USING DROSOPHILA AS A MODEL SYSTEM TO STUDY HUMAN
INCLUSION BODY MYOPATHY TYPE 3 DISEASE

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DEDICATION

For my mother and father, who offered me unconditional love and support throughout the course of this thesis.
ABSTRACT OF THE THESIS

Using Drosophila as a Model System to Study Human Inclusion Body Myopathy Type 3 Disease

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Hereditary inclusion-body myopathy type 3 (IBM-3) is caused by a single amino acid Glu706Lys substitution in the SH1 helix of the myosin head. The SH1 domain has been proposed to play a key role in the conformational changes that occur in the myosin head during force generation which is coupled to ATP hydrolysis. We are using an integrative approach to study the structure-function relationship of the myosin SH1 domain in the Drosophila model system. We constructed a gene encoding myosin with the single amino acid mutation and expressed it in place of wild-type myosin heavy chain by germline transformation and crossing into a line that lacks myosin in its flight and jump muscles. Our results suggest that the E699K mutation (corresponding the E706K in humans) in the SH1 helix of the myosin head severely affects myofibril structure and function in homozygous flies. Myofibrillar disarray is detected in E699K indirect flight muscle at 2 hours of age, and more severe defects are seen at 3 weeks. Some vacuoles are observed, as found in the human disease. The E699K ultrastructure data agree with the locomotion assay, as the E699K flies are unable to beat their wings and a wings-up phenotype was present in 2 day old flies. The jump ability of young flies was also severely impaired compared to controls. The mutation depressed calcium as well as basal and actin-activated MgATPase ($V_{\text{max}}$) by $\sim 75\%$ compared to wild-type PwMhc2 myosin. Also, the mutation decreases in vitro motility of actin filaments by $\sim 80\%$. Thus, the homozygous E699K MHC mutation severely affects muscle structure and function in Drosophila. In the heterozygous condition, the myofibrillar structure shows normal thick and thin filament packing in young and older flies. The jumping ability of heterozygotes is increased significantly when compared with the homozygotes, but not as good as that of the control. The indirect flight muscle of heterozygotes cannot support flight. These results suggest that one copy of E699K with one copy of wild-type Mhc in the Mhc\textsuperscript{10} background affects myosin function but not IFM myofibril assembly and structure.
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INTRODUCTION

Muscle is the contractile tissue of animals which produces force and causes motion. Muscles are classified as either striated or smooth. Myosin is a major structural component of muscle and is considered to be the molecular motor that converts free energy derived from the hydrolysis of ATP into mechanical work. Defects in myosin structure are known to cause human muscle disease (Burghardt, Neff, Wieben, & Ajtai, 2010). Human hereditary inclusion-body myopathy type 3 (IBM-3) is caused by a single amino acid Glu706Lys substitution in the SH1 helix of the myosin head (Martinsson et al., 2000). In this study, I used *Drosophila* as a model system to study this disease.

MUSCLE FIBER STRUCTURE

Striated muscle is named for its repeating pattern of light and dark stripes, and includes cardiac and skeletal muscle. In human, the skeletal muscle can be further broken down into two categories: Type I and Type II. Type I fibers appear red in color due to the presence of the oxygen binding protein myoglobin. These fibers use oxidative metabolism and contract slowly with high force that is suited for endurance. Type II fibers appear white due to the absence of myoglobin. These fibers are able to contract quickly while producing a relatively small amount of force, and they use both oxidative metabolism and anaerobic metabolism (Reviewed by Zierath & Hawley, 2004).

The sarcomere is the fundamental unit of a striated muscle's myofibril, consisting of two Z-disks at opposite ends with an M-line in the center (Figure 1, blue arrow). The region between the Z-disk and the M-line is composed of thick and thin filaments (Figure 1, blue and pink lines). Thick filaments are composed of myosin, and thin filaments are composed of polymeric actin, also known as F-actin. Myosin has a head and a rod, and it is aligned tail-to-tail at both sides of the M-line. In thick filaments, myosin is assembled by its rod in an anti-parallel order. F-actin is polar with a plus end and minus end. The plus end of each thin filament is anchored on the Z-disk, and the minus end is extended toward the central M-line. The composition of the sarcomere can be divided into an A (anisotropic) band, and I (isotropic) band (Figure 1, brown arrows). The A band is
composed of thick filaments, while the I band is composed of thin filaments. The region where A band and I band overlap is usually considered part of the A band or is designated the A/I zone. In many vertebrate muscles, each thick filament is surrounded by six thin filaments arranged in a hexagon (reviewed by Squire, 1997). As described below, as a result of ATP-dependent movement of the myosin head, thin filaments slide relative to the thick filaments, and the Z-disks are forced to move towards the M-line causing muscle shortening.

**MYOSIN STRUCTURE AND THE ROLE OF THE SH1 HELIX**

Myosins constitute a large superfamily of proteins with at least 18 classes that share a motor domain which interacts with actin, hydrolyzes ATP and produces force (The Myosin Home Page, http://www.mrc-lmb.cam.ac.uk/myosin/myosin.html). Myosins
are generally classified into two categories: conventional myosins and unconventional myosins. Conventional myosins are composed of two myosin heavy chains (MHC), two essential light chains (ELC) and two regulatory light chains (RLC). MHC is a 200 kD protein consisting of a globular head, an alpha-helical rod and a non-helical C-terminal tailpiece. The myosin heads have ATPase activity and can attach to actin filaments to generate cross-bridge cycles. The muscle contraction is powered by the energy released from ATP hydrolysis with the movement of the myosin filaments past the actin filaments (Sellers, 2000).

Kinetic studies show that during the actomyosin contractile cycle, the motor domain undergoes a number of conformational changes in order to transduce ATP hydrolysis energy into mechanical work. The differential affinity of myosin for actin in the cycle is often described as causing the strong actin-binding state or the weak actin-binding state. The strong binding state is also called rigor or the nucleotide-free state, because the lever arm orientation is similar to that observed in electron microscopy (EM) images of actomyosin in the “rigor” state (Bauer, Holden, Thoden, Smith, & Rayment, 2000). When ATP binds to the rigor state actomyosin, the complex enzymatic process begins and myosin undergoes a conformational change to a weak-binding state. In this state, myosin hydrolyzes ATP and rapidly attaches and detaches from actin. After release of phosphate, myosin changes to a strong binding state. The power stroke that generates force and movement is likely to occur during the transition from the weak to strong binding state. When ADP releases from myosin, the myosin remains strongly bound to actin in the “rigor” state and the contractile cycle is accomplished (Figure 2, reviewed by VanBuren, Guilford, Kennedy, Wu & Warshaw, 1995).

![Figure 2. Scheme for the actomyosin cycle. A, actin; M, myosin; Pi, inorganic phosphate.](image)

The myosin head (subfragment one, or S1) consists of a motor domain and a lever arm domain. The lever arm amplifies the small conformational changes of the motor and generates force. The motor domain has four major subdomains: the N-terminal
subdomain, the upper 50-kD subdomain, the lower 50-kD subdomain, and the converter subdomain. The N-terminus lies near the start of the lever, and its function is unknown. The lower 50-kD subdomain is also called the actin-binding subdomain since it constitutes the major part of the actin-binding site. The upper 50-kD subdomain consists of a central seven-stranded β-sheet separating the ATP binding site from the actin binding site. The cleft between the upper and lower 50-kD subdomains is called the 50-kD cleft, which mediates the communication between the actin-binding site and the nucleotide-binding site via the γ-phosphate pocket. The converter subdomain is hypothesized to rotate by 65-70° upon the transition from the nucleotide-free state to the pre-power stroke state. It is believed that this rotation could amplify the small structural changes in the motor to the swivel of the lever arm and movement of F-actin (reviewed by Geeves, Fedorov, & Manstein, 2005). The converter is connected to the nucleotide binding site and actin binding site by two parallel helices: the SH1 helix and the relay helix (Figure 3A). With actin binding, the γ-phosphate pocket opens to release phosphate and the relay helix transmits linear force originating from the active site to the converter subdomain which rotates the lever-arm (Burghardt et al., 2010).

The SH1 helix is believed to play a key role during the conformational changes of myosin force generation. Amino acid alignment shows that the SH1 helix is 100% conserved in both conventional and unconventional myosins among different species (Martinsson et al., 2000; Figure 3C). The crystal structure of Drosophila melanogaster myosin, which is the subject of this study, has not been determined yet. Therefore, the homology modeling method is used to illustrate the protein structure (http://www.swiss-model.com). Drosophila homology modeling structures from the pre-power stroke and post-power stroke templates of scallop myosin show that the SH1 helix region melts during the power stroke (PDB: 1kk8 and 1qvi, Figure 3B). The unwinding of the SH1 helix could explain the rotation of the converter subdomain during the power stroke. The SH1/SH2 region is highly flexible at different actomyosin states, since several bifunctional sulfhydryl reagents (BSR) can cross-link Cys707 (SH1) and Cys697 (SH2) with different lengths (from 5 to 12Å) (Huston, Grammer, & Yount, 1988). Myosin with SH1-SH2 cross-linked also shows weakened ATPase activity and lower actin-binding affinity and exhibits rotational disorder comparable to that of the post-hydrolysis state.
Figure 3. (A) Overview of the *Drosophila* myosin structure by homology modeling method (http://www.swiss-model.com). The SH1 helix is labeled in red. The four major subdomains are labeled in green: Upper 50-kD subdomain, Lower 50-kD subdomain, the converter subdomain and N-terminal subdomain. The actin-binding site and ATP-binding site are labeled in blue. The homology model is generated by Swiss-Prot Homology Modeling and QuickPhyre. 3D structure is generated by PyMol. Template: crystal structure of scallop in pre-power stroke state. ExPDB: 1qviA. (B) The SH1 helix melts during the power stroke. Left panel, Pre-power stroke SH1 domain, right panel, post-power stroke SH1 domain. Glutamic acid is mutated to lysine and labeled in stick mode by Pymol mutation wizard. Model generated by Pymol. Pre-power stroke template: 1qviA, post-power stock template: 1kk8A. (C) Comparison of amino acid sequences in the conserved SH1 helix region of myosin class II in various species and organisms (data derived from GeneBank, NCBI). From top to bottom are human wild type, human IBM-3, chicken, *Drosophila* wild type and our mutant E699K. An arrow indicates the residue E706 in human myosin, which was mutated in IBM-3 MHC-IIa myopathy, and in residue E699 in our *Drosophila* model. Color codes for the amino acids: yellow, nonpolar; green, uncharged polar and glycine; red, acidic; and blue, basic.
(A·M·ADP·Pi, Figure 2) (Thompson, Naber, Wilson, Cooke, & Thomas, 2008). Also, chemical modification of the SH1 helix resulted in a dramatic decrease of myosin ATPase activity and loss of the motor function in the \textit{in vitro} motility assays (Bobkova, Bobkov, Levitsky, & Reisler, 1999). Mutations in this region like R689H (Iwai & Chaen, 2007) and G709V also affect myosin motor function and reduce myosin thermal stability. Therefore, it is proposed that the SH1 helix is a kind of clutch that controls coupling between the converter and the motor.

**CLINICAL STUDIES OF IBM-3**

Darin and coworkers first described a multigenerational Swedish family with a novel myopathy inherited as an autosomal dominant (Darin, Kyllerman, Wahlstrom,, Martinsson & Oldfors, 1998). This myopathy was classified as inclusion-body myopathy type 3 (IBM-3) and the clinical characteristic can be summarized as follows:

1. The clinical course was nonprogressive in childhood, but most adults experienced deterioration of muscle function, starting from 30 to 50 years of age.

2. The major histopathology change in skeletal muscle from childhood to adult patients was focal disorganization of myofilaments. In adults with progressive muscle weakness, the muscle biopsies showed dystrophic changes and rimmed vacuoles with cytoplasmic and intranuclear inclusions of 15 to 20 nm filaments.

3. In muscle biopsies, typical findings show inclusion bodies, rimmed vacuoles and accumulation of aberrant proteins similar to those found in senile plaques of Alzheimer's brain disease.

Martinsson and colleagues determined that IBM-3 is linked to G-to-A transition at nucleotide 2116 of the \textit{MYH2} gene in human fast skeletal muscle IIA (MyHC IIa), resulting in a Glu706-to-Lys (E706K) mutation in the highly conserved SH1 helix region of the motor domain on chromosome 17 (located at 17p13.1) (Martinsson et al., 2000).

Tajsharghi and coworkers analyzed skeletal muscle tissue from six members of the family reported by Martinsson (Martinsson et al., 2000; Tajsharghi et al., 2002). Two young patients aged 6 and 7 years old showed minor pathologic changes, and the mRNA level of MyHC IIa isoform was undetectable. However, in four older adults, aged 36 to 61 years, the wild type and E706K MyHC IIa alleles were equally expressed (Tajsharghi et al., 2002). Characteristic morphologic features include disorganization of the intermyofibrillar network, focal absence of mitochondria, disorganization of
myofilaments, frequent central nuclei, increased interstitial connective tissue, and 15 to 21 nm filamentous inclusions associated with rimmed vacuoles (Tajsharghi et al., 2002).

Li and colleagues performed *in vitro* motility assays on the myosin isolated from the muscle samples of IBM-3 patients (Li et al., 2006). Their results show that actin-sliding velocity is slower with the heterozygous myosin (E706K mutant and wild type myosin, equally expressed at the mRNA level) than that with the wild-type myosin. However, their results are not statistically reliable due to limited access to the tissue.

One interesting finding in the clinical study is that E706K mutation in MyHC IIa affects muscle structural integrity of all muscle cells irrespective of MyHC isoform expression (Li et al., 2006; Tajsharghi et al., 2002). One possible explanation could be that the proteolytic degradation products of myofibrillar proteins might have a “toxic” effect on muscle cell structure leading to impaired function and eventually apoptotic effects (Xu, Gu, Belknap, White, & Yu 2006). Since the motor function of E706K myosin is probably compromised, the dysfunctional myosin could cause the degradation of other sarcomeric structural proteins. When the “toxic” degradations accumulate with aging, muscle in adults may be more vulnerable compared with muscle in young individuals. Another explanation could be that IBM-3 is a “polygenic” disease, which means other genes and proteins may play important roles as modifiers of the symptoms (Li et al., 2006).

**C. elegans and Dictyostelium Models**

Tajsharghi and coworkers introduced *C. elegans* as a disease model to study the E706K mutation (Tajsharghi, Pilon, & Oldfors, 2005). They performed site-directed mutagenesis and generated *Mhc* DNA with an equivalent E706K mutation. They injected the DNA into the *C. elegans* embryos with a myosin heavy chain null background and investigated the ultrastructure and motility of the transgenic worms. They found that the E706K mutation does not severely affect *C. elegans* body wall myosin assembly properties, but does badly affect the motility. Zeng and colleagues performed transient kinetic measurements of the actomyosin ATPase activity in the Dictyostelium non-muscle myosin II (Zeng et al., 2004). The Dictyostelium myosin with an analogous E706K mutation demonstrated decreased actin-activated ATPase and an increased duty ratio.
Results from both studies indicate that the myosin motor function might be compromised by the E706K mutation.

**THE DROSOPHILA MODEL SYSTEM**

We chose *Drosophila melanogaster* as the model system to study the IBM-3 disease. *Drosophila* is idea model system for biology research for the following reasons:

1. Short generation time. The generation time of Drosophila is usually 11 to 12 days, with about seven to eight days in the egg and larval stages, and four to five days in the pupal stage.

2. High fecundity. On average, each healthy female can generate 200 new adults within 14 days and lay 300-400 eggs throughout its life.

3. Drosophila genome is fully sequenced. Several online bioinformatics database tools like FlyBase are user-friendly and easy to access (Http://www.flybase.org).

4. Genetics is easy to manipulate. It is relatively simple to perform crosses of each genotype and select a specific phenotype by using well-characterized genetic markers.

5. Easy and reliable transgenic methods. Over the years, a range of transgenic tools have been developed that allow easy genetic manipulation such as the GAL4-UAS system, protein traps and fusions, RNA interference and so forth. Most of these Drosophila transgenesis methods are based on a native transposon called the “P element”. The P element DNA construct is made to carry the gene sequence of interest. The DNA can be micro-injected into the embryos and becomes randomly and stably inserted into the genome. Our lab has been successfully using this method for several years.

In addition to these advantages, the *Drosophila* model system is specifically suitable to study “myopathy” muscle diseases like IBM-3. First of all, the *Drosophila Mhc* gene exists as a single copy per haploid genome that encodes all muscle MHC isoforms through alternative RNA splicing, which greatly simplifies genetic manipulation (Bernstein, Mogami, Donady, & Emerson, 1983). The *Drosophila Mhc* gene has a total of 19 exons, and five of them are alternative sets of exons (exon 3, 7, 9, 11 and 15). Only one homologous exon can be included in the mature transcript of either embryonic muscle isoform (EMB) or indirect flight muscle isoform (IFM). Exon 18 is a single alternative exon, either included or excluded. The E699 residue is located in exon 10 of *Mhc*, which is not an alternatively spliced exon.

In addition, MHC-null organisms are available to avoid endogenous MHC *in vivo*. The *Mhc* allele contains a single base substitution in the splice site upstream of exon
10a. These flies are viable organisms that are unable to express MHC in the IFM or jump muscle (Collier, Kronert, O'Donnell, Edwards, & Bernstein, 1990). Since these muscles are not essential for Drosophila survival in a laboratory environment, the mutant Mhc transgene can be moved into the Mhc\textsuperscript{10} background to obviate the masking effect of wild-type myosin. The mutant myosin can be isolated from their IFM to perform in vitro motility and ATPase analysis (Miller et al., 2005; Swank et al., 2003). Therefore, Mhc\textsuperscript{10} is an ideal genetic background for the transgenic expression of “myopathy” Mhc genes.

Last but not least, locomotion assays and transmission electron microscopy (TEM) can be used as straightforward ways to analyze the function and structure of skeletal muscles in the transgenic Drosophila. We use a flight index to measure the function of IFM, which is determined by the composite score of whether a fly is capable of flying up, horizontally, down or not at all (Drummond, Hennessey, & Sparrow, 1991). We also use jump distance to measure the jump muscle function, which is determined by measuring how far a fly can jump without wings from an elevated platform onto a paper containing concentric rings (Swank et al., 2002). TEM can be used to visualize the ultrastructure of the developing and aging IFM and jump muscle from the homozygous and heterozygous Glu699Lys flies. Furthermore, mechanical assays can be performed on both IFM and TDT fibers (Swank et al., 2006).

In this study, we produced a mutant Drosophila line expressing Glu699Lys myosin. We performed the protein expression assay to determine the mutated protein expression level, the locomotion and TEM assays to determine the structure and function of mutant Drosophila skeletal muscles, and the ATPase assay and in vitro motility assays to test the motor function of the mutated myosin. By using genetic approaches to study the molecular mechanism of IBM-3, we may be able to identify therapeutic agents for treating this myopathy and possibly other myosin-based inclusion body diseases.
MATERIALS AND METHODS

CREATION OF THE E699K CONSTRUCT

The E699K mutant Mhc gene was generated by W. A. Kronert using site directed mutagenesis. Overall, the construct containing the entire Drosophila melanogaster myosin heavy chain gene with the E699K mutation was cloned into a P element vector with the miniwhite gene, w^+, as a selectable eye-color marker (Swank et al. 2000). The construction of this mutant myosin gene was initiated by subcloning a 0.6 kb Bam HI – Eag I fragment that includes the exon 10 region. Site directed mutagenesis was performed on this subclone using Stratagene’s QuickChange II kit (Stratagene, La Jolla, CA). Upon sequence confirmation of the E699K site-directed mutagenesis product, the mutated subclone fragment was inserted to replace the wild-type version in a 2.5 kb Pst I – Eag I subclone containing the exon 9 through 11 region. The resulting subclone was cut with Eag I replaced back into the wild-type construct PwMhc2 at its Eag I site. The resulting clone was digested with Eag I, and the 12 kb fragment of the 3’ end of the Mhc gene was removed from its vector by Eag I digestion and inserted into the linearized 5’ fragment using DNA ligase. Ligation sites were confirmed by DNA sequencing, as were all splice junctions and coding regions of the final E699K plasmid. The E699K construct was purified using QIAfilter Plasmid Maxi Kit (Qiagen Inc, CA) according to the manufacturer's instructions.

DROSOPHILA TRANSFORMATION

BestGene, Inc. (Chino Hills, CA; http://www.thebestgene.com) produced transgenic lines by P element-mediated transformation using the method of Spradling and Rubin (1982). Descriptions of technique details can be found in Cripps and Bernstein (2000). In the procedure, the E699K plasmid at 0.4 μg/μl along with the helper plasmid Δ2-3 at 0.08 μg/μl were injected into embryos (generation G0) collected from the yw Drosophila strain. G0 larvae were raised in vials and crossed with the yw strain. The offspring (G1 generation) from this mating were screened for orange eye color, indicating the presence of the miniwhite (w^+) marker and E699K transgene. These offspring were sent to our lab. In the first injection, 1200 embryos were injected but only 3 transformed lines were viable. We sent the cDNA to
BestGene for a second round of injections, and this time 1000 embryos were injected with 12 viable lines obtained.

**Drosophila Genetic Crosses**

Transformant flies were crossed with $w^{1118}$; CyO/Bl; TM2/TM6B balancer flies (Lindsley & Zimm, 1992) to map the chromosomal location of each line using standard techniques. All the 15 transformant lines obtained from BestGene were genetically mapped to determine the chromosomal location of the transgene. For each individual line, male transgenic flies were crossed with virgin female flies of the balancer line. The presence of red eyes on only female offspring indicated $P$ element insertion on the X chromosome. Among these 15 lines, 3 lines were mapped on the X chromosome. For mapping insertions on other chromosomes, the male offspring were selected by red eye color ($w^+$) and curly wing (CyO) and crossed back to the balancer line. The progeny were selected for red eyes and scored for the segregation of the markers. The absence of $w$; CyO/Bl;TM2/w$^+$ or TM6B/w$^+$ transgenic progeny indicated a $P$ element insertion on chromosome 2, while the absence of the $w$; CyO/w$^+$;TM2/TM6B markers in transgenic progeny denoted integration on chromosome 3. The insertion was on the fourth chromosome if none of above situations appeared. Among these lines, three lines mapped to the third chromosome. Lines containing insertions on the X and third chromosome were subsequently crossed into the $Mhc^{10}$ background, a null allele for the indirect flight and jump muscle myosin isoforms (Collier et al., 1990). The homozygous $E699K$ fly lines were generated in the $Mhc^{10}$ background. Two of the X-linked lines were homozygous lethal in the $Mhc^{10}$ background. Therefore, four viable homozygous $E699K$ lines in the $Mhc^{10}$ background were named $E699K$-2, $E699K$-3, $E699K$-5 and $E699K$-11. These transgenic fly lines and the $PwMhc2$ control fly lines were used in all further studies.

**Confirmation of the $E699K$ Mutation**

For each transgenic fly line, total RNA isolated from indirect flight muscle was used for RT-PCR cDNA synthesis. RNeasy Mini Plus Kit (Qiagen, CA) was used to isolate the total RNA from 30 upper thoraces of virgin female flies following the protocol of the manufacturer, and the contaminating DNA was digested on the silica-gel-based spin column by the Qiagen gDNA column from the kit. RNA was eluted from the column by addition of 40 µl of RNase-free water. The concentration of RNA was determined by measuring the
absorbance at 260 nm (reading of 1 OD unit equals to 40 μg/ml RNA) in the spectrophotometer. The 30 upper thoraces typically yielded 6 μg of total RNA. Primers used in the following synthesis were designed by the Primer3 online software (version 0.4.0, http://frodo.wi.mit.edu/primer3).

Synthesis of the first strand of cDNA was performed using 1.5 μg of total RNA with 2 μmol of primer exon 10(-) by First Strand cDNA Synthesis Kit (BioLab, MA) according to the manufacturer’s instructions. Total RNA was heated at 70 °C for 5 min, incubated at 42 °C for 1 hour with reverse transcriptase, and this was followed by 5 min enzyme inactivation at 95 °C. After inactivation, the first strand cDNA was diluted with 30 μl of water. Synthesis of double-stranded cDNA was performed using 2 μl of the first strand cDNA with 2 μmol of primer exon 10(+), 2 μmol of primer exon 13(-) (Table 1), and the One-Step RT-PCR System (Roche, CA) according to the manufacturer’s instructions. The thermocycler program used was: 95 °C for 2 min; 25 rounds of 95 °C for 30 sec; 50 °C for 30 sec; 72 °C for 40 sec; and 72 °C for 10 min. RT-PCR products were purified using the PCR Clean Up Kit (Qiagen, CA). The PCR products were run on a 0.8% agarose gel, purified using the QIAEX II Gel Extraction System (Qiagen, CA) and sequenced at San Diego State University Microchemical Core Facility using the primers exon 10(+) and exon 13(-).

<table>
<thead>
<tr>
<th>Table 1. Primers Used in E699K RT-PCR Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>Exon 10(+)</td>
</tr>
<tr>
<td>Exon 13(-)</td>
</tr>
<tr>
<td>Exon 10(-)</td>
</tr>
</tbody>
</table>

Note: (+) or (-) indicating forward or reverse primers
QUANTIFICATION OF TRANSGENE EXPRESSION LEVELS

Protein expression levels of transgenes were determined by standard one-dimensional SDS-PAGE analysis (Laemmli et al., 1970). Six upper thoraces from 2 day old virgin female flies from each transgenic line were individually homogenized in 30 μl of sample buffer. PwMhc2 flies were used as a control. All samples were heat treated in boiled water for 3 min. Samples were run on 10- or 12-well pre-cast 10% polyacrylamide Tris-glycine gels (BioRad). Two wells on the edge of each gel were loaded with protein ladder as size markers, the other wells were loaded with 5 μl, 10 μl and 15 μl of the PwMhc2 line sample as the loading standard, and the remaining wells were loaded with 10 μl of transgenic fly line sample. Gels were stained with Coomassie blue, destained, scanned, and analyzed using the NIH ImageJ program (http://rsb.info.nih.gov/nih-image). The myosin to actin ratio for each sample was determined and expressed in relative proportions to the average myosin to actin ratio for control PwMhc2 samples.

ULTRASTRUCTURE

Thoraces from different age (less than 1 day old, two days old and three weeks old) female E699K flies were isolated and prepared for transmission electron microscopy according to the established protocol (Cripps et al., 1999). The upper thorax tissue was fixed in 2% glutaraldehyde, 3% paraformaldehyde, 100 mM sucrose in 100 mM sodium phosphate buffer, with pH 7.2 on ice for two days. Thoraces were washed several times with 100 mM sodium phosphate wash buffer and fixed secondarily in 1% osmium tetroxide in 100 mM sodium phosphate buffer, pH 7.2 for 2 hrs on ice in a dark room. Samples were washed several times with water and dehydrated with a series of washes containing increasing concentrations of acetone, from 50% acetone, 75% acetone, 90% acetone to 100% acetone. Samples were then infiltrated with Embed812 resin (purchased from Microscopy Science) by incubation in resin-acetone mixtures with increasing concentrations of resin from 50% to 100%. Each mixture was left on the samples for 24 hrs. Samples were placed into molds with fresh resin and heated in a 60°C oven under vacuum for 48 hrs to polymerize the resin.

Images were collected on a Tecnai 12 transmission electron microscope. Images of transverse sections of IFM were collected at 11,500x or 40,000x magnification. Images of IFM longitudinal sections were collected at 4,300x or 11,500x magnification.
JUMP AND FLIGHT TESTING

For jump testing, newly eclosed female flies with their wings cut off were placed into plastic vials containing fly food, and aged 2 days at 25°C. The jump testing apparatus was set up by putting a 10 cm tall inverted plastic vial onto a piece of paper containing concentric rings, spaced 0.5 cm apart. Each fly were encouraged with a paint brush to jump from the edge of the vial to the paper. The jump distance of each was documented by the farthest jump distances out of three trials per fly. At least 50 flies were tested from each line.

Flight testing was performed at room temperature (Drummond et al., 1991). Flies were collected on the day of eclosion, placed into vials and aged for 2 days. Each individual fly was released into the center of a flight test chamber and its ability was recorded as flying up toward a light (U), horizontal (H), downward (D), or not at all (N). The flight index value was defined by 6 for U, 4 for H, 2 for D, and 0 for N (Tohtong et al., 1995). The flight index average was calculated by adding all the value numbers and dividing by the sample size. At least 50 flies were tested for each line.

MYOSIN ISOLATION AND PURIFICATION

Myosin was purified from dorso-longitudinal IFMs (DLMs; Swank et al., 2001). DLMs were isolated from more than 150 young female transgenic flies, and incubated in a glycerol and 2% Triton X-100 solution. Myosin was extracted and purified by a series of high salt suspensions and low salt precipitations. Purified myosin was run on a 10% polyacrylamide gel to verify the purity of the product. The protein concentration was determined by the absorbance of 280 nm (1 OD280/0.53= 1 mg/ml) (Margossian and Lowey, 1982). ATPase and in vitro motility assays were performed immediately following myosin preparation.

ACTIN PREPARATION

Since Drosophila yields too little actin for the in vitro motility and ATPase assays, chicken skeletal muscle actin was used. Details are described by Miller et al. (2009). Briefly, G-actin was isolated from chicken pectoralis muscle acetone powder which was dehydrated by acetone. After increasing salt concentration, G-actin was extracted and isolated from the muscle acetone powder. The G-actin concentration was determined by the absorbances at 290 nm and 310 nm using the equation (OD290-OD310)/0.64 equals 1 mg/ml (Kron, Uyeda,
Warrick, & Spudich, 1991). For the in vitro motility assay, G-actin was polymerized and labeled with rhodamine-phalloidin before analysis.

**IN VITRO MOTILITY ASSAY AND ATPASE ASSAY**

Actin in vitro sliding motility assay and ATPase assay were done immediately after myosin was purified. Assays were performed by Anju Melkani and Dr. Girish Melkani. Methods and analyses followed the protocols that were previously published (Kronert et al., 2008; Swank et al., 2001; Swank et al., 2003). At least three sets of assays were performed for each line studied.

**WESTERN BLOT**

The western blot assay was performed by modification of the protocol described in Finley et al. (2003) and Simonsen et al. (2008). The E699K-5 and PwMhc2 flies were collected at four different ages: 2-days old, 1-week old, 2-weeks old and 3-weeks old. Aging was performed in a 25°C incubator. At the end of each time point, the flies were immediately stored in a -70°C freezer. After the collection of 3-week old flies, the upper thoraxes from four female flies from each time point were cut at 4 °C and homogenized gently using the small pestle in freshly prepared 1% Triton-X100 buffer (75 μl). Before removal, the pestle was rinsed with an additional 75 μl of Triton-X100 buffer and the samples placed on ice (final volume 150 μl). The tissue homogenate was centrifuged (14,000 rpm) for 10 min at 4 °C. The individual supernatant fractions were collected into fresh tubes and saved as the Triton-X100 soluble fraction. After the removal of supernatant, 100 μl of 2% SDS buffer was added to the remaining protein pellets, and centrifuged (14,000 rpm) for 10 min at 4 °C. The resulting supernatant fractions were collect and saved as the SDS soluble fractions. The protein concentration of each SDS soluble sample was determined using a detergent compatible Lowry protein assay (BioRad, CA). Each sample was boiled for 10 min at 100 °C in the presence of protein loading buffer (4x, BioRad, CA). 20 μg of total protein for each SDS soluble sample was loaded per lane on a 10% precast SDS-PAGE gel (BioRad, CA), and electrophoresed at 80V with constant current. The SDS gel was electroblotted onto beta-probe blotting membrane (Bio-Rad) for 2 hours using 200 mAmmps current at 4°C. Western membranes were blocked with gentle shaking in 1x TTBS (Tris-Tween Buffered Saline) containing 5% powdered milk for one hour. They were rinsed several times in 1x TTBS and
then probed with anti-ubiquitin antibodies (provided by Dr. Kim Finley, SDSU Bioscience Center) overnight at 4 °C. On the next day, the blots were washed several times in 1x TTBS and incubated in anti-mouse HRP secondary antibody (Promega, CA) for one hour at room temperature. The blots were then rinsed in 1x TTBS and incubated in anti-mouse HRP secondary antibody (Promega, CA) for one hour at room temperature. The blots were then rinsed in 1x TTBS and developed using Immun-Star ECL reagents (BioRad, CA) and GS-800 digital imaging systems (BioRad, CA) with Quantity One imaging analysis software (BioRad, CA). The blots were then stripped, reblocked and probed with the anti-beta-actin antibody (Promega, CA). The relative amounts of IUP proteins from individual samples were quantified and corrected using actin as a loading control using the ImageJ program (http://rsbweb.nih.gov/ij/).
RESULTS

The purpose of this research is to use *Drosophila melanogaster* as a model system to study the IBM-3 disease. We obtained four lines expressing the E699K mutation (corresponding to E706K mutation in IBM-3 disease of humans) in the Mhc<sup>10</sup> background. After verification of wild-type levels of protein expression and confirmation of the E699K mutation in the IFM, we examined the ultrastructure and function of the affected muscles, the level of insoluble aggregates in the IFM during aging, and the induced actin motility and ATPase of transgenic E699K myosin.

**GENERATION OF TRANSGENIC FLY LINES IN THE Mhc<sup>10</sup> BACKGROUND**

DNA containing the entire *Drosophila melanogaster* myosin heavy chain gene with the E699K mutation was cloned into the P element vector. The miniwhite gene (w<sup>+</sup>) was used as the selectable eye-color marker for the genetic mapping (see Materials and Methods for details). We sequenced the whole coding region and verified that no cloning artifacts were introduced (using established lab protocol, data not shown).

Transgenic lines that express E699K transgenes were obtained by micro-injecting embryos with DNA constructs described in Materials and Methods. The E699K construct was injected along with a helper plasmid that expressed a transposase which is required for the transgene to insert into *Drosophila* chromosomes. For the first injection, only 3 transformed fly lines were obtained from 1,200 injected embryos. Since we need to obtain at least 3 individual lines to confirm the mutation phenotype, we sent the construct for a second round of injections, and 12 transformed fly lines were obtained from 1,000 injected embryos.

For each transformed fly line, we used the standard genetic mapping method to determine the location of the transgene (see Materials and Methods). Since the second chromosome is the site of the endogenous Mhc gene, transformed fly lines for which the transgene inserted on this chromosome were excluded from further study. Two recessive lethal transgenic lines were excluded from further study as well. In one of the transgenic lines, the sibling flies of the same age showed various eye colors, indicating that more than one transgene was inserted into the genome and this line was excluded from further study.
The remaining four E699K transgenic fly lines, named [E699K]-2, -3, -5, and -11, met the requirements for future research (Table 2).

Table 2. Transgene Linkage in E699K Fly Lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Transgene Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>[E699K]-2</td>
<td>X</td>
</tr>
<tr>
<td>[E699K]-5</td>
<td>3</td>
</tr>
<tr>
<td>[E699K]-3</td>
<td>3</td>
</tr>
<tr>
<td>[E699K]-11</td>
<td>3</td>
</tr>
</tbody>
</table>

To eliminate endogenous MHC expression from the transgenic flies, we crossed each into the Mhc10 background, which has a point mutation in the intron prior to exon 15a, resulting in it being MHC null for the IFM and tergal depressor of the trochanter (Collier et al., 1990). This genetic background allows us to isolate and purify transgenic E699K myosin free of endogenous MHC contamination for the ATPase and motility assays. As described in the following section, the three 3rd chromosome linked transgenic homozygous lines in the Mhc10 background were used in the various assays discussed in this thesis. Additionally, a fly line transformed with a wild-type genomic Mhc gene, so-called PwMhc2, was used in this study as a transgenic control (Swank et al., 2000).

**Transgenes Express Wild-type Levels of MHC**

We used SDS-PAGE to determine whether transgene expression yielded normal levels of MHC (Figure 4). The MHC expression levels relative to actin accumulation in the upper thoraces of 2 hour old flies were determined for E699K-2, E699K-3, E699K-5, E699K-11 transgenic lines. Levels of MHC expression were essentially identical with that of the PwMhc2 control in all transgenic lines tested except for the E699K-2 line (Table 3). In particular, E699K-3, E699K-5 and E699K-11 female flies were selected for detailed ultrastructural analyses due to their high levels of E699K myosin expression.
Figure 4. Protein expression levels: Protein from the upper thorax of 2 hour old flies of control (PwMhc2), Mhc-null control (Mhc<sup>10</sup>), and E699K-3,-5,-11 and -2 transgenic flies (all labeled above the gel) were analyzed by SDS-PAGE and stained with Coomassie blue. We used flies that had just eclosed (2 hours old) to minimize the impact of any myosin degradation. Myosin and actin bands are labeled on the right hand side. The MHC to actin ratio was measured by comparing the intensity of the bands. Values are compared to PwMhc2 (control) by ImageJ software.

<table>
<thead>
<tr>
<th>Line name</th>
<th>% Protein Expr. ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PwMhc2 (control)</td>
<td>100±2.4(6)</td>
</tr>
<tr>
<td>Mhc&lt;sup&gt;10&lt;/sup&gt;</td>
<td>13±3.2 (5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E699K-2</td>
<td>82±5.2 (5)</td>
</tr>
<tr>
<td>E699K-3</td>
<td>92±6.3 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E699K-5</td>
<td>95±4.8 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E699K-11</td>
<td>89±4.6 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Student's <i>t</i> test <i>p</i> < 0.05, statistically different from control.
<sup>b</sup> Not statistically different from control (<i>p</i> > 0.05, Student's <i>t</i> test).

SEM, standard error of the mean; n= sample number.
**TRANSGENE TRANSCRIPTION VERIFICATION**

To verify that E699K MHC was translated from the transgene transcripts, total mRNA isolated from the upper thoraces of E699K-3, E699K-5 and E699K-11 transgenic fly lines was used for RT-PCR cDNA synthesis. Typically, 40 upper thoraces from virgin female flies yielded approximately 6 µg of total RNA. Double-stranded cDNAs that include the E699K coding region in exon 10 were synthesized from 1.5 µg of total RNA by RT-PCR. The PCR products were purified and then sequenced at the San Diego State University Microchemical Core Facility (see Materials and Methods). The E699K mutations in lines E699K-3, E699-5 and E699K-11 were verified (Figure 5).

![K699 (GAA-->AAA)](image)

**Figure 5.** E699K-5 mutation confirmed in the transgenic lines. Chromatogram was displayed by Chromas (Version 2.33 http://www.technelysium.com.au/chromas.html).

To confirm the correct exon splicing pattern of E699K lines, we used primers that flank alternative exon regions and performed RT-PCR by established lab protocols as previously described in Suggs et al. (2007) and Kronert et al. (2008). Briefly, total RNA was isolated from upper thoraces of 2-day-old female adult flies of wild type (yw) and E699K, and partial cDNAs from portions of Mhc transcripts flanking alternative exons were synthesized by the RT-PCR method. The purified cDNA was digested with restriction endonucleases to the IFI-specific alternative exon: the fragment containing exon 3b was digested with Bgl II; exon 7d with Eco RI; exon 9a with Pst I; exon 11e with Bam HI; and exon 15a with Pvu I. The cDNA fragments were run on an agarose gel, stained with ethidium bromide and compared with yw. For example, the exon 10/exon 13 primer pair was used to amplify the region from exon 10 through a portion of exon 13, creating a 1059 bp product, and the presence of alternative exon 11e was confirmed by digesting the fragment with Bam-
HI, which generated 180 bp, 383 bp and 496 bp sub-fragments. If exon 11e was not present, then the cDNA fragment would generate only two fragments instead of three. Since exon 18 lacks useful restriction endonuclease sites, the presence or absence of exon 18 was determined by sequencing the purified RT-PCR product produced by an exon 18-specific primer, and the product sequence was compared to the sequence of the yw IFM isoform. As expected, the DNA analyses confirmed the presence of normal splicing of IFM isoform-specific alternative exons 3b, 7d, 9a, 11e, 15a and 18 in E699K-3, E699K-5 and E699K-11 lines (data not shown).

**THE E699K MUTATION SIGNIFICANTLY IMPAIRS JUMP AND FLIGHT MUSCLE FUNCTION**

To determine the flight and jump muscle function of homozygous E699K flies in the Mhc\textsuperscript{10} background, flight and jump assays were performed with 2-day-old adult female flies at 22 °C (see Materials and Methods). Because Mhc\textsuperscript{10} flies lack the ability to accumulate MHC isoforms in the IFM and TDT (Collier et al., 1990), the introduction of E699K allows for its exclusive expression of mutated myosin in these two muscle types. The flight performance of each fly was converted into a flight index (FI) with a value from 0 to 6.0, with 6.0 equal to upward flight and 0 equal to flightless (Tohtong et al., 1995). As a control, the flight index of the PwMhc2 line was 4.3, in which 56% of the flies could reach the flight index of 6.0. Test results show that homozygous E699K flies completely lack flight abilities when compared to the PwMhc2 flies (Table 4). The E699K flies were unable to beat their wings and a wings-up phenotype was present in 2-day-old flies, indicating that the IFM might be compromised. The jump ability of young flies was also severely reduced compared to controls (less than 0.6 cm vs. 5.4 cm control value), suggesting that the TDT muscle might also be seriously impaired by the E699K MHC mutation (Table 4).

The heterozygous condition (one mutant copy and one normal copy) corresponds to the genetic makeup of human IBM-3 patients (Tajsharghi et al., 2002). In order to determine the IFM and TDT function in the heterozygous mutation flies, we crossed the E699K flies with the wild type (yw, indicated by +) flies. Before the offspring eclosed, the parents from the cross were removed to make sure the genotype of the offspring corresponded to E699K/+ (Mhc\textsuperscript{10}/+; E699K/-) (where “-“ indicates absence of a transgene). We used the offspring of yw and PwMhc2 as the control, indicated by PwMhc2/+ (PwMhc2/-; Mhc\textsuperscript{10}/+). The flight
<table>
<thead>
<tr>
<th>Line</th>
<th>No. flies tested</th>
<th>Up (%)</th>
<th>Horizontal (%)</th>
<th>Down (%)</th>
<th>Not at all (%)</th>
<th>Flight index ± SD</th>
<th>Jump distance ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E699K-2</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>98</td>
<td>0</td>
<td>0.4 ± 0.2 (100)²</td>
</tr>
<tr>
<td>E699K-3</td>
<td>103</td>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>99.1</td>
<td>0</td>
<td>0.3 ± 0.2 (103)²</td>
</tr>
<tr>
<td>E699K-5</td>
<td>112</td>
<td>0</td>
<td>0</td>
<td>1.8</td>
<td>98.2</td>
<td>0</td>
<td>0.6 ± 0.1 (112)²</td>
</tr>
<tr>
<td>E699K-11</td>
<td>105</td>
<td>0</td>
<td>0</td>
<td>3.8</td>
<td>96.2</td>
<td>0.1</td>
<td>0.3 ± 0.1 (105)²</td>
</tr>
<tr>
<td>PwMhc2</td>
<td>100</td>
<td>56</td>
<td>15</td>
<td>15</td>
<td>14</td>
<td>4.3 ± 0.3</td>
<td>5.4 ± 0.2 (100)</td>
</tr>
</tbody>
</table>

SD, standard deviation.
SEM, standard error of the mean; n = sample number.
Flight and jump measurements were performed at room temperature (22 °C). Flight abilities are calculated as percentages.
Homozygous E699K transgenic flies and PwMhc2 control flies were assayed for the ability to fly up (U), horizontally (H), down (D) or not at all (N). Flight index equals 6U/T + 4H/T + 2D/T + 0N/T, where T is the total number of flies tested.
²Student's t test showed significant difference between PwMhc2 and E699K-2, 3, 5 and 11 in the jump distance (p < 0.001)
tests were performed on 2 day old female heterozygous $E699K^+/+$ lines and the $PwMhc2^+/+$ control line (Table 5). The 2 day old flies in all three heterozygous lines were unable to fly upward at 22 °C, however horizontal flight and downward flight were observed infrequently, resulting in the flight index less than 0.9 when compared to a FI=5.2 of the control line (Table 5). The weak flight index in all three heterozygous lines suggested that one copy of wild-type $Mhc$ could not rescue the flightless phenotype of $E699K$ Drosophila (in terms of flying upward). For the jumping test, our results show that the jumping ability of all three $E699K^+/+$ lines was significantly increased when compared with that of the homozygotes (Table 4 and Table 5, 5.1 cm compared with 0.6 cm for $E699K^/-5$, etc.), although their jumping ability was not fully rescued as that of the $PwMhc2^+/+$control (Table 5, $p<0.05$ in all three lines compared to control). In summary, the locomotion assay indicated one wild-type copy of $Mhc$ could not fully rescue the heterozygous $E699K$ TDT function, and IFM function is not rescued in terms of upward flight ability, which suggested that mutation $E699K$ in the SH1 helix of myosin is a dominant mutation for TDT and IFM function in Drosophila.

Table 5. Effect of Heterozygous $E699K^+/+$ Genotype on Jump and Flight Ability

<table>
<thead>
<tr>
<th>Line and Genotype</th>
<th>Jump Distance (cm)</th>
<th>Flight Index (2 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SEM (n)</td>
<td>mean ± SD (n)</td>
</tr>
<tr>
<td>$PwMhc2^+/+$</td>
<td>6.3±0.2 (51)</td>
<td>5.2±0.5 (50)</td>
</tr>
<tr>
<td>$E699K-3^+/+$</td>
<td>5.3±0.4(55) $^{a,b}$</td>
<td>0.5±0.3 (51) $^c$</td>
</tr>
<tr>
<td>$E699K-5^+/+$</td>
<td>5.1±0.4 (53) $^{a,b}$</td>
<td>0.9±0.2(48) $^c$</td>
</tr>
<tr>
<td>$E699K-11^+/+$</td>
<td>4.6±0.4 (55) $^{a,b}$</td>
<td>0.3±0.3 (51) $^c$</td>
</tr>
</tbody>
</table>

SEM, standard error of the mean; n = sample number.

$^a$Student's $t$ test showed all three $E699K^+/+$ lines’ jump distance to be significantly increased from that of the homozygotes (Table 4) ($p < 0.001$)

$^b$Student's $t$ test showed that all three $E699K^+/+$ lines’ jump distance to be significantly different from that of the $PwMhc2^+/+$control line ($p<0.05$)

$^c$Student's $t$ test showed all three $E699K^+/+$ lines’ FI to be significantly different from that of the control line ($p < 0.001$)
**E699K Mutation Causes IFM Defects**

To address whether expression of E699K MHC produced ultrastructural defects, the IFMs of young adults (less than 24 hour old) from the three highest expressing fly lines were examined by transmission electron microscopy (TEM) and compared to those of the PwMhc2 transgenic control flies in the Mhc10 background (Figure 6). The myofibril ultrastructure of PwMhc2 in the Mhc10 background shows wild-type hexagonal packing of thick and thin filaments containing well organized Z-bands (Figures 6A and 6B). Fibers of young homozygotes (2 hours old) from all three E699K line flies show severe ultrastructural disarray, with disruption of myofibril integrity and broken Z-bands, and the sarcomeric structure is poorly organized (Figures 6C to 6H). Myofibril structure of 2 day old E699K-5 shows severe ultrastructural deterioration which is similar to 2 hour old E699K-5 (Figure 7B and 7D). Fibers of 3 week old E699K-5 show further disruption, with loss of myofibril shape and hexagonal packing of thick and thin filaments compared to PwMhc2 and E699K-5 2 day old adults (Figure 7F and 7H). We observed abnormal vacuoles in various sizes and shapes from young and older E699K flies (Figure 7I and 7J). Interestingly, mitochondrial structures were observed inside some of the vacuoles of older E699K-5 flies (Figure 7J, arrow head). Thus, the E699K mutation in the myosin SH1 helix domain severely affects IFM myofibril structure, which likely contributes to the flightless phenotype.

For the heterozygous condition, we used the offspring of yw and PwMhc2 as the control as we did for the flight and jump tests. The myofibril ultrastructure of 2 hour old PwMhc2/+ and E699K-5/+ flies show normal hexagonal packing of thick and thin filaments and have identical sarcomere structures, with normal Z-lines and M-lines (Figure 8A compared to 8C, and 8B compared to 8D). In the flight test, we observed that one copy of wild-type Mhc could not rescue the flightless phenotype (Table 5); thus, we investigated the myofibril integrity of 7 day old E699K-5/+ flies. The ultrastructure of 7 day old heterozygous E699K-5/+ flies shows normal hexagonal packing of thick and thin filaments with well organized Z-bands (Figure 8E and 8F), suggesting that one copy of E699K with one copy of wild-type MHC in the Mhc10 background did not affect myofibril assembly and structure, but rather myosin function (See Discussion).
Figure 6. IFM ultrastructure in PwMhc2 flies and E699K transgenic lines in the Mhc<sup>10</sup> background. Longitudinal (left panels) and transverse (right) views of the IFM from 2 hour old female flies. Severe myofibril ultrastructural deterioration was observed in all of the mutant lines, E699K-3 (C and D); E699K-5 (E and F), and E699K-11 (G and H). All longitudinal views are at the same magnification, bar =1 µm. All transverse views are the same magnification, bar =0.5 µm.
Figure 7. IFM ultrastructure and the abnormal vacuoles in the $E699K-5$ line compared to the control. (A) Transverse section from $PwMhc2$ 2 day old adult. (B) Transverse section from $E699K-5$ 2 day old adult. (C) Longitudinal section from $PwMhc2$ 2 day old adult. (D) Longitudinal section from $E699K-5$ 2 day old adult. Myofibril structure of 2 day old $E699K-5$ shows severe ultrastructural deterioration compared to $PwMhc2$. (E) Transverse section from $PwMhc2$ 3 week old adults. (F) Transverse section from $E699K-5$ 3 week old adult. (G) Longitudinal section from $PwMhc2$ 3 week old adult. (H) Longitudinal section from $E699K-5$ 3 week old adult. $E699K-5$ shows further disruption in myofibril shape and hexagonal packing of thick and thin filaments compared to $PwMhc2$ and $E699K-5$ 2 day old adults. Abnormal vacuoles observed from 2 day old (I) and 3 week old (J) $E699K-5$ adults. The mitochondria-like structures are indicated by arrow heads. The scale bars represent 0.5 μm.
Figure 8. IFM ultrastructure in heterozygous PwMhc2/+ and E699K-5/+ transgenic lines in the Mhc^10 background. Longitudinal (left panels) and transverse (right) views of the IFM in female flies. PwMhc2/+ flies were generated by crossing PwMhc2; Mhc^10/Mhc^10 flies with yw flies, and the myofibril structure from 2 hour old flies was used as the control for the heterozygous IFM ultrastructure (A and B). E699K-5/+ flies were generated by crossing Mhc^10/Mhc^10; E699K-5/E699K-5 flies with yw flies. Myofibril structure of E699K-5/+ appeared identical to the control in both 2 hour (C and D) and 7 day old flies (E and F). All longitudinal views are the same magnification, bar =1 µm. All transverse views are the same magnification, bar =0.5 µm.
**IN VITRO ACTIN SLIDING VELOCITY IS DECREASED WITH E699K-5 MYOSIN**

We used an *in vitro* motility assay to examine the sliding velocity of fluorescently labeled actin filaments generated by *E699K* myosin and the *PwMhc2* wild-type control (see Materials and Methods for details). The *PwMhc2* myosin moves actin filaments at a velocity of 6.38 μm/s at 22 °C (Table 6). In contrast, *E699K* myosin drives actin filament movement at a rate of 1.35 μm/s (Table 6). Based on two independent preparations, actin sliding velocity stimulated by *E699K* myosin was decreased by 80% when compared to the control. The p-value is less that 0.05 by Student’s *t* test, suggesting that this difference is statistically significant.

<table>
<thead>
<tr>
<th>Line name</th>
<th>Actin velocity (μm⋅s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PwMhc2</em> (control)</td>
<td>6.38 ± 0.23 (4)</td>
</tr>
<tr>
<td><em>E699K-5</em></td>
<td>1.35± 0.14 (2)</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation with numbers in parenthesis indicating the number of individual preparations.

*Statistically different from *PwMhc2* (*p* <0.05, Student’s *t* test).

**ATPASE ACTIVITIES ARE DECREASED IN E699K-5 MYOSIN**

We used an ATPase assay to determine the *E699K* myosin enzymatic activity compared to the *PwMhc2* control. All ATPase activities of the myosin from the *E699K-5* line were significantly lower than the control. The reduction of the basal Mg-ATPase activity of *E699K-5* was 75%, and for actin-activated ATPase activity, the value was reduced by 80% (Table 7). We hypothesize that the *E699K* mutation increases the time myosin spends in a strongly actin bound state, leading to decreased ATPase rate (see Discussion).
Table 7. Actin-stimulated Myosin ATPase Activity for the Myosin Purified from PwMhc2 and E699K-5 Flies.

<table>
<thead>
<tr>
<th>Myosin</th>
<th>Basal Ca-ATPase ± SD (s⁻¹)</th>
<th>Basal MgATPase ± SD (s⁻¹)</th>
<th>Actin-stimulated V_max ± SD (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PwMhc2</td>
<td>10.34 ± 0.73 (n = 6)</td>
<td>0.26 ± 0.02 (n = 6)</td>
<td>1.86 ± 0.33 (n = 6)</td>
</tr>
<tr>
<td>E699K-5</td>
<td>1.50 ± 0.53 (n = 3)*</td>
<td>0.07 ± 0.01 (n = 3)*</td>
<td>0.32 ± 0.06 (n = 3)*</td>
</tr>
</tbody>
</table>

* Statistically different from PwMhc2 myosin (p < 0.001, Student's t test)

**E699K Increases Ubiquitin-tagged Protein Aggregation During Aging**

Autophagy (“self-eating”) involves lysosomal import and degradation of cytosolic material. Previous study shows that protein degradation via the UPS (ubiquitin–proteasome system) and autophagy-lysosome system play critical roles in muscle metabolism and signaling pathways (Sandri, 2010). Hallmarks of the human E699K mutation phenotype are aggregates and inclusion bodies in muscle tissue (Li et al., 2006). To measure the level of protein aggregation in E699K Drosophila IFM we used a two-part protein extraction and Western analysis technique (Simonsen et al., 2008; see Materials and Methods for details). The first step involves the sequential fractionation of proteins obtained from upper thoraces of PwMhc2 and E699K-5 flies. Triton-X100 was used as a mild nonionic detergent for the soluble proteins, and the remaining proteins contained within the pellet were extracted with 10% SDS. The second step in the procedure involves Western blot analysis of the SDS-soluble fraction to detect the aggregated proteins that carry the molecular marker ubiquitin. The insoluble ubiquitinated proteins (IUP) from the SDS-soluble fraction represent aggregate-like proteins that are targeted by ubiquitin to be eliminated by the proteasome and endosomal/lysosomal systems (Simonsen et al., 2008). The Western blot shows age-dependent increases of IUP in both PwMhc2 control and E699K-5 (Figure 9). We used the ImageJ program to quantitatively analyze the IUP pattern by normalizing to the beta-actin loading control. The results show that the intensity of IUP increased during aging in both lines, and that the IUP from E699K-5 showed levels above control values at the same time
Figure 9. Western blot analysis of IUP (Insoluble Ubiquitinated Protein) in upper thoraces from *PwMhc2* (control) and *E699K-5* show age-dependent aggregation. The flies were collected at four different ages: 2-days old, 1-week old, 2-weeks old and 3-weeks old, indicated by 2d, 1w, 2w, and 3w, respectively (see Materials and Methods for details). The SDS-soluble aggregates were probed by ubiquitin antibody, as show on the top panel. Anti-beta-actin antibody was used as the loading control, as shown under the anti-ubiquitin blot. The ImageJ program was used to quantitatively analyze the IUP pattern by normalizing to the beta-actin loading control; bar value indicates the intensity as shown in the lower panel.

points (Figure 9, lower panel). Thus, Western blotting shows that ubiquitin-tagged aggregates accumulated in the *E699K-5* line at higher levels than in the control during aging.
DISCUSSION

In this thesis, we used an integrative approach to study the structure-function relationship of the E699K myosin in the Drosophila model system. The eventual goal was to shed light on therapeutic approaches to human hereditary inclusion-body myopathy type 3 (IBM-3). Our results suggest that the E699K mutation in the SH1 helix of the myosin head severely affects myofibril structure and function in homozygous flies. Myofibrillar disarray was detected in E699K indirect flight muscle at 2 hours of age, and more severe defects are seen at 3 weeks. The E699K ultrastructure data agree with the locomotion assay, as the E699K flies were unable to beat their wings and a wings-up phenotype was present in 2 day old flies. The jump ability of young flies was also severely impaired compared to controls. The mutation depressed calcium as well as basal and actin-activated MgATPase ($V_{\text{max}}$) by $\sim 75\%$ compared to wild-type PwMhc2 myosin. Also, the mutation decreased in vitro motility of actin filaments by $\sim 80\%$. Thus, the homozygous E699K MHC mutation severely affects muscle structure and function in Drosophila. In the heterozygous condition, the myofibril structure showed normal thick and thin filament packing in young and older flies. The locomotion assay indicated one wild-type copy of Mhc could not rescue the heterozygous E699K TDT and IFM function. These results suggest that one copy of E699K with one copy of wild-type Mhc in the Mhc$^{10}$ background affects myosin function but not IFM myofibril structure.

STRUCTURE-FUNCTION RELATIONSHIP OF THE SH1 HELIX IN MHC

The SH1 helix is proposed to perform like a clutch that controls coupling between the converter and the motor during the myosin power stroke. Drosophila homology modeling structures show that the SH1 helix region melts during the power stroke (Figure 3B), which could explain the rotation of the converter subdomain during the power stroke. The SH1-SH2 helix is 100\% conserved in both conventional and unconventional myosins among different species (Martinsson et al. 2000; Figure 3C), suggesting the importance of this region during evolution. Also, previously published data show that chemical modification of
the SH1 helix resulted in a dramatic decrease of myosin ATPase activity and loss of the motor function in \textit{in vitro} motility assays (Bobkova, Bobkov, Levitsky, & Reisler, 1999). SH1-SH2 cross-linked myosin shows weakened ATPase activity and lower actin-binding affinity, and exhibits rotational disorder (Thompson et al., 2008). Other mutations in this region, like R689H (Iwai & Chaen, 2007) and G709V (Kad, Patlak, Fagnant, Trybus, & Warshaw, 2007) affect myosin motor thermal stability. The analogous $E699K$ mutation in the \textit{Dictyostelium} non-muscle myosin II showed decreased actin-activated ATPase and an increased duty ratio (Zeng et al., 2004). Also, an arginine residue mutation at the end of the SH1 helix (R714) can cause hypertrophic cardiomyopathy when mutated to serine (see review of Burghardt et al., 2010; Figure 10 labeled in cyan).

Figure 10. Interaction between relay helix and SH1-SH2 helix domains of myosin in pre-power stoke state. E699 is labeled in magenta, R714 is labeled in cyan, and E499 is labeled in yellow. The SH1-SH2 helix is labeled in red and relay helix is labeled in blue. The Swiss model homology modeling method was used to illustrate the protein structure (http://www.swiss-model.com, based on scallop pre-power stroke structure). Pymol v1.3 was used to produce the figure (http://www.pymol.org).
The Lymn and Taylor model proposed that myosin carries out a cycle of “power” and “recovery” strokes during different kinetic states (Lymn & Taylor, 1971; see review of Geeves et al., 2005). While the power stroke occurs during the transition from the weak to strong binding state by the rotation of the lever arm which leads to the production of mechanical force, the recovery stroke is the reverse process when the lever arm swings back to prepare for the next power-stroke. The recovery stroke is simpler for the computer simulation than the power stroke since it does not involve actin binding. Therefore, Fischer and his colleagues published a series of papers to outline the mechanism of the recovery stroke (Fischer, Windshugel, Horak, Holmes, & Smith, 2005; Gyimesi et al., 2008; Koppole, Smith, & Fischer, 2006; Koppole et al., 2007; Mesentean, Koppole, Smith, & Fischer, 2007; Schwarzl, Smith, & Fischer, 2006). Their results propose that after the force-generating power-stroke and the detachment of the actin filament, myosin swings back its lever-arm by ~65 degrees by a so-called “seesaw” mechanism. In this mechanism, the converter domain undergoes two distinct and successive motions: while in a first phase the converter rotates 25 degree after closing of the Switch-2 loop (upon the ATP binding) via the relay helix, the motion of the converter is further amplified by 40 degree via the piston motion of the SH1 helix in the second phase. Molecular modeling of Drosophila myosin shows that the relay helix is parallel with the SH1 helix (Figure 10, relay helix labeled in blue and SH1-SH2 helix labeled in red). The relay helix is partially unwinding during the recovery stroke and works as the communication pathway between the nucleotide-binding pocket and the converter domain (Koppole et al., 2007). Colleagues in our lab had generated an R759E mutation at the site of the converter domain that interacts with the relay loop, which significantly disrupted the myosin ATPase activity and actin motility (Kronert et al., 2010). Based on the recovery stroke modeling results (Fischer et al., 2005; Koppole et al., 2007), we propose that the closing of the Switch-2 loop causes the partial unwinding of the relay helix by the transient interaction of E499 and R714 on the SH1 helix. This communication with the converter domain drives the lever arm through a 25° rotation. The interaction of the SH1 helix and relay helix induces the piston motion of the SH1 helix and results in a further amplified 40° rotation of the converter and lever arm (Figure 10). The E699K mutation could cause a surface charge change of the SH1 helix and might impair the piston-motion mechanism of the SH1 helix during the recovery stroke, resulting in malfunction of the myosin motor.
EFFECTS OF E699K ON MYOFIBRIL STRUCTURE AND FUNCTION IN DROSOPHILA IFM

For the homozygous E699K condition in the Mhc10 background, we observed a completely flightless phenotype and extremely weak jump ability (Table 4). Myofibrils of newly eclosed 2 hour old and 2 day old flies show severe ultrastructural disarray, with disruption of myofibril integrity and poorly organized sarcomeric structure (Figure 6C to 6H, Figures 7B and 7D). The impaired fibers degenerate as flies age, as 3 week old E699K-5 flies show further disruption with loss of myofibril shape and hexagonal packing of thick and thin filaments, and poor sarcomeric structure (Figures 7F and H). The degeneration of myofibrils could be explained by the results of the anti-ubiquitin western blot, which suggests that ubiquitin-tagged aggregates accumulated during aging (Figure 9, compare the bar levels of 2d and 3w). We hypothesize that the aggregates were induced by the misfolding of E699K myosin. We also observed abnormal vacuoles in various sizes and shapes from young and older E699K flies (Figure 7I and 7J), with mitochondria inside some of the vacuoles (Figure 7J, arrow heads). Similar structures are observed in dilated cardiomyopathy, where degenerated muscle fibers had autophagic vacuoles containing intracellular organelles, such as mitochondria. The ubiquitin-proteasome and autophagy-lysosome pathways are the two major methods for protein clearance in skeletal muscle (Knaevelsrud & Simonsen, 2010). The western blot with ubiquitin antibody shows that aggregates increase during aging. However the presence of even higher levels of aggregates in the mutants suggests that E699K myosin might overload the ubiquitin-proteasome and autophagy pathways for protein and organelle clearance. These pathways are stimulated in degenerative diseases such as cachexia (see review of Attaix, Combaret, Bechet, & Taillandier, 2008). On the other hand, previous study showed that inhibition of lysosome-dependent degradation causes myopathies like Danon and Pompe diseases, and autophagy inhibition is thought to play a role in many myopathies with abnormal mitochondria or with inclusion bodies (Levine & Kroemer, 2008; Temiz, Weihl, & Pestronk., 2009). The exact physiological function of autophagy in skeletal muscle is still being addressed.

For the heterozygous condition, the myofibril ultrastructure of 2 hour old E699K-5/+ flies shows normal hexagonal packing of thick and thin filaments, and the fibers did not show any degeneration in 7 day old flies (Figure 8). However, one copy of wild-type Mhc
could not rescue the flightless phenotype, and the TDT function was not fully rescued (Table 5). The flightless phenotype suggests that one copy of E699K severely affects the myosin motor function, although the myofibril structure was normal. This is in accordance with the clinical studies in young IBM-3 individuals who showed normal sarcomeric structure (Li et al., 2006; Martinsson et al., 2000). By overexpressing the E710K mutant (E699K in Drosophila) in the wild-type background of Caenorhabditis elegans, the worms showed severe paralysis but apparently normal formation of thick filaments in the body wall muscle (Tajsharghi et al., 2005).

We did not observe inclusion bodies in the heterozygous condition. One possible explanation is that IBM-3 is a polygenic disease in which other genes and proteins may play important roles as modifiers of the symptoms (Li et al., 2006). There are three myosin isoforms expressed in human skeletal muscles, two fast (type IIa and IIx) and one β/slow (type I) myosin, and the E699K mutation is only observed in the MYH2 gene which encodes the fast type IIa muscle isoform (Martinsson et al., 2000). However, myofibril structural abnormalities were observed not only in IIa fibers, but also in other fiber types as well (Li et al., 2006). Thus, other proteins might be responsible for this disease. Mutations of the GNE gene, which encodes the rate-limiting enzyme of the sialic acid biosynthetic pathway are causative of some cases of hereditary inclusion body myopathy (see review of Huizing & Krasnewich, 2009). Therefore further clinical studies should be performed on IBM-3 patients to address the possible polygenic nature of the disease.

**In vitro Studies of E699K**

We used the in vitro motility assay and ATPase assay to address the effect of the E699K mutation on myosin motor function. We found that E699K myosin severely decreased the ATPase activities when compared with the PwMhc2 control, with Ca-ATPase and basal Mg-ATPase activity lowered by ~75%, and actin-stimulated V\textsubscript{max} reduced by ~80%. In terms of actin sliding velocity, E699K decreased it from 6.38 to 1.35 µm•s\(^{-1}\) when compared with the PwMhc2 control, which is a ~80% reduction. One possible explanation of actin-stimulated V\textsubscript{max} reduction may be that E699K myosin spends more time in the strong binding state, which could significantly reduce the transition speed between actomyosin states.
The values obtained in the actin-activated Mg-ATPase assays were performed with chicken actin, due to the technical limitations of isolating large amounts of actin from *Drosophila* IFM. However, the 4-fold decrease in the actin-activated Mg-ATPase rate compared with the control strongly suggests that the reduced ATPase activity is caused by the *E699K* mutation and not the actin isoform (Swank et al., 2003). Our data are supported by transient kinetic measurements of actomyosin ATPase activity in the analogous *E699K Dictyostelium* non-muscle myosin II (Zeng et al., 2004), which showed decreased actin-activated ATPase and an increased duty ratio. Our *in vitro* motility result is also in agreement with a clinical study on the motility of myosin isolated from the muscle samples of IBM-3 patients (Li et al., 2006), which show that actin-sliding velocity is slower with the heterozygous myosin (*E699K* myosin and wild type myosin) than that with wild-type myosin.
FUTURE STUDIES

To address the biomedical and biophysical properties of \textit{E699K} myosin, several measurements could be performed in the future. Mechanics studies of single fibers from indirect flight muscles can be used to determine work, power output, isometric tension and stiffness in young homozygous and heterozygous flies (Swank et al., 2006). Isometric force measurement in the optical trap can be used to determine the ability of \textit{E699K} and control myosins to produce force under similar experimental condition (Moore et al., 2001). It will be interesting to compare these values to the \textit{in vitro} ATPase and motility experiments to see how the \textit{E699K} mutation affects the myosin chemo-mechanical cycle.

Since autophagy inhibition is thought to play a role in many myopathies with abnormal mitochondria (Temiz et al., 2009), qRT-PCR could be used to measure the change of ATG (autophagy related genes) (Simonsen et al., 2008) transcript levels in the mutant lines. The qRT-PCR data could be coupled with IUP Western blot analysis, which provides insight into the role of autophagic clearance of aggregate-like substrates in the skeletal muscle (Finley et al., 2003).

Further, since some inclusion body neuropathies can be alleviated by chaperone induction (beta-crystallin, Hsc70, Hsp90), specific chaperones or small molecule chaperone inducers may be used as a therapeutic approach to reduce myofibril disruption and muscle dysfunction in the IBM-3 disease (see review of Kim, Lowe, & Hoppe, 2008).
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