs</td>
<td>20</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>Serotonin antagonist</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>Antibacterial/anti-infective/antiseptic</td>
<td>187</td>
<td>673</td>
</tr>
<tr>
<td>10</td>
<td>Dietary supplements: vitamins/amino acids/simple carbohydrates</td>
<td>40</td>
<td>135</td>
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<tr>
<td>11</td>
<td>Anthelmintic/antiparasitic</td>
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<tr>
<td>12</td>
<td>Antifungal/antimycotic; triazole</td>
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<td>115</td>
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<td>13</td>
<td>Atypical antipsychotic; multiple targets; thienobenzodiazepine derivative</td>
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<td>12</td>
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<td>14</td>
<td>Antipsychotic; phenothiazine derivative</td>
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<td>15</td>
<td>Antipsychotic; haloperidol analog</td>
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<td>16</td>
<td>Antipsychotic; other structure</td>
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<td>18</td>
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<td>H2 antihistamine</td>
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<td>19</td>
<td>Leukotriene antagonist/H1 antihistamine</td>
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<td>20</td>
<td>Alpha adrenergic agonist</td>
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<td>21</td>
<td>Alpha adrenergic antagonist</td>
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<td>Beta adrenergic antagonist</td>
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<td>Antidepressant; tricyclic</td>
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<td>Antidepressant; other structure</td>
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<td>Cholinergics, including acetylcholinesterase inhibitors; Muscarinic receptor agonists; Nicotinic receptor agonists</td>
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<td>Antimalarial</td>
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<td>31</td>
<td>Antiprotocoazol/coccidiostat</td>
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<td>32</td>
<td>Antihyperlipidemics</td>
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<td>RAAS system; renin inhibitor</td>
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<td>34</td>
<td>DHPR antagonist; dihydropyridine derivative</td>
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<td>35</td>
<td>Caffeine/xanthine analogs</td>
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<td>Chelating agent</td>
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<td>Ergot alkaloids; ergoline derivatives</td>
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<td>Miscellaneous</td>
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</tbody>
</table>

Note: Some drug categories were subcategorized based on structural similarity. These categories have shared bioactivity and are indicated by the same colour.