# Modeling Human MEGF10 Myopathy in *Drosophila melanogaster*

A thesis

Submitted by

**Bahar Matin**

In partial fulfillment of the requirements
for the degree of

Master of Science

in

Pharmacology and Drug Development

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

Date

May, 2015

Advisors:

Alan Kopin
Isabelle Draper

**Abstract**

MEGF10 myopathy or EMARDD (Early onset of Myopathy, Areflexia, Reparatory Distress and Dysphagia) is a rare human congenital muscle disease caused by mutations in MEGF10. This single transmembrane receptor is expressed in skeletal muscles, the retina and in CNS glial cells, and is conserved through evolution. The *Drosophila melanogaster* (i.e. fruit fly) homolog of human MEGF10 is Drpr. Conservation of MEGF10 from insect to human, together with the versatility of fly genetics, makes *Drosophila* a good model organism to investigate mechanisms underlying human MEGF10 pathogenesis. In this study, a loss-of-function mutant *Drosophila* model of MEGF10 myopathy (which displays muscle defects reminiscent of those seen in EMARDD patients) was utilized to further clarify the link between MEGF10/Drpr, and signaling pathways involved in muscle development and regeneration. To achieve this objective, the expression level of candidate partners of MEGF10/Drpr that comprise the Notch and Wnt signaling cascades was assessed in Drpr null flies, (vs. controls). These studies revealed a decrease in the expression levels of arm and Pi3K68D, the fly orthologs of human β-catenin and Pi3K, respectively, which are two important effectors in the Wnt pathway. In addition, decreased expression of Dl, the fly homolog of human Delta and a ligand for Notch, was also observed. In parallel to the Drpr loss-of-function mutant, MEGF10/Drpr gain-of-function *Drosophila* models were generated and characterized in our laboratory. Mutant flies that express mouse Megf10 display increased pre-adult lethality. The rare flies that survive to adulthood showed a shortened lifespan, reduced motor function and morphological abnormalities in the legs. These phenotypes are recapitulated in flies that overexpress other members of the MEGF family

of receptors, e.g. each of the three known fly Drpr protein isoforms, or mouse Megf12. Together our results illustrate the importance of balanced MEGF10/Drpr levels *in vivo* to sustain normal muscle physiology and survival. Furthermore, we have begun to unravel a role for MEGF10/Drpr via its interaction with the Wnt and Notch signaling pathways, which are major regulators of muscle progenitor cells proliferation/differentiation. Future studies will help further delineate the signaling cascades downstream of MEGF10/Drpr. Extending from these investigations, candidate therapeutic strategies that increase the levels of Wnt and/or Notch signaling may be considered as treatment options for MEGF10 myopathy. To that extent, our MEGF10/Drpr mutant *Drosophila* models of MEG10 myopathy can be utilized as *in vivo* screening platforms to expedite the identification of candidate therapeutics.

This work is part of a collaborative project with Dr. Peter Kang (Associate Professor and Chief, Division of Pediatric Neurology, University of Florida, USA).

**Table of Contents**

**List of Tables**

**List of Figures**

**List of Abbreviations**

| | |
|---|---|
| Act5C | Actin 5C |
| ALS | Amyotrophic Lateral Sclerosis |
| AMP | Adult Muscle Precursors |
| Arm | Armadillo, fly homolog of human β-catenin |
| C | Centigrade |
| Cad74A | *Drosophila* homolog of human Cadherin 23 |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| Dkk-1 | Dickkopf-1 |
| Dl | Delta, (*Drosophila* homolog of human Delta) |
| Drpr | Draper |
| Drpr-I | Drpr Isoform, Drpr-PB, (Flybase ID: FBpp0072681, NCBI Reference Sequence: NP_728660.2) |
| Drpr-II | Drpr Isoform, Drpr-PA, (Flybase ID: FBpp0072680, NCBI Reference Sequence: NP_477450.1) |
| Drpr-III | Drpr Isoform, Drpr- PC, (Flybase ID: FBpp0301579, NCBI Reference Sequence: NP_001246549.1) |
| Ds | Dachsous, (*Drosophila* homolog of human Cadherin 23) |
| DSL | Delta/Serrate/LAG-2 |
| EGF | Epidermal Growth Factor |
| EMARDD | Early onset of Myopathy, Areflexia, Reparatory Distress and Dysphagia |
| ft | Fat, (*Drosophila* homolog of human Cadherin 23) |

| | |
|---|---|
| Fz | Frizzled |
| Gal4 | A yeast transcription activator protein |
| GSK3β | Glycogen Synthase Kinase 3β |
| ITAM | Immune-receptor Tyrosine-based Activation Motif |
| KO | Knockout |
| kug | Kugelei, (*Drosophila* homolog of human Cadherin 23) |
| LPR5/6 | Low-density Lipoprotein Receptor-related protein 5/6 |
| Megf10 | Mouse homolog of human MEGF10 |
| MEGF10 | Multiple Epidermal Growth Factor-like domain 10 |
| Megf12/Jedi | Mouse homolog of human MEGF12 |
| MEGF12/JEDI | Multiple Epidermal Growth Factor-Like Domain 12 |
| min | Minute |
| Myf5 | Myogenic regulatory factor 5 |
| MyoD | Myogenic regulatory factor 3 |
| N | Notch, (*Drosophila* homolog of human Notch) |
| PAX7 | Paired box protein 7 |
| PCR | Polymerase Chain Reaction |
| Pi3k | Phosphatidylinositol 3-kinase |
| Pi3K68D | Phosphotidylinositol 3 kinase 68D, *Drosophila* homolog of human Pi3kCa |
| Pi3K92E | Phosphotidylinositol 3 kinase 92E, *Drosophila* homolog of human Pi3kCa |
| PI3KCa | phosphatidylinositol 3-kinase Ca |

| | |
|---|---|
| RNAi | RNA interference |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SBHA | Suberoyl Bis-Hydroxamic Acid |
| sec | Second |
| SFRP | Secreted Frizzled-Related Protein |
| TCF/LEF | T-Cell Factor/Lymphoid Enhancer Factor |
| UAS | Upstream Activation Sequence |
| VPA | Valproic Acid |
| wg | Wingless, (*Drosophila* homolog of human Wnt) |

## Introduction

### 1.1 The role of the MEGF10 receptor in physiology and disease

Human MEGF10 or 'multiple epidermal growth factor-like domain 10' is a single transmembrane receptor. Recently mutations in *MEGF10* have been linked to a rare muscle disease in humans, a novel congenital myopathy named Early onset of Myopathy, Areflexia, Reparatory Distress and Dysphagia (EMARDD), or MEGF10 myopathy. This disease presents features of both muscular dystrophy and congenital myopathy (1-4).

MEGF10 is expressed in the central nervous system (CNS), retina and skeletal muscles. In the CNS, this protein is abundant in astrocytes and myelinating oligodendrocytes (5). MEGF10 acts as a phagocytic receptor and mediates glial engulfment of apoptotic neurons (6), is involved in amyloid-β uptake (7) and synapse remodeling by astrocytes in both the developing and adult CNS (8, 9). In the retina MEGF10 promotes the spacing of interneurons into mosaics (10). In muscles, MEGF10 is expressed in skeletal myoblasts and progenitor quiescent satellite cells, where it suppresses the myoblast differentiation program and plays a role in enhanced proliferation of PAX7 positive satellite cells, muscle repair and regeneration ((11) and table 1).

At the structural level, the MEGF10 protein consists of an N-terminus EMI domain and multiple extracellular EGF-like domains, followed by an intracellular non-canonical immune-receptor tyrosine-based activation motif (ITAM) (5, 6, 10). Each of the EGF-like domains possess 8 cysteine residues and two of the missense mutations that result in EMARDD, C326R and C774R, alter one of these cysteine residues (4).

1.2 The MEGF10 protein is conserved through evolution

Key structural features of MEGF10 are conserved from invertebrates to human (5, 10). Similarity between human MEGF10 and its *Drosophila melanogaster* homolog, Drpr has previously been reported (6, 30, 31), and residues that are targeted by mutations in human are conserved in the fly homologous protein. Drpr is encoded by the *draper, drpr (CG2086)* gene (6, 30), and is expressed in glia as well as in muscle at the postsynaptic neuromuscular junction ((32) and table 1). Drpr has roles in neuronal corpse glial engulfment during programmed cell death (30, 33), phagocytosis of degenerating axons and their debris (34) and remodeling of neuromuscular junction synapses (32). Notably, *Drpr* is the sole fly gene homolog of mammalian *MEGF10, MEGF11* and *MEGF12* (aka *JEDI)* (31). The *Drpr* gene encodes multiple protein isoforms. The three that are most characterized to date are Drpr-PB (Flybase ID: FBpp0072681), Drpr-PA (Flybase ID: FBpp0072680) and Drpr-PC (Flybase ID: FBpp0301579), referred to as Drpr-I, -II and –III, respectively.

**Table 1:** Human MEGF10 vs. *Drosophila* Drpr (in muscle).

| | MEGF10, Human muscle | Drpr, *Drosophila* muscle |
|---|---|---|
| Muscle cell type | Skeletal myoblasts (11)<br>Satellite cells (1, 2, 11)<br>Neuromuscular junction (2) | All skeletal muscles<br>Muscle progenitors (results from Drpr RNAi experiments) (12)<br>Neuromuscular junction (13) |
| Mediated function in muscle | Enhances satellite cell proliferation/ suppresses myoblast differentiation<br>Regulates muscle repair and regeneration (11) | Maintenance of normal muscle function<br>Down regulation results in abnormal muscle physiology (12) |

1.3 Animal models of MEGF10 myopathy

*Rodent model:* Preliminary studies carried out in the laboratory of our collaborator on the project, Dr. Peter Kang (University of Florida), have shown that the Megf10 knockout (*Megf10*$^{-/-}$) mouse (10) fails to recapitulate any of the muscle alterations observed in the human disease (Dr. P. Kang, personal communication). We hypothesize that the absence of phenotype in the muscle of Megf10$^{-/-}$ knock out mice might be due to functional redundancy provided by other members of MEGF family, Megf11 and Megf12. This is in contrast in what is observed in human where MEGF10 deficiency leads to the development of muscle disease.

*Drosophila* model:

(i) Advantages of the *Drosophila* model organism:

Down-regulation and/or overexpression of genes in *Drosophila melanogaster* is practiced widely, due to the variety of tools available for genetic manipulations in this model organism. One of the useful tools used to generate loss of function or gain of function *Drosophila* models of disease is the Gal4-UAS bipartite system. This binary system which originates from yeast is used extensively in *Drosophila* to manipulate gene expression in specific tissue or cell types (or in the whole organism) (14). In transgenic flies, the Gal4 gene inserted into the fly genome encodes a transcription factor and is expressed only in cells where the promoter placed upstream of Gal4 dictates. UAS stands for Upstream Activating Sequence, a promoter which is recognized by Gal4. Binding of Gal4 to UAS leads to expression of the gene (or RNAi sequence) cloned downstream of UAS. To down-regulate or overexpress a specific gene, Gal4 driver lines

are crossed with flies carrying the UAS transgene. The corresponding double transgenic progeny carry both UAS and Gal4 and can be used to study the effects of altered gene expression *in vivo* (14). Studies of the pathophysiology of diseases can be thus expedited in *Drosophila* (15, 16).

Fly mutants have also been used to set *in vivo* drug screening platforms aimed at identifying candidate therapeutic compounds. To date, multiple fly models of human neuromuscular diseases (e.g. spinal muscular dystrophy, actin myopathies, lamin-associated myopathies, dystrophinopathies, dystroglycanopathies) have been generated and characterized, and have provided insight into the molecular mechanisms underlying the corresponding human disease (17-24).

The versatility of fly genetics, together with the identification in flies of Adult Muscle Precursor cells (AMPs) that resemble satellite cells of higher organisms (25, 26), makes *Drosophila melanogaster* a well-suited model system for the study of muscle development and neuromuscular diseases. More specifically, what makes *Drosophila* a good model for human MEGF10 myopathy is the overlap in structure and reported function (e.g. regulation of glial cells engulfment) between human MEGF10 and fly Drpr. Many gaps remain however in our understanding of the molecular functions of MEGF10 in muscle cells. Our goal is to further investigate the molecular mechanisms regulated by MEGF10 in muscle, by using the corresponding *Drosophila* models that were generated and/or characterized in our laboratory (as detailed below).

(ii) MEGF10/Drpr loss of function mutant fly:

Recently a Drpr loss-of-function mutant fly (27) has been characterized by our group and the observed phenotypes were shown to recapitulate key features of the human muscle

disease (12). Drpr null mutant flies (Drpr$\Delta 5^{-/-}$) offer a valuable model to investigate the mechanisms underlying MEGF10 associated muscular disease. These fly mutants display a range of phenotypes including a shorter life span and locomotor defects with abnormal positioning and rigidity in the legs (compared to their heterozygous siblings and to control flies). Histological analysis of the muscle has shown hyalinization, loss of striation and vacuolization in the fibers. Drpr mutant flies also show extensive vacuolization and degeneration in the brain. The brain phenotype however contrasts with observations reported for MEGF10 myopathy patients who do not display any known cognitive impairments, structural brain abnormalities or visual defects. The CNS phenotype in *Drosophila* could be explained by the fact that Drpr is the sole homolog of MEGF10, MEGF11, and MEGF12; and that in mammals MEGF11 and/or MEGF12 may help compensate for MEGF10 shortage in the brain (12). Of note, the phenotypes observed in the *drpr* null mutant fly were also observed in a *drpr* RNAi mutant fly model (where drpr is down regulated in the entire organism) which was generated in a different genetic background. The Drpr KO/knockdown fly models of MEGF10 myopathy can be utilized as complementary genetic tools to further investigate the mechanisms underlying the disease, identify protein partners of MEGF10/Drpr, as well as study the signaling cascades downstream of these receptors.

(iii) MEGF10/Drpr gain of function mutant fly:

Recent studies in our laboratory have shown that ubiquitous overexpression of mouse *Megf10* or fly *drpr*, in a *drpr* wild-type genetic background, results in adult fly lethality and is deleterious to the developing organism (Figure 1). By using Gal4 driver lines that

specify expression of transgenes in selected cell types or tissue, it was determined that expression of Drpr in skeletal muscles, but not in glia, in the heart or in motor neurons, leads to lethality (Figure 2).

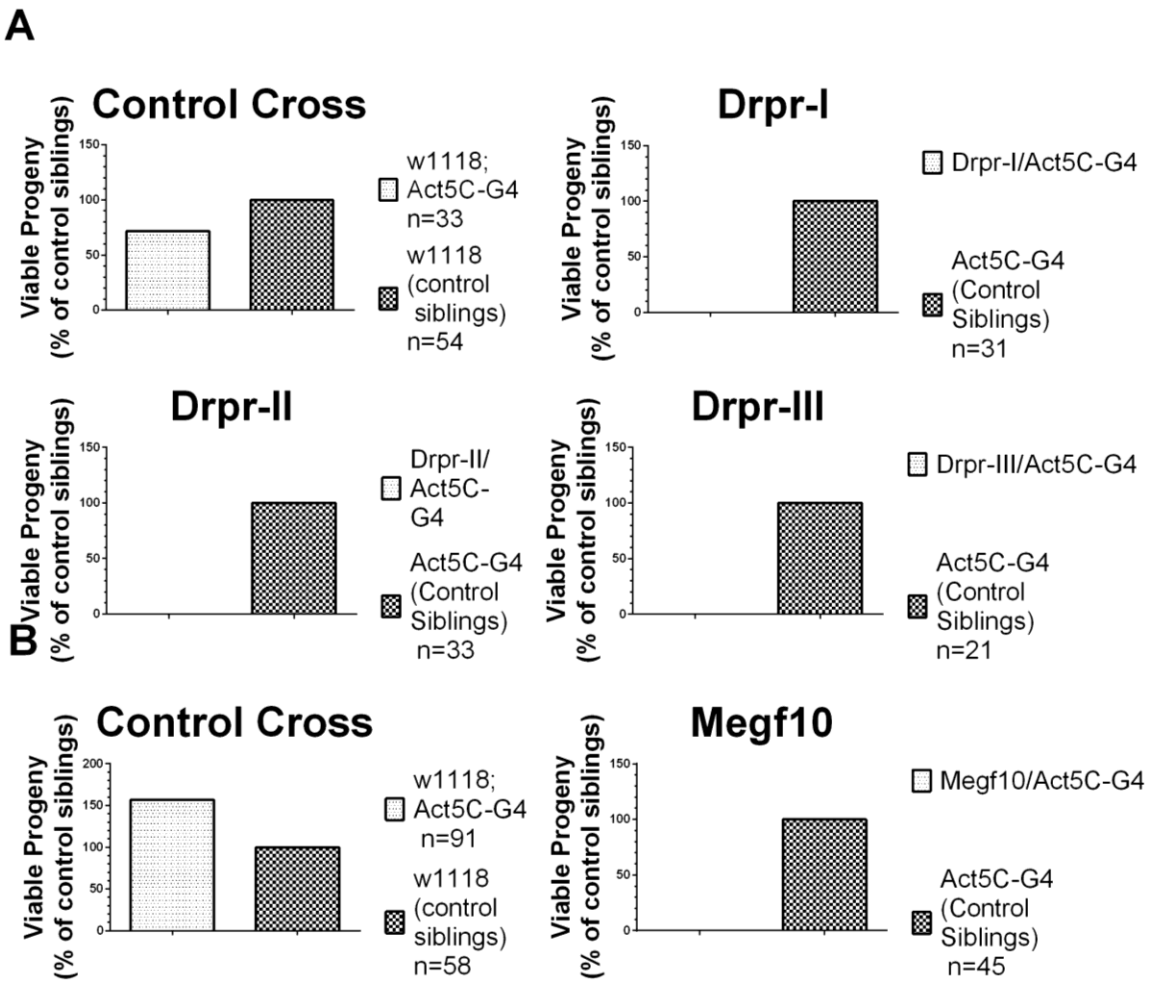


**Figure 1**: Overexpression of the fly, or mammalian, MEGF10 homolog in Drosophila is harmful to the developing organism. **A:** Ubiquitous overexpression of each of the three fly Drpr isoforms (i.e. I, II or III) in *Drosophila* results in pre-adult lethality. **B:** Ubiquitous expression of mouse Megf10 in *Drosophila*, also leads to lethality. All progeny was generated at 25°C.

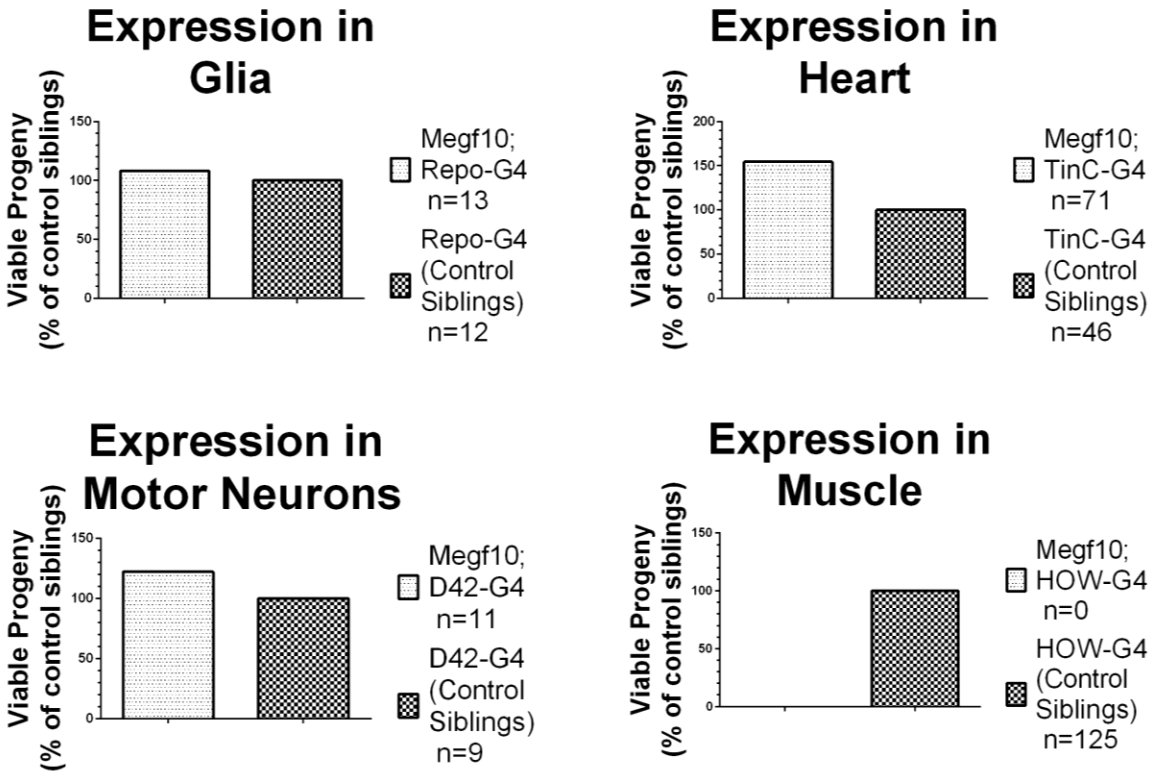


**Figure 2:** Expression of Megf10 in *Drosophila* muscle but not in glia, in the heart or in motor neurons, leads to lethality. Repo-Gal4, TinC-Gal4, D42-Gal4 and HOW-Gal4 are Gal4 driver lines that target transgene expression specifically to glia, the heart, motor neurons and muscle, respectively. Dark boxes correspond to the experimental progeny, and lighter boxes to control siblings that define the 100 % expected progeny).

1.4 Signaling pathways linked to MEGF10/Drpr mediated function

It is assumed that alteration in MEGF10/ Draper expression levels leads to deregulation of downstream signaling pathways. Evidence has been found by our group and others that supports a link between MEGF10/ Drpr to effectors of the Wnt (11) and Notch (11, 28, 29) signaling pathways (as summarized in the inset below; and figures 3, 4 and 5).

MEGF10 link with Notch and Wnt signaling pathways:

There exists functional correlation between MEGF10 regulation of satellite cell proliferation (1, 11) and Notch/Wnt signaling mediated regulation of satellite cell function (11, 30).

MEGF10 link with Notch:

(i) Sequence similarity was found between Notch ligands (e.g. Delta) and MEGF10/Drpr in the DSL-like domain ((29) and our results, figure 4). (ii) Previous studies have demonstrated decreased expression of Notch in Megf10 deficient C2C12 mouse myoblast cell lines (11)

MEGF10 link with Wnt:

Preliminary data from our group has identified two effectors in the Wnt pathway, i.e. cadherin 23 and Pi3KCa, as binding partners of Megf10.

Thus the following effectors/ candidate partners of MEGF10 were selected for studies:

■ in the Notch pathway N and Dl (*Drosophila* orthologs of human Notch and Delta, respectively, Figure 3). Delta is a ligand for the Notch receptor.

■ in the Wnt pathway armadillo and Pi3K68D (*Drosophila* orthologs of human β–catenin and Pi3KCa, respectively, Figure 5). β–catenin is the main effector in the Wnt canonical pathway.

The Notch signaling pathway contributes to the proliferation pattern of satellite cells and myoblasts (31), is inhibited in non-myogenic cells (32) and also modulates the homing of satellite cells and their adhesion to myofibers by regulating expression of basal lamina

components and adhesion molecules, like Megf10 (28). In addition, preliminary studies carried out in the laboratory of our collaborator, Dr. Peter Kang (University of Florida) has recently found that cadherin 23 and Pi3KCa, which belong to the Wnt pathway are binding partners of MEGF10. Notably, the Wnt signaling pathway is involved in homeostasis of adult muscle. It regulates activation of satellite cells and myogenic differentiation in adult skeletal muscles (33, 34), as well as proliferation and differentiation of myoblasts in myogenic lineage (30, 35).

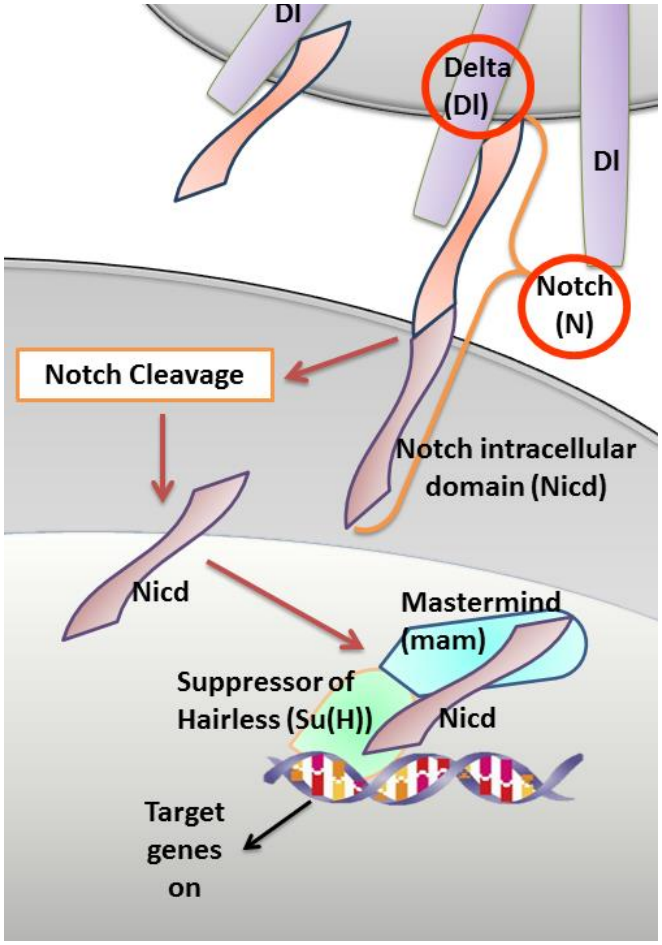


**Figure 3:** Simplified schematic view of the Notch signaling pathway in *Drosophila melanogaster*. Activation of the Notch (N) transmembrane receptor by its ligand Delta (Dl), results in cleavage of the receptor and release of the notch intracellular domain (Nicd). In the nucleus, Nicd interacts with the DNA binding protein Suppressor of Hairless (Su(H)) and coactivator Mastermind (mam). This interaction initiates the transcription of Notch signaling target genes (36, 37). Figure design adapted from (37). Red circles indicate effectors in the pathway that we selected for our analysis.

```
Dl              CDLNYYGSG--CAKFCRPRDDSFGHSTCSETGEIICLTGWQGDYC
Mus Dll1        CDEHYYGEG--CSVFCRPRDDAFGHFTCGDRGEKMCDPGWKGQYC
DrprI           CCDGYIASAGECVPHC---SEPCQHGRCISPEKCKCDHGYGGPAC
DrprII          CCDGYIASAGECVPHC---SEPCQHGRCISPEKCKCDHGYGGPAC
DrprIII         CCDGYIASAGECVPHC---SEPCQHGRCISPEKCKCDHGYGGPAC
Jedi            CCRGYYESRGACVPLC---AQECVHGRCVAPNQCQCAPGWRGGDC
Megf10          CCPGFYESRDMCVPHC---ADKCVHGRCIAPNTCQCEPGWGGTNC
                *   :  .   *   *    :   *  *       *  *: *  *
Consensus       C          C   C        H  C       C  G  G  C
```

**Figure 4:** The canonical DSL domain of the Notch ligands Delta (Dl, Drosophila) and Delta-like-1 (Dll1, mouse), shows significant similarity with a comparable motif found in mouse Megf10, mouse Megf12 (Jedi) and Drosophila Drpr isoforms -I, -II, -III. Abbreviations: Dl: *Drosophila melanogaster* Delta (NP_477264.1), mDll1: mouse Delta like 1 (AAH65063.1) homolog of Delta, DSL-like Domain: Delta/Serrate/LAG-2 like Domain.

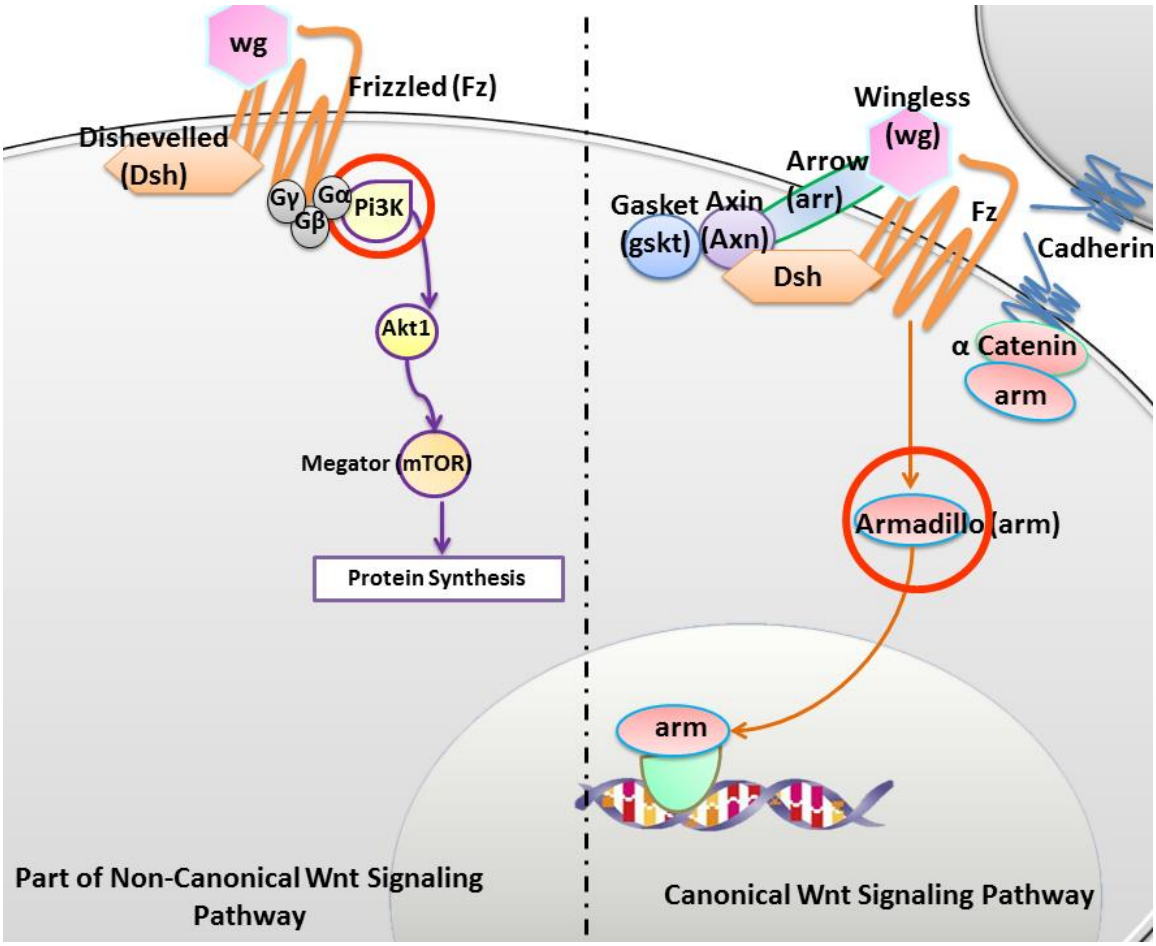


**Figure 5:** Simplified schematic view of the Wnt signaling pathway in *Drosophila melanogaster*. Right Panel: in canonical Wnt signaling, biding of Wingless (wg) to both its receptor Frizzled (Fz), and co-receptor Arrow (arr), activates disheveled (Dsh) and

leads to the recruitment of Axin (Axn) to arr. In the absence of wg, Axn degrades Armadillo (arm, the *Drosophila* homolog of β-catenin). Recruitment of Axn to arr leads to accumulation of arm in the cytoplasm. Arm then translocates into the nucleus, where it complexes with transcription factors and induces transcription of target genes. Left panel: in one of the non-canonical Wnt signaling pathways, Wingless (wg) stimulates Frizzled (fz) (independently of arr) and initiates the AKT/mTOR pathway by activating phosphatidylinositol 3-kinase (Pi3k). This in turn results in increased protein synthesis (30, 36). Figure design adapted from (30). Red circles indicate effectors in the pathway that we selected for our analysis.

Considering the important role of satellite cells in muscle repair, and the abundant expression of MEGF10 in these progenitor cells, the identification and characterization of MEGF10/Drpr binding partners could help define novel therapeutic entries to treat EMARDD as well as other muscle diseases.

## 1.5 Objective of this thesis

To complement the genetic studies carried out in the lab, I initiated molecular analyses focused on a restricted number of candidate pathways downstream of MEGF10/ Drpr. The rationale for selecting candidate pathways/ proteins is outlined in the inset "MEGF10 link with Notch and Wnt signaling pathways" (page 8). To carry out these studies, I used the *drpr* null fly model of MEGF10 myopathy, as well as the *Megf10/ drpr* gain-of-function mutant flies characterized in the lab. These studies were aimed at identifying novel binding partners of MEGF10/ Drpr, and at providing mechanistic insights into the function regulated by this family of receptors in muscle.

To identify candidate partners of MEGF10, we chose to assess the expression levels of canonical signaling molecules belonging to the Notch and Wnt signaling pathways (Figures 3 and 5), using our fly models of MEGF10 myopathy. These pathways play a critical role in muscle development and regeneration (11, 30, 38-48), and were identified

by our group and others as relevant to MEGF10 mediated function (11, 28, 29). The role of MEGF10 in the activation of satellite cell proliferation and inhibition of their differentiation was shown to be mediated at least in part by Notch (11). Also, the DSL domain of Notch ligands has significant similarity with a comparable motif in MEGF10, MEGF11 and MEGF12 (figure 2 and (29)), which gives us the rationale for studying expression levels of Notch signaling pathway effectors. In addition to Notch, two factors in the Wnt signaling cascade, a well-established regulatory pathway for myogenesis (11, 30, 40, 42, 43, 48), were demonstrated to bind MEGF10 *in vitro*, in co-immunoprecipitation and Western blot analyses carried out in the lab of our collaborator Dr. P. Kang/UF: Cadherin 23 and Pi3KCa. Cadherin 23 is a single-pass transmembrane protein essential to cell adhesion. It binds to β-catenin and participates in canonical Wnt signaling (49). Pi3KCa is a phosphatidylinositol 3-kinase. Pi3K activation in the non-canonical Wnt pathway results in muscle hypertrophy (30, 42, 43, 50) (Figure 5).

The factors that were selected for our study include, in the Notch pathway: N and Dl (which are *Drosophila* homologs of human Notch and Delta, respectively Figure 3); and in the Wnt pathway: arm and Pi3K68D (which are *Drosophila* homologs of human β–catenin and Pi3KCa, respectively, Figure 5). I designed primer pairs specific to each gene and assessed their expression level - *in vivo*, in response to altered Drpr expression. These studies were carried out by using reverse transcription polymerase chain reaction (RT-PCR) on total RNA isolated from Drpr null mutant ($Drpr\Delta5^{-/-}$) adult male flies and their (age- and gender-matched) Drpr heterozygous ($Drpr\Delta5^{-/+}$) siblings.

In addition, I used gain of function models of MEGF10/Drpr function. Although the mutations that underlie MEGF10 myopathy likely impair or abolish the normal function

of the corresponding protein, it is not known what would be the consequences of putative gain of function mutations in MEGF10. Our complementary fly models provide useful tools to begin to reconstitute the signaling pathway modulated by MEGF10/Drpr *in vivo*. The long term goal of this study is to develop new therapeutic strategies for MEGF10 myopathy, which is caused by mutations in the MEGF10 receptor.

## Materials and methods

2.1 *Drosophila* stocks and culture

The drprΔ5 mutant fly line [genotype: $w^{-}$*; sp/CyO*$^{act::GFP}$*; drprD5*$^{rec8\ (9)}$*/TM6, sb, Tb, e*; where $w^{-}$*; sp/CyO*$^{act::GFP}$*; drprD5*$^{rec8\ (9)}$*/drprD5*$^{rec8\ (9)}$ null are adult viable] was a gift from Marc R. Freeman (University of Massachusetts Medical School, Boston, MA). The *UAS-megf10* and *UAS-Jedi Drosophila* lines were generated and generously provided by Mary Logan (Oregon Health and Science University, Portland, OR). The genetic background strain $w^{1118}$ (FBal0018186), Canton-S (FBst0000001) and Gal4 driver lines Actin5C-Gal4 (*y1 w*; P[Act5C-Gal4] 25FO1/CyO*, y+; FBst0004414), how-Gal4 (w*; P[GawB]how24B; FBst0001767) and D42-Gal4 (w*; P[GawB]D42; FBst0008816) were purchased from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The repo-Gal4 driver line (expression in glia) was donated by Mary Roberts (F. Rob Jackson laboratory, Tufts University School of Medicine, Boston, MA). The TinC-Gal4 driver line was donated by Dr. Matthew J. Wolf (Duke University, Durham, NC). All strains were raised at 25$^{o}$C in a 12 h light: 12 h dark cycle on standard *Drosophila* media. To generate flies that overexpress *Mus musculus* Megf10 or

Megf12/Jedi, flies carrying the corresponding UAS-cDNA transgene were crossed with Actin5C-Gal4 driver flies at 25°C or at 18°C (the strength of the Gal4/UAS system is temperature sensitive, and more efficient at higher temperatures).

2.2 Assessment of transcript levels

RT-PCR was used to assess the transcription level of the following genes: *Mus musculus* Megf10, *Mus musculus* Jedi/Megf12, *Drosophila* Act5C (housekeeping gene), drpr (i.e. Megf10 homolog in fly), N (i.e. Notch ortholog in fly), Dl (i.e. Delta ortholog in fly), arm (i.e. β-catenin ortholog in fly) and Pi3K68D, Pi3KCa ortholog in Drosophila. Total RNA was isolated from a pool of eight 5-day-old adult male drprΔ5 homozygous null, drprΔ5 heterozygous (four independent biological replicates) and Canton-S (i.e. wild-type control) *Drosophila*. For *Drosophila* that express mouse Jedi ubiquitously (and corresponding controls), RNA was prepared from a pool of five 5-day-old adult males. For *Drosophila* that express mouse Megf10 ubiquitously (and corresponding controls), a pool of two males and three females (5-day-old) adult flies were used. On note, a large number of Jedi and Mef10 expressing flies die before reaching the adult stage, and the lethality phenotype is more pronounced in Meg10 transgenic flies. We thus used the rare Megf10 expressing mutant escapers, regardless of gender, (and a matching number of males/females control flies). RNA was extracted with the RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) following the manufacturer's recommendations. DNA contamination was removed prior to the reverse transcription using a DNA-free kit (Ambion). Complementary DNA was generated using a Superscript First-Strand kit (Life Technologies) following the manufacturer's recommendations. The conditions for RT

included cycles of 25°C × 10 min, 42°C × 20 min, 99°C × 5 min. Alternatively, Genomic DNA was isolated from pooled adult male Jedi- or Megf10- expressing *Drosophila* using the QIAamo DNA Micro Kit (Qiagen), following the manufacturers' recommendations. PCR was performed following these conditions: pre-incubation at 94°C × 10 min, followed by 35 cycles of amplification: 94°C × 30 sec, 60°C × 30 sec, 72°C × 1 min. The reaction was completed with a ten-minute incubation at 72°C. Primers were synthesized at the Tufts University Molecular Core (Tufts University, Boston, MA). All primers were designed specifically for cDNA, (of note, transgenic flies that express mouse Megf10, or mouse Jedi, carry the corresponding cDNA transgene) (Table 2). When assessing the level by PCR analysis, a minus RT condition was always added to the analysis to control for possible genomic DNA contamination. Results were analyzed, and PCR band intensity quantified, using Image J software (NIH). Transcript levels were expressed as a ratio of the control (housekeeping) gene Actin5C. Statistical analysis was performed using GraphPad Prism, using a one sample t test. (GraphPad Software, Inc., CA).

**Table 2:** List of primer pairs

| **Target cDNA** | **Primer sequence** |
|---|---|
| Act5C forward | 5'-CAGCCAGCAGTCGTCTAATCC-3' |
| Act5C reverse | 5'-CGACAACCAGAGCAGCAACTT-3' |
| Drpr forward | 5'-AGGACCTGGAATCCACTGC-3' |
| Drpr Reverse | 5'-GCCTGAAAAGGGCTCACATA-3' |
| Megf10_1 forward | 5'-CTGCCGATTCCTATCAGATC-3' |
| Megf10_1 reverse | 5'-GCTCACTGTAGGTTCGACTT-3' |
| Megf10_2 forward | 5'-GTTGTTCACCTGGGTACACTG-3' |
| Megf10_2 reverse | 5'-AGAGCAGTCAGTCCCTTTGA-3' |
| Jedi_1 forward | 5'-GACTGTGTACCGTCAGGTGG-3' |
| Jedi_1 reverse | 5'-GGTCCATGGAAACCCTCTGG-3' |
| Jedi_2 forward | 5'-CCATTCTTCCTGCCACCCAT-3' |
| Jedi_2 reverse | 5-AACAGTGCTATCAGGGCCAC-3' |
| arm forward | 5'-AAATTCCTTGGACGCGGTGTG-3' |

| | |
|---|---|
| arm reverse | 5'-CCAAAGCGGCTACCATCTGA-3' |
| Pi3k68D forward | 5'-ACGAGGACTACTCCCGTGTG-3' |
| Pi3k68D reverse | 5'-CGTGTACAAGTTTTCCGCCG-3' |
| N forward | 5'-AGTGTCCTTTGGGCTTCGAC-3' |
| N reverse | 5'-TTGCCCTCGAAACCTTTGGG-3' |
| Dl forward | 5'-GTTCCGGCAGCTTTGAGTTG-3' |
| Dl reverse | 5'-TGGCGCATTTGGGTATGTGA-3' |

**Results**

3.1 Sequence alignment of multiple Drosophila and mammalian homologs of human MEGF10 highlights conservation in selected extracellular domains of the receptor.

Drpr in *Drosophila* and Megf10 in mouse are homologs of human MEGF10. Jedi is the homolog of human MEGF12 in mouse. Sequence alignment (CLUSTAL 2.1, figure 6) of Drpr-I/Drpr-PB (1031 aa, http://www.ncbi.nlm.nsih.gov/protein, accession number NP_728660.2), Drpr-II/Drpr-PA (594 aa, http://www.ncbi.nlm.nsih.gov/protein, accession number NP_477450.1), Drpr-III/Drpr-PC (528 aa, http://www.ncbi.nlm.nsih.gov/protein, accession number NP_001246549.1), Mus musculus Megf10 protein (1147 aa, http://www.ncbi.nlm.nih.-gov/protein, accession number AAH75647.1) and mouse Megf12/Jedi protein (1034 aa, http://www.ncbi.nlm.nih.gov/protein, accession number AF444274_1) revealed a high degree of conservation in the extracellular EMI domain (N-terminus), and Delta/Serrate/LAG-2 (DSL)-like domain that is usually found in Notch ligands, as well as in four EGF-like/ laminin-like domains close to the transmembrane region of the protein. We postulate that each of the conserved motifs plays an important role in mediating the function of this family of receptors.

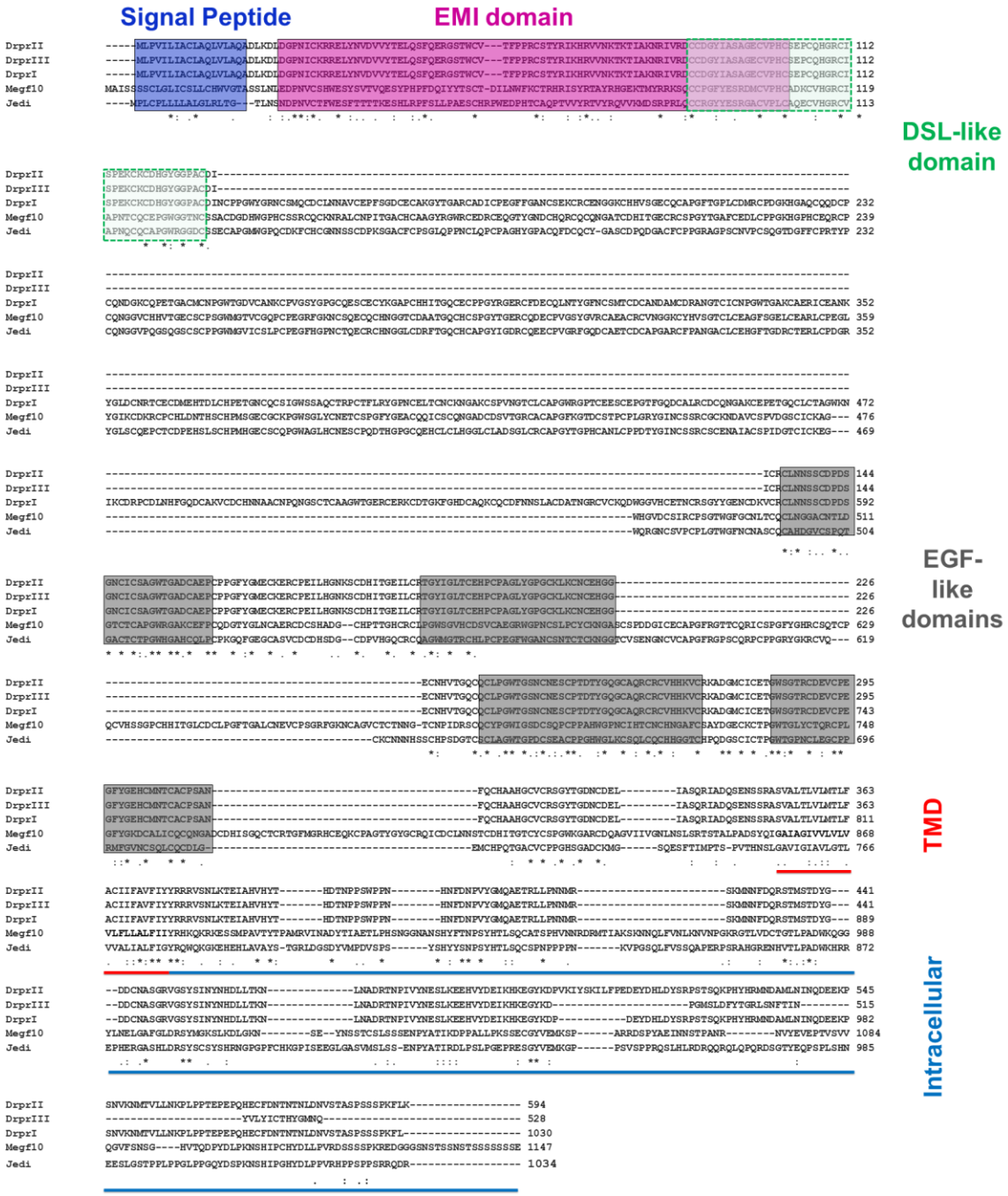


**Figure 6:** Sequence alignment of the three known *Drosophila* Drpr protein isoforms and the homologous proteins in mouse. *Drosophila* Drpr protein isoforms I, II and III are homologous to mammalian MEGF10 and MEGF12 (JEDI). The highest degree of similarity is found within the N-terminal EMI domain (magenta), the DSL like Domain (green) as well as in selected distal EGF-like domains (grey). Abbreviations: DSL-like Domain: Delta/Serrate/LAG-2 like Domain, EGF-like domain: Epidermal Growth Factor-like domain, TMD: transmembrane domain.

3.2 Decreased expression of genes involved in Wnt and Notch signaling pathways in *Drosophila* model of Loss of function mutation of Drpr/*Megf10*

RT-PCR analysis of total RNA from mutant Drpr$\Delta 5^{-/-}$ null and Drpr$\Delta 5^{+/-}$ heterozygous flies from four different RNA isolations (corresponding to four biological replicates) showed that the expression of important effectors in the Wnt signaling pathway, bcatenin (arm) and PI3K (Pi3K68D), as well as that of Delta (Dl, the ligand for Notch) significantly decreases in Drpr null vs. heterozygous flies ($p$ value$<0.05$). We confirmed in parallel that Drpr RNA is not detected in Drpr$\Delta 5$ null flies, while the corresponding RNA can be amplified from Drpr$\Delta 5^{+/-}$ heterozygotes. The expression of the Notch (N) receptor did not show a significant difference between the null and heterozygous flies (Figure 7). Canton-S wild type flies were tested as an additional control to Drpr$\Delta 5$ heterozygotes, however only one biological replicate was assessed, and no statistical analysis is available for this subgroup.

3.3 Expression of mouse Jedi/Megf12 in *Drosophila*

Crossing UAS-Jedi female flies with Actin5C-Gal4 male flies at 25°C results in some "escaper flies" which survive to adulthood and show decreased motor activity and abnormal positioning or rigidity of the legs. RT-PCR analysis of genomic DNA as well as of total RNA isolated from the corresponding Megf12/Jedi transgenic adult *Drosophila* confirms the presence, and expression, of the mouse transgene in these mutant flies (Figure 8).

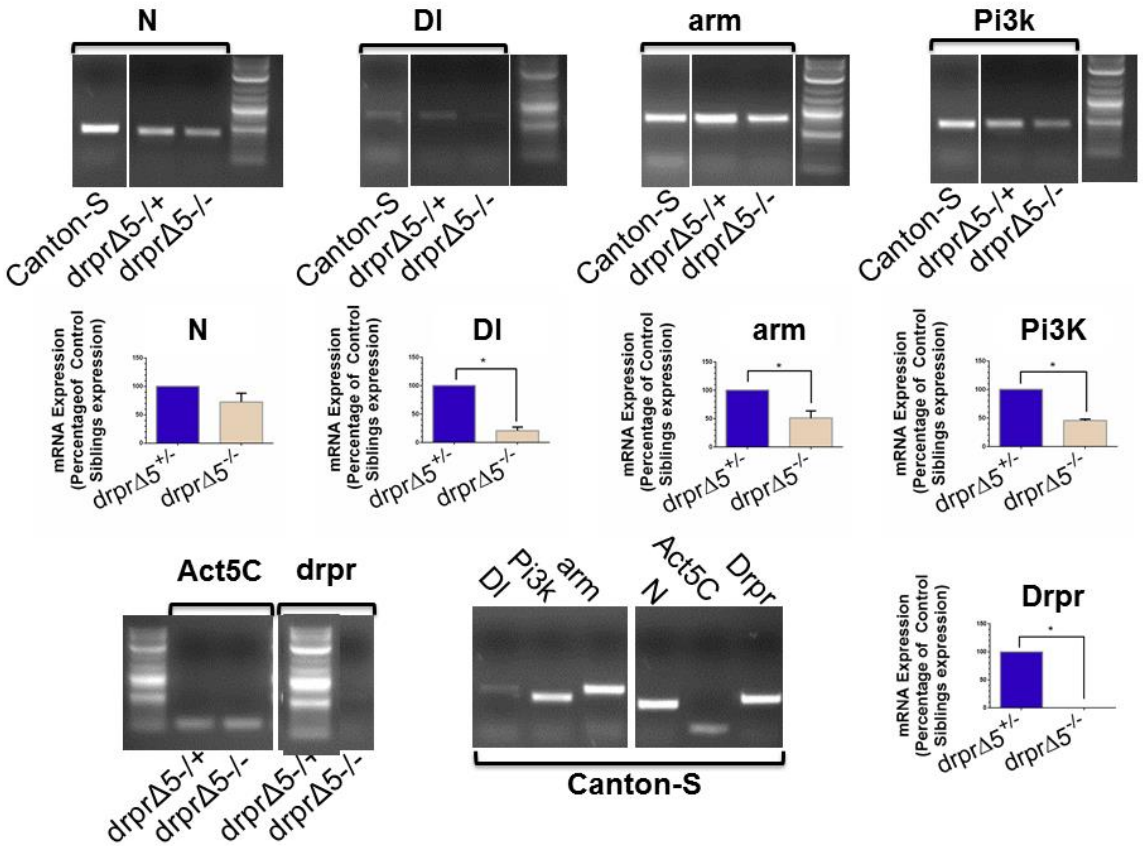


**Figure 7:** Semi-quantitative RT-PCR analysis of total RNA isolated from drpr mutant and control flies. This analysis shows that loss of function mutations in *Drpr* decreases the expression levels of candidate partners of Megf10/Drpr genes in the Wnt and Notch signaling pathways. Expression level of candidate genes was assessed in four biological replicated of Drpr$\Delta 5^{-/-}$ null flies and Drpr$\Delta 5^{+/-}$ heterozygotes. Canton-S wild type flies were used as an additional control. Data was normalized relative to expression of the Act5C (housekeeping, control) gene. Expression level was expressed as percent of expression in Drpr$\Delta 5^{+/-}$ heterozygous flies. Data is shown as Mean ± SEM of 4 data points for armadillo, Pi3K68D, N and Drpr. n=8 flies per genotype per replicate. * indicates a *p value<0.05*. Abbreviations: arm: armadillo/β-catenin, Dl: Delta, N: Notch, Pi3K: Pi3K68D, cDNA: complementary DNA.

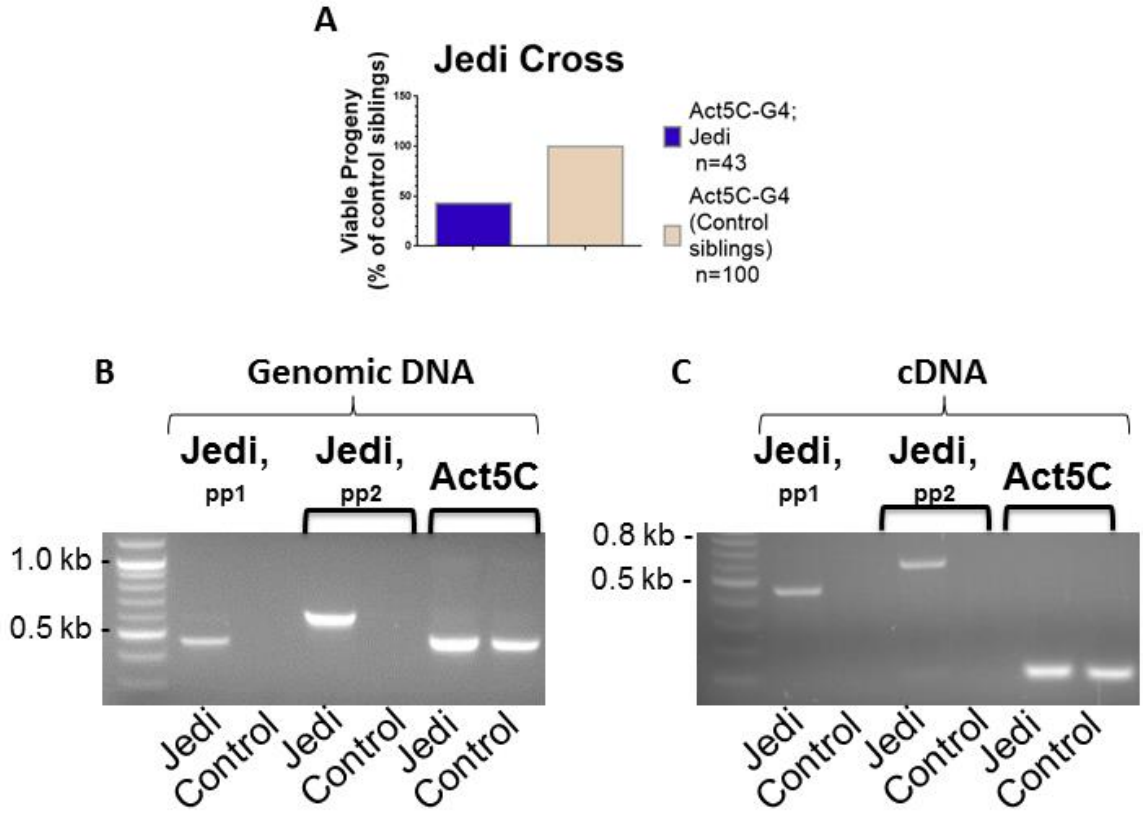


**Figure 8:** PCR analysis of fly genomic DNA and total RNA confirms the presence and expression of the mouse Jedi transgene in *Drosophila*. **A:** Some progeny that express Jedi escape lethality. **B:** PCR analysis of fly genomic DNA. **C:** RT-PCR analysis of total RNA. n=5 flies per genotype (B, C). cDNA: complementary DNA, Control flies: Flies from the control cross with genotype $w^{1118}$; Act5C-G4, Jedi flies: Flies expressing the mouse Jedi, pp: primer pair.

3.4 Expression of fly drpr and mouse Megf10 in *Drosophila*

Ubiquitous overexpression in *Drosophila* of any of the three most characterized Drpr isoforms (I, II and III), or expression of mouse Megf10, (which is obtained by crossing either UAS-Drpr I, UAS-Drpr II,

UAS-Drpr or UAS-Megf10 female flies with Actin5C-Gal4 male flies at 25°C) results in adult lethality (Figure 4). However, crossing Act5C-Gal4 flies with UAS-Megf10 flies at a lower temperature, i.e. 18°C, leads to the emergence of few 'escaper flies' that ubiquitously express mouse Megf10. RT-PCR analysis confirmed the presence and

expression of the corresponding mouse transgene in these escapers (Figure 9). The Megf10 expressing *Drosophila* displayed abnormalities in their legs and wings (figure 9) as well as locomotor defects and a shorter lifespan (not shown).

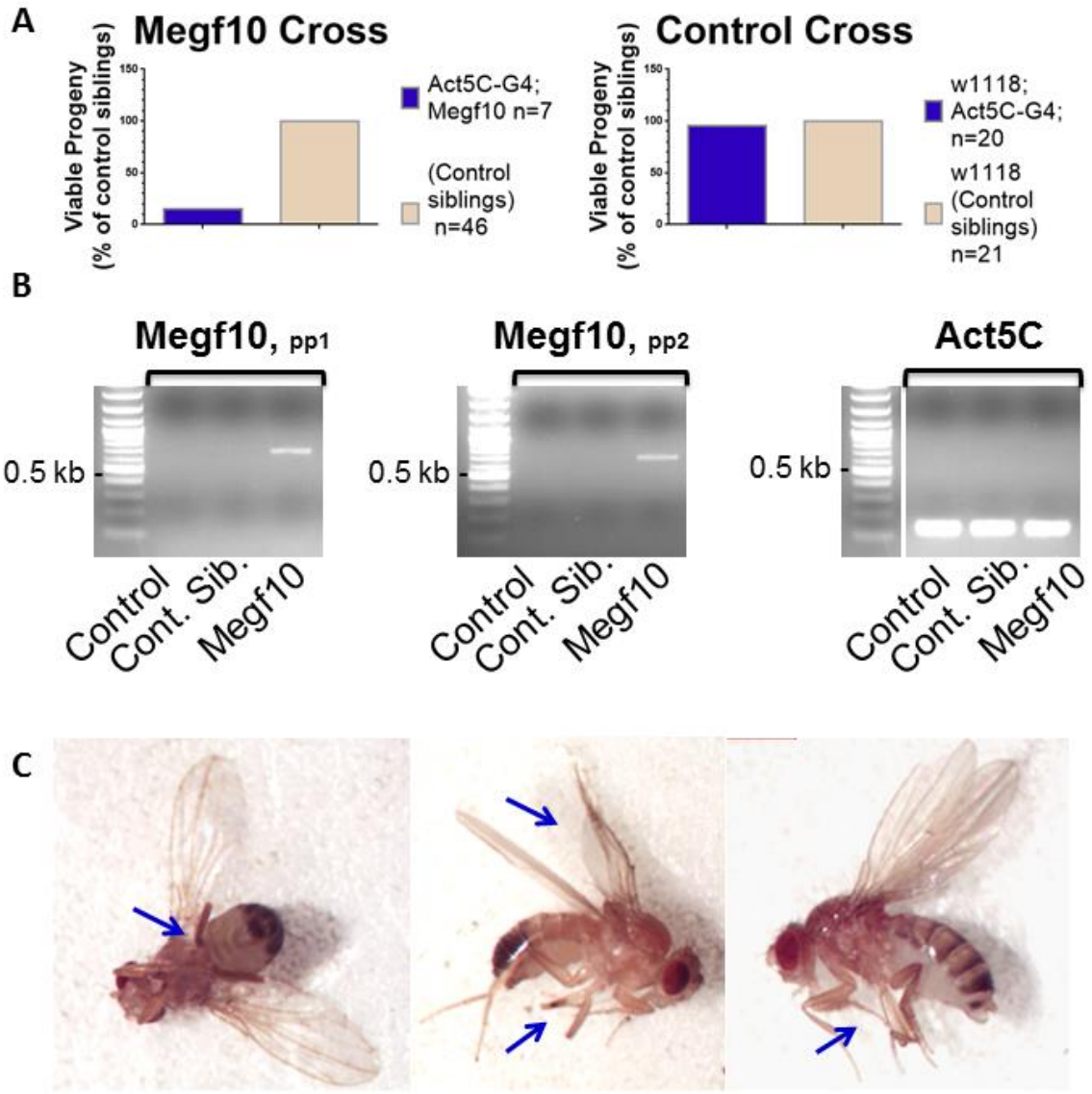


**Figure 9:** Ubiquitous expression of mouse Megf10 in Drosophila results in mutants that display marked morphological abnormalities in the appendages. A: A limited number of flies that express mouse Megf10 escape lethality at 18oC. B: RT-PCR analysis of total RNA confirms expression of mouse Megf10 in corresponding transgenic flies. n=5 per genotype. C: Megf10 expressing transgenic flies show pronounced abnormalities in the legs (sometimes severed after the tibia) and wings (arrows). Abbreviations: cDNA: complementary DNA, Control: w1118; Act5C-G4 flies, obtained from the control cross, Cont. Sib: flies that carry, but do not express, the Megf10 transgene, pp: primer pair.

**Discussion and Future Directions**

Megf10, multiple epidermal growth factor like domain 10, is a single transmembrane receptor expressed in skeletal muscle cells and neuronal glial cells in mammals and flies. Mutations in *MEGF10* cause MEGF10 myopathy, a rare autosomal muscular dystrophy in humans (1-4).

Currently there is no confirmed mouse model of MEGF10 myopathy. To further understand the role of MEGF10 in muscle, we have generated and/or characterized complementary loss of function and gain of function fly models of MEGF10 deregulation. These include flies deficient in the *Drosophila* homolog of MEGF10, i.e. *Drpr* null as well as *drpr* RNAi flies. In addition either fly Drpr, or one of its mammalian homologs (mouse Megf10, mouse Megf12/JEDI) has been expressed in transgenic flies, in the *drpr* wild-type genetic background. Both down-regulation and overexpression of MEGF10/Drpr in flies result in reduced viability and decreased motor function. The lethality phenotype is more pronounced in gain of function mutants where the great majority of flies die before eclosion. Drpr/Megf10 overexpressing flies that escape lethality display rigid limbs that are dragging or held upright, a phenotype which is highly reminiscent of that observed in *drpr* loss of function (i.e. null) mutant flies.

Together our genetic analysis of loss and gain of function MEGF10/ Drpr mutant flies indicate that the levels of this transmembrane receptor are tightly regulated *in vivo* and changes in its expression impairs normal muscle physiology and is not well tolerated by the developing organism. Parallels can be made with published results from studies focused on Notch signaling, which demonstrated that both loss of function mutation, as well as gain of function mutation in this pathway can lead to the development of similar

pathologies (e.g. aortic valve disease, hearing loss (51)). In addition, Panchumarthi has demonstrated that overexpression in *Drosophila* of Serrate (a Notch ligand) results in defects that phenocopy those seen in Serrate loss-of-function mutants (52). We also show that overexpression of any member of the MEGF10/ Drpr family of receptors that are available in the lab (i.e. Drpr isoforms I, II and III, mouse Megf10 and mouse Meg12) in *Drosophila* results in similar lethality /leg phenotypes.

Sequence alignment of these proteins (using all Drpr isoforms, mouse Megf10 and mouse Megf12/Jedi, Figure 6) shows conservation in specific domains of the protein, notably in the extracellular EMI and DSL domains situated at the N-terminus, as well as in four distal EGF-like domains localized prior to the transmembrane domain. The EMI domain is postulated to participate in protein-protein recognition (53) and the DSL domain participates in the recognition of the Notch receptor (29). The overall structural similarity of MEGF10/JEDI to the Notch ligands Jagged and delta (Figures 2 and 6), in particular in the extracellular portion, suggests that these transmembrane receptors may regulate Notch signaling. It would be of interest to investigate whether short peptides derived from the N-terminus portion of Drpr/MEGF10 (more specifically the sequence encompassing the EMI and/or DSL-like domain), can modify Notch signaling *in vitro*, as well as in *Drosophila* where physiological outcomes such as viability, locomotor activity and muscle histology can be assessed (54). Our complementary fly models can be used as useful tools to investigate the role of MEGF10 in the regulation of the Wnt and Notch signaling cascades, as well as to pursue structure-function studies.

Our results indicate that key components in the Notch and Wnt signaling pathways are down regulated in *drpr* loss of function mutant flies.

4.1 Effect on Notch signaling

Our findings correlate with work by others that shows that Megf10 acts on the notch signaling pathway to activate the proliferative potential of satellite cells and suppresses their differentiation (11). It is well-established that dynamic regulation of Notch signaling is necessary to balance self-renewal and differentiation of satellite cells (38, 39). Notably Holterman's group has documented expression of Megf10 in the committed progenitor population of satellite cells where Delta-like-1, a ligand of the Notch receptor, is also expressed. Although Megf10 and Delta share similar structural features, co-expression of these two signaling molecules in e committed progenitor population of quiescent satellite cells (11) suggests an absence of functional redundancy (i.e. in MEGF10 myopathy patients, Delta does not compensate). It was also shown that Megf10 knockdown leads to premature differentiation of primary myoblasts and a decline in the levels of Notch 1, 2 and 3 receptors (as well as in other downstream components of the Notch signaling pathway), but no significant difference was observed in the expression levels of notch ligands, including Delta1 (11). Of note, although both Holterman's group as well as our study demonstrate cross talk between MEGF10 mediated function and the Notch pathway, our results (decreased in Delta levels and no change in Notch, in Drpr deficient flies) are the reverse of those obtained by Holterman (decrease in Notch levels and no change in Delta, in cells that down-regulate Megf10 –as assessed by PCR analysis (11)). Additional experiments using *in vitro* as well as *in vivo* models of Megf10 deficiency are needed to further probe the link between Megf10 and Notch.

4.2 Effect on Wnt signaling

We also see a decrease in expression levels of key component of the Wnt pathway in drpr null mutant flies (vs. heterozygous siblings). Wnt proteins have multiple roles in myogenesis: (i) conversion of proliferating satellite cells (i.e. precursors) into fibers in aged muscles (through the β-catenin canonical pathway) (40, 48), (ii) satellite cell expansion resulting in enhanced muscle repair and (iii) induction of hypertrophy in muscles (through activation of Pi3K) (30, 42, 43). Preliminary data from our group has identified two effectors in the Wnt pathway, i.e. cadherin 23 and Pi3KCa, as binding partners of Megf10. In addition, MEGF10 has been shown to regulate the levels of important myogenic factors, Myf5 and MyoD (11). These proteins act downstream of β-catenin in the canonical Wnt signaling pathway(45-47).

In future investigations we propose to utilize our Drpr *Drosophila* model of the disease as a screening platform. These studies are aimed at identifying potential pharmacologic modifiers of MEGF10 myopathy. Hit compounds that are identified using our *in vivo* screen may also have potential for the treatment of other muscular diseases (where muscle progenitors are recruited to repair the degenerating tissue). Our targeted screen would include selected drugs that activate either the Notch, or the Wnt, signaling pathways (Table 3).

4.3 Candidate drugs that activate Notch

(i) Histone deacetylase inhibitors: Valproic acid (VPA), a fatty acid used primarily in the treatment of seizure disorders and bipolar disorder (55), has been studied for its capability to activate Notch 1 (and act as a tumor suppressor in neuroendocrine and thyroid cancers (56, 57)). The mechanism of VPA's mechanism in Notch activation remains unknown.

**Table 3:** Candidate MEGF10 myopathy therapeutic agents that activate the Wnt and Notch pathways. Candidate therapeutics in grey cells are suited for preclinical/ clinical studies in mammals only (there is no corresponding homolog in *Drosophila*).

| | | Mechanism | Reference |
|---|---|---|---|
| Notch Signaling Activators | Valproic Acid | unknown, probably related to VPA's histone deacetylase activity | (56-61) |
| | Resveratrol | Up-regulation of a Notch isoform | (62) |
| | Peptide: CDDYYYGFGCNKFCRPR | Human Jagged-1 DSL domain | (63) |
| Wnt Signaling Activators | Wnt7a and its derivatives | Stimulates the Wnt receptor (Fz) | (30, 41, 43, 44, 64, 65) |
| | GSK3β inhibitors, example: Lithium (66, 67) | Inhibits GSK3β from phosphorylating β catenin | (66-70) |
| | Riluzole | Unknown | (71) |
| | DKK1 monoclonal antibodies | target of TCF in the Wnt canonical pathway, participating in a negative feedback loop | (68, 72-79) |
| | Sclerostin monoclonal antibodies, example: AMG 785 (80) | blocks sclerostin which inhibits the canonical Wnt signaling | (79-85) |
| | SFRP Inhibitors, example: Piperidinyl iphenylsulfonyl sulfonamides (86) | Targets SFRPs, which are Wnt antagonists | (86, 87) |

Another candidate drug in this subgroup is suberoyl bis-hydroxamic acid (SBHA) which has also been shown to activate Notch signaling (59-61).

(ii) Resveratrol: this compound has been confirmed as a Notch activator from a pool of 7264 candidate compounds (62).

(iii) A 17-aminoacid peptide Notch activator containing the highly conserved DSL domain of Notch ligands (63).

4.4 Candidate drugs that activate Wnt

Stimulating the Wnt signaling pathway can be achieved by stimulating Wnt receptors, upregulating the activity of pathway effectors or inhibiting the endogenous antagonists of this cascade:

(i) Activators of the Wnt receptor include Wnt7a (and derivatives), one of the major Wnt proteins in muscle regeneration (43). Notably natural secretion of Wnt proteins in the body minimizes an immunological reaction. Generating recombinant Wnts however has been difficult (30, 44, 64, 65). A truncated version of Wnt7a, i.e. Wnt7a-C-terminus variant, (which lacks the pamitoylation) has been generated and proven to be fully functional in skeletal muscles (41). Other pharmacological activators of Wnt signaling have also been found, including 44 compounds from a pool of 1857 that activate Wnt signaling through β-catenin. Among those is riluzole, a drug approved for amyotrophic lateral sclerosis (ALS) (71).

(ii) Inhibitors of Wnt signaling antagonists include antibodies (developed against endogenous Wnt antagonists).

Many monoclonal antibodies that would be candidates for human therapeutics cannot be an option for a drug screen in *Drosophila*; one example is anti dickkopf-1 (Dkk-1) antibody (68, 72, 74, 79, 84). DKK-1, a negative regulator of canonical Wnt signaling (79) has no known fly homolog. Likewise, anti-Sclerostin antibodies (75, 80) will not be functional in *Drosophila* either; Sclerostin which binds to LPR5/6 and inhibits the canonical Wnt signaling through β-catenin (85) has no known homolog in *Drosophila*.

Another therapeutic entry is through the inhibition of glycogen synthase kinase 3β (GSK3β), which phosphorylates β catenin downstream of canonical Wnt signaling. Corresponding candidate drugs include lithium (*both in vitro* and animal studies have demonstrated the inhibitory effect of lithium on GSK3β (66, 67)), as well as Clozapine (68, 69). Pharmacological inhibition of GSK3β, however, might have side effects in human and caution needs to be taken due to oncogenic risks (68, 79).

A promising therapeutic avenue also includes agents that block SFRPs (Secreted Frizzled-Related Proteins). SFRP proteins, which comprise the largest family of Wnt antagonists, have structural similarities with the Frizzled receptor. Diphenylsulfonyl sulfonamide which inhibits human SRRP-1 was identified within a library of ~440,000 compounds, using a cell-based assay that measured activation of canonical Wnt signaling (87). Another study led to identification of an entire novel class of inhibitors of SFRPs called piperidinyl diphenylsulfonyl sulfonamides, showing good binding affinity at the Wnt receptor (86). SFRP has no identified homolog in *Drosophila*, (88, 89). However, *Drosophila* wingless was shown to bind to vertebrate SFRP (90), which suggests the presence of a functional homolog of SFRPs in *Drosophila*.

4.5 Future Directions

Currently, we are pursuing the identification and characterization of partners of MEGF10/Drpr that comprise key signaling pathways engaged in muscle development and regeneration (i.e. the Wnt and Notch pathways). In addition to the candidate partners discussed in the present report, we propose to investigate (using our fly models of MEGF10 myopathy) the expression levels of other candidates as follows. (i) In the Wnt pathway: Cad88C, ft, ds, kug (which are additional fly homologs of mammalian Cadherin), Pi3K92E (Pi3KCa fly homolog), Basket (Junk fly homolog), Rac1 (Rac1 fly homolog), Src42A and Src64B (Src fly homologs) and (ii) in the Notch pathway: hairy (homolog of human Hes-1). The corresponding primers have been designed for each of these genes. For the most promising MEGF10/Drpr candidate partner, the protein expression levels will be assessed in confirmatory Western blot analysis. These

complementary studies will enable us to further reconstitute the signaling cascade downstream of MEGF10/Drpr, and potentially identify molecular targets that may offer alternative entry points to treat the disease. Lastly, we propose to use our Drpr *Drosophila* models of MEGF10 myopathy as a screening platform to assess the potential of candidate therapeutic agents for this disease and other related muscular dystrophies.

## References


1. Boyden SE, Mahoney LJ, Kawahara G, Myers JA, Mitsuhashi S, Estrella EA, et al. Mutations in the satellite cell gene MEGF10 cause a recessive congenital myopathy with minicores. Neurogenetics. 2012;13(2):115-24.
2. Logan CV, Lucke B, Pottinger C, Abdelhamed ZA, Parry DA, Szymanska K, et al. Mutations in MEGF10, a regulator of satellite cell myogenesis, cause early onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD). Nature genetics. 2011;43(12):1189-92.
3. Pierson TM, Markello T, Accardi J, Wolfe L, Adams D, Sincan M, et al. Novel SNP array analysis and exome sequencing detect a homozygous exon 7 deletion of MEGF10 causing early onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD). Neuromuscular disorders : NMD. 2013;23(6):483-8.
4. Hartley L, Kinali M, Knight R, Mercuri E, Hubner C, Bertini E, et al. A congenital myopathy with diaphragmatic weakness not linked to the SMARD1 locus. Neuromuscular Disorders.17(2):174-9.
5. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28(1):264-78.
6. Scheib JL, Sullivan CS, Carter BD. Jedi-1 and MEGF10 signal engulfment of apoptotic neurons through the tyrosine kinase Syk. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012;32(38):13022-31.
7. Singh TD, Park SY, Bae JS, Yun Y, Bae YC, Park RW, et al. MEGF10 functions as a receptor for the uptake of amyloid-beta. FEBS letters. 2010;584(18):3936-42.
8. Chung W-S, Clarke LE, Wang GX, Stafford BK, Sher A, Chakraborty C, et al. Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature. 2013;504(7480):394-400.
9. Wu HH, Bellmunt E, Scheib JL, Venegas V, Burkert C, Reichardt LF, et al. Glial precursors clear sensory neuron corpses during development via Jedi-1, an engulfment receptor. Nature neuroscience. 2009;12(12):1534-41.
10. Kay JN, Chu MW, Sanes JR. MEGF10 and MEGF11 mediate homotypic interactions required for mosaic spacing of retinal neurons. Nature. 2012;483(7390):465-9.
11. Holterman CE, Le Grand F, Kuang S, Seale P, Rudnicki MA. Megf10 regulates the progression of the satellite cell myogenic program. The Journal of Cell Biology. 2007;179(5):911-22.
12. Draper I, Mahoney LJ, Mitsuhashi S, Pacak CA, Salomon RN, Kang PB. Silencing of drpr leads to muscle and brain degeneration in adult Drosophila. The American journal of pathology. 2014;184(10):2653-61.
13. Fuentes-Medel Y, Logan MA, Ashley J, Ataman B, Budnik V, Freeman MR. Glia and Muscle Sculpt Neuromuscular Arbors by Engulfing Destabilized Synaptic Boutons and Shed Presynaptic Debris. PLoS Biology. 2009;7(8):e1000184.
14. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993;118(2):401-15.

15. Lloyd TE, Taylor JP. Flightless flies: Drosophila models of neuromuscular disease. Annals of the New York Academy of Sciences. 2010;1184:e1-20.
16. Draper I. Model organisms offer new possibilities for discovery of therapeutics. Drug discovery today Technologies. 2013;10(1):e61-4.
17. Chang HC-H, Dimlich DN, Yokokura T, Mukherjee A, Kankel MW, Sen A, et al. Modeling Spinal Muscular Atrophy in Drosophila. PLoS ONE. 2008;3(9):e3209.
18. Dialynas G, Flannery KM, Zirbel LN, Nagy PL, Mathews KD, Moore SA, et al. LMNA variants cause cytoplasmic distribution of nuclear pore proteins in Drosophila and human muscle. Human molecular genetics. 2012;21(7):1544-56.
19. Goldstein JA, Kelly SM, LoPresti PP, Heydemann A, Earley JU, Ferguson EL, et al. SMAD signaling drives heart and muscle dysfunction in a Drosophila model of muscular dystrophy. Human molecular genetics. 2011;20(5):894-904.
20. Rajendra TK, Gonsalvez GB, Walker MP, Shpargel KB, Salz HK, Matera AG. A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. J Cell Biol. 2007;176(6):831-41.
21. Sevdali M, Kumar V, Peckham M, Sparrow J. Human congenital myopathy actin mutants cause myopathy and alter Z-disc structure in Drosophila flight muscle. Neuromuscular disorders : NMD. 2013;23(3):243-55.
22. Shcherbata HR, Yatsenko AS, Patterson L, Sood VD, Nudel U, Yaffe D, et al. Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. The EMBO journal. 2007;26(2):481-93.
23. Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, et al. Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. Aging cell. 2008;7(2):237-49.
24. Wairkar YP, Fradkin LG, Noordermeer JN, DiAntonio A. Synaptic defects in a Drosophila model of congenital muscular dystrophy. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28(14):3781-9.
25. Figeac N, Jagla T, Aradhya R, Da Ponte JP, Jagla K. Drosophila adult muscle precursors form a network of interconnected cells and are specified by the rhomboid-triggered EGF pathway. Development. 2010;137(12):1965-73.
26. Maqbool T, Jagla K. Genetic control of muscle development: learning from Drosophila. Journal of muscle research and cell motility. 2007;28(7-8):397-407.
27. Freeman MR, Delrow J, Kim J, Johnson E, Doe CQ. Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. Neuron. 2003;38(4):567-80.
28. Brohl D, Vasyutina E, Czajkowski MT, Griger J, Rassek C, Rahn HP, et al. Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. Developmental cell. 2012;23(3):469-81.
29. Krivtsov AV, Rozov FN, Zinovyeva MV, Hendrikx PJ, Jiang Y, Visser JW, et al. Jedi--a novel transmembrane protein expressed in early hematopoietic cells. Journal of cellular biochemistry. 2007;101(3):767-84.
30. von Maltzahn J, Chang NC, Bentzinger CF, Rudnicki MA. Wnt signaling in myogenesis. Trends in cell biology. 2012;22(11):602-9.
31. Wen Y, Bi P, Liu W, Asakura A, Keller C, Kuang S. Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. Molecular and cellular biology. 2012;32(12):2300-11.

32. Caine C, Kasherov P, Silber J, Lalouette A. Mef2 interacts with the Notch pathway during adult muscle development in Drosophila melanogaster. PLoS One. 2014;9(9):e108149.
33. Fujimaki S, Hidaka R, Asashima M, Takemasa T, Kuwabara T. Wnt protein-mediated satellite cell conversion in adult and aged mice following voluntary wheel running. The Journal of biological chemistry. 2014;289(11):7399-412.
34. Doi R, Endo M, Yamakoshi K, Yamanashi Y, Nishita M, Fukada S, et al. Critical role of Frizzled1 in age-related alterations of Wnt/beta-catenin signal in myogenic cells during differentiation. Genes to cells : devoted to molecular & cellular mechanisms. 2014;19(4):287-96.
35. Alexander MS, Kawahara G, Motohashi N, Casar JC, Eisenberg I, Myers JA, et al. MicroRNA-199a is induced in dystrophic muscle and affects WNT signaling, cell proliferation, and myogenic differentiation. Cell death and differentiation. 2013;20(9):1194-208.
36. Mbodj A, Junion G, Brun C, Furlong EE, Thieffry D. Logical modelling of Drosophila signalling pathways. Molecular bioSystems. 2013;9(9):2248-58.
37. Zacharioudaki E, Bray SJ. Tools and methods for studying Notch signaling in Drosophila melanogaster. Methods. 2014;68(1):173-82.
38. Conboy IM, Rando TA. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Developmental cell. 2002;3(3):397-409.
39. Jiang C, Wen Y, Kuroda K, Hannon K, Rudnicki MA, Kuang S. Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. Disease models & mechanisms. 2014;7(8):997-1004.
40. Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, et al. Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science (New York, NY). 2007;317(5839):807-10.
41. von Maltzahn J, Zinoviev R, Chang NC, Bentzinger CF, Rudnicki MA. A truncated Wnt7a retains full biological activity in skeletal muscle. Nature communications. 2013;4:2869.
42. von Maltzahn J, Renaud JM, Parise G, Rudnicki MA. Wnt7a treatment ameliorates muscular dystrophy. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(50):20614-9.
43. von Maltzahn J, Bentzinger CF, Rudnicki MA. Wnt7a-Fzd7 signalling directly activates the Akt/mTOR anabolic growth pathway in skeletal muscle. Nature cell biology. 2012;14(2):186-91.
44. Bentzinger CF, von Maltzahn J, Dumont NA, Stark DA, Wang YX, Nhan K, et al. Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. J Cell Biol. 2014;205(1):97-111.
45. Pan W, Jia Y, Wang J, Tao D, Gan X, Tsiokas L, et al. Beta-catenin regulates myogenesis by relieving I-mfa-mediated suppression of myogenic regulatory factors in P19 cells. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(48):17378-83.
46. Tajbakhsh S, Borello U, Vivarelli E, Kelly R, Papkoff J, Duprez D, et al. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse

paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development. 1998;125(21):4155-62.
47. Armstrong DD, Esser KA. Wnt/beta-catenin signaling activates growth-control genes during overload-induced skeletal muscle hypertrophy. American journal of physiology Cell physiology. 2005;289(4):C853-9.
48. Polesskaya A, Seale P, Rudnicki MA. Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. Cell. 2003;113(7):841-52.
49. Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. Cold Spring Harbor perspectives in biology. 2010;2(2):a002915.
50. Vadlakonda L, Pasupuleti M, Pallu R. Role of PI3K-AKT-mTOR and Wnt Signaling Pathways in Transition of G1-S Phase of Cell Cycle in Cancer Cells. Frontiers in oncology. 2013;3:85.
51. Andersson ER, Lendahl U. Therapeutic modulation of Notch signalling--are we there yet? Nature reviews Drug discovery. 2014;13(5):357-78.
52. Panchumarthi S. The Drosophila Serrate is Required for Synaptic Structure and Function at Larval Neuromuscular Junctions. The University of Arizona.; 2010.
53. Doliana R, Bot S, Bonaldo P, Colombatti A. EMI, a novel cysteine-rich domain of EMILINs and other extracellular proteins, interacts with the gC1q domains and participates in multimerization. FEBS letters. 2000;484(2):164-8.
54. Mitsuhashi S, Mitsuhashi H, Alexander MS, Sugimoto H, Kang PB. Cysteine mutations cause defective tyrosine phosphorylation in MEGF10 myopathy. FEBS letters. 2013;587(18):2952-7.
55. Henry TR. The history of valproate in clinical neuroscience. Psychopharmacology bulletin. 2003;37 Suppl 2:5-16.
56. Greenblatt DY, Cayo MA, Adler JT, Ning L, Haymart MR, Kunnimalaiyaan M, et al. Valproic Acid Activates Notch1 Signaling and Induces Apoptosis in Medullary Thyroid Cancer Cells. Annals of surgery. 2008;247(6):1036-40.
57. Mohammed TA, Holen KD, Jaskula-Sztul R, Mulkerin D, Lubner SJ, Schelman WR, et al. A Pilot Phase II Study of Valproic Acid for Treatment of Low-Grade Neuroendocrine Carcinoma. The Oncologist. 2011;16(6):835-43.
58. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, Ning L, Haymart M, Kunnimalaiyaan M, et al. Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist. 2007;12(8):942-51.
59. Adler JT, Hottinger DG, Kunnimalaiyaan M, Chen H. Combination therapy with histone deacetylase inhibitors and lithium chloride: a novel treatment for carcinoid tumors. Annals of surgical oncology. 2009;16(2):481-6.
60. Adler JT, Hottinger DG, Kunnimalaiyaan M, Chen H. Histone deacetylase inhibitors upregulate Notch-1 and inhibit growth in pheochromocytoma cells. Surgery. 2008;144(6):956-61; discussion 61-2.
61. Ning L, Greenblatt DY, Kunnimalaiyaan M, Chen H. Suberoyl bis-hydroxamic acid activates Notch-1 signaling and induces apoptosis in medullary thyroid carcinoma cells. Oncologist. 2008;13(2):98-104.

62. Pinchot SN, Jaskula-Sztul R, Ning L, Peters NR, Cook MR, Kunnimalaiyaan M, et al. Identification and Validation of Notch Pathway Activating Compounds through a Novel High-Throughput Screening Method. Cancer. 2011;117(7):1386-98.
63. Kannan S, Sutphin RM, Hall MG, Golfman LS, Fang W, Nolo RM, et al. Notch activation inhibits AML growth and survival: a potential therapeutic approach. The Journal of Experimental Medicine. 2013;210(2):321-37.
64. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 2003;423(6938):448-52.
65. Willert K, Nusse R. Wnt proteins. Cold Spring Harbor perspectives in biology. 2012;4(9):a007864.
66. Zhu Z, Yin J, Guan J, Hu B, Niu X, Jin D, et al. Lithium stimulates human bone marrow derived mesenchymal stem cell proliferation through GSK-3beta-dependent beta-catenin/Wnt pathway activation. The FEBS journal. 2014;281(23):5371-89.
67. Edwards CM, Edwards JR, Lwin ST, Esparza J, Oyajobi BO, McCluskey B, et al. Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma bone disease and reduces tumor burden in bone in vivo. Blood. 2008;111(5):2833-42.
68. Zimmerman ZF, Moon RT, Chien AJ. Targeting Wnt Pathways in Disease. Cold Spring Harbor perspectives in biology. 2012;4(11):a008086.
69. Roh MS, Seo MS, Kim Y, Kim SH, Jeon WJ, Ahn YM, et al. Haloperidol and clozapine differentially regulate signals upstream of glycogen synthase kinase 3 in the rat frontal cortex. Experimental & molecular medicine. 2007;39(3):353-60.
70. Engler TA, Henry JR, Malhotra S, Cunningham B, Furness K, Brozinick J, et al. Substituted 3-imidazo[1,2-a]pyridin-3-yl- 4-(1,2,3,4-tetrahydro-[1,4]diazepino-[6,7,1-hi]indol-7-yl)pyrrole-2,5-diones as highly selective and potent inhibitors of glycogen synthase kinase-3. Journal of medicinal chemistry. 2004;47(16):3934-7.
71. Biechele TL, Camp ND, Fass DM, Kulikauskas RM, Robin NC, White BD, et al. Chemical-genetic screen identifies riluzole as an enhancer of Wnt/beta-catenin signaling in melanoma. Chemistry & biology. 2010;17(11):1177-82.
72. Bu G, Lu W, Liu CC, Selander K, Yoneda T, Hall C, et al. Breast cancer-derived Dickkopf1 inhibits osteoblast differentiation and osteoprotegerin expression: implication for breast cancer osteolytic bone metastases. International journal of cancer Journal international du cancer. 2008;123(5):1034-42.
73. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. The New England journal of medicine. 2003;349(26):2483-94.
74. Fulciniti M, Tassone P, Hideshima T, Vallet S, Nanjappa P, Ettenberg SA, et al. Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood. 2009;114(2):371-9.
75. Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, et al. DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. Oncogene. 2004;23(52):8520-6.

76. Glantschnig H, Hampton RA, Lu P, Zhao JZ, Vitelli S, Huang L, et al. Generation and selection of novel fully human monoclonal antibodies that neutralize Dickkopf-1 (DKK1) inhibitory function in vitro and increase bone mass in vivo. The Journal of biological chemistry. 2010;285(51):40135-47.
77. Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, et al. Dickkopf-1 is a master regulator of joint remodeling. Nature medicine. 2007;13(2):156-63.
78. Komatsu DE, Mary MN, Schroeder RJ, Robling AG, Turner CH, Warden SJ. Modulation of Wnt signaling influences fracture repair. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2010;28(7):928-36.
79. Kim JH, Liu X, Wang J, Chen X, Zhang H, Kim SH, et al. Wnt signaling in bone formation and its therapeutic potential for bone diseases. Therapeutic Advances in Musculoskeletal Disease. 2013;5(1):13-31.
80. Padhi D, Jang G, Stouch B, Fang L, Posvar E. Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2011;26(1):19-26.
81. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, et al. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. The Journal of biological chemistry. 2005;280(20):19883-7.
82. Li X, Warmington KS, Niu QT, Asuncion FJ, Barrero M, Grisanti M, et al. Inhibition of sclerostin by monoclonal antibody increases bone formation, bone mass, and bone strength in aged male rats. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2010;25(12):2647-56.
83. Li X, Ominsky MS, Warmington KS, Morony S, Gong J, Cao J, et al. Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2009;24(4):578-88.
84. Agholme F, Li X, Isaksson H, Ke HZ, Aspenberg P. Sclerostin antibody treatment enhances metaphyseal bone healing in rats. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2010;25(11):2412-8.
85. Ominsky MS, Vlasseros F, Jolette J, Smith SY, Stouch B, Doellgast G, et al. Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2010;25(5):948-59.
86. Moore WJ, Kern JC, Bhat R, Bodine PV, Fukyama S, Krishnamurthy G, et al. Modulation of Wnt signaling through inhibition of secreted frizzled-related protein I (sFRP-1) with N-substituted piperidinyl diphenylsulfonyl sulfonamides: part II. Bioorganic & medicinal chemistry. 2010;18(1):190-201.

87. Bodine PV, Stauffer B, Ponce-de-Leon H, Bhat RA, Mangine A, Seestaller-Wehr LM, et al. A small molecule inhibitor of the Wnt antagonist secreted frizzled-related protein-1 stimulates bone formation. Bone. 2009;44(6):1063-8.
88. Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. Journal of cell science. 2008;121(Pt 6):737-46.
89. Esteve P, Sandonis A, Ibanez C, Shimono A, Guerrero I, Bovolenta P. Secreted frizzled-related proteins are required for Wnt/beta-catenin signalling activation in the vertebrate optic cup. Development. 2011;138(19):4179-84.
90. Uren A, Reichsman F, Anest V, Taylor WG, Muraiso K, Bottaro DP, et al. Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. The Journal of biological chemistry. 2000;275(6):4374-82.